Structural and functional characterization of peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*

by

Lezaan Prinsloo

28002688

Submitted in partial fulfilment of the degree *Magister Scientiae* Biochemistry

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

South Africa

05/11/2013

Submission declaration:

I, Lezaan Prinsloo, declare that the dissertation, which I hereby submit for the degree *Magister Scientiae* in the Department of Biochemistry, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:.....

DATE:.....06/02/2014.....

Plagiarism statement

UNIVERSITY OF PRETORIA
FACULTY OF NATURAL AND AGRICULTURAL SCIENCES
DEPARTMENT OF BIOCHEMISTRY

Full name: Lezaan Prinsloo Student number: 28002688

Title of the work: Structural and functional characterization of peptides derived from the carboxy-terminal of a defensin from the tick *Ornithodoros savignyi*

Declaration

1. I understand what plagiarism entails and am aware of the University's policy in this regard.
2. I declare that this dissertation (e.g. essay, report, project, assignment, dissertation, thesis etc) is my own, original work. Where someone else's work was used (whether from a printed source, the internet or any other source) due acknowledgement was given and reference was made according to departmental requirements.
3. I did not make use of another student's previous work and submit it as my own.
4. I did not allow and will not allow anyone to copy my work with the intention of presenting it as his or her own work.

Signature _____ Date 06/02/2014

CONTENTS

ACKNOWLEDGEMENTS

I am grateful to the following people and institutions without which I would not have been able to complete my MSc degree:

- My supervisor, Dr. A.R.M. Gaspar, Department of Biochemistry, University of Pretoria, for her guidance, constructive criticism, encouragement and willingness to discuss any query;
- Prof. A.W.H. Neitz, Department of Biochemistry, University of Pretoria, my co-supervisor for his support, helpful suggestions and inspirations;
- Prof. M.J. Bester, Department of Anatomy, University of Pretoria, for co-supervising this project, for guidance in innovative topics and her never-ending optimism;
- Prof. Y. Sayed, Protein Structure-Function Research Unit, School of Molecular and Cell Biology, University of Witwatersrand, for his guidance in circular dichroism spectroscopy;
- The University of Stellenbosch for their generous donation of the *S. aureus* (U3300) and *M. luteus* (NCTC 8340) bacterial strains;
- The University of Kwazulu Natal for their generous donation of the *S. aureus* (3775) and bacterial strain;
- Dr M. Beukes, Department of Biochemistry, University of Pretoria, for his generous donation of the *M. luteus* (10240) and *P. aeruginosa* (10145) bacterial strains;
- June Serem, Department of Anatomy, University of Pretoria, for her assistance in cytotoxicity, hemolysis and statistical analysis;
- Mrs. S. van Wyngaard, Department of Biochemistry, University of Pretoria, for her hard work and dedication in this department;
- My friends, family and fellow students for their continual support throughout my studies. Thank you for making the difficult times bearable;
- The National Research foundation, the Medical Research Council and the University of Pretoria for their financial support.

SUMMARY

In this study the structural characteristics and antibacterial activities of two peptides derived from the carboxy-terminal of a tick defensin were investigated. Two defensin isoforms (OsDef1 and OsDef2) were previously identified in the midgut of the tick, *Ornithodoros savignyi*. Both OsDef1 and OsDef2 were found to be active against Gram-positive bacteria but showed no antibacterial activity against Gram-negative bacteria. OsDef2 was found to be slightly more active than OsDef1 and was, therefore, used as the template for the design of smaller antimicrobial peptides. Two peptide analogues were synthesised using the carboxy-terminal sequence of OsDef2 and differed in that in the one peptide the cysteine residues were present (Os) and in the other the cysteine residues were omitted (Os-C). Structurally, Os contained more α-helical properties than Os-C, whereas Os-C was more β-sheeted when prepared in 25 mM SDS. Both Os and Os-C showed no antibacterial activity when tested in Luria-Bertani broth or Mueller-Hinton broth indicating that the activities of Os and Os-C were influenced by the presence of broth salts and proteins. When tested in sodium phosphate buffer, both Os and Os-C exhibited Gram-positive and Gram-negative antibacterial activity. Os was slightly more active than Os-C against 3 of the 4 tested strains, with minimum bactericidal concentrations (MBCs) ranging from 0.94 µg/ml to 3.75 µg/ml. Os retained bactericidal activity against both *Staphylococcus aureus* and *Escherichia coli* when tested in the presence of 100 mM NaCl or 30% human serum. Os-C retained activity against *E. coli* in the presence of NaCl but became inactive in 30% human serum against both bacterial strains. At the MBCs, Os exhibited faster killing kinetics than Os-C killing both *Bacillus subtilis* and *E. coli* within 5 min, whereas Os-C took up to 120 min and 60 min, respectively. SYTOX Green permeabilization assays showed that both Os and Os-C caused permeabilization of *E. coli* membranes after 30 min exposure. At high peptide concentrations, both Os and Os-C were shown to interact with plasmid DNA. Both Os and Os-C exhibited no cytotoxic effects against SC-1 and Caco-2 cell lines, even at peptide concentrations 32 times higher than the highest MBC.

LIST OF ABBREVIATIONS

9Pbw2	:	9-mer peptide analogue from a defensin isolated from *Protaetia brevitarsis*
AAPH	:	2,2'- azobis-2-methyl-propanimidamide,dihydrochloride
AMPs	:	Antimicrobial peptides
Caco-2	:	Human colon cancer cell line
CD	:	Circular dichroism
CFU	:	Colony forming unit
DMEM	:	Dulbecco's modified Eagle medium
DTT	:	Dithiothreitol
EDTA	:	Ethylenediaminetetraacetic acid
EMEM	:	Eagle's minimum essential medium
EtBr	:	Ethidium bromide
FCS	:	Foetal calf serum
HDPs	:	Host defence peptides
LB broth	:	Luria-Bertani broth
LPS	:	Lipopolysaccharides
MBC	:	Minimum bactericidal concentration
MH	:	Mueller-Hinton broth
MIC	:	Minimum inhibitory concentration
MRSA	:	Methicillin resistant *Staphylococcus areus*
MS	:	Mass spectrometry
NaP	:	Sodium phosphate buffer (10 mM, pH 7.4)
OD_{600}	:	Optical density measured at 600 nm

OmC	:	Defensin isoform C of *Ornithodoros moubata*
OMΔC	:	Defensin analogue of *Ornithodoros moubata* in which cysteine residues were omitted
Os	:	Synthetic peptide derived from the carboxy-terminal of *Ornithodoros savignyi* defensin isoform 2 with cysteine residues present
Os-C	:	Synthetic peptide derived from the carboxy-terminal of *Ornithodoros savignyi* defensin isoform 2 with cysteine residues omitted
OsDef1	:	*Ornithodoros savignyi* defensin isoform 1
OsDef2	:	*Ornithodoros savignyi* defensin isoform 2
P4	:	Longicin derivative
SC-1	:	Mouse embryonic fibroblasts cell line
SDS	:	Sodium dodecyl sulphate
SEM	:	Standard error of the mean
TFE	:	Trifluoroethanol

LIST OF FIGURES

Page

LIST OF TABLES

Page

Chapter 1: Introduction

Chapter 2: Materials and methods

Chapter 3: Results

CHAPTER 1: INTRODUCTION

1.1 Rational of study

The immune system of all multicellular organisms consists of innate and, in vertebrates, adaptive immune responses (Campbell & Reece, 2005). Innate immune responses include the release of peptides referred to as host defence peptides (HDPs) into and around the infected area (Brown & Hancock, 2006). Antimicrobial peptides (AMPs) are HDPs that play a major role in the defence against pathogens (Campbell & Reece, 2005). Although capable of protecting the host from various pathogens this protection by the immune system is sometimes inadequate, resulting in the onset of disease such as infection. In these instances additional measures, such as the use of antibiotics, are required. The frequent use and misuse of antibiotics to treat bacterial infections are causing an increase in bacterial resistance. A few examples of antibiotic resistant bacteria are strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* that are resistant towards several conventional antibiotics (Allahverdiyev *et al*., 2013 & Sievert *et al*., 2013). Sievert *et al*. (2013) reported that for the period of 2009 to 2010, 15.6%, 11.5% and 7.5% of all hospital acquired infections were caused by antibiotic resistant *S. aureus*, *E. coli* and *P. aeruginosa* strains, respectively. This resistance of bacteria towards currently available antibiotics is fuelling the need for the discovery of new antibacterial agents. Antimicrobial peptides (AMPs) are found in vertebrates, invertebrates and in plants (Kindt *et al*., 2007) and these peptides show promise as antibacterial agents. AMPs have been reported to exhibit a different mode of action to that of antibiotics and have shown lower incidence of bacterial resistance (Lazarev and Govorun, 2010). Various AMPs and their derivatives have already been subjected to clinical trials, some showing success (Lazarev and Govorun, 2010 & Fox, 2013). Invertebrates, relying on only an innate immunity, have been shown to be a rich source of AMPs of which the defensins form a large subgroup. Various AMPs, including defensins, have been isolated from ticks as well as other cheliceriformes such as scorpions and horseshoe crabs. In this study the structural characterization and antibacterial properties of peptides derived from the carboxy-terminal of a defensin of the tick, *Ornithodoros savignyi*, were investigated.

1.2 Host immunity against invading pathogens: Adaptive and innate immune responses

The immune system is an organism's defence against pathogens without which the organism cannot survive. This defence can be categorised into two parts: adaptive and innate immune responses (Campbell & Reece, 2005). The former is a specialised form of defence for it uses

immunoglobulins to neutralise antigens to which it is primed. The adaptive immune system is slow to respond to invading pathogens, taking up to 5 days, whereas the response time of the innate immune system is mere minutes to hours (Kindt *et al.*, 2007).

Although the innate immune system is less specialised, it is the organism's primary defence against microbial pathogens and acts in response to microbes even before adaptive immunity is activated. When a pathogen enters the body, the innate immune system is immediately activated. Innate immunity can be categorised into two components, namely the humoral and cellular components. The latter comprises of specialised cells such as: macrophages, natural killers cells, monocytes, neutrophils, and dendritic cells (Janeway *et al.*, 2001). These cells have various functions in the innate immune system including processes such as phagocytosis, antigen presentation, release of cytokines and interferons, lysis of viral infected cells, and synthesis of AMPs (Kindt *et al.*, 2007). The humoral component consists of cytokines and peptides which, together with the cellular components contribute to the innate immune response.

The peptides associated with innate immunity are collectively referred to as host defence peptides (HDPs) and have been shown to play a major role in the innate immune response of the host. Several of these peptides have been studied and found to have a variety of roles in the immune response of organisms as reported in the review by Gordon & Romanowski (2005). HDPs can have a direct effect where the peptide, itself, can exhibit antimicrobial properties, or it can have a synergistic effect where the peptide causes the activation of other immune responses or mediate the release of other immune regulatory molecules. These peptides are referred to as HDPs because of their wide range of activities including immune suppression, immune activation and enhancement as well as direct antimicrobial activities (Brown & Hancock, 2006). However, a specific group of these peptides displays solely antimicrobial effects and will in this study be referred to as AMPs.

1.3 Antimicrobial peptides as defences in innate immunity

Several anatomical barriers form part of the innate immune response. External barriers such as the skin and mucous membranes are examples of anatomical barriers that shield the inner part of the body against pathogens. Apart from being protective shields, these barriers can synthesize AMPs and proteins, thereby producing biochemical defences (Janeway *et al.*, 2001 and Kindt *et al.*, 2007). To date there are 2271 AMPs listed in the Antimicrobial Peptide Database (available at: http://aps.unmc.edu/AP/main.php) that vary in length, secondary structure composition,

charge and activity. Generally, AMPs are cationic, small, amphipathic peptides, however, anionic peptides have also been identified (Lai *et al*., 2004). Brogden (2005) described how AMPs are grouped according to their secondary structure characteristics and amino acid composition. These categories are summarized in Table 1.1.

