Bioactivity of a synthetic peptide derived from a defensin from the tick *Ornithodoros savignyi* Audouin (1827)

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Submitted in partial fulfilment of the degree *Magister Scientiae*

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Pretoria

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Summary

With the emergence of drug resistant bacteria, the need for new antimicrobial agents is growing. Antimicrobial peptides (AMPs) are natural antibiotics which have a broad spectrum of activity and also due to a great diversity in structure and chemical formula, it is less likely that bacteria will develop resistance to these agents. In addition to their antimicrobial roles, AMPs and/or their derivatives display immunomodulatory functions and thus have the potential for a wide range of medical applications.

Ticks are rich sources of bioactive molecules which may serve as templates for the development of multifunctional peptides. The purpose of this study was to evaluate a synthetic peptide (OsΔC) derived from the carboxy-terminus of Ornithodoros savignyi defensin isoform 2 (OsDef2). In the derived peptide (OsΔC) the cysteine residues were omitted, an extra tryptophan was added to the N-terminus and the C-terminus was amidated. OsΔC was evaluated in terms of its antibacterial, cytotoxic and antioxidant activities as well as nitric oxide scavenging ability.

OsΔC showed strong antibacterial activity against Gram-negative Escherichia coli and Pseudomonas aeruginosa with a minimal bactericidal concentration (MBC) of 0.40 µM. The MBC for the peptide against Gram-positive Bacillus subtilis and Staphylococcus aureus was 0.81 µM and 12.8 µM, respectively. Circular dichroism (CD) analysis showed that OsΔC maintained a predominantly random coil structure in water and 505% trifluoroethanol (TFE), whereas in 25 mM sodium dodecylsulfate (SDS) it adopted a more α-helical structure. OsΔC showed no significant cytotoxicity when evaluated against human erythrocytes, mouse fibroblasts (Sc-1 cell line) and human colon cancer cells (Caco-2 cell line). Oxygen radical absorbance capacity (ORAC) and trolox equivalence antioxidant capacity (TEAC) assays were used to determine its antioxidant potential and in both of the assays, OsΔC showed strong antioxidant activity when compared to equal concentrations of glutathione. Intracellular oxidant scavenging capacity was confirmed in the Caco-2 cell line using the 2',7'-dichlorfluorescein-diacetate (DCFH-DA) assay and indicated that OsΔC, at a concentration of 12.5 µM, was able to offer approximately 20% protection to Caco-2 cells. Reactive nitrogen species may also lead to nitrosative stress and nitric oxide (NO) scavenging activity would be beneficial in preventing the consequence of this effect. NO scavengers may also serve as anti-inflammatory agents. The Griess
reaction was used to measure the NO scavenging capacity of OsΔC and showed that at a concentration of 12.5 µM, approximately 89% of NO was scavenged.

Overall OsΔC was found to possess potent antimicrobial, antioxidant and NO scavenging activities, while no toxicity of the peptide to mammalian cells was observed. OsΔC thus shows potential as a multifunctional peptide and further research into each of the peptide’s bioactivities is needed to determine its full potential.
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<tr>
<td>ABTS</td>
<td>2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)</td>
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<tr>
<td>AMP</td>
<td>Antimicrobial peptides</td>
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<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<td>CFU</td>
<td>Colony forming units</td>
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<td>CV</td>
<td>Crystal violet</td>
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<td>Crystal violet stain</td>
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<td>DCFH-DA</td>
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<td>DIF</td>
<td>Dorsal-related immune factor</td>
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<td>GSH</td>
<td>Glutathione</td>
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<td>HBD</td>
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<td>HNP</td>
<td>Human neutrophil peptide</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani broth</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
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<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
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<td>MBC</td>
<td>Minimum bactericidal concentration</td>
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<tr>
<td>Mel</td>
<td>Melittin</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>NaP</td>
<td>Sodium phosphate buffer</td>
</tr>
<tr>
<td>NED</td>
<td>N-naphthyl-ethylenediamine dihydrochloride</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PGN</td>
<td>Peptidoglycan</td>
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<td>PRP</td>
<td>Pattern recognition protein</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SA</td>
<td>Sulphanilamide</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SNP</td>
<td>Sodium nitroprusside</td>
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<td>TE</td>
<td>Trolox equivalents</td>
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<td>Trolox equivalence antioxidant capacity</td>
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<tr>
<td>TFE</td>
<td>Trifluoroethanol</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-interleukin-1 receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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Chapter 1: Introduction

1.1 Problem statement

Due to the extensive use of antibiotics and antibacterial products the emergence of resistant strain bacteria has become a great concern, leading to the urgent need for new antibiotics. There is evidence that up to 70% of bacteria-causing infections in hospitals are resistant to at least one of the commonly used antibiotics (St Georgiev et al., 2008) and that newer strains have become multi resistant. In 2010, the Infectious Diseases Society of America launched its “10x20” initiative, aimed at global commitment to development of 10 new antibacterial drugs by 2020 (Boucher et al., 2009). Antimicrobial peptides (AMPs) are an emerging solution to this problem. They are natural antibiotics that are found in both vertebrates and invertebrates and are key components of the innate immune system that protects a host from invading pathogens (Nguyen et al., 2011). It is difficult for pathogens to develop resistance to AMPs as AMPs have been found to have various targets, the main target being the bacteria membrane but also intracellular targets (Sato and Feix, 2006)

Ticks are a rich source of bioactive compounds (Nuttall, 1998; Ribeiro and Francischetti, 2003; Ribeiro et al., 1990) and are valuable sources of novel proteins and peptides which may serve as templates for the development of multifunctional peptides. A major family of AMPs that has been characterized are defensins, which play a significant role in the innate immune system of many organisms and are the most studied AMPs in ticks (Ganz and Lehrer, 1995; Gillespie et al., 1997; Taylor, 2006; Sonenshine and Hynes, 2008; Kopacek et al., 2010). Defensins have great potential for a wide range of therapeutic applications. Synthetic derivatives of defensins have shown broad spectrum activities (Varkey et al., 2006; Nakajima et al., 2003; Tsuji et al., 2007), immunomodulatory functions (Kim and Kaufmann, 2006) and are potential leads for development of novel antibiotics with a wide range of applications.

1.2 Innate immunity

The immune system is a defence mechanism to protect the organism from foreign matter or pathogens. A basic requirement of the immune system is the ability to determine which matter is non-self (Steiner, 2004). There are two major parts to immunity, innate and acquired. Innate immunity is the first response and is a fast cellular response to the pathogen, as components of the innate system are always
active within the organism. The acquired immune system is made up of specialised white blood cells and antibodies, requires a few days for its activity to develop due to the complexity of the system. A key distinguishing factor is that acquired immunity includes immunologic memory (Lavine and Strand, 2002).

Due to the lack of an adaptive immune response invertebrate animals rely on innate immune responses to defend against potential pathogens. The innate immune response of *Drosophila melanogaster* is considered a suitable model for the invertebrate immune response (Figure 1.1) and has served as a basis for further research into the innate immune systems of other animals (Tzou et al., 2002).

![Figure 1.1: Overview of the immune response in Drosophila melanogaster (Tzou et al., 2002)](image)

In invertebrates the first line of defence is the integument (the outer shell) and the gut, followed by a complex system including cellular and humoral responses. Phagocytosis, melanization, and encapsulation form part of the cellular responses (Figure 1.1). The synthesis and secretion of AMPs by the fat-body constitute the bulk of the humoral response (Kounatidis and Ligoxygakis, 2012). The cell mediated system in insects is a very simplified version of the vertebrate cellular responses, due to the fact that it lacks antigen-antibody complexes and memory cells. Hemocytes are cells involved in the immune response of invertebrates and increase with an increase of foreign matter/infection. There are three basic types of hemocytes; crystal cells, plasmocytes and lamellocytes (Kounatidis and Ligoxygakis, 2012). Plasmocytes are responsible for
removing microorganisms and apoptotic cells by phagocytosis and hence cause their removal from circulation. Production of lamellocytes, is increased when foreign matter is detected, that is too large for the plasmocytes to eliminate (Kounatidis and Ligoxygakis, 2012). Lamellocytes act by encapsulation of foreign matter by surrounding them in layers of cells (Nappi et al., 1995). At the site of injury or within the lamellocyte-capsule, the production of a black pigment is the result of a pathway that converts tyrosine to melanin (Söderhäll and Cerenius, 1988). Melanization is the isolation of pathogens by the crystal cells, which are suggested to be involved in rapid wound sealing. Larvae lacking crystal cells have a reduced ability for clot formation and decreased wound healing (Kounatidis and Ligoxygakis, 2012; Bidla et al., 2005).

Identifying pathogens is vital to the survival of the host and differentiating between self and non-self prevents the immune system from attacking itself. This is achieved by pattern recognition proteins (PRPs), binding to certain pathogen-associated molecular patterns (PAMPs), which are found on microbes (Royet, 2004). Examples of PAMPs (Figure 1.1) include lipopolysaccharide (LPS), peptidoglycan (PGN), β-1,3-glucan, mannan and lipoteichoic acid (LTA) (Medzhitov and Janeway, 1997). Response to Gram-positive bacteria and fungi occurs via Toll receptor activation (Tzou et al., 2002). The extracellular domain of the Toll receptor (a transmembrane protein) consists of an extracellular domain of leucine-rich repeats (LRRs), while the intracellular domain is referred to as the Toll-interleukin-1 receptor (TIR) due to its homology to the interleukin-1 receptor (Hoffmann and Reichhart, 2002). Recognition of LTA and PGN leads to the initiation of a serine protease cascade which ends in the cleavage of a protein called Spaetzle, which cleaves the Toll receptor by binding to the LRRs. The TIR domain interacts with other intracellular proteins such as Tube and Pelle. This leads to the phosphorylation of the cactus protein resulting in its disassociation from the cactus-dorsal complex, which also releases dorsal-related immune factor (DIF). The latter translocates to the nucleus where it binds to a response element upstream of the AMP gene promoter, leading to transcription of the AMP gene. In addition to the fat body, AMPs are also produced in other areas such as the hemocytes, the cuticular epithelial cells, the gut, the salivary gland and the reproductive tract (Hultmark, 1993; Vilmos and Kurucz, 1998).
1.3 Antimicrobial peptides

It was originally thought that lysozyme alone was responsible for the AMP humoral reaction in invertebrates, but a breakthrough in 1980 proved otherwise. Three inducible AMPs were found in the hemolymph of giant silk moths. One peptide was similar to lysozyme but the other two, called cecropins and attacins, showed bacteriolytic ability (Hultmark et al., 1980). These sparked interests in AMPs and today a large variety of peptide classes have been identified (Chrudimska et al., 2010). To date, according to the Antimicrobial Peptide Database, November 2013, there are 2308 AMPs in the database, 82% of which are antibacterial ones and 57% of which have an unknown 3D structure (Wang and Wang, 2009).

AMPs consist of gene-encoded, ribosomally synthesized oligopeptides or proteins present in most organisms, or non-ribosomally synthesized peptide antibiotics, produced by most bacteria and fungi. The first class is subdivided into two major classes based on whether these AMPs are produced by bacteria or eukaryotes. Bacterial defence peptides and proteins are referred to as bacteriocins while the term “antimicrobial peptides” is used for eukaryotic derived peptides (Wiesner and Vilcinskas, 2010; Ganz and Lehrer, 1999). Bacteriocins are a heterogenous group consisting of very small, highly modified peptides and proteins. These molecules are generally highly active at very low concentrations but only active on a restricted spectrum of bacteria closely related to the producer. AMPs range from 12 to 100 amino acid residues and generally exhibit broad spectrum activity but at higher concentrations (Wiesner and Vilcinskas, 2010; Ganz and Lehrer, 1999).

Table 1.1 shows some of the physicochemical properties which are used to differentiate AMPs.
Table 1.1: Physicochemical properties of antimicrobial peptides (Adapted from Brogden, 2005)

<table>
<thead>
<tr>
<th>Property</th>
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<tr>
<td>Size</td>
<td>Varies from 6 amino acid residues to greater than 180.</td>
</tr>
<tr>
<td>Composition</td>
<td>Often contain basic amino acid residues such as Lys and Arg. Some have hydrophobic residues such as Ala, Leu, Phe and Trp and other residues like Ile, Tyr and Pro. Some contain amino acid repeats and others vary in ratios of hydrophobic to charged residues.</td>
</tr>
<tr>
<td>Charge</td>
<td>Anionic peptides contain more Asp and Glu, while the cationic peptides contain more Arg and Lys. Anionic peptides complexed with zinc and highly cationic peptides are often more active than neutral peptides or those with lower charge.</td>
</tr>
<tr>
<td>Conformation and Structure</td>
<td>Peptides can have a variety of secondary structures such as alpha helices, relaxed coils or anti-parallel sheets. Some α-helical peptides have shown more activity than peptides with less-defined secondary structures. The gamma core motif which is two anti-parallel β-sheets with a short turn between them (in defensin-like molecules) is often very active.</td>
</tr>
<tr>
<td>Hydrophobicity</td>
<td>The level of hydrophobicity determines the ability of the peptide to insert into the membrane lipid bilayer.</td>
</tr>
<tr>
<td>Amphipathicity</td>
<td>Describes the amount of hydrophilic amino acid residues aligned on one side and hydrophobic amino acid residues aligned along the opposite side of a helical molecule.</td>
</tr>
</tbody>
</table>

Three main groups have been identified and within these groups the peptides may also be anionic or cationic. Anionic peptides, are small (5 - 70 amino acids), produced in millimolar concentrations, usually carry a negative charge of -1 or -2 and tend to be rich in Glu and Asp (Harris et al., 2009). These AMPs are active against Gram-positive and Gram-negative bacteria. A disadvantage of this class is that they often require cations, such as zinc as co-factors for activity. It is suggested that their targets are intracellular, such as the ribosomes (Brogden et al., 1996).

The cationic group, make up the majority of AMPs. These AMPs are also short, less than 40 amino acid residues and typically carry a net positive charge between +2 and +9. This charge is due to a general lack of Glu or Asp (acidic residues) and high numbers of cationic amino acids (Lys and/or Arg). Hydrophobic residues account for 30-50% of the peptide and allow for an amphiphilic structure, when the peptide interacts with membranes. Alterations of the hydrophobicity to charge ratio can result in changes in the selectivity/activity (Zelezetsky et al., 2005). These peptides have cysteine residues that form disulphide bridges which can result in the peptide
containing a hinge region (Chrudimska et al., 2010). Cationic peptides show broad structural diversity but share the characteristics of being cationic at physiological conditions, contain a similar primary amino acid sequence and allowing the formation of an amphipathic structure (Maloy and Kari, 1995; Saberwal and Nagaraj, 1994).

1.3.1 Linear and amphipathic α-helical peptides

Cecropins are the most abundant of linear peptides and were the first AMPs to be isolated. Insect cecropins are small, 35-39 residues, cysteine free and show activity against Gram-positive and Gram-negative bacteria with no hemolytic activity at antimicrobial concentrations. Cecropins have a helix-bend-helix motif with an amidated C-terminus which contributes to its antimicrobial activity. They are unordered in aqueous solutions but adopt a stable α-helical structure in hydrophobic environments. The N-terminal helix (residues 5-21) is basic, amphipathic and longer than the hydrophobic C-terminal helix (residues 24-37). The activity of these peptides is affected by charge, helicity, size, hydrophobic moment and hydrophobicity (Bulet and Stöcklin, 2005) (See Table 1.2).

1.3.2 Amino acid rich peptides

Proline-rich

Two subfamilies, the short (less than 20 amino acids) and long chain (longer than 20 amino acids) peptides ranging from 14-39 amino acids, with the short chain peptides showing greater selectivity for Gram-negative bacteria and the long chains having activity against both Gram-positive and Gram-negative. Unlike the α-helical or β-sheet (cysteine stabilized) peptides which have fast killing times, the short-chain Pro rich peptides require hours to kill (Bulet and Stöcklin, 2005). Some examples include cecropins, magainin, apidaecin and abaecin (Bulet and Stöcklin, 2005) (See Table 1.2).

Glycine-rich

These polypeptides are much larger, ranging from 60-186 residues and show activity against Gram-negative bacteria and sometimes Gram-positive bacteria as well. Attacins and sarcotoxins share a large Gly rich domain while sarcotoxin also has a Pro rich domain. Diptericins have both Pro and Gly rich domains and are considered chimeric molecules (Bulet and Stöcklin, 2005) (See Table 1.2).
Histidine-rich

His-rich peptides show amphipathic and helical structures. A quarter of their amino acids are His residues and they exert their antimicrobial action by membrane disruption (Conde et al. 2012). Histatins are small cationic His–rich peptides and show potent antibacterial and antifungal activity (De Smet and Contreras, 2005) (See Table 1.2).

Tryptophan-rich

The main example of Trp rich peptides is the linear indolicin. It has a globular secondary structure in water but adopts a wedge shape when in contact with micelles. It has the ability to permeate bacterial membranes and can inhibit DNA synthesis (Conde et al. 2012) (See Table 1.2).

1.3.3 Cysteine-containing peptides

There are three classes of peptides varying in the number of cysteine residues; α-helical/β-sheet, triple-stranded β-sheet and β-hairpin-like structures. Hairpin structures are formed in peptides with only one disulphide bridge (two cysteine residues), more cysteine residues allow for disulphide bridges and more complex folding patterns (Bulet and Stöcklin, 2005). One of the major peptides from this class is the defensins (See Table 1.2).
Table 1.2: Examples of different cationic peptides, their source and antimicrobial spectrum (Adapted from Vizioli and Salzet, 2002)

<table>
<thead>
<tr>
<th>Structure and examples</th>
<th>Organism</th>
<th>Antimicrobial spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linear α-helical peptides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecropins</td>
<td>Insects</td>
<td>Bacteria, fungi, virus, protozoa, metazoa</td>
</tr>
<tr>
<td>Magainin</td>
<td>Amphibians</td>
<td>Bacteria, protozoa</td>
</tr>
<tr>
<td>Abaecin and apidaecins</td>
<td>Bee</td>
<td>Bacteria</td>
</tr>
<tr>
<td><strong>Linear, amino acid rich peptides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro- rich</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drosocin</td>
<td>Fruit fly</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Pyrrhocorisin</td>
<td>Hemipteran</td>
<td>Bacteria, fungi</td>
</tr>
<tr>
<td>Gly- rich</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dipterics</td>
<td>Dipterans</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Attacin &lt;sup&gt;1&lt;/sup&gt;</td>
<td>Cecropia moth</td>
<td>Bacteria</td>
</tr>
<tr>
<td>His- rich</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histatins</td>
<td>Humans</td>
<td>Bacteria, fungi</td>
</tr>
<tr>
<td>Tyr-rich</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indolicin</td>
<td>Cattle</td>
<td>Bacteria</td>
</tr>
<tr>
<td><strong>Cysteine-containing peptides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Single disulphide bond</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thanatin</td>
<td>Hemipteran</td>
<td>Bacteria, fungi</td>
</tr>
<tr>
<td>Brevinins</td>
<td>Frog</td>
<td>Bacteria</td>
</tr>
<tr>
<td><strong>Two disulphide bonds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protegrin I</td>
<td>Horseshoe crab</td>
<td>Bacteria, fungi, virus</td>
</tr>
<tr>
<td>Androctonin</td>
<td>Scorpion</td>
<td>Bacteria, fungi</td>
</tr>
<tr>
<td>Tachyplesin II</td>
<td>Pig</td>
<td>Bacteria, fungi, virus</td>
</tr>
<tr>
<td><strong>Three disulphide bonds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-defensins</td>
<td>Mammals</td>
<td>Bacteria, fungi</td>
</tr>
<tr>
<td>β-defensins</td>
<td>Mammals</td>
<td>Bacteria, fungi</td>
</tr>
<tr>
<td>Defensins</td>
<td>Insects</td>
<td>Bacteria, fungi, protozoa</td>
</tr>
<tr>
<td><strong>More than three disulphide bonds</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defensins</td>
<td>Plants</td>
<td>Fungi</td>
</tr>
<tr>
<td>Gambicin</td>
<td>Mosquito</td>
<td>Bacteria, fungi, protozoa</td>
</tr>
<tr>
<td>Drosomycin</td>
<td>Fruit fly</td>
<td>Fungi</td>
</tr>
</tbody>
</table>

<sup>1</sup> Hultmark (1993)

1.4 Defensins

Defensins are the most conserved class of AMPs, found in a wide range of organisms (some examples shown in Figure 1.2) such as plants, mammals, insects, molluscs and several tick species (Broekaert <i>et al.</i>, 1995; Selsted <i>et al.</i>, 1985; Matsuyama and Natori, 1988; Lowenberger <i>et al.</i>, 1995; Viljakainen and Pamilo, 2005; Charlet <i>et al.</i>, 1996; Johns <i>et al.</i>, 2001; Tsuji <i>et al.</i>, 2007; Chrudimska <i>et al.</i>, 2010; Hynes <i>et al.</i>. 2005; Fogaca <i>et al.</i>, 2004). They are small cationic peptides of approximately 4 kDa, averaging 34 to 43 residues in length with pl values ranging from 8.0-8.5. These peptides are known to be active against most Gram-positive bacteria and have high affinity for the major phospholipids of bacteria (Gillespie <i>et al.</i>, 1997).
Defensins have shown bactericidal, antifungal and antiviral activities and are associated with many cells and tissues involved in the host defense (Brandenburg et al., 2012). They have been shown to accumulate at a higher rate and to a larger extent at sites of infection compared to inflamed but non-infected tissues, indicating that these peptides are able to distinguish between microorganisms and host tissue with regard to inflammation and to accumulate at the infection sites (Welling et al., 2001). Depletion or removal of the AMPs has resulted in higher susceptibility to severe and frequent infections (Ganz et al., 1988; Pütsep et al., 2002; Moser et al. 2002; Huang et al., 2007). HNP-1 (a human α-defensin) inhibits replication of HIV and the influenza virus (Zhang et al., 2004; Salvatore et al., 2007) and can inactivate papillomavirus, herpes simplex virus, cytomegalovirus, and adenovirus (Buck et al., 2006; Daher et al., 1986). Human β-defensin can block HIV-1 and influenza virus replication (Bergman et al., 2007; Braida et al., 2004; Liang et al., 2010). Some defensins inhibit HIV entry into the cells by binding/antagonising either the viral proteins or cellular receptors (Gallo et al., 2006, Feng et al., 2006). The underlying mechanisms of virus inhibition are unknown but antiviral activity seems to be dependent on interaction of the peptides with viral proteins (Brandenburg et al., 2012). Mechanisms of antifungal activity by the peptides are similar to antibacterial mechanisms and occur by cell lysis, binding and disruption of outer membrane, interference with cell wall synthesis and depolymerisation of the actin cytoskeleton (Koo et al., 2004; De Lucca and Walsh, 1999). Defensins also have shown antiparasitic activity by disrupting cell membrane integrity (McGwire et al., 2003).

Common between insect defensins are 6 cysteine residues which help stabilize the molecule through disulphide bridges. Three domains are present in most defensins; an N-terminal loop (which has some flexibility), a central α-helix (amphipathic) and an anti-parallel β-sheet on the C-terminus. Two disulphide bonds link the α-helix to a strand of the β-sheet and one disulphide bond links the other strand to the N-terminal loop.
1.5 Mechanism of action

It is suggested that a number of AMPs exert their toxicity by permeabilizing the lipid matrix of cell membranes (Zasloff, 2002; Hancock and Sahl, 2006; Matsuzaki, 1999) but intracellular targets have also been suggested (Park et al., 1998; Subbalakshmi and Sitaram, 1998; Kragol et al., 2001).

1.5.1 Targeting the microbial cell membrane

This mode of action of cationic AMPs relies upon the interaction with the cell membrane of the microorganism (Figure 1.3). Present in eukaryotic membranes are phosphatidylcholine, phosphatidylethanolamine, sphingomyelin together with sterols, ergosterol and cholesterol which normally have no net charge. Due to the neutral charge of the eukaryotic membrane, no ionic interactions occur between AMPs and the cell membrane (Yeaman and Yount, 2003). Prokaryotic bacteria have negatively charged cytoplasmic membranes and a high electrical potential gradient. These
characteristics are provided in part, by the presence of hydroxylated phospholipids phosphatidyglycerol, phosphatidylserine and cardiolipin, which sustain a net negative charge (Yeaman and Yount, 2003). This negatively charged membrane allows for an electrostatic interaction between the cationic peptide and dense anionic charges of the bacterial membranes, leading to aggregation of AMPs onto the bacterial membrane. An advantage of this mechanism is that it reduces cytotoxicity towards mammalian cells and makes it more difficult for the bacteria to develop resistance as this would require the bacteria to change the properties of their membranes (Laverty et al., 2011). The hydrophobic residues of AMPs are important for integration into the bacterial membrane (Wiesner and Vilcinskas, 2010).

![Diagram showing attraction of AMPs with the bacterial cell membrane](http://2011.igem.org/Team:St_Andrews/switch)

Figure 1.3: Diagram showing attraction of AMPs with the bacterial cell membrane

When comparing the outer membrane structure of a Gram-negative and Gram-positive bacterium, there is an additional outer lipid membrane on Gram-negative bacteria and a self-promoted uptake pathway has been proposed for the action of cationic peptides. The outer membrane is composed of LPS for which peptides have a high affinity. It is proposed that as the AMPs bind, they compete with divalent cations such as
magnesium and calcium ions for their relative binding sites (Chen and Cooper, 2002). As these ions are important for the stability of the cell membrane surface, the removal of them leads to disruption of the outer membrane, resulting in the formation of pores allowing more peptides and other small molecules to pass through. This hypothesis also explains how cationic AMPs work together with conventional antibiotics and this synergy as also been proven with Gram-positive bacteria (Giacometti et al., 2000) and fungi (Kumar et al., 2005).

There are a few proposed mechanisms (Figure 1.4) by which AMPs interact with or enter the bacterial cells, but the four main mechanisms are the aggregate model (Figure 1.4 A), the toroidal pore model (Figure 1.4 B), the barrel-stave model (Figure 1.4 C) and the carpet model (Figure 1.4 D).