Table 1.1 Antimicrobial peptide categories (Adapted from Brogden, 2005)

Category	Distinguishing characteristic	Example	Reference
Cationic			
α-Helical (linear)	Lack Cys residues; rich in Lys and/or Arg	Magainin 2	Zasloff, 1987
With Cys residues	Contain 1 disulphide bond	Brevinins	Basir *et al*., 2000
	Contain 2 disulphide bonds	Protegrins	Kokryakov *et al*., 1993
	Contain 3 disulfide bonds	Human defensins	Lehrer, 2004
Rich in specific amino acids	Rich in either Trp, Pro, Arg, Phe and Gly	Indolicidins	Selsted *et al*., 1992
Anionic			
Without Cys residues	Rich in anionic amino acids (Asp and Glu)	Maximin H5	Lai *et al*., 2004
With Cys residues	Contain Cys residues; rich in Asp and Glu	SPAG11/isoform HE2C	von Horsten *et al*., 2004

Net positive charges and α-helical properties are important factors of most AMPs (Rahman *et al*, 2009), enabling these peptides to associate with the negatively charged outer membranes of microorganisms. The positive charges are due to the presence of arginine and lysine in their primary structures. Dathe *et al.* (2001) investigated the influence of charge on the antibacterial activity and hemolytic properties of magainin 2, an AMP isolated from African clawed frog skin (Zasloff, 1987). The authors synthesized magainin 2 analogues with varying amounts of lysine residues and found that the antibacterial activities of the magainin 2 analogues increased as the overall net charge was increased from +3 to +7. It was reported, however, that the hemolytic activities of the peptide analogues increased as the charge was increased above +5. Another group, Zang *et al*. (1999), investigated the effect of α-helical properties on the antibacterial and hemolytic activities of a synthetic peptide. This was achieved by varying the amount of proline residues in the amino acid sequence of this peptide, thereby introducing kinks in the secondary structure. The authors found that the parent peptide, without proline residues, exhibited the highest antibacterial activity but was also the most hemolytic. The peptide derivative that contained one proline residue showed a decrease in antibacterial activity but was less hemolytic

than the parent peptide. The double proline derivative was the least active against Gram-positive and Gram-negative bacteria but was non-hemolytic. One can, therefore, assume that the charge and α-helical property of an AMP is linked to its antimicrobial and hemolytic activity.

AMPs play an essential role in innate immunity (Hancock, 2001) and are found in diverse species including humans, frogs, insects as well as plants (Kindt *et al*., 2007). AMPs have been shown to be active against Gram-positive and Gram-negative bacteria, viruses and fungi (Reddy *et al*., 2004). Table 1.2 provides some examples of AMPs isolated from various organisms as well as the wide spectrum of antimicrobial activity of these peptides. The wide activity spectrum observed for AMPs might be due to their diverse mechanism of action. This mechanism includes, for most AMPs, the interaction of the cationic AMP with the negatively charged bacterial cell membrane.

Table 1.2 Identified antimicrobial activities of antimicrobial peptides isolated from various sources

AMP	Source	Active against	Reference
HBD-3	Human, skin	[a]G+, [b]G- and yeast	Harder *et al*., 2001
LL-37	Human	Influenza virus	Barlow *et al*., 2011
Cecropin P1	Pig, intestine	G+ and G-	Lee *et al*., 1989
Magainin 2	Frog, *Xenopus laevis*, skin	G+, G- and fungi	Zasloff, 1987
Nigrocin-2GRb	Frog, *Rana graham*, skin	G+, G- and fungi	Conlon *et al*., 2006
Mytimacin-AF	Snail, *Achatina fulica*, mucus	G+, G- and fungi	Zhong *et al*., 2013
Arasin 1	Spider crab, *Hyas araneus*, hemocytes	G+ and G-	Stensvag *et al*., 2008
Kenojeinin 1	Skate, *Raja kenojei*, fermented skin	G+, G- and yeast	Cho *et al*., 2005
Aurelin	Jellyfish, *Aurelia aurita*, mesoglea	G+ and G-	Ovchinnikova *et al*., 2006
Defensins	Tick, *Ornithodoros moubata*, hemolymph	G+	Nakajima *et al*., 2001
Ixosin	Tick, *Ixodes sinensis*, salivary glands	G+, G- and fungi	Yu *et al*., 2006
Cn-AMPs	Green coconut, *Cocos nucifera*, water	G+ and G-	Mandal *et al*., 2009

[a]Gram-positive bacteria
[b]Gram-negative bacteria

Hancock and Rozek (2001) observed that the same AMP had different antimicrobial activity against different strains of bacteria indicating that AMPs recognise specific pathogen associated molecular patterns. Different AMPs have also been shown to act differently with the same bacterial strain (Isogai *et al*., 2009) indicating that the target sites and/or mechanism of action of

diverse AMPs are different (Wu *et al*., 1999). These findings suggest that the structures of AMPs, as well as that of the bacterial cell envelopes, are important factors to consider when attempting to unravel the mode of action of AMPs.

1.4 Mode of action of cationic antimicrobial peptides

The primary target of bacteria for AMPs is the bacterial cell envelope. It has been reported that the initial attraction of cationic AMPs towards bacterial cells is of electrostatic nature. Gram-positive and Gram-negative bacteria differ in the composition of their cell envelopes (Madigan *et al*., 2009). Gram-positive bacteria contain a thick peptididoglycan layer and a plasma membrane. In contrast, the Gram-negative bacterial cell envelope contains an outer membrane, a thin peptidoglycan layer and an inner membrane (Fig. 1.1). Peptidoglycan consists of repeating units of N-acetyl glucosamine-N-acetyl muramic acid linked by pentapeptide bridges (Vollmer, 2008). The peptidoglycan layers present in Gram-positive bacterial cell envelopes are threaded with teichoic acids that consist of repeating glycerol phosphate, glycosyl phosphate or ribitol phosphate units. The outer membrane present in Gram-negative bacteria is made up of lipopolysaccharides (LPS) whereas the inner membrane, in both Gram-negative and Gram-positive bacteria, consists of phospholipid bilayer (Silhavy *et al*., 2010).

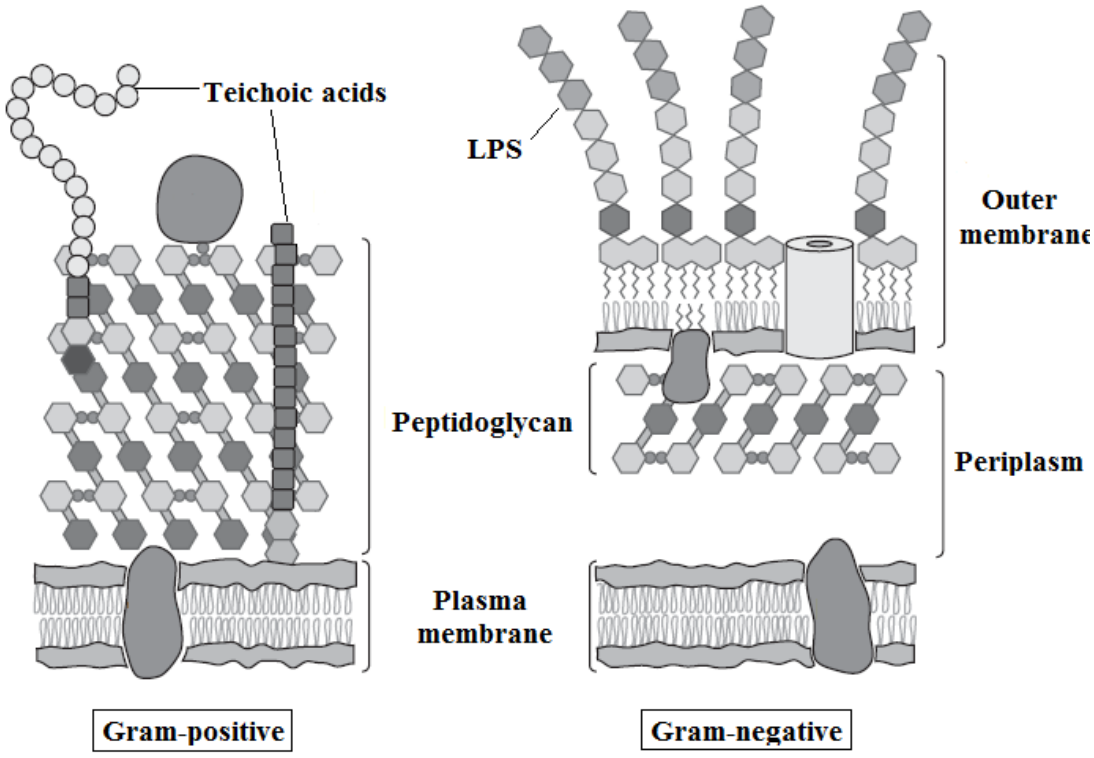


Figure 1.1: Gram-positive and Gram-negative bacterial cell envelopes. Image adapted from Silhavy *et al*., 2010.

Plasma membranes consist primarily of sphingolipids, cholesterol and phosphoglycerides. In mammals, the exoplasmic leaflet of plasma membranes consists primarily of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine (Fig. 1.2 A-C), whereas bacterial membranes consist primarily of negatively charged phosphatidylserine and phosphatidylglycerol (Fig. 1.2 D and E). Therefore, bacterial cell membranes are negatively charged at pH 7 whereas mammalian cell membranes are neutral. Brogden (2005) explained that cationic AMPs are known to be electrostatically attracted to membranes containing negatively charged phospholipids and target specifically bacterial cells.

Mammalian

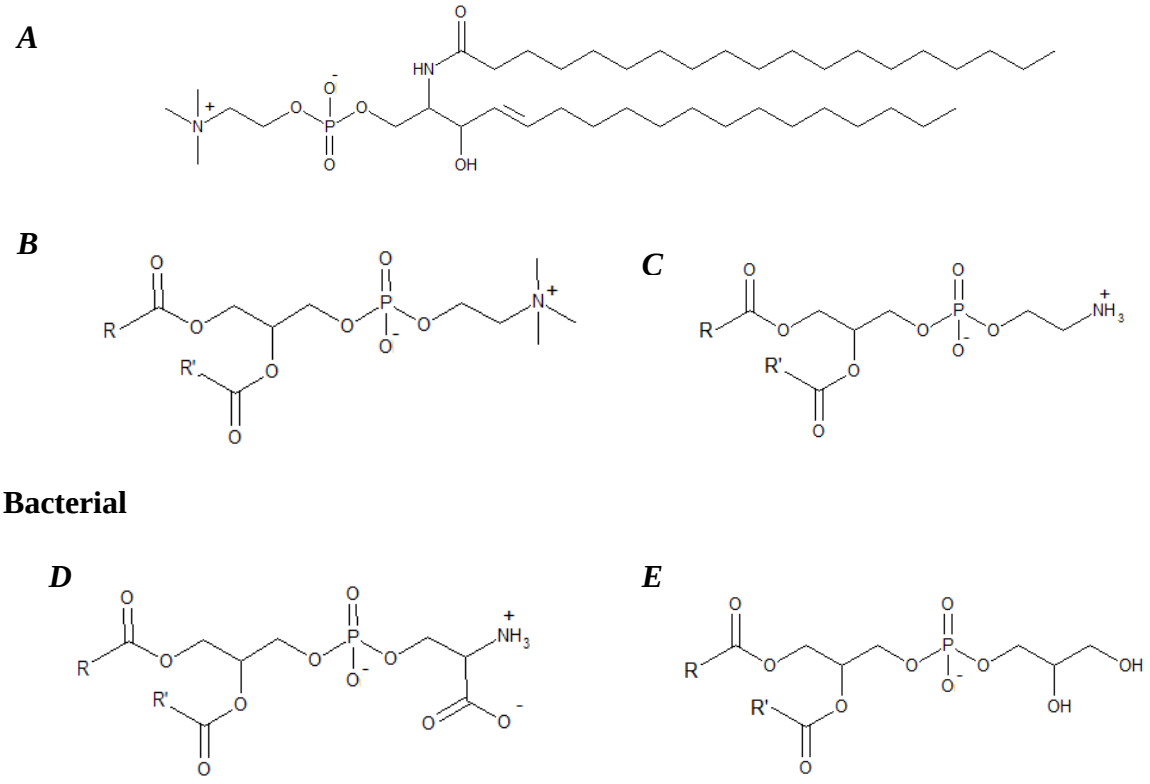


Figure 1.2: Comparison of the phospholipids found in bacterial and mammalian plasma membranes. Shingomyelin (A), phosphatidylcholine (B) and phosphatidylethanolamine are found in mammalian cell membranes whereas phosphatidylserine (D) and phosphatidylglycerol (E) are found in bacterial cell membranes. (Lodish *et al*., 2008)

The additional outer membrane is the reason why Gram-negative bacterial infections are more difficult to treat (Hancock & Rozek, 2001). However, some Gram-negative bacteria are more susceptible towards AMPs due to the self-promoted uptake system of these bacteria (Piers *et al*., 1993). Cationic AMPs have been shown to kill microbes efficiently and to play an important role

in host innate immune responses (Hancock & Rozek, 2001). These AMPs can target the outer membrane of Gram-negative bacteria, displacing the Mg^{2+} cations that stabilize the interaction between negatively charged LPS, thereby destabilizing the outer membrane. As the interaction between AMPs and the bacteria is electrostatic, excess cations may inhibit AMP activity and consequently bacterial killing. Thus, the presence of cations, especially divalent cations, might decrease the antimicrobial activity of AMPs.