There are different ways in which AMPs associate on the bacterial cells. Some insert into the membrane and aggregate to form transmembrane pores with their hydrophilic surfaces on the inside and their hydrophobic surfaces interact with the lipids of the membrane surrounding it (Figure 1.4 C). Some sit on the surface of the membrane as monomers or aggregate to form bundles of peptides. Some AMPs can enter the membrane completely and lie within the membrane (Shai, 1995).
In the aggregate model (Figure 1.4 A), AMPs gather on the surface and form an aggregate which spans the membrane, made up of peptide and lipid micelle complexes but have no particular orientation (Laverty et al., 2011).

The toroidal pore (or worm-hole) model (Figure 1.4 B), suggests that AMPs associate with the lipid head groups, insert perpendicularly and stretch through the membrane forming a lipid monolayer which bends continuously through the pore. The inner surface of the pore is then lined with both peptides and lipid head groups, the AMPs remain bound to the polar heads during pore formation and result in the inner and outer membrane forming a continuous monolayer (Wiesner and Vilcinskas, 2010; Laverty et al., 2011).

In the barrel-stave model (Figure 1.4 C), AMPs aggregate forming dimers and multimers, similar to the carpet model but proposes that the peptides are inserted into the membrane to form a pore. The hydrophobic regions of the AMPs align with the acyl chains of the membrane lipids and the hydrophilic regions form the inner surface of the
pore channel (Wiesner and Vilcinskas, 2010; Laverty et al. 2011). As little as 3 peptide molecules can allow for the formation of pores (Shai and Oren, 2001).

In the carpet model (Figure 1.4 – D), AMPs aggregate in parallel onto the membrane surface, covering it in a carpet-like manner until a critical threshold concentration is reached, at which disruption of the membrane occurs and leads to the formation of micelles (Wiesner and Vilcinskas, 2010; Laverty et al., 2011).

Following exposure to the pore-forming and membrane disrupting AMPs, cell death may result from loss of electrochemical gradients across the membrane, loss of metabolites and lysis of the cells. Molecular electroporation has also been proposed as a method, where AMPs are able to create electrostatic potential across the lipid bilayer that is sufficient for pore formation. A certain charge density must be reached which is dependent on the amount of cationic amino acids in the AMP (Miteva et al., 1999). Another proposed model is the sinking raft model in which the peptides sink into the lipid bilayer and may create transient pores (Dawson and Liu, 2008; Pkorny and Almeida, 2004).

1.5.2 Intracellular targets of AMPs

There are other mechanisms for microbial cell death other than membrane damage and with many peptides, membrane damage is not the principle mechanism (Park et al., 1998). Some AMPs are able to translocate into the cytoplasm and act on intracellular targets (Figure 1.5), such as inhibition of DNA or RNA synthesis (Boehr et al., 2003), targeting of the mitochondria, stimulation of autolytic enzyme cascades (Ginsburg and Koren, 2008) interfering with cell wall enzymes to cause lysis of bacteria (Ginsburg, 2004) and affecting the cell metabolism (Boman et al., 1993; Subbalakshmi and Sitaram, 1998; Gennaro et al., 2002; Wiesner and Vilcinskas, 2010; Laverty et al., 2011). For example, buforin II which has a linear proline hinge and an amphipathic α-helix, has been proven to cross the cell membrane without loss of the membrane potential and inhibit E. coli by binding to DNA and RNA (Park et al., 1998).
Figure 1.5: Mechanisms of action for antimicrobial peptides and possible targets within the cell (Laverty et al., 2011)
1.6 Tick defensins

Ticks show strong humoral responses to invading organisms that work together with the cellular responses. The three main actions of the humoral response are encapsulation, hemagglutination and induced synthesis of AMPs and proteins. AMPs are potent effector molecules of the humoral response and are synthesized and released in large amounts following blood feeding (Chrudimska et al., 2010; Taylor, 2006).

Defensins are the major class of AMPs in ticks (Sonenshine and Hynes, 2008) and the most widely studied class of AMPs in ticks. They are small cationic peptides, approximately 4kDa for the mature peptides and have been identified in nearly twenty hard and soft tick species, expressed in the midgut, fat body and salivary glands (Saito et al., 2009; Sonenshine and Hynes, 2008). The first report of defensin in ticks was a partial amino acid sequence from Ornithodoros moubata (van der Goes van Naters-Yasui et al., 2000). Nakajima et al. (2001, 2002a and b), later obtained the full sequence of four defensin isoforms from this tick. Nakajima et al. (2003) reported that the synthetic defensin isoform A showed strong antibacterial activity against Gram-positive bacteria, had low hemolytic ability and kill by causing lysis of the cytoplasmic membrane. Defensin mRNA was found to be strongly upregulated in the midgut following blood meals (Nakajima et al., 2001). A defensin-like peptide from the hard tick Amblyomma hebraeum was found to be active against Gram-positive and Gram-negative bacteria (Lai et al., 2004). A peptide (longicin) identified in Haemaphysalis longicornis, was found to have antibacterial, antifungal and anti-parasitic activity (Tsuji et al., 2007). Similar activities were found using a shorter synthetic peptide (P4), derived from the carboxy-terminal of longicin (Rahman et al., 2010). Two defensin isoforms (def1 and def2) from the hard tick Ixodes ricinus were found to be active only against Gram-positive bacteria and presented with low toxicity to human cells. Def2 differed from def1 by a single amino acid residue which also increased the cationicity by one positive charge and def2 was found to be more active than def1 (Chrudimska et al., 2011).
1.7 Multiple functions of AMPs

In addition to their antimicrobial role, AMPs are involved in multiple facets of immunity. They have been shown to have immunomodulatory (Auvynet and Rosenstein, 2009) antioxidant (Huang et al., 2012) activities, and affect the inflammatory response (van de Does et al., 2010). AMPs have also been associated with maintaining the skin barrier (and possibly its restoration), re-epithelialization of wounds (Hirsch et al., 2009), wound healing (Sorensen et al., 2003), mediation of angiogenesis and vasculogenesis (Koczulla et al., 2003) and recently some anticancer activity (Lee et al., 2008) (see Figure 1.6).

AMPs are primarily chemotactic for immune and non-immune cells (Brandenburg et al., 2012). α- and β-Defensins recruit phagocytes, neutrophil granulocytes, immature dendritic cells, memory T-cells and monocytes to sites of inflammation (Lai and Gallo, 2009). Defensins also activate masts cells for degranulation, prostaglandin production and intracellular Ca\(^{2+}\) mobilization (Chen et al., 2007). AMPs have also been shown to induce chemokine production (Niyonsaba et al., 2006) as well as alter pro- and anti-inflammatory cytokine expression inducing both expression and regulating expression to protect the organism during an excessive inflammatory response (Brandenburg et al., 2012, Davidson et al., 2004; van der Does et al., 2010; Mookherjee and Hancock, 2007). The AMP’s effects on the immune response aid in the uptake, processing and presentation of antigens and stimulate expansion of the T- and B-lymphocytes (Brandenburg et al., 2012). Figure 1.6 shows a broad overview of the induction and potential role of AMPs in the immune response.

Research has shown that defensins accumulate at a higher rate and extent at sites of injury, when compared to non-infected but inflamed tissues. This indicates that peptides distinguish between microorganisms and the host tissues and can accumulate at sites of infection (Welling et al., 2000, Brandenburg et al., 2012). Studies have shown that the antimicrobial activity is affected by cationic concentrations, serum and anionic molecules, however their immunomodulatory activities are less sensitive (Nijnik et al., 2010).
There are a number of papers on the immunomodulatory effects of peptides and also specifically on the multiple functions of defensins in the immune response (Steinstraesser et al., 2009; Ganz 1987 and 2003; Auvynet and Rosenstein 2009; Bowdish et al., 2005; Scott and Hancock 2000). Immunomodulation is a complex process and specific processes related to immunomodulation such as, antioxidant and anti-inflammatory effects will be discussed in more detail.

1.7.1 Peptides with antioxidant activity

Oxidation is an important process that occurs in vertebrates and humans and also leads to the formation of free radicals. ROS are important intermediate products of oxidative metabolism (Hipler et al., 2000). During respiration there is an unavoidable formation of ROS, free radicals such as super oxide anions (O$_2^-$) and hydroxide radicals (OH$^*$), and also non-free radicals such as hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (1$^1$O$_2$). Excess ROS can also be produced by stress to the cell, such as ischemia/reperfusion, exposure to ionizing and ultraviolet radiation and/or inflammation (Hipler et al., 2000).

Oxidative stress from ROS causes cellular damage, and affects DNA, proteins, lipids and other small cellular molecules (Lee et al., 2006) and has been linked to numerous human diseases such as stroke, diabetes, cancer as well as chronic wounds and septic shock (Najafian and Babji, 2012).
An antioxidant is defined as any substance that when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of the substrate (Park et al., 2001). Antioxidants and other repair systems of the cells counter/control the damage and amount of free radicals and other reactive species which are constantly being produced within the cell (Cell Biolabs, product manual 2012). Antioxidants help by donating a hydrogen atom or electron to the radicals and end the chain reactions. Some well-known antioxidants are vitamin A and E, and glutathione. A number of papers have shown antioxidant activity with peptide hydrolysates from a variety of sources (Ahn et al., 2012; Memarpoor-Yazdi et al., 2012; Taha et al., 2013; Zambrowicz et al., 2012; Gomez-Guillen et al., 2010). This shows that many peptides contain amino acids that contribute to antioxidant activity of a peptide. The antioxidant activity of peptides is associated with the presence of certain amino acids such as Val, Leu, Pro, Asp, His and Tyr (Sarmadi and Ismail, 2010; Rajapakse et al., 2005; Chen et al., 1995, 1996, 1998; Je et al., 2005), as well as specific sequences such as the Ile-Arg amino acid sequence found in defensin isoform 2 from O. savignyi (Prinsloo et al., 2013). Huang et al. (2012) found antioxidant activity with a fragment of a defensin from Ipomoea batatas (sweet potato) roots and reported that the CFCTKPC peptide fragment was the strongest antioxidant peptide tested in the study and suggested that the activity was related to the presence of cysteine residues. A peptide fraction from a crab (Ocypode macrocera), which showed antibacterial activity, also presented with antioxidant activity, nitric oxide scavenging capacity and ferrous iron chelating ability (Sivaperumal et al., 2013).

1.7.2 Peptides with anti-inflammatory activity

Inflammation is a defence mechanism initiated by invasion of pathogens or by tissue injury caused by biological, chemical, or physical damage. Macrophages are activated and release inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines such as TNF-α, IL-6, and -1β, to enhance defence capacity (Je and Kim, 2012). NO is an important signalling molecule in vasodilation, neurotransmission and host immune defence (Pacher et al., 2007). Stuehr and Marletta (1985) reported that mouse macrophages produce nitrite and nitrate in response to bacterial LPS. Bacterial products such as LPS, LTA, PGN and DNA activate macrophages (Karupiah et al., 1993), lead macrophages to produce large amounts of NO and other pro-inflammatory cytokines which further upregulate the inflammatory response. Although a fairly simple molecule, the enzyme that produces NO, nitric oxide synthase, is one of the most
complex enzymes in nature (Sun et al., 2003). There are three types; neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS).

However, overproduction of NO and other mediators are strongly linked with a number of inflammatory diseases such as septic shock, hemorrhagic shock, multiple sclerosis, rheumatoid arthritis and atherosclerosis (Bertolini et al., 2001). Another important reaction that occurs with NO is the reaction with superoxide anion radical to produce peroxynitrite (ONOO⁻) which is a strong oxidant (Beckman et al., 1990) that can cause lipid peroxidation and modification of structural proteins (Haddad et al., 1994). Overproduction of NO occurs when the cell’s ability to neutralize and eliminate NO, exceeds its generation and leads to nitrosative stress (Alisi and Onyeze, 2008). Nitrosative stress may cause damage to proteins or DNA and result in cell injury and death (Sun et al., 2003).

Inhibition of NO is one of the strategies proposed for therapeutic intervention in inflammatory diseases (Cho et al., 2011) and would also reduce nitrosative stress. Nonselective inhibitors of NOS inhibit all three enzymes, but have shown mixed effects. They result in decreases of NO production, but may also cause increased blood pressure in animal models of sepsis (Kim and Greenburg, 2002), depressed cardiac function and increased mortality (Lopez et al., 2004), decreased renal blood flow, increased capillary leakage, intestinal damage and exacerbated pulmonary hypertension (Spain et al., 1994). Selective inhibition of iNOS should avoid some of these problems and has shown some positive results in animal models (Ichinose et al., 2003). However, due to the complex physiological effects of NO, selective inhibition of iNOS has also shown mixed results. It has shown improvements in blood pressure but with adverse effects on cardiac output and impaired oxygen delivery (Wolfe and Dasta, 1995; Petros et al., 1994). The next approach would be to reduce the amount of NO available by making use of NO scavengers which would not affect the functioning of the eNOS and iNOS or the beneficial roles of NO (Shah et al., 1998). Yang et al. (2009), found peptides in the skin of an amphibian that had antimicrobial, antioxidant activity and some had anti-inflammatory activity. A peptide fraction from O. macrocera, which presented with antioxidant activity also showed NO scavenging capacity (Sivaperumal et al., 2013). There are papers on the inhibition of NO production by peptides via inhibition of the NOS enzymes but almost none on the NO scavenging capacity of specific AMPs.
1.8 Therapeutic application of AMPs

AMPs possess many desirable features of a new class of antibiotics and can be used with conventional antibiotic therapy. AMPs have shown synergy with classical antibiotics, neutralise endotoxins, are active in animal models and have shown similar minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) (Giuliani et al., 2007). The broad antimicrobial spectrum of AMPs opens up other areas for their uses, such as imaging probes for bacterial and fungal infections (Welling et al., 2001), enhancing the activity of existing antibiotics, probably by helping them gain access into bacterial cells (Giacometti et al., 2000). For reviews of AMPs and clinical implications and uses, see Guani-Guerra et al. (2010), Giuliani et al. (2007), Marr et al. (2006) and Brogden and Brogden (2011).

AMPs being developed as anti-infective agents with indicated advantages and disadvantages are shown in Table 1.3.

Table 1.3: Advantages and disadvantages of peptides as therapeutic drugs
(Adapted from Huth and Dietrich, 2007)

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Difficult to develop resistance to</td>
<td>- Manufacturing:</td>
</tr>
<tr>
<td>- Diverse range of compositions</td>
<td>High manufacturing costs</td>
</tr>
<tr>
<td>- High specificity and activity</td>
<td>Short half-life</td>
</tr>
<tr>
<td>- Low toxicity</td>
<td>- Physiological:</td>
</tr>
<tr>
<td>- Small size, i.e. better tissue penetration</td>
<td>Rapidly metabolized</td>
</tr>
<tr>
<td>- Modifications can aid stability (unnatural amino acids, C-terminal amidation, etc)</td>
<td>Poor ability to cross physiological barriers</td>
</tr>
<tr>
<td>- Less immunogenic than recombinant proteins and antibodies</td>
<td>Low oral bioavailability</td>
</tr>
<tr>
<td>- May have synergistic effects with other antibiotics</td>
<td>Possible side effects</td>
</tr>
</tbody>
</table>

Research is being directed towards development of modified peptides with unique properties which have less negative effects. Examples of this research include AMP mimetics, hybrids of AMPs, AMP congeners, stabilizing AMPs, conjugates of AMPs and immobilised AMPs (Brogden and Brogden, 2011).

AMPs can enhance the potency of current antibiotics by facilitating entry in microorganisms (Giacometti et al., 2000). Three basic mechanisms by which AMPs
assist the immune response is enhancing recruitment of immune cells, promoting activation of the recruited cells and polarizing them to achieve the required response (Brandenburg et al., 2012). As mentioned earlier AMPs have shown a number of other activities which aid in wound healing, another greatly beneficial aspect of AMPs to be developed further. An example of which is with the acne lesions caused by Propionibacterium acnes, which can cause severe inflammation. AMPs with antibacterial and anti-inflammatory properties would be of significant benefit. One such peptide MBI-594AN (derived from an indolicidin peptide from Migenix), is antimicrobial against P. acnes but is also able to suppress the cytokines stimulated by P. acnes (McInturff et al., 2005).

1.9 Background to the study

The tick is a rich source of bioactive molecules and using it as a model for tick investigations, several anti-hemostatic components have been described (Maritz-Olivier et al., 2007) as well as a number of AMPs. The tick used in this study, O. savignyi, is a livestock parasite endemic to arid and semi-arid regions in Africa (Hoogstraal, 1985). Recently two defensin isoforms (OsDef1 and OsDef2) were identified from the midgut of O. savignyi (unpublished data).

Defensin isoform 1:

\[
\text{PRVRRGYGCPFNQYQCHSEC} \underline{\text{SGIRGYRGYCKGAFKTCKCY}}
\]

Defensin isoform 2:

\[
\text{PRVRRGYGCPFNQYQCHSHC} \underline{\text{KGIKYKGGYCKGAFKTCKCY}}
\]

The underlined sections indicate the mature defensin isoform sequences and the sections in bold show the carboxy-terminal sequences, different amino acids in the sequences are indicated by the arrows. OsDef1 and OsDef2 were shown to only be active against Gram-positive bacteria (Prinsloo et al., 2013). However, Os, the carboxy-terminal peptided derived from OsDef2, was active against Gram-positive and Gram-negative bacteria.

Shorter AMPs would be greatly beneficial because of the high cost of production involved with peptides. A number of tick defensins and defensin derived peptides (discussed in section 1.7) have shown antimicrobial activity. It has also been shown that mammalian defensins have retained their antimicrobial activity, regardless of the
order of disulphide linkages or even the presence of disulphide bonds (Campopiano et al., 2004; Hoover et al., 2003; Klüver et al., 2005; Maemoto et al., 2004; Wu et al., 2003). Varkey et al. (2006) showed that peptides derived from the C-terminal region of arthropod defensins from *Pyrhocoris apterus* (a firebug) and *O. moubata*, in which the cysteines were omitted, were active against both Gram-positive and Gram-negative bacteria. The parent peptide of *O. moubata* defensin A was also only active against Gram-positive bacteria (Nakajima et al., 2001). Table 1.4 shows the parent and derived peptide sequences used by Varkey et al. (2006). These authors added tryptophan to the N-terminus, to monitor the interaction of the amidated C-terminus with lipid vesicles. Amidation of the C-terminus removes a negative charge and helps stabilize the peptide as it helps mimic natural peptides and protecting the peptide from degradation by enzymes. Fifty percent of mammalian peptide hormones and more than eighty percent of insect hormones have an amidated C-terminus (Eipper and Mains, 1988).

**Table 1.4: Original peptides and carboxy-terminal sequences synthesized with changes in the charge of the peptides** *(adapted from Varkey et al., 2006)*

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa</td>
<td>ATC DILSFQSOWVTPNHAGCALHCVIKGYKGGQCKITVCRCRR</td>
<td>+5</td>
</tr>
<tr>
<td>PaΔC</td>
<td>WVIKGYKGGQKITVHR-amide</td>
<td>+6</td>
</tr>
<tr>
<td>OmC</td>
<td>GYGC PFNQYQCHSHCSGIRGYKGGYCGLFKQTCNCY</td>
<td>+4</td>
</tr>
<tr>
<td>OmΔC</td>
<td>WSGIRGYKGGYKGLFKQTNCY-amide</td>
<td>+5</td>
</tr>
</tbody>
</table>

*Pa* and *Om* are defensins from *P. apterus* and *O. moubata*, respectively. *PaΔC* and *OmΔC* are the synthetic analogues. The cysteine residues in the parent peptides are underlined.

In this study, OsΔC was derived from the C-terminus of OsDef2, a Trp residue was added to the N-terminus and the C-terminus was amidated (Table 1.5). The *PaΔC* peptide used in Varkey et al. (2006) was used as a control peptide for antibacterial assays.
Table 1.5: Original and derived peptide used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Net charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsDef2^A</td>
<td>GYGCPFNQYQCCHSHCKGIRGYKGGYCKGAFKQTCKCY</td>
<td>+6</td>
</tr>
<tr>
<td>OsΔC^B</td>
<td>W--KGIRGYKGGY--KGAFKQT--K--Y-amide</td>
<td>+7</td>
</tr>
</tbody>
</table>

A- Original *O. savignyi* defensin isoform 2
B- Peptide used in this study designed in the same manner as Varkey *et al.* (2006)

1.10 Aims of the study

The objective of this study was to investigate whether OsΔC exhibited multiple bioactivities. More specifically the aims were to determine whether the synthetic peptide:

1. Displays antibacterial activity against Gram-positive and Gram-negative bacteria.
2. Undergoes conformational changes when exposed to a membrane-mimicking environment.
3. Is toxic to mammalian cells.
4. Exhibits antioxidant activity and *in vitro* cellular protection against oxidative damage.
5. Possesses NO scavenging activity.

1.11 Output


*Performed CD analysis as well as interpretation of CD data
Chapter 2: Materials and Methods

2.1 Materials

The peptides Os∆C and Pa∆C were synthesized by GenScript USA. The purity and molecular mass of the peptides were determined by reverse-phase HPLC and mass spectrometry, respectively (see Appendix for data). Na₂HPO₄ and NaH₂PO₄ used in the sodium phosphate buffer (NaP) and melittin were acquired from Sigma Aldrich USA. *Escherichia coli* (ATCC 700923), *Bacillus subtilis* (13933), *Staphylococcus aureus* (U3300) and *Pseudomonas aeruginosa* (ATCC 10145), were used for the antibacterial assays. Chemicals and spectropolarimeter used for circular dichroism (CD) analysis were provided by the School of Molecular and Cell Biology, University of Witwatersrand. Cell lines and chemicals used for the cytotoxicity and antioxidant assays were provided by the Department of Anatomy, University of Pretoria.

Stock peptide solutions were prepared in water and sterilized by filtration through a 0.45 μm filter. Peptide concentrations were determined by measuring the absorbance of tyrosine and tryptophan residues at 280 nm, using the following equation:

\[
c = \frac{Abs_{280} \times df \times MW}{x(E_{Trp}) + y(E_{Tyr})}
\]

where \(c\) is the peptide concentration in mg/ml; \(MW\) is the molecular weight of the peptide; \(df\) is the dilution factor; \(E_{Trp}\) and \(E_{Tyr}\) are the extinction coefficients for tryptophan and tyrosine which are 5560 and 1200 AU/mole/ml, respectively, and \(x\) and \(y\) are the number of tryptophan and tyrosine residues, respectively.
2.2 Determination of antibacterial activity

For the determination of the minimal bactericidal concentration (MBC) of OsΔC and PaΔC, a modified method of the one used by Varkey et al. (2006) was followed. Bacteria were grown aerobically in Luria broth (LB) at 37°C. Overnight cultures were diluted 100x and allowed to proliferate to mid-log phase bacteria. A straight line equation of optical density vs. CFU was determined for each bacterial strain. Determination of the bacterial equations was performed by, firstly growing the bacteria overnight at 37°C, diluted 100x and incubated, and allowed to grow to an OD$_{600}$ of 0.5. The bacterial sample was serially diluted 2-fold to 128x and also 10-fold to $10^{9}$. The OD$_{600}$ of each 2-fold dilution was determined and the 10-fold dilutions were plated onto an LB plate and incubated overnight at 37°C, to determine the number of CFU in the original sample. The OD$_{600}$ readings and corresponding CFU numbers were plotted and the equation of the graph was determined. The equation was used to dilute the bacterial samples to the required $10^{6}$ CFU/ml concentration.

The mid-log phase bacteria were diluted to approximately $10^{6}$ CFU/ml. The bacteria were centrifuged at 14100 g for 90 sec and re-suspended in NaP buffer (10 mM pH 7.4) for Gram-negative bacteria or 1% LB in NaP buffer for Gram-positive bacteria. Washed cultures were diluted to approximately $1 \times 10^{6}$ CFU in either NaP buffer for Gram-negative bacteria or 1% LB in NaP buffer for Gram-positive bacteria, after which 90 μl of bacterial suspension was incubated with 10 μl of peptide (concentration range of 120-0.06 μg/ml), in polypropylene tubes for 2 h at 37°C, in a shaking incubator. The incubated samples were diluted 500 times in either NaP buffer (Gram-negative bacteria) or 1% LB in NaP buffer (Gram-positive bacteria), thereafter 100 μl of the samples were plated out on LB-agar plates and incubated for 16 h at 37°C. The growth control and sterile control contained water and buffer only. The MBC was defined as the concentration at which complete killing was observed.
2.3 Secondary structure determination

The secondary structures for OsΔC and PaΔC were predicted using PSIPRED (available at bioinf.cs.ucl.ac.uk/psipred/). Os-C, an analogue of OsΔC, non-amidated and without N-terminal tryptophan, was used for comparison (Prinsloo et al., 2013).

The effects of three different solvents on peptide secondary structures were determined using far-UV CD spectroscopy. CD is one of the most commonly used techniques for estimating secondary structures of proteins and polypeptides in solution (Gopal et al., 2012). Plane polarized light consists of two circularly polarized components of equal magnitude, one that rotates counter-clockwise and one that rotates clockwise. CD refers to the differential absorption of these two components (Kelly et al., 2005). A CD signal is generated when a chromophore is chiral (optically active), made possible by one of three situations, being intrinsically chiral because of its structure, being covalently linked to a chiral centre or being placed in an asymmetric environment by virtue of its 3-D structure adopted by the molecule (Kelly et al., 2005). In proteins important chromophores are the peptide bonds (absorb below 240 nm), aromatic side chains (absorb between 260 and 320 nm) and disulphide bonds (absorption centred around 260 nm) (Kelly et al., 2005). It distinguishes between unordered (random-coil) and ordered (α-helix or β-sheet) structures (Gopal et al., 2012). Figure 2.1 shows the profiles of pure secondary structures.