Cationic amphipathic AMPs associate with the plasma membrane of both Gram-positive and Gram-negative bacteria (Brogden, 2005) where the electrostatic and hydrophobic interactions with the membrane phospholipids, and LPS in Gram-negative bacteria, cause the AMPs to be inserted into the phospholipid membrane of these microorganisms. AMPs carry out their antibacterial activities by one of various mechanisms. Electrostatic interactions enable the peptides to be inserted in a parallel position with the phospholipids, into the membrane (Matsuzaki *et al*., 1998).

There are specific steps needed for killing to occur, the first being that the AMP must be attracted towards the negatively charged membrane of the targeted microorganism. Secondly, the peptide must attach to the microorganism after which, it will be inserted into the membrane causing the membrane to become more permeable (Brogden, 2005). Permeabilization of the bacterial plasma membrane is the most prominent mode of action that is observed for AMPs (Wu *et al*., 1999). Another proposed mechanism of action of AMPs includes targeting intracellular sites that can influence bacterial protein synthesis thereby causing disruption in cellular function such as cell wall synthesis and DNA replication (Park *et al*., 1998).

Several models can be used to explain the permeabilization effect of AMPs. In the carpet model (Fig. 1.3A), peptides gather on the surface of the membrane thereby covering the membrane as a carpet and at high concentrations dissolve the membrane in a detergent-like manner forming micelles (Brogden, 2005). The toroidal pore mechanism is one in which the AMPs associate with the bacterial membranes causing the lipid polar head groups to bend with the hydrophilic parts of the AMPs resulting in aqueous pores that consist of the hydrophilic lipid heads and the AMPs (Fig. 1.3B). In the barrel-stave model the hydrophobic domain of the peptide associates with the lipid core of the membrane, causing the hydrophilic domain of the peptide to form a channel (Fig. 1.3C). The formation of trans-membrane pores or channels subsequently causes the loss of ions and, therefore, the loss of the electrochemical gradient across the membrane.

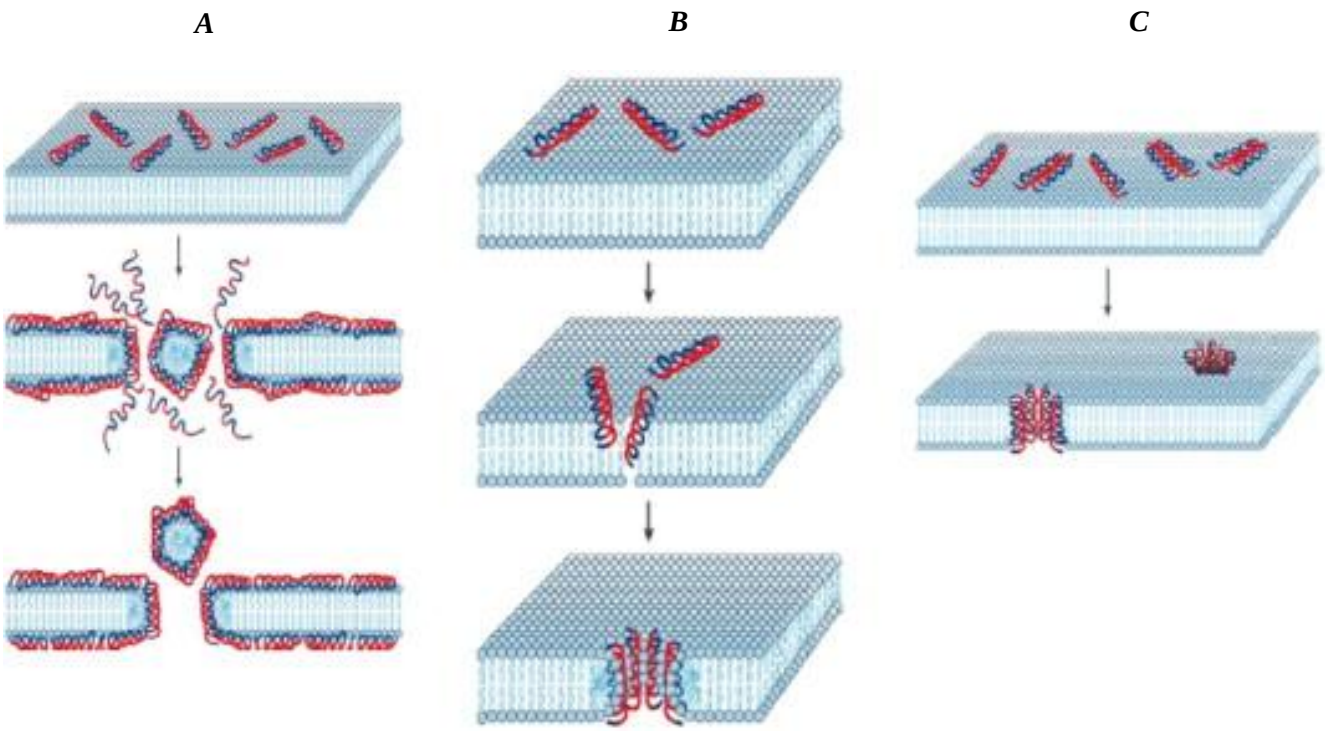


Figure 1.3: Mode of action of antimicrobial peptides. *A*) Carpet model, *B*) toroidal pore model and *C*) the barrel-stave model. (Brogden, 2005)

Conventional antibiotics target specific sites within bacteria causing bacterial death. Tetracycline, for example, binds to the small (30S) ribosomal subunit in the mRNA complex thereby inhibiting protein synthesis (Schnappinger & Hillen, 1996). Specific target site enables bacteria to become resistant toward antibiotics. Due to this diverse and non-specific mode of action, AMPs are less likely to cause bacterial resistance and are, therefore, promising candidates for new therapeutic agents against bacterial infections.

1.5 Antimicrobial peptides as potential therapeutic drugs

Various AMPs have been considered for therapeutic applications and have already entered clinical drug trials (Table 1.3). There are several advantages in using AMPs instead of antibiotics in treatment of bacterial infections, namely: (i) bacteria develop less resistance towards AMPs than towards antibiotics; (ii) AMPs are active against a larger spectrum of bacteria (both Gram-positive and Gram-negative bacteria); (iii) AMPs have shown activity in even nanomolar concentrations; and (iv) AMPs can be chemically modified in many ways (Lazarev & Govorun, 2010). Certain AMPs have even shown to have additional activities such as anti-endotoxic and/or anti-inflammatory actions (Marr *et al.*, 2006). There are, however, restrictions to using

AMPs as therapeutic agents. These restrictions include the high costs involved in the production of AMPs, the vulnerability of peptides towards proteolytic degradation as well as the loss of activity under physiological conditions (Peters *et al.*, 2010). The ability of AMPs to be chemically modified can, however, alleviate these drawbacks. By synthesizing shorter peptide fragments the cost involved would also be lowered.

Table 1.3 Sources, application and clinical trial results of tested antimicrobial peptides (Compiled from Lazarev & Govorun, 2010 and Fox, 2013)

AMP or drug	Source or characteristics	Treatment of	Clinical phase
Magainin	African clawed frog, *Xenopus laevis*, skin	Diabetic foot ulcers	3
OP-145	Derived from LL-37	Middle ear infection	2
Omiganan (MBI – 226)	Analogue of of indolicidin	Rosacea	2
Novexatin	Cyclic, cationic peptide	Fungal toenail infections	1 & 2
Lytixar	Synthetic peptide	Nasal MRSA infections	1 & 2

Various naturally occurring AMPs have been extensively studied for their antimicrobial and therapeutic potential and an important class of these is the defensins.

1.6 Defensins: a major class of antimicrobial peptides

Defensins are a major group of cationic AMPs found in a variety of organisms including humans, invertebrates and plants (Ganz, 2003 and Reddy *et al.*, 2004). These peptides are plentiful in human cells and tissues that are linked to the host's immune response (Ganz, 2003) where these AMPs act directly against invading microorganisms (Hancock, 2001). These arginine-rich peptides contain six to eight disulfide linked cysteine residues that are natively found to have an anti-parallel β-sheet (Ganz, 2003). In vertebrates there are two main subfamilies, namely α- and β-defensin, but θ-defensins have also been identified in non-human primates (Tang *et al.*, 1999). The α- and β-subfamilies contain large amounts of positively charged amino acids (lysine and arginine) and differ in cysteine linkages where α-defensins contain Cys1-Cys6, Cys2-Cys4 and Cys3-Cys5 disulfide bonds and β-defensins Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6 pairings. These different cysteine pairings lead to differences in the tertiary structures of α and β-defensins, as depicted in Fig. 1.4, however, both sub-families contain triple-stranded anti-parallel β-sheets.

The cytoplasmic membrane of invading microorganisms is a proposed target for defensins (Nakajima *et al.*, 2003). The suggested killing mechanisms of defensins include membrane permeabilization of bacteria. One method of this permeabilization of the bacterial membrane is the formation of ion channels induced by defensins (Lichtenstein, 1991; Cociancich *et al.*, 1993). The antimicrobial activity of defensins has been shown to decrease when the concentration of monovalent and divalent cations (Cociancich *et al.*, 1993) and/or when plasma proteins are increased (Ganz, 2003). The reason for this decrease may be because of the competitive displacement of defensins by cations and/or plasma proteins. Defensins are also active against various viruses (Hancock, 2001) including human immunodeficiency virus (HIV), the herpes virus, the vesicular stomatitis virus, and the influenza A virus (Zang & Hancock, 2000). These AMPs are also active against fungi, as well as protozoa (Ganz, 2003; Bulet *et al.*, 1999). For these reasons, defensins are of particular interest in antimicrobial drug discovery.