Unordered peptides are usually indicated by a single negative band below 200nm. α-Helical structures have a positive band around 192 nm, with two negative bands around 208 nm and 222 nm. A positive band around 195 nm with a negative band around 217 nm is indicative of β-sheet structures (Gopal et al., 2012). Cationic peptides in aqueous solutions usually have unordered conformations but they are amphipathic molecules and can adopt folded states in hydrophobic environments (Gopal et al., 2012). Determining the changes in secondary structures may give some insight into the structure-activity relationship and could explain any differences in activity as a result of the modifications made to the peptide.
The CD spectroscopy analysis was performed at the University of Witwatersrand with a Jasco J-810 spectropolarimeter, over the 180-250 nm range and a path length of 0.2 cm. The scans were carried out at 20°C with a scan speed of 200 nm/min, using a data pitch of 0.1 nm, at a bandwidth of 0.5 nm and the results are the average of 10 scans. Peptides were prepared in 3 different solutions: 50% trifluoroethanol (TFE), 25 mM sodium dodecyl sulphate (SDS) or in water, at a final peptide concentration of 50 μM. Data from the scans were presented as mean residue ellipticity vs. wavelength. Any data acquired for the CD spectra that were above 800 volts were omitted, due to the unreliability of data beyond that point. The following equation was used to convert the data to mean residue ellipticity which incorporates the concentration and number of amino acid residues:

\[
[\theta]\text{mre} = \frac{100 \times \theta}{Cn l}
\]

Where \([\theta]\text{mre}\) is the mean residue ellipticity (deg.cm².dmol⁻¹), \(\theta\) is the reading acquired in mdeg, \(C\) is the concentration of peptide in mM, \(n\) is the number amino acid residues and \(l\) is the pathlength (cm).
2.4 Determination of the effect of the peptides on mammalian cells

2.4.1 Erythrocyte hemolytic assay

If an AMP is to be developed for potential human use, it is necessary to show that it is non-toxic and does not cause erythrocyte hemolysis or cellular death. The erythrocyte membrane is a typical bilayer membrane and hemolytic assays are common first steps in investigating possible cytotoxic effects of compounds. The hemolysis assay is rapid and easy to perform. Damage to the erythrocyte membrane causes hemolysis and leakage of cellular contents which includes the major cellular protein, haemoglobin, that shows strong absorbance at 570 nm. This assay has been used to study the cytotoxicity of AMPs. By using human erythrocytes, the effect is studied in the same species in which these AMPs will eventually be tested on, in clinical trials.

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). Using a sterile needle connected to a 5 ml EDTA vacuum extraction tube, 5 ml of blood was collected from a vein. Erythrocytes were collected by centrifugation at 2500 g and the plasma and buffy coat were removed. The erythrocytes were washed with NaP buffer (100 mM, pH 7.4). A 10 μl volume of erythrocytes diluted in 80 μl buffer were exposed to 10 μl of the peptides OsΔC or PaΔC (final concentration range 0.25-100 μM) or 10 μl buffer (negative control) or 2% SDS (positive control), and incubated for 30 min at 37°C. SDS is an anionic surfactant commonly used for the disruption of cell walls. The samples were then removed from the incubator and centrifuged at 2500 g for 3 min. A 75 μl volume of the supernatant was collected and placed into a 96 well plate and the absorbance was measured at 570 nm. The results were calculated and expressed as % hemolysis, relative to the SDS control, using the following formula:

\[
\% \text{ hemolysis} = \left( \frac{Abs_{\text{peptide}} - Abs_{(0\% \text{ control})}}{Abs_{(100\% \text{ control})} - Abs_{(0\% \text{ control})}} \right) \times 100
\]

2.4.2 Cell culture based determination of cytotoxicity

The effects of the peptides were investigated on SC-1 (embryo, Mus musculus fibroblast) and Caco-2 (adult, Homo sapiens, epithelial, colorectal adenocarcinoma) cell lines using the crystal violet (CV) assay. CV is positively charged (Figure 2.2) and binds to negatively charged molecules such as nucleic acids and the amino acid...
residues of proteins such as Glu and Asp. Following exposure to the peptide for 24 h, the cells are fixed and stained with CV. Toxicity can inhibit cell division, causes detachment of the cell or causes cell lysis and decreases the cell content, each of which will result in the decrease of CV staining. The CV is extracted and colour intensity quantified.

![Chemical structure of crystal violet compound](http://www.sigmaaldrich.com/catalog/product/sigma/ht90132?lang=en&region=ZA#)

**Figure 2.2: The chemical structure of crystal violet compound**

The maintenance and plating of the SC-1 and Caco-2 cell cultures (in DMEM 10% fetal calf serum and 1% antibiotics) used in the experiments were maintained by Ms. JC Serem of the Department of Anatomy, University of Pretoria. In this study cells were plated at a cell concentration of 3x10⁴ per 200 µl in 96 - well flat bottom plates and were incubated for 24 h at 37°C and 5% CO₂ to allow the cells to attach to the tissue culture surface before conducting experiments. Cells were incubated with the synthetic peptides OsΔC and PaΔC at 10, 25 and 50 µM concentrations. Melittin (25 µM) was used as a positive control, for 24 h at 37°C and 5% CO₂. Following exposure, 10 µl of a 20% paraformaldehyde solution was added to the cell culture medium, as a fixative. The fixative and medium were removed and the plates were dried well until the dried layer of cells could be observed. The cells attached to the bottom of the plate were stained by adding 200 µl of a 0.1% (w/v) CV solution prepared in 200 mM of formic acid (pH 3.5) to each well for 30 min. The plate was washed with ddH₂O and dried, the
bound dye was extracted with 50 μl of a 10% acetic acid solution. The absorbance was
determined at 630 nm and the data were expressed as percentage compared to the
control (no peptide added).

2.5 Antioxidant and nitric oxide scavenging activity

There are two types of antioxidant assays of which the one is based on hydrogen atom
transfer (HAT) and and the other is based on electron transfer (ET). Both HAT and ET
based methods measure the radical (or oxidant) scavenging capacity, instead of the
preventative antioxidant capacity (Huang et al., 2005). An example of an ET-based
assay is the trolox equivalence antioxidant capacity (TEAC) method. ET-based assays
measure the ability of an antioxidant in the reduction of an oxidant, which changes
colour. It involves one redox reaction with the oxidant also serving as an indicator of
the end of the reaction. The degree of the change in colour is correlated with the
sample concentrations. Most HAT-based assays are a competitive reaction
mechanism, where the known antioxidant and sample (being tested) compete for
thermally generated peroxyl radicals through the decomposition of azo compounds.
Present in these assays is a synthetic free radical generator, an oxidizable molecular
probe and an antioxidant. The competitive reaction kinetics is monitored and the
quantification is derived from kinetic curves. An example of this is the oxygen radical
absorbance capacity (ORAC) assay, which is considered as the international standard
method for the quantification of antioxidant activity.

2.5.1 Trolox equivalence antioxidant capacity assay

The TEAC assay was first reported by Miller and Rice-Evans in 1993 and later
improved. The general reaction for an ET based assay is:

Probe (oxidant) + e⁻ (from antioxidant) → Reduced probe +oxidized antioxidant

The oxidant used in the TEAC assay is 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic
acid) (ABTS) and is generated by reacting a strong oxidizing agent (eg potassium
persulfate) with the ABTS salt. The assay measures the relative ability of the
antioxidant to scavenge the ABTS** generated, as compared with a trolox (water
soluble vitamin E analogue) standard. The method used here is a based on the
methods of Awika et al. (2003), modified for multiple sample analysis by Serem and Bester, (2012). The sample is again quantified in comparison to a known antioxidant, trolox. The assay is inexpensive, easy to use and is a fast reaction (Awika et al., 2003).

The ABTS** was freshly generated by adding 3 mM of potassium peroxodisulfate \((K_2S_2O_8)\) solution to 8 mM ABTS and the mixture was left to react in the dark for at least 12 h at room temperature. The working solution was prepared by diluting ABTS stock solution with 0.2 M phosphate buffer, pH 7.4. Trolox (a vitamin E analogue and known antioxidant) was used as a standard, with a concentration range of 0 – 1000 µM. A 290 µl volume of the working solution was added to 10 µl of the control, trolox standard or peptide (12.5, 25, 50 and 100 µM). Glutathione (GSH) and melittin (at the same concentrations as the peptide) were used as reference samples for comparison. GSH is a naturally occurring antioxidant peptide that protects important cellular components from damage caused by ROS species (Pompella et al., 2003). The reaction mixtures were left to stand at room temperature and the absorbance readings were taken at 734 nm after 30 min. To eliminate the possible effects of interference each sample served as its own control, i.e. all components, no ABTS added. The results were expressed in µM trolox equivalents (TE).

**2.5.2 Oxygen radical absorbance capacity assay**

The ORAC assay was first reported by Cao et al. (1993) and contains a radical generator to supply a steady flow of peroxyl radicals in an air-saturated solution. The antioxidant competes with the probes for radicals and inhibits or slows down the probe oxidation (Huang et al., 2005). Components of the assay include a radical initiator, normally, 2,2’-azobis-(2-methyl-propanimidamide)dihydrochloride (AAPH), a molecular probe to monitor progress (fluorescein) and the sample to be tested (possible antioxidant). Serial dilutions of standard, trolox, are used to construct a standard curve. In the control (Figure 2.3A), the reading remains constant, while in the standard (Figure 2.3 B, C, D and E increasing concentrations of trolox result in a delayed loss of fluorescein. The sample’s antioxidant properties (delaying the loss of fluorescein) are measured as a TE. These are compared by calculating the area under curves (AUCs) (Huang et al., 2005). Therefore, quenching of the radical by the antioxidant is quantified using a known antioxidant, trolox. The assay integrates both degree and time of the antioxidant reaction and is standardized which allows for data comparison with other laboratories (Awika et al., 2003).
33

Figure 2.3: Schematic diagram of the ORAC assay (A) control, only fluorescein. (B-E) are increasing trolox concentrations

Procedures were based on a modified method of Ou et al. (2002). AAPH was used as a peroxyl radical generator, trolox as standard (0 – 1000 μM) and fluorescein as a fluorescent probe. Phosphate buffered saline (PBS) was used as a blank. To 160 μl 0.139 nM fluorescein working solution, 40 μl of PBS (0.2 M Na₂HPO₄, 0.2 M NaH₂PO₄·H₂O, 0.15 M NaCl, pH 7.4), or trolox standard or peptide (12.5, 25, 50 and 100 μM) were added. GSH and melittin (at the same concentrations as the peptide) were used as reference samples for comparison. This was followed by the addition of 40 μl 0.11 μM AAPH. Samples were mixed and the microplate was placed into the plate reader and incubated at 37°C. The fluorescence was measured every 5 min for 4 h. The assay protocol included: measurement start time of 0.0 sec, 10 flashes per cycle, 300 sec cycle time, 485 nm for the excitation filter and 520 nm for the emission filter. The results were expressed as μM TE per gram sample.

2.5.3 Cell based antioxidant assay

The 6-carboxy-2’,7’-dichlorofluorescein-diacetate (DCFH-DA) assay, is a cell based antioxidant assay used to evaluate the ability of antioxidants to protect cells against ROS damage (Figure 2.4). The non-fluorescent DCFH-DA first diffuses across the cell membrane where it is hydrolysed by intracellular esterases to the non-fluorescent DCFH which is trapped inside the cells (Chen et al., 2005). In the presence of AAPH (the ROS generator), DCFH is oxidized to the highly fluorescent DCF. The fluorescent intensity is proportional to the amount of ROS/RNS in the cell (Hipler et al., 2000; Blasa et al., 2010). Addition of an antioxidant will inhibit the formation of DCF and a reduction in fluorescence will be observed. The advantage of this assay is that it is simple, rapid and allows usage in a multiwell format analysis in a serum free environment.

Total protective effects against oxidative damage caused by AAPH were determined, as described by Serem and Bester (2012). GSH is not able to cross the cell membrane and therefore is not used as a control (Wellner et al., 1984). A stock solution of 750 μM
DCFH-DA was prepared in PBS and diluted 10x to the working solution of 75 μM. Caco-2 cells were plated at a concentration of 2x10^4 cells/ml and 100 μl in 96 well plates. DCFH-DA working solution (50 μl) was added to each well, to a final concentration of 25 μM, and cell culture plates were maintained for a further 1 h at 37°C. The medium containing the DCFH-DA solution was carefully removed. Cell culture plates were washed once with PBS and were blotted dry. Peptide (25 μl) (final concentration of 12.5, 25 and 100 μM) was added to each well of the cell culture plates followed by 25 μl AAPH (final concentration of 7.5 mM). Change in fluorescence was measured immediately over 0 – 60 min, every 2 min. The effect of the peptides alone on the cells was also determined. The gradient of the change in fluorescence was calculated, and the data were expressed as % cellular damage where AAPH alone causes 100% damage.
2.5.4 Nitric oxide scavenging assay

The ability of a peptide to scavenge NO is beneficial and provides a first level of evaluation related to possible anti-inflammatory activity. NO scavengers may decrease the level of NO at sites of inflammation without affecting the production of NO elsewhere in the body. Determination of the NO scavenging capacity of the peptides was achieved by using sodium nitroprusside (SNP), which spontaneously degrades to produces NO and acts as a NO generator. The NO, in aqueous solutions, is converted
to nitrite (NO\textsubscript{2}) (Wink et al., 1993). The levels of nitrite can be quantified with the Griess reaction (Figure 2.5). In the assay SNP is incubated with the peptide and generates the highly unstable NO, which converts to nitrite. Sulphanilamide (SA, a diazotizing agent) in an acidic medium is added to the nitrite to form a diazonium salt. A coupling reagent, N-naphthyl-ethylenediamine dihydrochloride (NED), is added to form a stable azo compound (Sun et al., 2003). The product is purple in colour and the absorbance is linearly proportional to the concentration of nitrite in the sample. GSH and trolox were used as controls and have shown NO scavenging activity in previous studies (Vriesman et al., 1997; Naik and Vaidya, 2011). PBS is used as the 100% control and the other samples are compared to it. Any scavenging of NO by peptides will result in less nitrite available hence less for conversion to the azo compound and less intense colour.

![Figure 2.5: Overview of Griess reaction assay (Sun et al., 2003)](image)

SNP (5 mM, 80 µl) diluted in PBS, 0.2 M, pH 7.2, is added to 20 µl peptide (final concentrations of 12.5 µM and 25 µM, diluted in dddH\textsubscript{2}O) and incubated for 1 h at room temperature in the dark. Thereafter 50 µl 0.33% sulphanilamide (w/v) (diluted in 20% glacial acetic acid) was added and incubated for a further 10 min. NED (0.1% w/v, 50 µl) solution (diluted in 20% glacial acetic acid) was added and the absorbance was read at 570 nm. NaNO\textsubscript{2} was used to prepare a standard curve over a range of 0 to 0.1 mM (R\textsuperscript{2}=0.9998).
Chapter 3: Results

The purpose of this study was to investigate the antibacterial, cytotoxic and antioxidant activities as well as NO scavenging capacity of peptide OsΔC. The physicochemical properties of the synthetic peptides used in this study are presented in Table 3.1. PaΔC was used as a control for the antibacterial assays (Varkey et al., 2006). Comparing peptides OsΔC and PaΔC, OsΔC is three residues longer, carries one extra positive charge and is less hydrophobic. Melittin, a cationic peptide isolated from bee venom (Tosteson et al., 1985) was used as a control in the cytotoxicity, antioxidant and NO scavenging assays.

Table 3.1: Physicochemical properties of the synthetic peptides used in this study

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Length</th>
<th>Net charge</th>
<th>Molecular Mass (Da)</th>
<th>pI</th>
<th>&lt;H&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaΔC</td>
<td>WVIKGYKGGQKITVHR-amide</td>
<td>17</td>
<td>+6</td>
<td>2025.42</td>
<td>12.44</td>
<td>0.261</td>
</tr>
<tr>
<td>OsΔC</td>
<td>WKGIRGYKGGYKGAQTKY-amide</td>
<td>20</td>
<td>+7</td>
<td>2335.72</td>
<td>10.84</td>
<td>0.155</td>
</tr>
<tr>
<td>Os-C</td>
<td>KGIRGYKGGYKGAQTKY</td>
<td>19</td>
<td>+6</td>
<td>2150.50</td>
<td>10.58</td>
<td>0.045</td>
</tr>
<tr>
<td>Melittin</td>
<td>GIGAVLKVLTGLPALS IKRKRQQ</td>
<td>26</td>
<td>+5</td>
<td>2847.40</td>
<td>12.43</td>
<td>0.511</td>
</tr>
</tbody>
</table>

a Iso-electric point.  
b Hydrophobicity. Data obtained from HeliQuest [heliquest.ipmc.cnrs.fr].  
c Analogue of OsΔC, non-amidated and without the N-terminal tryptophan, used as a control in the determination of secondary structure.

3.1 Determination of antibacterial activity

The synthetic peptides PaΔC and OsΔC were tested for antibacterial activity and found to be bactericidal against both Gram-positive and Gram-negative bacteria (Table 3.2). The control peptide, PaΔC was equally active against both B. subtilis (Gram-positive bacteria) and E. coli (Gram-negative bacteria) with a MBC value of 0.46 μM. OsΔC showed antibacterial activity towards Gram-positive bacteria, with MBC values of 0.81 μM and 12.8 μM for B. subtilis and S. aureus, respectively. However, OsΔC was more active against Gram-negative bacteria, with a MBC value of 0.40 μM for both E. coli and P. aeruginosa.
Table 3.2: Minimum bactericidal concentration of peptides

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MBC (μg/ml)</th>
<th>PaΔC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>OsΔC</th>
<th>Os-C&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>0.94 (0.46 μM)</td>
<td>1.90 (0.81 μM)</td>
<td>3.75 (1.74 μM)</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>ND</td>
<td>30 (12.8 μM)</td>
<td>15 (6.98 μM)</td>
<td></td>
</tr>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>0.94 (0.46 μM)</td>
<td>0.94 (0.40 μM)</td>
<td>3.75 (1.74 μM)</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>ND</td>
<td>0.94 (0.40 μM)</td>
<td>1.90 (0.88 μM)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>PaΔC was the control peptide  
<sup>b</sup>MBC values for Os-C from Prinsloo et al. (2013)  
ND – not determined  
Values are representative of 2 independent experiments preformed in triplicate

3.2 Secondary structure determination

Cationic peptides in aqueous solutions usually have unordered conformations but they are amphipathic molecules and can adopt folded states in hydrophobic environments (Gopal <i>et al.</i>, 2012). Determining the changes in secondary structures may give some insight into the structure-activity relationship and could explain any differences in activity as a result of the modifications made to the peptides. The secondary structures of the synthetic peptides were determined using Far-UV CD spectroscopy. In Table 3.3, the secondary structures of the synthetic peptides PaΔC, OsΔC and Os-C, as predicted by PSIPRED, an online secondary structure predicting program, is presented. Predominantly random coiled structures are predicted for the OsΔC and Os-C peptides, while the PaΔC peptide is predicted to have much less random coil content and more β-sheet structure.

The Far-UV CD profiles obtained for PaΔC, OsΔC and Os-C, and the effects of three solvents SDS (a membrane mimicking solvent), TFE (a secondary structure promoting solvent) and water on the structures of the peptides, are shown in Figure 3.1. Evaluation of the spectra is based on the shape and values at specific wavelengths. The PaΔC peptide retained a large degree of random coil content (unordered) in all the solutions. The amount of random coil content of PaΔC decreased slightly (seen by the decreases in the negativity of the peak) in 50% TFE (Figure 3.1 B) and 25 mM SDS
(Figure 3.1 C) as compared to water (Figure 3.1 A), and may have adopted more α-helical content. The negative peaks for PaΔC might indicate the presence of some β-sheet content when in TFE and water and possibly the presence of some α-helical content in the SDS.

### Table 3.3: Predicted secondary structures of PaΔC, OsΔC and Os-C

<table>
<thead>
<tr>
<th>Peptide</th>
<th>α-Helical</th>
<th>β-Strand</th>
<th>Random coil</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaΔC</td>
<td>0</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>OsΔC</td>
<td>0</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>Os-C</td>
<td>0</td>
<td>21</td>
<td>79</td>
</tr>
</tbody>
</table>

Predicted using PSIPRED [available online: bioinf.cs.ucl.uk/psipred/]

The OsΔC and Os-C peptides were found to have mostly random coil content in water (Figure 3.1 A). However OsΔC appears to have some α-helical structure in water (Figure 3.1 A) as the negative peak is closer to 200 nm, as compared to the structure in SDS, and a slight shoulder formation around 215 nm. OsΔC changed slightly in 50% TFE (Figure 3.1 B) where it retains much of its random coil content and an increase in α-helical content. This is seen by the decrease in the content of the negative peak and a shift in the negative shoulder around 212-215 nm. The Os-C peptide lost much of the random coil content and adopted a more α-helical structure in the 50% TFE solution (Figure 3.1 B). In 25 mM SDS (Figure 3.1 C) both the OsΔC and Os-C peptides have adopted more α-helical structures and possibly some β-sheet content.
Figure 3.1: Far-UV CD spectra of synthetic peptides OsΔC, PaΔC and Os-C in (A) Water (B) 50% TFE and (C) 25 mM SDS.
3.3 Determination of the effect of the peptides on mammalian cells

3.3.1 Erythrocyte hemolytic assay

To investigate the specificity of the peptides, their effects were tested on human red blood cells, mouse fibroblast cells (SC-1 cell line) and human colon cancer cells (Caco-2 cell line). The ability of PaΔC and OsΔC peptides to induce hemolysis is shown in Figure 3.2. At the concentration range (0.25–100 μM), both peptides showed very little to no hemolytic activity compared to SDS, the 100% hemolysis control.

![Figure 3.2](image)

**Figure 3.2: Hemolysis of human erythrocytes by synthetic peptides.** Erythrocytes were incubated with the peptides (0.25-100 μM). Values are expressed as means ±SEM obtained from two independent experiments performed in triplicate.

3.3.2 Cell culture-based determination of cytotoxicity

The cytotoxicity of the peptides towards mammalian cells was further investigated with the CV staining assay in the SC-1 and Caco-2 cell lines (Figure 3.3 A and B). Both cell lines were exposed for 24 h to 10-50 μM OsΔC and PaΔC, with the control, melittin, at a concentration of 25 μM.
Figure 3.3: Cytotoxicity of synthetic peptides PaΔC and OsΔC on SC-1 cells (A) and Caco-2 cells (B), exposed for 24 h. Melittin was used as the positive control. Values are expressed as means ±SEM obtained from two independent experiments performed in triplicate. * Compared to the control (PBS) significant different (p≤0.001).

For the SC-1 (Figure 3.3 A) and Caco-2 (Figure 3.3 B) cell lines, both peptides showed no difference in % cell numbers at the concentration range tested, compared to the control. Melittin with well described toxicity (Tosteson et al., 1985; Raghuraman and Chattopadhyay, 2007) was used as a positive control. Melittin caused an approximate 20% reduction in % cell number when compared to the untreated cells (PBS).
3.4 Antioxidant and nitric oxide scavenging activity

Having shown that the synthetic peptides PaΔC and OsΔC have strong antibacterial activity and showed no cytotoxicity towards human erythrocytes, and SC-1 or Caco-2 cell lines, the potential of these peptides to possess multifunctional activity was investigated. Analysis of the peptides with BIOPEP [http://www.uwm.edu.pl/biochemia/index.php/pl/biopep], identified antioxidant dipeptide sequences in melittin (LK) and OsΔC (IR) but none in PaΔC. The antioxidant activity of the peptides was determined using the TEAC (Figure 3.4) and ORAC assays (Figure 3.5). Two assays were used as they test two different methods of oxidant scavenging, described earlier in Chapter 2 (2.5.1 and 2.5.2). GSH and melittin were included as controls. GSH is a known antioxidant peptide and melittin is an antibacterial peptide.

3.4.1 Trolox equivalence antioxidant capacity assay

The TEAC assay is an ET-based assay and measures the ability of the antioxidant to reduce an oxidant. Antioxidant activity of the peptides were measured and compared in TE. To determine the TE of the samples, a 0-800 µM trolox range was used for the standard curve (R^2=0.9905). The highest antioxidant activity at 100 µM was found for OsΔC. A clear dose dependent response was seen with the relative antioxidant capacity increasing with peptide concentration (Figure 3.4). From Figure 3.4 the ratio of antioxidant activity to peptide concentration (µM TE/µM peptide) could be determined. For OsΔC, PaΔC, melittin and GSH these ratios were calculated to be 6.75, 5.41, 2.74 and 2.11 µM TE/µM peptide, respectively. From this data, it could be calculated that the antioxidant activities of OsΔC, PaΔC and melittin were 3.20, 2.56 and 1.29 times larger than that of GSH, respectively. Also the antioxidant activity of OsΔC and PaΔC were 2.46 and 1.97 times larger than that of melittin, respectively.
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Figure 3.4: Antioxidant activity of synthetic peptides OsΔC and PaΔC measured with the TEAC assay compared with melittin and GSH. Peptides were tested over a concentration range of 12.5-100 μM. Values are expressed as means ±SEM obtained from three independent experiments performed in triplicate.