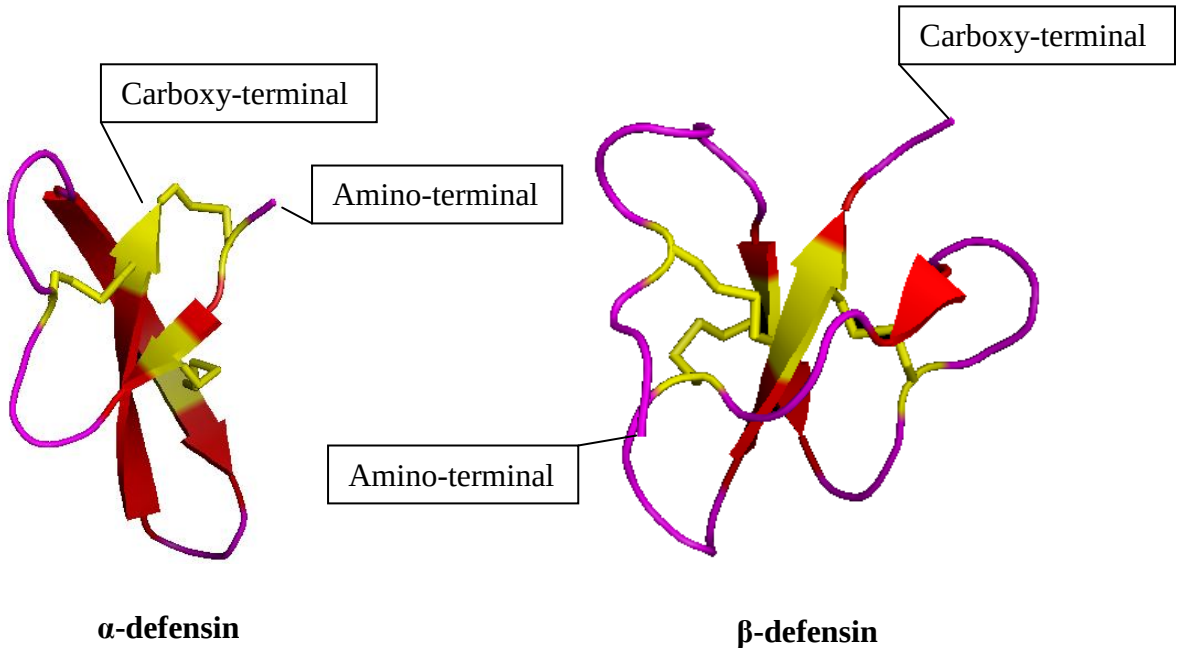


Figure 1.4: Tertiary structures of human α-defensin and β-defensin. Images obtained by using PyMOL modelling of PDB files obtained from the Protein Data Bank in Europe (available at: http://www.ebi.ac.uk/pdbe/searchResults.html?display=both&term). Loops, β-sheets and disulfide bonds are indicated in purple, red and yellow, respectively.

Defensins, as well as other AMPs, isolated from invertebrates, including arthropods and insects, have been extensively studied. Invertebrates are resilient towards most micro-organisms including bacteria and fungi (Hoffmann, 1995). The innate immune system of invertebrates is, therefore, adapted to protect the insect against various pathogens. The two major families of AMPs present in insects are cecropins and defensins (Hoffmann, 1995 and Taylor, 2006).

Cecropins are active against Gram-positive, as well as Gram-negative bacteria (Hoffmann, 1995), whereas insect defensins are more active against Gram-positive bacteria (Hoffmann & Hetru, 1992).

Invertebrate defensins are small peptides (4 kDa) and contain 3 separate domains (Fig. 1.5): an amino-terminal loop; a central amphipathic α-helix and an anti-parallel β-sheet at the carboxy-terminal (Hoffmann, 1995). The α-helix is linked to the β-sheet and the amino-terminal via disulphide bridges (Ganz, 2003; Hoffmann, 1995).

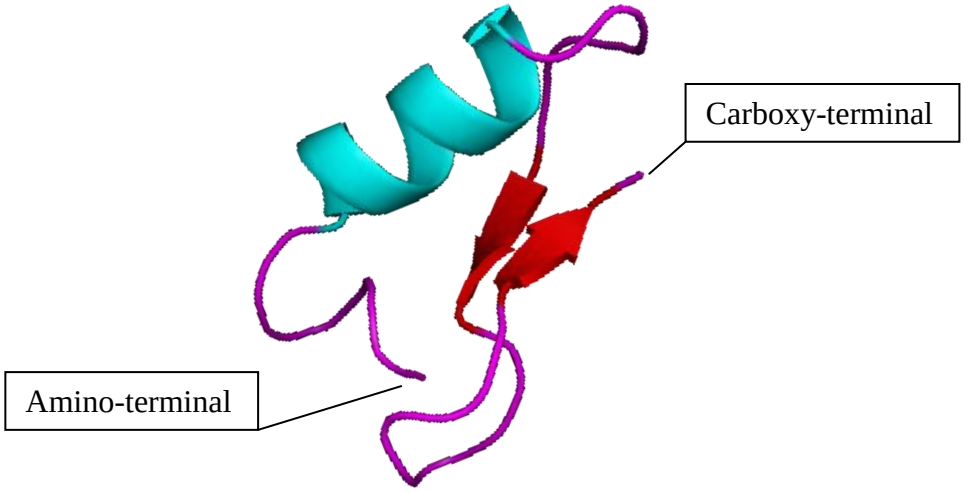


Figure 1.5: Tertiary structure domains of invertebrate defensins. Amino-terminal loop, α-helix and β-sheets indicated in purple, turquoise and red, respectively.

Invertebrate defensins contain 6 or 8 cysteine residues forming part of 3 or 4 disulphide bridges (Ganz, 2003). According to Bulet *et al.* (2004), there are two forms of cysteine pairings found within invertebrate defensins: Cys1-Cys4, Cys2-Cys5, Cys3-Cys6; and Cys1-Cys8, Cys2-Cys5, Cys3-Cys6 and Cys4-Cys7 (Fig. 1.6).

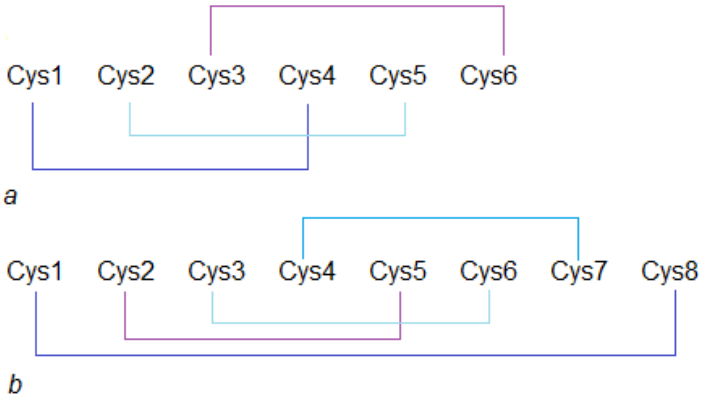


Figure 1.6: Cysteine pairing found within invertebrate defensins. Only cysteine residues are shown here for comparative purposes. Cysteine pairing of invertebrate defensin containing 6 cysteine residues (a) and 8 cysteine residues (b).

1.6.1 Tick defensins

Ticks are blood feeding external parasites of mammals, reptiles and birds. They are capable of transferring blood-borne diseases amongst various hosts without being affected themselves (Sonenshine & Hynes, 2008) due to their innate immune system. AMPs play a major role in the innate immunity of ticks and various tick AMPs have already been isolated (or synthesized) and studied (Table 1.4).

Table 1.4 Antimicrobial peptides isolated from ticks

Tick species	AMP	Source[a]	Antimicrobial activity			Reference
			G+	G-	Fungi	
Ornithodoros moubata	Defensins A-D	MG	+	-	-	Nakajima *et al*, 2001, 2002
Rhipicephalus haemaphysaloides	Ramp	SG				Zhang *et al*., 2011
Amblyomma hebraeum	Peptide 1	HL	+	+	-	Lai *et al.*, 2004
	Peptide 2	HL	+	+	-	
	Hebraein	HL	+	+	+	
Boophilus microplus	Bovine α-haemoglobin segment	MG	+	-	+	Fogaça *et al.*, 1999
	Ixodidin	HCs	+	-	-	Fogaça *et al.*, 2006
Dermacentor variabilis	Varisin	HL	+	+	-	Sonenshine *et al.*, 2002
Haemaphysalis longicornis	Defensins	MG & SG	+	+	-	Zhou *et al.*, 2007
	Longicin	MG	+	+	+	Tsuji *et al.*, 2007
	Longicornsin	SG	+	+	+	Lu *et al.*, 2010
	Defensin	AG				Zheng *et al*., 2012
Ixodes ricinus	Defensin	Whole tick	+	-	-	Rudenko *et al.*, 2007
Ixodes scapularis	ISAMP	HCs, FB, SG	+	+	-	Pichu *et al*,, 2009
Ixodes sinensis	Ixosin	SG	+	+	+	Yu *et al.*, 2006
	Ixosin	SG	+	+	+	Liu *et al.*, 2008

HL: hemolymph; MG: mid-gut; HCs: hemocytes; SG: salivary glands; FB: fat body; AG: accessory glands

Defensins are the major group of AMPs in ticks and have been found in a range of tick species (Isogai, 2009). Tick defensins are rich in cysteine and cationic residues (Sonenshine & Hynes, 2008) and primarily active against Gram-positive bacteria (Cociancich *et al.*, 1993 and Nakajima

et al., 2003). The carboxy-terminals tend to be similar, whereas the amino-terminals tend to vary greatly amongst different defensin types (Varkey *et al.*, 2006). The positive charges of tick defensins are due to the arginine and/or lysine residues located at the carboxy-terminal region. Tick defensins have been shown to be active against a large range of Gram-positive bacteria (Nakajima *et al.*, 2003; Chrudimská *et al.*, 2011) but are less active against Gram-negative bacteria and fungi (Varkey *et al.*, 2006).

1.6.2 Synthetic tick defensins and derivatives

Various studies have been focussed on synthetic tick defensins and their derivatives. For example, Chrudimská *et al*. (2011) investigated the antimicrobial and hemolytic activities of two synthetic defensin isoforms of the hard tick *Ixodes ricinus*. The authors found that both synthetic isoforms (reduced state) are active against Gram-positive bacteria but that Gram-negative bacteria, fungi and viruses are insensitive to the defensins. Another group, Nakajima *et al*. (2003) studied the antimicrobial activities and mode of action of synthetic defensin isoform A (oxidised form) of the soft tick *Ornithodoros moubata* and found that this peptide is active against only Gram-positive bacteria causing bacterial membrane permeabilization after 60 min exposure. Both these studies focussed on the full-length synthetic tick defensins and both research groups found that the synthetic peptides displayed the same antimicrobial activities than that reported for the native defensins.

Chemical synthesis of AMPs is costly and increases as the lengths of the peptides increase. Therefore, shorter AMPs are more attractive as possible therapeutic agents. Tsuji *et al*. (2007) conducted a study in which they synthesized overlapping fragments of a defensin-like peptide, longicin. The authors found that the carboxy-terminal analogue, P4, retained the bactericidal, fungicidal and parasiticidal activities. P4 consisted of 21 amino acid residues whereas the parent peptide, longicin, consisted of 52 amino acids. The authors showed that P4 had similar; if not increased, antimicrobial activities compared to longicin thereby indicating that the residues responsible for the activity of longicin are located on the carboxy-terminal of the peptide.

In a similar study, Varkey *et al*. (2006) investigated the antibacterial activities of peptides derived from the carboxy-terminal of arthropod defensins. The authors synthesized these analogue peptides omitting the cysteine residues thereby further decreasing the length of the peptides. They found that the peptide derived from the carboxy-terminal of *O. moubata* defensin isoform C (OM∆C) was more active than the parent peptide (OmC) displaying activity against

Gram-positive and Gram-negative bacteria, whilst the *O. moubata* defensin C was reported to be active against only Gram-positive bacteria (Nakajima *et al*., 2002). Overall the authors found that the antibacterial activity could be improved by synthesizing only the carboxy-terminal domain of the defensin peptide. By synthesizing the shorter peptide fragment the costs involved were lowered and by removing the cysteine residues uncertainty of the oxidation state of the cysteine residues and the possible cysteine pairing was eliminated.

1.7 Background of study

Previous research, conducted by N. Olivier during his MSc study, identified by conventional chromatography four fractions from the hemolymph of immune challenged *Ornithodoros savignyi* ticks displaying Gram-positive antibacterial activity. Edman sequencing of one of the fractions resulted in an amino-terminal amino acid sequence with high homology to scorpion defensins. Mariëtte Botha (Honours study) used the partial sequence to design primers for the cDNA cloning of the defensin from the midgut of *O. savignyi*. This led to the identification of two full-length defensin isoforms (OsDef1 and OsDef2) of 42 amino acid residues long, with the mature peptide represented by 37 of these residues. Both isoforms exhibited the cysteine residues characteristic to defensins. The amino acid sequences for the mature forms of OsDef1 and OsDef2 differ only at positions 16 and 22 (Table 3.1). In position 16, serine is present in OsDef1 and lysine in OsDef2, whereas in position 22, OsDef1 has an arginine and OsDef2 a lysine residue [N. Olivier *et al*., manuscript in preparation].

The overall objective of this study was to investigate whether the identified tick defensins could serve as templates for the design of shorter peptides exhibiting a wider spectrum of antibacterial activity. Before the shorter peptides could be tested, it was imperative to firstly confirm whether the identified defensin isoforms were indeed active. To this end both synthetic isoforms were tested for antibacterial activity. OsDef2 was chosen as the template because although both synthetic forms were found to be active against Gram-positive bacteria, OsDef2 (net charge at pH 7 of +6) was slightly more active than OsDef1 (+5). Two peptides were synthesized from the carboxy-terminal template of OsDef2. These peptide analogues differed with respect to the presence (Os) and the absence (Os-C) of the cysteine residues.

The aims were to:

1. Elucidate the secondary structures of synthetic peptides.
2. Determine the antibacterial activities of synthetic OsDef1 and OsDef2.

3. Compare the antibacterial activities of Os and Os-C.
4. Investigate the effects NaCl and human serum on the antibacterial activities of Os and Os-C.
5. Perform killing kinetics of Os and Os-C.
6. Investigate membrane permeabilization as a possible mode of action of Os and Os-C.
7. Determine whether Os and Os-C bind DNA.
8. Investigate the effect of Os and Os-C on eukaryotic cells.

1.8 Outputs

Publication:

PRINSLOO L, NAIDOO A, SEREM J, STEYN H, SAYED Y, BESTER M, NEITZ AWH & GASPAR ARM (2013). Structural and functional characterization of peptides derived from the Carboxy-terminal of the soft tick, *Ornithodoros savignyi*, defensin isoform 2. *Journal of Peptide Science* 19, 325-332.

Conferences:

PRINSLOO L, NEITZ AWH & GASPAR ARM (2012). Antibacterial properties of linear peptides derived from the c-domain of a defensin from the tick *Ornithodoros savignyi*. 23 rd South African Biochemistry and Molecular Biology (SASBMB) congress, Drakensberg, KwaZulu-Natal, 29 January -1 February 2012 (P-06, p. 122).

PRINSLOO L, NAIDOO, A, SEREM JC, TAUTE H, BESTER MJ, NEITZ AWH & GASPAR ARM (2012). Functional characterization of synthetic peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*. II International Conference on Antimicrobial Research. Lisbon, Portugal, 21-23 November 2012 (p.210)

CHAPTER 2: MATERIALS AND METHODS

2.1 Peptides and peptide preparation

During this study the structural and functional characteristics of synthetic peptides were investigated. The names, sources, sequences and uses of these peptides, as well as that of peptides used as controls, are given in table 2.1.

P4, Os and Os-C were purchased from GenScript (USA) and OsDef1, OsDef2 and 9Pbw2 were purchased from LifeTein (USA). The purity and molecular mass of the peptides were determined, by the suppliers, using reverse-phase HPLC and mass spectrometry, respectively. Melittin and magainin 2 were purchased from Sigma Aldrich (RSA). As the lyophilised peptides can contain anything between 10% and 70% bound salts and water by weight it was necessary to determine the concentrations of the peptides. The concentrations of the peptides were determined using the molar extinction coefficient of the chromophoric residues: tyrosine (1200 AU/mmole/ml) and tryptophan (5560 AU/mmole/ml), and the following equation:

$$c = \frac{A \times df \times Mr}{\sum \mathcal{E} of\ Tyr + \sum \mathcal{E} of\ Trp}$$

where *c* is the peptide concentration in mg/ml; *A* the absorbance of the peptide measured at 280 nm; *df* the dilution factor; *Mr* the relative molar mass of the peptide in g/moles and Ɛ the molar extinction coefficient in AU/mmole/ml.

Peptides were dissolved in water and gently inverted to ensure that the peptides dissolved completely. Stock samples at concentration of 1.2 mg/ml were prepared, filter sterilized (0.45 µm membrane) and stored in 20 µl aliquots, in polypropylene tubes, at -20 ºC.

2.2 Bacterial stains

Micrococcus luteus (NCTC 8340), *Staphylococcus aureus* (U3300), *Bacillus subtilis* (13933), *Escherichia coli* (ATCC 700928) and *Pseudomonas aeruginosa* (ATCC 10145) bacterial strains were used during this study. Bacteria were aerobically grown in Luria-Bertani (LB) broth (1% (*w/v*) NaCl [0.2 M], 1% (*w/v*) Tryptone, 0.5% (*w/v*) yeast, pH 7.5) or Mueller-Hinton (MH) broth (0.2% (*w/v*) beef infusion solid, 0.15% (*w/v*) starch, 1.75% (*w/v*) casein hydrolysate; pH 7.4) at 37 ºC.

Table 2.1 Peptides used in this study

Peptide name	Source	Amino acid sequence	Use	Reference
OsDef1	Defensin isoform 1 of *O. savignyi*	GYGCPFNQYQCHSHCSGIRGYRGGYCKGAFKQTCKCY	Investigation of structural and antibacterial characteristics	[N. Olivier, manuscript in preparation]
OsDef2	Defensin isoform 2 of *O. savignyi*	GYGCPFNQYQCHSHCKGIRGYKGGYCKGAFKQTCKCY	Investigation of structural and antibacterial characteristics	[N. Olivier, manuscript in preparation]
Os	Carboxy-terminal of OsDef2	KGIRGYKGGYCKGAFKQTCKCY	Investigation of structural characteristics and antibacterial properties	Prinsloo *et al*., 2013
Os-C	Carboxy-terminal of OsDef2 without Cys residues	KGIRGYKGGY-KGAFKQT-K-Y	Investigation of structural characteristics and antibacterial properties	Prinsloo *et al*., 2013
Controls				
Melittin	Honey bee venom, *Apis mellifera*	GIGAVLKVLTTGLPALISWIKRKRQQ-amide	Positive control in: • secondary structure determination, known to be α-helical; • SYTOX Green assay, known to cause membrane permeabilization; • cytotoxicity, known to be hemolytic and cytotoxic.	Vogel & Jähnig (1986); Van den Bogaart *et al*. (2008)
Magainin 2	African clawed frog	GIGKFLHSAKKFGKKAFVGEIMNS	Negative control for SYTOX Green assay, reported not to cause membrane permeabilization	Bourbon *et al*. (2008)
9Pbw2	Beetle larvae, *Protaetia brevitarsis*	RLWLAIKRR-amide	Negative control in SYTOX Green assay, reported not to cause membrane permeabilization	Shin *et al*. (2009)
P4	Defensin derivative form *H. longicornis*	SIGRRGGYCAGIIKQTCTCYR	Positive control in antibacterial assay of Os and Os-C	Tsuji *et al*. (2007)

Bacterial concentrations were determined using bacterial standard curve equations. Briefly, a single bacterial colony was inoculated in LB broth and grown overnight at 37 °C and 200 rpm. The overnight *E. coli* or *P. aeruginosa* culture was diluted (100-fold) in LB broth and grown to an OD_{600} of 0.5-0.6. The bacterial cells were washed twice and re-suspended in sodium phosphate (NaP) buffer (10 mM, pH 7.4). Two-fold serial dilutions of the bacteria were made and the OD_{600} measured. Ten-fold serial dilutions were made of the washed, re-suspended bacteria after which the 10^5 to 10^9 dilutions were plated on LB-agar plates. Plates were placed at 37 °C for 16 hrs and the formed bacterial colonies counted. These counts, together with the OD_{600} readings of the two-fold serial dilutions, were used to construct the bacterial standard curves.

The standard curves of *S. aureus* and *B. subtilis* were determined in a similar fashion to that of *E. coli* and *P. aeruginosa* with the substitution of 1% LB in NaP (*v/v*) for the washing and dilution steps.

2.3 Peptide characterization

Physicochemical properties of the peptides were obtained using ProtParam (http://web.expasy.org/protparam/). The secondary structures of the synthetic peptides were predicted using PSIPRED computer-based program (http://bioinf.cs.ucl.uk/psipred/).

2.4 Circular dichroism secondary structure determination

Circular dichroism (CD) spectroscopy was performed in order to elucidate the secondary structures of the synthetic peptides. At the far-UV region, 190-240 nm, the peptide bonds absorb polarised light. Different secondary structures give rise to the differences in the absorbance of right and left rotating polarised light. CD spectra, can therefore, be used to determine the secondary structures of peptides. Fig. 2.1 illustrates the spectra expected for peptides consisting purely of β-sheets, α-helices or random coils. By comparing the experimentally obtained CD spectra with the spectra shown in Fig. 2.1, the secondary structures of the synthetic peptides investigated in this study could possibly be elucidated. If the CD spectrum shows a high positive elipticity at 190 nm and 2 troughs (negative ellipicity) at 210 nm and 220 nm the peptide will be determined to be α-helical. A high positive ellipicity at 200 nm and a low ellipicity value at 210 nm will indicate that the peptide consists out of a β-sheet secondary structure. A large negative

elipticity value at 195 nm and positive ellipicity at wavelengths longer than 210 nm will be indicative of a random coiled secondary structure.

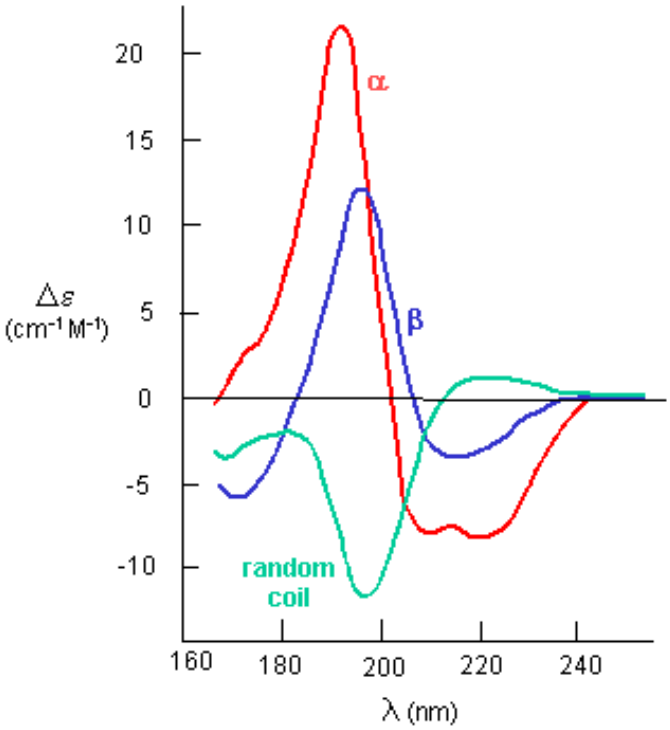


Figure 2.1: CD spectra of β-sheet (dark blue), α-helical (red) and random coiled (turquoise) secondary structures. Imaged obtained from Jakubowski, 2013.

CD spectroscopy was performed at University of Witwatersrand, Protein Structure-Function Research Unit, School of Molecular and Cell Biology. The effect of four different solvents on peptide secondary structures were determined, using a Jasco J-810 spectropolarimeter (Jasco Inc., USA), over the 180-250 nm range and a path length of 0.2 cm. The scans were carried out at 20 ºC with a scan speed of 200 nm/min, using a data pitch of 0.1 nm and at a bandwidth of 0.5 nm. Peptides were prepared in either water, NaP buffer, 50% (*v/v*) trifluoroethanol (TFE) or 25 mM sodium dodecyl sulfate (SDS) to a final peptide concentration of 50 µM. The results obtained are the average of 10 scans corrected for solvent effects. The signals were converted to mean residue ellipticity, [θ], by using the following equation:

$$[\theta] = \frac{(100 \times \theta)}{Cnl}$$

where *C* is the peptide concentration in millimolar, θ is the measured ellipticity in millidegree, n is the number of residues and l is the path length (cm). Any data acquired for the CD spectra that was greater than 800 volts was removed, due to the unreliability of data beyond that point.

This equation enabled the comparison between the secondary structures of the peptides, normalizing the CD data by factoring in the concentration and the number of residues of each peptide.

2.5 3-Dimentional structure construction

The 3-D structures of OsDef1 and OsDef2 were constructed by using the PyMOL software program. After subjecting the amino acid sequences of the peptides to Phyre[2] (Protein Homology/analogy Recognition Engine V 2.0) with intensive modelling, available at: http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index, the sequences, in .pdb format, were viewed in PYMOL and the 3-D structures obtained.