3.4.2 Oxygen radical absorbance capacity assay

The ORAC assay is a competitive reaction system as the antioxidant and sample both compete for generated radicals. To determine the TE of the samples, a 0-800 μM trolox range was used for the standard curve (R²=0.9943). PaΔC, OsΔC, GSH and melittin all revealed antioxidant activity with PaΔC and GSH displaying a dose dependant response (Figure 3.5). The strongest antioxidant was the OsΔC peptide, which at 25 μM had already reached the maximum capacity of the trolox standard range. Melittin performed poorly as an antioxidant at 12.5 and 25 μM concentrations, having negative values. This resulted in a poor correlation between μM peptide and μM TE. Therefore, the ratio of antioxidant activity to peptide concentration (μM TE/μM peptide) could not be determined accurately. An estimate of the μM TE/μM peptide values (from this data) for OsΔC, PaΔC, melittin and GSH were approximately 20.63, 13.63, 8.61 and 11.42 μM TE/μM peptide, respectively. Using these values, the antioxidant activity of OsΔC and PaΔC are 1.81 and 1.19 times larger than that of GSH, respectively, and was 2.40 and 1.58 times larger than that of melittin, respectively.
3.4.3 Cell-based antioxidant assay

Chemical-based assays showed antioxidant activity for OsΔC and PaΔC. This activity was further evaluated using a cell-based model to determine if this translates into the protection of cells against oxidative damage. This provides a more realistic indication of the antioxidant properties of these peptides. Cellular protection can be investigated with the DCFH-DA assay, which monitors ROS production in the cytosol. The peptide must enter the cell to scavenge radicals because that is where the non-fluorescent DCFH is converted to the fluorescent DCF, by the action of ROS. Figure 3.6 shows the antioxidant activity of the peptides with the DCFH-DA assay using Caco-2 cells. Caco-2 cells with AAPH alone caused oxidative damage, which is measured as 100%. Peptides alone, in the absence of AAPH caused a small amount of oxidative damage. Melittin caused 4% damage at a concentration of 100 μM. In the presence of AAPH, at all concentrations, an approximate 20% protection against oxidative damage was observed. However the observed effect is a function of the number of cells plated, i.e. the more cells used the larger the measured cellular protective effect would be. The gradients of % oxidative protection of OsΔC and PaΔC indicated a minor dosage related effect, with an increasing concentration of OsΔC leading to a decrease in
oxidative protection (negative gradient). An increase in oxidative protection is observed with PaΔC (positive gradient).

Figure 3.6: Antioxidant activity of the synthetic peptides melittin (Mel), PaΔC and OsΔC, measured with the DCFH-DA assay. Activity was determined over a concentration range of 12.5-100 μM. Melittin was tested at 100 μM. The effect of the peptides without AAPH was also determined. Values are expressed as means ±SEM obtained from three independent experiments performed in triplicate.

3.4.4 Nitric oxide scavenging assay

Due to the fact that the peptides showed strong antioxidant activity in the chemical assays and some activity in the cellular antioxidant assay, the peptides were also evaluated for their NO scavenging ability. Production of RNS may also lead to oxidative damage and NO scavenging activity may aid in protection from this. NO scavengers are also a proposed method of protection in inflammatory disorders (Shah and Billiar, 1998). In this assay SNP is used to produce NO which can be scavenged by molecules with NO scavenging activity. The samples are compared to the NO present in the PBS control, taken as 100% (Figure 3.7). NaNO₂ was used to prepare a standard curve over a range of 0 to 0.1 mM (R²=0.9998).
Strong NO scavenging activity was found for all the samples tested, showing more than 80% scavenging activity. OsΔC at 25 μM showed the strongest NO scavenging activity (89%). The activity of PaΔC was comparable to that of trolox.
Chapter 4: Discussion

AMPs derived from different sources such as plants, animals and insects are new antimicrobial agents that can be used to address the issue of drug resistant bacteria. A major problem with AMPs being used as therapeutic agents is the high cost involved in the production of peptides (McDermott, 2009). One of the strategies to overcome this problem is to shorten the sequences of the peptides by determining the most active region. Synthetic analogues of defensins lacking cysteine residues have been shown to retain their broad-spectrum activity (Varkey et al., 2006; Varkey and Nagaraj, 2005; Lundy et al., 2008). Nakajima et al. (2003) found that O. moubata defensin A was only active against Gram-positive bacteria. Varkey et al. (2006) investigated the C-terminal region of O. moubata defensin C with the cysteines removed and found that it was active against both Gram-positive and Gram-negative bacteria.

The peptides used in this study are all positively charged (Table 3.1), with OsΔC having the highest charge of +7, and melittin the lowest charge of +5, whereas PaΔC and Os-C both have charges of +6. Melittin (\(<H> = 0.511\)) is the most hydrophobic and Os-C (\(<H> = 0.045\)) is the least hydrophobic of the peptides used in this study. Varkey et al. (2006) observed that PaΔC (control peptide used in this study), was active against Gram-positive (lethal concentration of 2.5 μM) and Gram-negative (lethal concentration of 5.0 μM) bacteria. In this study PaΔC was found to be more active (MBC of 0.46 μM for both Gram-positive and Gram-negative bacteria) (Table 3.2). The difference in activity could be ascribed to the use of different strains of bacteria. The strains used by Varkey et al. (2006) were E. coli MG1655 and B. subtilis (NCIM 2162), whereas the strains used in this study were E. coli (ATCC 700928) and B. subtilis (ATCC 13933). The OsΔC peptide was found to be more active against Gram-negative bacteria (MBC of 0.40 μM) and least active against S. aureus (MBC of 12.8 μM) strain tested in this study (Table 3.2).

If the MBC values of OsΔC are compared to the MBC values of its analogue Os-C (Table 3.2), as determined by Prinsloo et al. (2013), OsΔC was found to be 2 times more active against P. aeruginosa and B. subtilis compared to Os-C. OsΔC was also 4 times more active against E. coli (0.40 vs. 1.74 μM), whereas Os-C was 2 times more active (15 vs. 30 μM) against S. aureus respectively. Comparing the sequence of
OsΔC to that of Os-C, the addition of Trp to the N-terminus, increased the hydrophobicity and amidation at the C-terminus added an additional positive charge. It has been shown that the C-terminal fragment of OsDef2 with cysteines removed, is a viable strategy for shortening peptides. This strategy has actually broadened the antibacterial spectrum of the peptide. The parent peptide, OsDef2, is only active against Gram-positive bacteria whereas the OsΔC peptide is active against Gram-positive and Gram-negative bacteria. One of the limitations associated with the use of AMPs is the high cost of synthesis. Shorter peptides which retain their activity are important in the development of AMPs as therapeutic agents, however, some shorter peptides have been shown to be cytotoxic (Leontiadou et al., 2006; Makovitzki et al., 2006).

One of the strategies used in an attempt to improve cell selectivity is optimization of physicochemical parameters (Matusuzaki, 2009). The disulphide bonds of β-sheet AMPs which stabilize both the β-hairpin and β-sheet structures may not be necessary for their antimicrobial activity as linear derivatives of tachyplesin, lactoferrin and defensins have retained their antimicrobial activity, and also lost their hemolytic activity (Gifford et al., 2005; Ramamoorthy et al., 2006; Varkey et al., 2006). Human β-defensin 3 contains an α-helical turn and a three stranded antiparallel β-sheet, stabilized by three disulphide bonds. Reducing the disulphide bonds did not alter the antimicrobial activity nor affect the overall formation of the native secondary structures (Wu et al., 2003; Klüver et al., 2005) but it did affect the chemotactic activities. Reduction of the disulphide bonds in human β-defensin 1 created an unstructured, flexible peptide which showed improved activity against Candida albicans and some Gram-positive bacteria (Schroeder et al., 2011). An increase in charge in some cases has been shown to increase antimicrobial activity without significantly affecting hemolytic activity (Zelezetsky and Tossi, 2006). A similar charge to activity relationship was seen in a study by Nguyen et al. (2011), with C-terminal analogues of macrophage inflammatory protein-3α. The analogue with greater charge showed more antibacterial activity against Gram-positive and Gram-negative bacteria. Charge is important for activity against Gram-negative bacteria, while hydrophobicity and hydrophobic moment increase activity against Gram-positive bacteria but also hemolytic activity (Dathe and Wieprecht, 1999; Wieprecht et al., 1996, 1997). End-tagging of peptides with oligo-tryptophan increases the hydrophobicity, which resulted in a decrease in toxicity to human cells and promoted peptide-induced lysis of phospholipid liposomes and killing of bacteria and fungi (Schmidtchen et al., 2011).
In this study the increase in charge together with the increase in hydrophobicity resulted in a more active peptide. This is not always the case as there is a fine balance between the amount of charge and the amount of hydrophobicity of a peptide that allows for attraction to bacterial membranes and insertion into it. Makovitski et al. (2006) reported that the balance between charge and hydrophobicity plays an important role just as in larger AMPs and showed that altering both the amino acid residues and chain length of a peptide to increase the hydrophobicity and/or charge, did not necessarily result in higher antimicrobial activity. It is difficult to predict or estimate what effect a physicochemical change will cause as many factors are involved in the activity of the peptide and they do not always act independently (Matsuzaki, 2009).

Many linear peptides (which lack cysteine residues) are unstructured in aqueous environments and adopt stable amphipathic conformations in membranous or membrane mimicking environments (Hwang and Vogel, 1998). CD is a commonly used method for investigating the secondary structures of proteins and peptides. The OsΔC and Os-C peptides presented with different activities, OsΔC showed lower MBCs with three of the four bacteria tested. A change in secondary structure, as a result of the addition of a Trp and amidation, might be the cause of the increased activity. The CD spectra of the peptides were studied in water, TFE and SDS solutions (Figure 3.1). TFE is a secondary structure promoting solution (Waring et al., 1995, Roccatano et al., 2002), while SDS is a membrane mimicking solution. The PaΔC peptide maintained a largely random coil structure conformation in all the media tested, with the amount of random coil structure decreasing slightly in TFE and slightly more in SDS. PaΔC may contain some β-sheet content in TFE and SDS media and some α-helical content in the SDS. Varkey et al. (2006) reported that the spectrum of PaΔC in water was characteristic of an unordered conformation. In SDS, the authors reported a mixed population of random coil and α-helical structure. In TFE, the spectrum suggested the presence of some β-hairpin and α-helical structure. The OsΔC peptide adopted a largely random coil structure in both the water and TFE media. Some β-sheet content may be present in water, the amount of which increased in TFE. In SDS, OsΔC adopted a more α-helical structure. Comparing the spectra of peptides OsΔC and Os-C, there is a clear difference. The Os-C peptide showed a much greater change in structure when in TFE and SDS solutions. The change to adopt less random coil and more α-helical content is seen in TFE and in SDS the peptide adopted a largely α-helical secondary structure (Figure 3.1).
The positive peaks seen may be the presence of turn formations but also may be caused by interference from aromatic residues. Os-C has a smaller positive peak than OsΔC and this may be a result of interference by the added Trp residue to OsΔC (Figure 3.1). Khan et al. (1989) found that the peaks for Tyr, Trp and Phe were present at 225, 224 and 214 nm, respectively. Although the position of each peak differs, Khan et al. (1989) found that together these residues resulted in a peak around 222 nm. The authors also noted that the CD spectra were influenced by the amount of Phe contained in the peptide, where the higher Phe content resulted in a shift of the peaks to shorter wavelengths. PaΔC has one Trp and one Tyr residue, whereas OsΔC has one Trp, three Tyr and one Phe residue and Os-C peptide has one Phe and three Tyr residues. The resulting peaks could mask some of the spectra and the presence of Phe could account for the slight shift in the peaks when compared to the standard peaks for α-helix and β-sheet formations.

Relating the structures adopted by each peptide to their antibacterial activities, as with the physicochemical properties, it is difficult to predict which structures will present with higher activity. OsΔC activity was stronger against three of the bacteria compared to Os-C and its structure is comparable to that observed for the Os-C peptide. The PaΔC structure is quite different from the Os-C and OsΔC structures but presented with higher activity than Os-C. The structures of OsΔC and Os-C are relatively similar in the different solutions, which suggest that the difference in activity may not be due to structural differences. Prinsloo et al. (2013) also investigated the antibacterial activity of Os, which was derived from the same parent peptide as the Os-C peptide, but Os still contained the three cysteine residues. The Os peptide was more active than the Os-C peptide and had similar activity to the OsΔC peptide against B. subtilis and P. aeruginosa, but OsΔC was 2x more active against E. coli. The OsΔC peptide would therefore be more suitable from a manufacturing point of view as it is shorter and implies that the activity is not dependent on the oxidation state of the thiols. The increased activity could be attributed to the increase in charge and hydrophobicity.

The peptides have shown strong antibacterial activity, but to be considered for further development as a therapeutic agent, they must also be non-toxic to mammalian cells. The cytotoxicity of the peptides were investigated by testing the effect of the peptides on human erythrocytes, mouse fibroblast cells (Sc-1 cell line) and human colon cancer cells (Caco-2 cell line). With the hemolytic assay the erythrocyte membrane represents a typical bilayer membrane and damage to the membrane will result in the release of
hemoglobin, which can be measured. The hemolytic assay serves as a rapid evaluation of the effect of the peptides on the typical mammalian bilayer membrane. The Caco-2 cell line is a physiologically relevant epithelial cell line and has been used as a model of the intestinal barrier for over 20 years. However, differences in culture conditions and different Caco-2 cell lines have made it difficult to compare results with literature (Sambuy et al., 2005). It does however still serve as a good representative of a typical mammalian cell. Melittin which was used as a control, is a component of bee venom and research has shown it to have strong cytotoxic activity against mammalian cells. It is an amphipathic, cationic, linear α-helical peptide (Table 3.1) containing 26 amino acids (Walsh et al., 2011) and exhibited cytotoxicity in erythrocytes (Tosteson et al., 1985), enterocytes (Maher and McLean, 2006) and other mammalian cells (Raghuraman and Chattopadhyay, 2007). The higher hydrophobic content of melittin contributes to its cytotoxicity to mammalian cells. Walsh et al. (2011) showed that comparison of the native melittin to deletion analogues of melittin with reduced hydrophobicity and amphipathicity, resulted in a reduction in the cytolytic action of melittin on mammalian cells.