2.6 Antibacterial activity assays

2.6.1 Micro-dilution broth assay

The antibacterial activities of OsDef1, OsDef2, Os and Os-C in LB and MH broth against both Gram-positive (*S. aureus, M. luteus* and *B. subtilis*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacterial strains were determined using the micro-dilution broth assay as described by Nakajima *et al*. (2003). Bacteria were grown overnight, diluted 100 times in either LB or MH broth and proliferated until an OD_{600} of 0.5 (logarithmic phase) was reached. Bacteria were then diluted to an OD_{600} of 0.001 in either LB or MH broth. Optical density (OD) was measured using an UV-visible recording spectrophotometer (SHIMADSU, Japan) and 1cm quartz cuvette. In a 96-well micro-titre plate (with lids, Nunc, Denmark), 80 µl bacteria (OD_{600} of 0.001) were incubated with peptide (10 µl), at a final concentration range of 0.06 to 120 µg/ml, for 24 hrs at 37 ºC whilst shaking at 150 rpm. Dithiothreitol (DTT, 10 µl at a final concentration of 1 mM) was added to the serially diluted peptides for 30 min with shaking, prior to incubating the peptides with bacteria, to ensure reduction of the cysteine residues. After 24 hrs the turbidity of the samples was read, using a multi-well plate reader (Multiskan Ascent, Labsystems) at 595 nm. The peptide concentration at which the bacterial samples showed no increase in turbidity (in comparison with the growth control) was reported as the minimum inhibitory concentration (MIC). The contents of the wells at the MIC, and two concentrations higher, were plated onto LB-agar plates (1.5% (*w/v*) bacteriological agar) and incubated at 37 ºC for 14 hrs. The lowest peptide concentrations at which no colonies were observed on the plates were reported to be the

minimum bactericidal concentrations (MBCs) of the peptides. Growth controls were bacteria incubated with DTT and water instead of peptide.

2.6.2 Colony forming unit assay

The colony forming unit (CFU) assay was performed to evaluate the antibacterial activities of Os and Os-C when incubated with NaP buffer (10 mM, pH 7.4). This assay was slightly modified from the method described by Tsuji *et al*. (2007) and Varkey *et al*. (2006). Bacteria were inoculated in LB broth, grown overnight, sub-cultured in LB broth and proliferated until logarithmic growth phase was reached. The bacteria were then washed, re-suspended and diluted to 1 x 10^6 CFU/ml in NaP buffer, for Gram-negative bacteria, or in 1% LB/NaP for Gram-positive bacteria. The diluted bacteria (90 µl) were then incubated with peptide (10 µl) spanning a final concentration range of 0.06 – 120 µg/ml for 2 hrs at 37 ºC whilst shaking at 150 rpm. Samples were then diluted (500 times) in NaP buffer or LB broth, for Gram-negative and Gram-positive bacteria, respectively, and plated onto LB-agar plates. Plates were left overnight at 37 ºC. The MBCs were defined as the peptide concentrations at which 98% bactericidal activity was observed. Growth controls were bacteria incubated with water instead of peptide. The positive control for this assay was P4, the longicin derived peptide previously studied by Tsuji *et al*., 2007.

The antibacterial activity of the reduced state of Os was determined using this method. Prior to incubating *E. coli* with the peptide, 10 mM (final concentration) DTT was added to the peptide (final concentration range of 0.06 µg/ml to 120 µg/ml) for 30 min to ensure reduction of the cysteine residues. Growth controls were bacteria incubated with water and DTT (10 mM).

The effects of the presence of 100 mM NaCl in the inoculation sample on the bactericidal activities of Os and Os-C were investigated using this method. For the salt effects NaP buffer containing 110 mM NaCl (final NaCl concentration in assay of 100 mM) was used to re-suspend and dilute the washed bacteria to 1 x 10^6 CFU/ml. The growth controls were bacteria, diluted in NaP buffer containing 110 mM NaCl, incubated with 10 µl water instead of peptide.

The effect of serum on the antibacterial activity of Os and Os-C was determined in the presence of 30% human serum. Pooled lyophilized human serum (Sigma Aldrich, RSA) was re-suspended in sterile water and stored at -20 ºC. For the serum effects 30 µl of human serum was added to the incubation sample with 10 µl peptide and 60 µl bacteria (final concentration of 1 x 10^6

CFU/ml). Growth controls were bacteria incubated with 30% (final concentration) human serum and water.

To evaluate the bactericidal kinetics bacteria were prepared and diluted as for the MBC determination and incubated at 37 ºC with the synthetic peptides, at their respective MBCs, whilst shaking at 150 rpm. At specific time intervals (0, 5, 10, 30, 60 and 120 min), 10 µl of the incubation samples were diluted (500-fold) and plated on LB-agar plates. Plates were left at 37 °C for 16 hrs. Effective killing time was defined as the time after which no colonies were formed.

2.6.3 Turbidity assays

This method was used for the initial screening of antibacterial activities of the peptides as it is a high-throughput method. It was adapted from the one initially reported by Ericksen *et al*. (2005). For the initial salt effect screening bacteria were inoculated, cultured, sub-cultured, proliferated and washed as described in section 2.6.2 after which 80 µl bacterial suspension (final concentration of 1 x 10^6 CFU/ml) were incubated with 10 µl of the synthetic peptides, at the respective MBCs of the peptides, in 96-well plates. To this incubation mixture, 10 µl of either NaCl (100 mM final concentration), $MgCl_2$ (final concentration of 2 mM) or $CaCl_2$ (final concentration of 2 mM) was added. After incubating for 2 hrs at 37 °C with shaking at 150 rpm, 2X LB broth (100 µl) was added to the samples which were incubated for 5 hrs under the same conditions. Plates were read at 595 nm, using an UV-spectrophotometer multi-well plate reader (Multiskan Ascent, Labsystem), and the absorbance values compared to that of the growth controls. Results were expressed as antibacterial activity compared to the activity of the positive control (bacteria incubated with peptide without the presence of salts). Growth controls were bacteria incubated in the presence of cations without peptide. As an additional growth control, bacteria were incubated without peptides and cations, thus in the presence of only NaP buffer. Positive controls were bacteria incubated with peptide, at the respective peptide MBC, without the presence of cations.

In order to determine if the control peptides used in the SYTOX Green assay (section 2.7) were active against *E. coli*, bacteria were proliferated, washed and diluted to 1 x 10^6 CFU/ml as previously described. A volume of 90 µl bacteria was then incubated with 10 µl peptide (final concentration range of 0.06 – 120 µg/ml) for 2 hrs at 37 ºC whilst shaking at 150 rpm. Thereafter 2X LB (100 µl) was added to the incubation mixture and incubated for another 5 hrs under the

same conditions. The 96-well micro-titre plates were read at 595 nm using a UV Spectrophotometer, and the absorbance values compared to that of the growth control (no peptide added).

2.7 SYTOX Green assay

In order to investigate whether Os and Os-C permeabilize *E. coli* cells the SYTOX Green assay was used. SYTOX Green (Fig. 2.2) is a nucleic acid stain that increases in fluorescence when bound to DNA and RNA at 504 nm excitation and 524 nm emission wavelengths (Fig. 2.3). It will easily enter cells with compromised cellular membranes, but not those whose membranes are intact (Roth *et al*., 1997). Thus, the fluorescence of the dye will be indicative of the permeability of the bacterial membrane and, in effect, describe a possible mode of action of the investigated peptides.

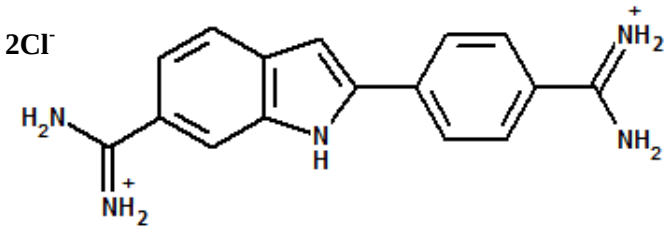


Figure 2.2: Chemical structure of SYTOX Green nucleic acid stain. Image obtained from SYTOX Green user manual.

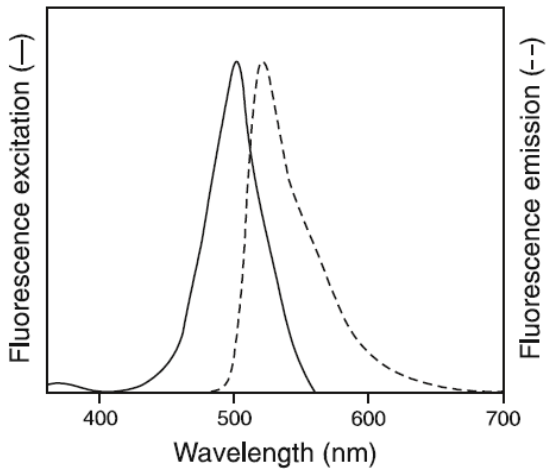


Figure 2.3: Excitation and emission wavelengths of SYTOX Green nucleic acid stain when bound to DNA. Image obtained from SYTOX Green user manual.

2.7.1 Preliminary SYTOX Green experiments

The SYTOX Green assay was adapted from the one described by Bourbon *et al*. (2008) and was performed in 96-well micro-titre plates (Nunc, Denmark). *E. coli* cells were proliferated, washed and diluted to 1.125 x 10^6 CFU/ml as previously described (section 2.6.2) and 80 µl incubated with 10 µl peptide (final concentration range of 0.06 – 120 µg/ml) and 10 µl SYTOX Green nucleic acid stain (final concentration of 5 µM, Invitrogen, USA) for 2hrs at 37 ºC whilst shaking at 150 rpm. The micro-titre plate was then read, using a filter with excitation and emission wavelengths of 483/538 nm, using a Fluoroscan Ascent FL (Thermo Labsystems). Melittin and magainin 2 were used as positive and negative controls, respectively. Basal fluorescence was obtained from bacterial cells incubated with SYTOX Green (final concentration of 5 µM) and water instead of peptide (untreated samples). Blank samples in NaP buffer were incubated with SYTOX Green and water. To establish whether the peptides contribute to the fluorescence, peptides were incubated with SYTOX Green in NaP buffer (without bacteria).

In another experiment, melittin and Os were incubated together. The experiment was performed as previously described with the exception that 70 µl bacteria (final concentration of 1 x 10^6 CFU/ml) was incubated with 10 µl Os, 10 µl melittin and 10 µl SYTOX Green dye (final concentration of 5 µM). The final concentration range of both Os and melittin was the same as the previous SYTOX Green assays.

2.7.2 Optimising the bacterial concentration for SYTOX Green fluorescence

The number of bacteria used in the previous permeabilization studies was not enough to obtain sufficient SYTOX Green fluorescence, thus the bacterial concentration was increased in order to obtain the desired fluorescence as was described by Makovitzki *et al*. (2006). In order to determine the bacterial concentration for optimal SYTOX Green fluorescence a range of bacterial concentrations was incubated with the corresponding effective peptide concentration ratios. The bacteria were prepared as described in section 2.6.2 and were serially diluted from 1.28 x 10^8 CFU/ml to 1 x 10^6 CFU/ml in NaP buffer. The peptides were also serially diluted, in water, so that the MBC ratio of each respective peptide stayed constant. Thus, if there were 128 times more bacteria, the MBC of the peptide was also increased with a factor of 128. Bacteria (90 µl) were incubated with peptide (10 µl) for 1 hr at 37 ºC whilst shaking at 150 rpm. SYTOX Green (10 µl, final concentration of 5 µM) was added to the incubated sample and the

fluorescence was measured at the same wavelength specified in section 2.7.1. Results were expressed as fluorescence ratio compared to the untreated (bacteria without peptide) samples.

2.7.3 Minimum bactericidal concentration determination of peptides in the presence of an increased bacterial concentration

The antibacterial activities of melittin, 9Pbw2, Os and Os-C, against the higher *E. coli* concentration, were determined using the turbidity assay as described in section 2.6.3. Briefly, logarithmic phase bacteria were washed, re-suspended and diluted to 6.4 x 10^7 CFU/ml in NaP buffer. The peptides were serially diluted, in water, over a final concentration range of 0.12 µg/ml to 240 µg/ml. The diluted bacteria (90 µl) were then incubated with 10 µl peptide in 96-well microtitre plates for 2 hrs at 37 ºC whilst shaking at 150 rpm. After the incubation period, 100 µl of 2X LB broth was added to the samples which were then incubated for 5 hrs. The turbidity of the samples was measured at 595 nm using a microtitre plate reader. Antibacterial activity was recorded as the peptide concentration at which no increase in the sample's turbidity was seen and was noted as the MBC.

2.7.4 Time study with SYTOX Green fluorescence

To investigate the kinetics of membrane permeabilization, *E. coli* were used as the targeted bacteria and was sub-cultured and proliferated as described in section 2.6.1. The bacteria were washed, twice, and re-suspended in NaP buffer and diluted to 6.4 x 10^7 CFU/ml (final concentration) in the same buffer. The diluted bacteria (80 µl) were added to 10 µl SYTOX Green and the basal fluorescence was measured as described previously in section 2.7.1. The addition of 10 µl peptide, at the respective MBCs, initiated the antibacterial reaction and the resulting SYTOX Green fluorescence was measured over specific time intervals. After 30 min, 50 µl EDTA (1 mM) and 50 µl Triton X-100 (0.5% *v/v*) were added to the incubated samples, in order to obtain maximum SYTOX Green fluorescence as described by Luca *et al*. (2013). EDTA (Fig. 2.4A) is a hexadentate ligand that is able to bind metal ions, thereby reducing the reactivity of the ions (Mg^{2+}) causing the membrane to become destabilized. Triton X-100 (Fig. 2.4B) is an amphipathic, non-ionic detergent that causes cellular lysis. Results were expressed as adjusted SYTOX Green fluorescence using the blank sample (SYTOX Green with NaP buffer and water) to normalise the output fluorescence.

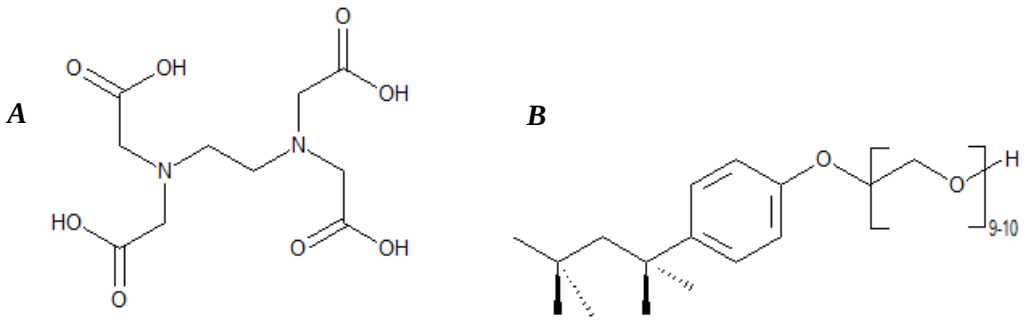


Figure 2.4: Chemical structures of *A*) EDTA and *B*) Triton X-100. Images obtained from Santa Cruz Biotechnology inc. [available at: http://www.scbt.com/]

2.7.5 Effects of EDTA and Triton X-100 addition on SYTOX Green fluorescence of Gram-positive bacterial strains

To further evaluate the effects of EDTA and Triton X-100 mediated lysis of Gram-positive bacteria, *S. aureus* and *B. subtilis* were used. In an experiment similar to that described in section 2.7.4 1 mM (final concentration) EDTA and 0.5% (*v/v*, final concentration) Triton X-100 were added to bacterial cells. Briefly, mid-log bacteria (OD_{600} of 0.6 for *S. aureus* and 0.4 for *B. subtilis*) were washed, re-suspended and diluted to 6.4 x 10^7 CFU/ml in 1% LB/NaP. SYTOX Green (10 µl, final concentration of 5 µM) was added to the diluted bacteria (90 µl) and the fluorescence measured at the same incubation time intervals as for the study conducted in section 2.7.4. After 30 min, 1 mM (final concentration) EDTA and 0.5% (*v/v*, final concentration) Triton X-100 were added to the incubated samples and the fluorescence measured for a further 10 min. Results were expressed as the adjusted SYTOX Green fluorescence by subtracting the blank (SYTOX Green with NaP buffer and water) to normalise the output fluorescence.

2.8 Plasmid binding assay

Besides causing cell wall lysis, positively charged molecules, such as cationic peptides, may, if the peptides traverse the membrane, bind to negatively charged bacterial DNA as their intracellular target thereby disrupting normal cellular function (Park *et al*., 1998). During agarose gel eletrophoresis DNA migrates from the negative pole to the positive through electrostatic attraction. If the cationic peptides bind to the negative phosphates of DNA, the migration of the DNA will be delayed. Ethidium bromide (EtBr) is a nucleic acid interchelator that fluoresces when viewed under UV light. It is used to view DNA bands on agarose gels. To investigate if Os and Os-C binds intracellular targets such as DNA, Os and Os-C were incubated with pBR322

plasmid DNA. Theoretically this can result in two effects: (i) reduced EtBr (Fig. 2.5) binding to plasmid DNA or (ii) reduced migration due to loss of negative charges. To evaluate this effect the method described by Hocquellet *et al*. (2010) was used with some modifications. Briefly, 5 µl of 50 µg/ml (final concentration) of a pBR322 vector DNA (Promega, USA) was incubated with 5 µl of peptide at a final concentration range of 0.06 µg/ml to 0.6 mg/ml (10-fold dilutions) for 2 hrs at 37 ºC. 2,2'-azobis-2-methyl-propanimidamide,dihydrochloride (AAPH, 0.003 mg/ml) was used as a positive control and DNA with only NaP buffer (10 mM, pH 7.4) used as a negative control. Equal amounts (10 µl) of gel loading buffer (0.13 % bromophenol blue and 40% sucrose) was added to the samples which were then loaded onto a 1% agarose gel (containing ethidium bromide) in TAE (4.84% (*w/v*) Tris base, 1.14% (*v/v*) acetic acid and 10 mM EDTA, pH 8.0). The gel was run for 2 hrs at 60V and 30 mAmps. Gels were viewed using a UV-trans illuminator (UVITEC, Cambridge, UK).

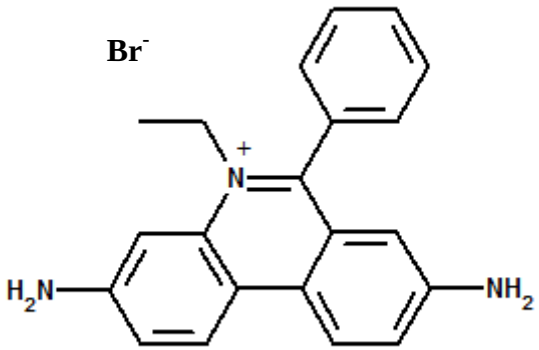


Figure 2.5: Chemical structure of ethidium bromide. Images obtained from Santa Cruz Biotechnology inc. [available at: http://www.scbt.com/].

2.9 Cytotoxicity

An ideal AMP for further therapeutic evaluation must have high antibacterial activity without being cytotoxic towards eukaryotic cells. To this end the cytotoxicity of the peptides against SC-1 (embryo, *Mus musculus*, fibroblast, doubling time 48 hrs) and Caco-2 (adult, *Homo sapiens*, epithelial, colorectal adenocarcinoma, doubling time 62 hrs) cell lines were investigated.

The cells were maintained in medium containing 10% foetal calf serum (FCS) and a 1% antibiotic solution, Eagle's minimal essential medium (EMEM/FCS) for SC-1 and Dulbecco's modified Eagle medium (DMEM/FCS) for Caco-2 cell lines. The cells were plated at 4 x 10^4 cells per ml in cells culture flasks and were maintained until confluency at 37 ºC at 5% CO_2 after

which the cells were passaged with a 5% trypsin solution prepared in 100 ml phosphate buffer saline solution. The cells were passaged by removing the medium from the confluent monolayer then adding 0.08 ml/ cm^2 of a 5% Trypsin solution before placing the flask at 37 ºC until the cells had detached. A 5 ml volume of medium was then added to the trypsin solution containing detached cells and transferred to a 15 ml centrifuge tube and the cells were collected by centrifugation at 7000xg for 2 min. The medium was removed and the cells were re-suspended in a 5 ml medium. The number of cells was determined by counting a 10 µl aliquot of cells using a haemocytometer. These cells were used for subsequent experiments or stored at -70ºC in a freezing medium at a cell concentration of 2 x 10^5 cells per ml. The freezing medium was prepared by adding 10% dimethyl sulfoxide (DMSO) and 80% FCS to EMEM or DMEM.

Crystal violet (CV) staining assay was used to determine cell number. CV is positively charged (Fig. 2.6) and binds to negatively charged DNA and proteins. Toxicity causes cell death or inhibits cell division. As a result cells can detach or growth can cease. This will cause a decrease in the extend of CV staining due to cell detachment.

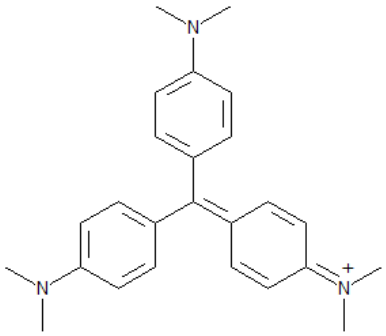


Figure 2.6: Chemical structure of crystal violet. Images obtained from Santa Cruz Biotechnology inc. [available at: http://www.scbt.com/].

To evaluate the effect of Os and Os-C on SC-1 and Caco-2 cells the method described by Chiba *et al*. (1998) was used. Briefly, cells were incubated with the synthetic peptides, at a concentration range of 1.9 to 120 µg/ml, for 24 hrs at 37 °C and 5% CO_2. Melittin, at the same concentration range, was used as a positive control. The cells were fixed for 20 min at 37 °C with the addition 10 µl of 20% (*v/v*) para-formaldehyde. The fixative and medium were removed and the plates dried. The cells were stained for 30 min at room temperature using CV dye solution (200 µl) consisting of 0.1% (*w/v*) CV in 200 mM formic acid, pH 3. After removing the dye, the cells were viewed using an Olympus IX71 inverted light microscope photos. Acetic acid (50 µl of 10% solution) was added to the cells, incubated for 30 min whilst shaking at room

temperature, to extract the dye and the absorbance of the resulting solution was measured at 630 nm using an UV spectrophotometer, (Bio Tek plate reader, Analytical and Diagnostic Products, RSA). Results were expressed as % cell number in comparison with the negative control (cells not treated with peptide).

3.10 Data and statistical analysis

All experiments were performed in duplicate or triplicate of three to four independent experiments. Data were statistically analysed using STATISTICA program. One-way ANOVA was performed, to obtain statistical significance with $p<0.05$, only when deemed necessary.

CHAPTER 3: RESULTS

3.1 Peptide characterization

The physicochemical characteristics (pI and theoretical charge) of the synthetic peptides investigated in this study were obtained by ProtParam, a computer-based program. The theoretical pI, molecular weight and the charge of the peptides are given in Table 3.1.

From Table 3.1 it is important to note that all the peptides used in this study have net positive charges ranging from +4 to +6, but vary greatly in length, molecular weight and amino acid composition. The percentage hydrophobic residues of the peptides ranged between 30% and 60%. The hydrophobic amino acids present in these peptides are: Leu, Ala, Val, Gly, Met, Pro, Ile and Phe. A Ser residue is located at the 16th amino acid position in OsDef1 whereas a Lys is located at that position in OsDef2. This substitution causes OsDef2 to have a higher net positive charge than OsDef1. The carboxy-terminal derivatives of OsDef2, Os and Os-C, contain all the positively charged amino acids from the parent peptide and have, therefore, the same net positive charge. Melittin and 9Pbw2, two control peptides, were amidated thereby increasing the net positive charge of these peptides. Melittin, Magainin 2, 9Pbw2 and Os-C lack cysteine residues. The differences in the amino acid composition between the two defensin isoforms, OsDef1 and OsDef2, are also indicated.