The complete cytotoxic profile of these peptides cannot be determined from these results but they may serve as rapid and simple screening assays for the ever growing number of AMPs being identified and produced. In the hemolysis assay almost no hemolytic activity was observed (Figure 3.2). AMPs with low specificity for eukaryotic membranes are not uncommon and the low hemolytic activity seen with the synthetic peptides OsΔC and PaΔC, correlates with previously studied peptides.

The assays with Sc-1 mouse fibroblast cells and Caco-2 human colon cells, showed no cytotoxic activity of the peptides (Figure 3.3). The positive control used was melittin which presented with approximately a 20% decrease in cells, with its activity being slightly higher with Sc-1 cells. The decrease is significant and Prinsloo et al. (2013) showed that it is a dosage dependant response. Nishimura et al. (2004) investigated the effect of human defensins, α-defensin (HNP-1) and β-defensins (hBD-1, -2, -3) on eukaryotic cell lines. At low concentrations the α-defensin showed enhanced cell proliferation with retinal epithelial cells and lung epithelial cells, but no proliferation with fibroblasts. The β-defensin showed very little effect at low and high concentrations. Proliferation of fibroblasts by a defensin has been reported before by Murphy et al. (1993). Wong et al. (2006) also showed that a plant defensin induced a mitogenic response from mouse splenocytes. In this study, a slight increase in the growth of
Caco-2 cells was observed. Statistically, the proliferation was not significant and may be due to non-specific staining of dead cells by the dye but the possibility of the peptides having a mitogenic response, should be further investigated. Allowing the cells to grow for a longer period of time in the presence of the peptides may show larger differences between control and exposed cells.

There is growing interest in peptides or protein fragments that exert physiological effects on body functions or conditions (Auvynet and Rosenstein, 2009; Shah, 2000; Salvatore et al., 2007; De Lucca and Walsh, 1999; Huang et al., 2012; Aarbiou et al., 2004; van der Does et al., 2010). In this study the potential antioxidant activity of the synthetic peptides was investigated. To determine the antioxidant activity of the OsΔC and PaΔC peptides, the ORAC and TEAC assays were used. The mechanisms for antioxidant activity differ between assays and hence different results are obtained depending on which one is used. For this reason more than one method should be used. The TEAC and ORAC assays are commonly used methods (Zheng et al., 2012). TEAC is based on electron transfer and is a colorimetric assay. ORAC is a hydrogen atom transfer assay and is a fluorometric assay. The results for both are expressed as µM TE. Two controls used were, GSH a tripeptide with known antioxidant activity, and melittin, a known AMP which has also shown cytotoxic properties. All the samples showed a dose dependant increase in µM TE values as the peptide concentration increased (Figure 3.4 and 3.5).

In the TEAC assay, OsΔC, PaΔC and melittin showed antioxidant activity which was stronger than the GSH control. Comparing the ratio of antioxidant activity to peptide concentration at µM TE/µM peptide, OsΔC, PaΔC and melittin were 3.20, 2.56 and 1.29 times larger than that of GSH respectively. The antioxidant values of OsΔC and PaΔC were 2.46 and 1.97 times larger than that of melittin, respectively. The values obtained for the ORAC assay were estimates as a poor correlation was obtained between µM TE and µM peptide. The estimated values for OsΔC and PaΔC were 1.81 and 1.19 times larger than that of GSH, respectively, and were 2.40 and 1.58 times larger than that of melittin, respectively. Although these are not accurate, it is an indication of the difference in activity. Further research into the mechanism of antioxidant action of the peptides, may provide some insight into the cause of the negative values obtained with melittin. Overall, OsΔC showed more antioxidant capacity than PaΔC for both the assays. Melittin showed stronger antioxidant activity than GSH in the TEAC assay but a lower value was obtained in the ORAC assay. A
closer look is needed into the reaction mechanisms to fully explain the reasons for the observed differences.

Numerous articles have shown that the amino acid sequence and composition as well as position in the sequence, are important for the antioxidant activity of a peptide. BIOPEP predicted that Os∆C has antioxidant potential because of the dipeptide sequence Ile-Arg and in melittin due to the Leu-Lys sequence. No such sequences were predicted for Pa∆C, however the peptide did show antioxidant activity which may be due to the presence of the antioxidant amino acids Tyr and Val. Some literature suggests that hydrophobic amino acids such as Val or Leu at the N-terminus are important and amino acids such as Pro, Asp, His and Tyr are required in the sequence (Sarmadi and Ismail, 2010; Rajapakse et al., 2005; Chen et al., 1998; Chen et al., 1996; Je et al., 2005; Chen et al., 1995). Aromatic amino acids can donate protons to radicals (Sarmadi and Ismail, 2010). Asp, Glu, Arg and Lys are believed to play a role in metal ion chelation (Rajapakse et al., 2005). Hydrophobic amino acids are important for enhancement of the antioxidant properties of peptides since they can increase the accessibility of the antioxidant peptides to hydrophobic cellular targets such as the polyunsaturated chain of fatty acids of biological membranes (Chen et al., 1998). The Os∆C and Pa∆C peptides have Trp on the N-terminus, the Os∆C peptide has 3 Tyr residues and the Pa∆C has one Tyr and two Val residues. The peptides both contain amino acids which are associated with antioxidant activity and the three Tyr residues could explain the higher antioxidant capacity observed with Os∆C. Evaluation of the antioxidant activity revealed that the peptides Os∆C and Pa∆C had strong antioxidant activity in the chemical based assays. This activity was further evaluated with a cell based antioxidant model.

The DCFH-DA assay shows the antioxidant capacity of the peptides within a cell, which in this study was the Caco-2 cell line. The conversion of non-fluorescent DCFH to the fluoresecent DCF, occurs by the action of ROS within the cell. To affect the production of DCF the peptides should scavenge available ROS inside the cell. AAPH is a free radical generator which is added to the cells and the assay measures each peptide’s ability to scavenge the radicals within the cell. The effect of the peptides without the addition of AAPH was also determined and a small increase in radicals was noted (Figure 3.6). A concentration dependant increase in oxidative stress was found when Caco-2 cells were incubated with a Trp-rich peptide fraction of milk protein, despite the fraction having shown strong antioxidant activity in an ORAC assay (Elisia et al. 2011).
The authors explained the observation being a result of the production of ROS from exposure of Trp-containing milk peptides, primarily generated by mitochondria as by-products of cellular metabolism. Even though Trp-containing peptides contributed to an increase in pro-oxidant activity, no cytotoxicity was observed. Elisia et al. (2011) attributed this to the activation of the Nrf-2 pathway, a cellular adaptive pathway which responds to cellular stress by activation of an antioxidant response.

In the samples with both peptide and AAPH, the antioxidant effects of the peptides were investigated and a decrease in the amount of radicals present was observed. At all their concentrations (12.5–100 μM), both OsΔC and PaΔC still showed approximately 20% protection (Figure 3.6). The gradients for the trend lines of the data for OsΔC and PaΔC showed that higher concentrations of OsΔC revealed a decrease in antioxidant activity but PaΔC higher concentrations, showed an increase. The observed effect is a function of the number of cells plated, if more cells are used the measured cellular protective effect would be greater. Greater cell numbers and further investigation into the antioxidant mechanism are needed.

There are two ways the production of NO may become detrimental to the body. Firstly, the over production of NO during inflammation and, secondly, it may lead to the generation of RNS. The role of NO in immune responses and inflammation is well known (Stuehr and Marletta, 1985; Karupiah et al., 1993). The inhibition of NO would be beneficial in chronic inflammatory diseases. Two strategies exist for inhibiting NO, the first is to inhibit the production of NO by inhibiting the enzymes responsible for its production (eNOS and iNOS). This however has presented with adverse effects (for both nonselective and selective inhibition of the enzymes) due to the beneficial role that NO also plays in the body (Hutcherson et al., 1990; Petros et al., 1994; Spain et al., 1994; Wolfe and Dasta, 1995; Kim and Greenberg, 2002; Lopez et al., 2004). The second option is to decrease the amount of NO available by making use of NO scavengers, which would not affect the functioning of eNOS or iNOS. The NO scavenging capacity of the synthetic peptides in this study was investigated (Figure 3.7). The results in this study demonstrated that all the samples offer some NO scavenging capacity. OsΔC and PaΔC performed only slightly better than melittin and GSH. Chen et al. (2005) showed that GSH depletion in hepatocytes resulted in the cells having higher levels of ROS, in response to NO exposure and suggested that it could be attributed to the loss of GSH scavenging activity for NO. There are a number of research articles documenting the anti-inflammatory activity of melittin by inhibition of
NOS and the LPS-induced accumulation of NO (Srivastava et al., 2012; Moon et al., 2007), but to our knowledge none on the direct NO scavenging capacity of melittin. Naik and Vaidya (2011) found high NO scavenging capacity (42%) for trolox (0.2 mg/ml). In comparison to melittin and trolox, strong NO scavenging capacity for both OsΔC and PaΔC peptides was found (Figure 3.7). Further research into the mechanism by which the peptides scavenge NO is required.

Studies on multiple functions of AMPs have mainly focused on antimicrobial activity and immunomodulation (Scott and Hancock, 2000; Metz-Boutigue et al., 2010; Yeung et al., 2011; Brandenburg et al., 2012;). Reports on peptides with antimicrobial and antioxidant activities have been from peptide fractions of hydrolysates (Bougatef et al., 2010; Alemán et al., 2011; Memarpoor-Yazdi et al., 2012; Najafian and Babji, 2012). This study has investigated the antioxidant activity of OsΔC, with antimicrobial activity and found strong activity for both. In addition to this the peptide has presented with NO scavenging activity and showed no damage to mammalian cells. Further investigation into the mechanisms of these activities is required. Based on the results observed in this study, the peptide OsΔC, shows promise as a multifunctional peptide which could be developed further as a therapeutic agent.
Chapter 5: Conclusion

This project has investigated the bioactivity of a synthetic peptide, OsΔC, derived from a tick defensin. The peptide exhibited strong antibacterial activity against both Gram-positive and Gram-negative bacteria, with slightly stronger activity towards the Gram-negative bacteria. Notably the original full length defensins OsDef1 and OsDef2 are only active against Gram-positive bacteria (Prinsloo et al., 2013). Comparing the antibacterial activity of OsΔC to Os-C, the extra Trp residue and additional positive charge resulted in a peptide with enhanced activity. The CD spectra showed that these modifications did alter the secondary structure of the OsΔC peptide slightly in the TFE and SDS media. Although a fine balance exists between the physicochemical properties and activity of an AMP, it is not always clear what effect a change in physicochemical character may have on the activity (Makovitski et al. 2006; Matsuzaki, 2009). Assays to determine the cytotoxic effects of the peptides against mammalian cells, showed no damage to red blood cells, mouse fibroblast (Sc-1) or human colon cells (Caco-2).

In addition to the strong antibacterial activity and low cytotoxicity, OsΔC and PaΔC also exhibited strong antioxidant activity when determined using the TEAC and ORAC assays. This antioxidant activity was confirmed using a cellular antioxidant assay. In addition, the peptides have also presented with NO scavenging activity. These peptides show promise as multifunctional peptides having antibacterial and antioxidant activity and could also serve as an anti-inflammatory agent through nitric oxide scavenging (Raghuraman and Chattopadhayay, 2007; Galli et al., 2012).

Other immunomodulatory activities need to be further investigated to determine the full potential of OsΔC. Some peptides have been shown to assist in wound healing by their effect on the production and control of other mediators involved in the process (Mookherjee et al., 2006; Hartman and Meisel, 2007; Yang et al., 2009; Zhang et al., 2008; van der Does et al., 2010). Notably the LPS-binding activity in reducing inflammation and the effect of the peptides on regulation of chemokines and cytokines should be investigated. Other aspects which should be investigated are the effects of salts on antibacterial activity, killing kinetics and the peptides’ mechanism of action. In the case of acne legions and sepsis, peptides with antibacterial and anti-inflammatory
properties would be beneficial and the antioxidant activity would control the production of ROS and prevent oxidative damage associated with infections (McInturff et al., 2005). Another use for peptides with this activity could be the coating of prosthetic implants to reduce infections while also creating an environment which promotes wound healing, e.g. by reduction of ROS.

The mechanisms by which the peptides, OsΔC and PaΔC, exert their antioxidant and anti-inflammatory activities should be investigated to further understand the observations in this study. Fragments of the peptides could be synthesized and tested, to further identify which amino acid residues and sequences are required for the antibacterial, antioxidant and NO scavenging activities. These activities together with the low toxicity towards mammalian cells suggest that the peptides could serve as future therapeutic agents. The activities shown here suggest that the peptides would be good candidates to develop for the treatment of wounds, infections and inflammation.

In conclusion, it was found that peptide OsΔC is a promising candidate for further evaluation for pharmaceutical applications. For this reason, further research into the use of this peptide to affect broader antimicrobial, antiviral and antifungal activities should be conducted.
Chapter 6: References


Cell Biolabs inc., OxiSelect Intracellular ROS Assay Kit (2009-2012), Product manual, Catalogue number STA-342


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Appendix

HPLC analysis of PaΔC
Mass spectrum analysis of PaΔC
HPLC analysis of OsΔC
Mass Spectrum analysis of OsΔC