The secondary structures of the synthetic peptides used during this study were predicted using PSIPRED, a computer-based program. The predicted results (Table 3.2) can then be used to compare to experimental data obtained from CD spectroscopy.

Table 3.1 Physicochemical properties of synthetic peptides used in this study

Peptide	Sequence	No. of amino acids	pI	MW[c]	Charge	Hydrophobic residues (%)
Investigated peptides						
[a]OsDef1	GYGC[1]PFNQYQC[2]HSHC[3]**S**GIRGY**R**GGYC[4]KGAFKQTC[5]KC[6]Y	37	9.06	4172.7	+5	32
[a]OsDef2	GYGC[1]PFNQYQC[2]HSHC[3]**K**GIRGY**K**GGYC[4]KGAFKQTC[5]KC[6]Y	37	9.20	4185.8	+6	32
Os	KGIRGYKGGYCKGAFKQTCKCY	22	9.67	2459.9	+6	36
[b]Os-C	KGIRGYKGGY-KGAFKQT-K-Y	19	10.2	2150.5	+6	42
Control peptides						
P4	SIGRRGGYCAGIIKQTCTCYR	21	9.50	2306.7	+4	38
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-amide	26	12.0	2847.4	+6	58
Magainin 2	GIGKFLHSAKKFGKKAFVGEIMNS	24	10.2	2595.1	+4	54
9Pbw2	RLWLAIKRR-amide	9	12.3	1211.2	+5	44

[a]Bold residues indicate the differences in the amino acid composition of isoforms OsDef1 and OsDef2, numbers indicate cysteine allocation
[b]The dashes indicate the omitted cysteine residues
[c]Molecular weight (Da) obtained from suppliers by MS analysis

Table 3.2 Predicted secondary structures of synthetic peptides

	Secondary structure constituent		
Peptide	**% α- Helix**	**% β-Strand**	**% Coil**
Melittin	88	0	12
9Pbw2	[a]nd	nd	nd
P4	0	62	38
OsDef1	19	19	62
OsDef2	22	16	62
Os	32	0	68
Os-C	0	21	79

[a]not determined; sequence is too short to be processed using PSIPRED

Melittin is reported to be predominantly α-helical (Vogel & Jähnig, 1986). This is confirmed in this study where melittin was predicted to be 88% α-helical (Table 3.2). OsDef1 and OsDef2 were predicted, due to structural similarities, to have similar secondary structures, both predominantly coiled with some α-helical and β-strand properties. It was predicted that Os and Os-C mostly consists of random coils and P4 of β-strands. When comparing Os with Os-C it can be seen that the former contains helical properties whereas Os-C has no helical structures but, however, has some β-strand properties (Table 3.2). Therefore, a difference between the CD spectra of the 3 peptides is expected. The reliability of these predictions is given in Fig. 3.1.

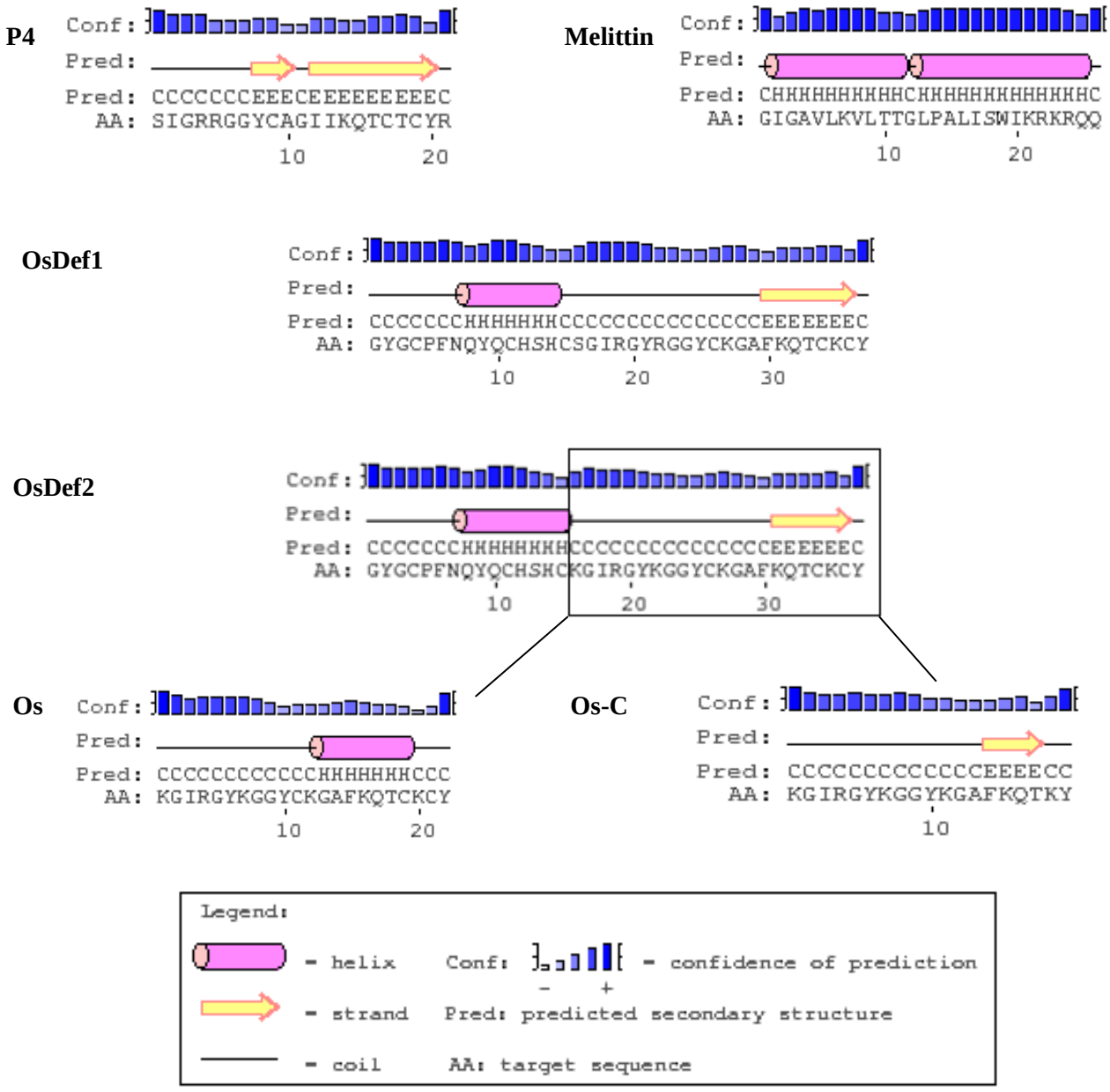


Figure 3.1: PSIPRED predictions of the secondary structures of the peptides. The images obtained from the PSIPRED predictions of the peptides are given indicating the amino acids that form part of the predicted α-helix (H), β-sheet (E) and coil (C) structure as well as the confidence level of this prediction. The boxed section of OsDef2 indicates sequence from which Os and Os-C were derived.

The confidence level of α-helical prediction for melittin is very high (Fig. 3.1) indicating that the prediction for melittin, is reliable. The α-helical property prediction for OsDef1 and OsDef2 was slightly more reliable than that of the β-sheet prediction. It should be noted, however, that the confidence levels of the prediction of the helical content for Os and the β-strand content for Os-C (Fig. 3.1) were very low, making the predictions in itself not reliable enough to draw conclusions on the secondary structures of the peptides. Therefore, CD spectroscopy was performed to elucidate the secondary structures of the peptides.

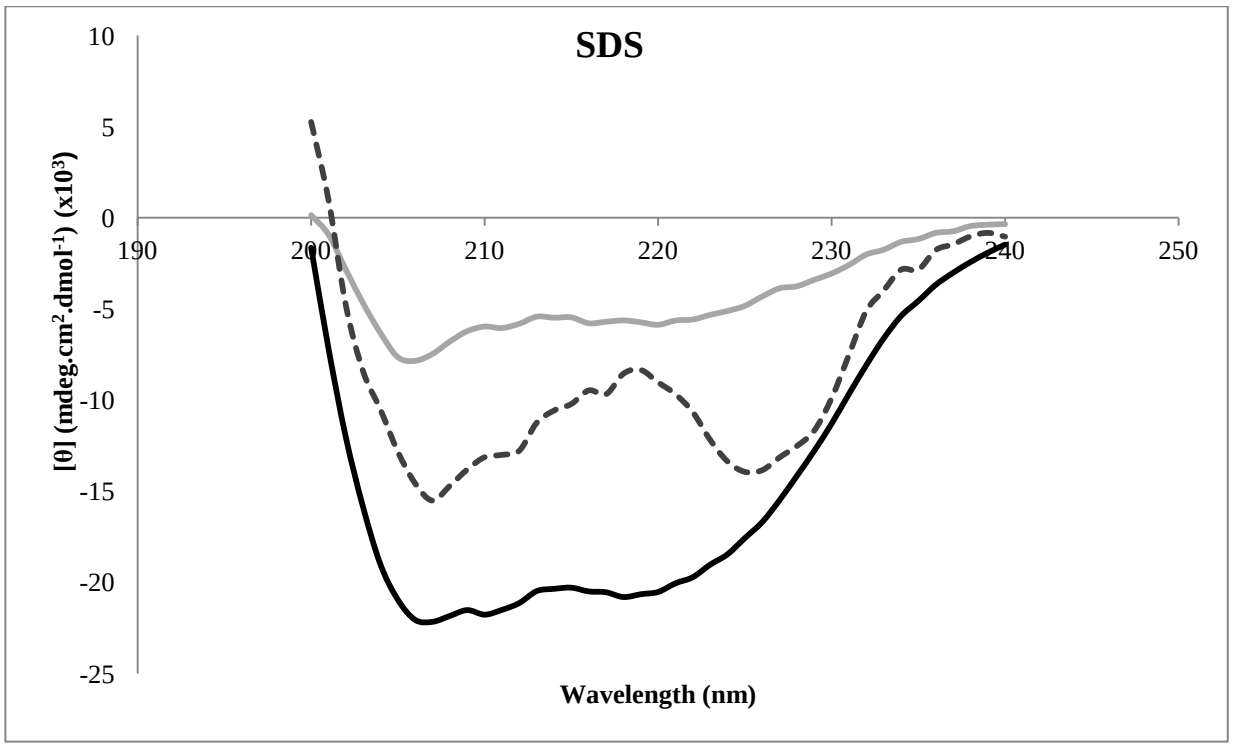
Since the secondary structures of proteins and peptides are dependent on the environment, the peptides were prepared in water and 25 mM SDS. The CD spectra of the peptides were determined in water as this is the medium in which the peptides were prepared. SDS was used as a membrane mimicking solution by forming micelles at concentrations higher than 9.5 mM (Khan & Shah, 2007).

The CD spectrum of P4 in SDS was similar to that reported by Rahman *et al*. (2009) who observed that P4 contains α-helical properties. Melittin had CD spectra distinctive of that of α-helical secondary structures when prepared in SDS and TFE (not shown). 9Pbw2 showed α-helical properties (Fig. 3.2a) in SDS which was also seen by Shin *et al*. (2009). When prepared in SDS Os and Os(red) had more α-helical properties than Os-C whereas in water Os, Os(red) and Os-C had no defined secondary structures (Fig. 3.2b).

3.2 3-Dimentional structure modelling

Homology searches of OsDef1 and OsDef2 were performed using Position-Specific Iterated BLAST (PSI-BLAST) from the NCBI website [available at: http://blast.ncbi.nlm.nih.gov/Blast.cgi]. From this search a fungal defensin, c21r5A, was identified as the most similar to OsDef1 and OsDef2 with 99.9% confidence. The sequences of OsDef1 and OsDef2 were processed by the Phyre^2 interactive website. The .pdb files obtained for the defensins were used in PyMOL computer-based program to obtain the 3-D structures (Fig. 3.3).

From Fig. 3.3 it is evident that both OsDef1 and OsDef2 contain an α-helix near the amino-terminal and an anti-parallel β-sheet at the carboxy-terminal. These secondary structures are stabilized by three disulfide linkages (Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6).



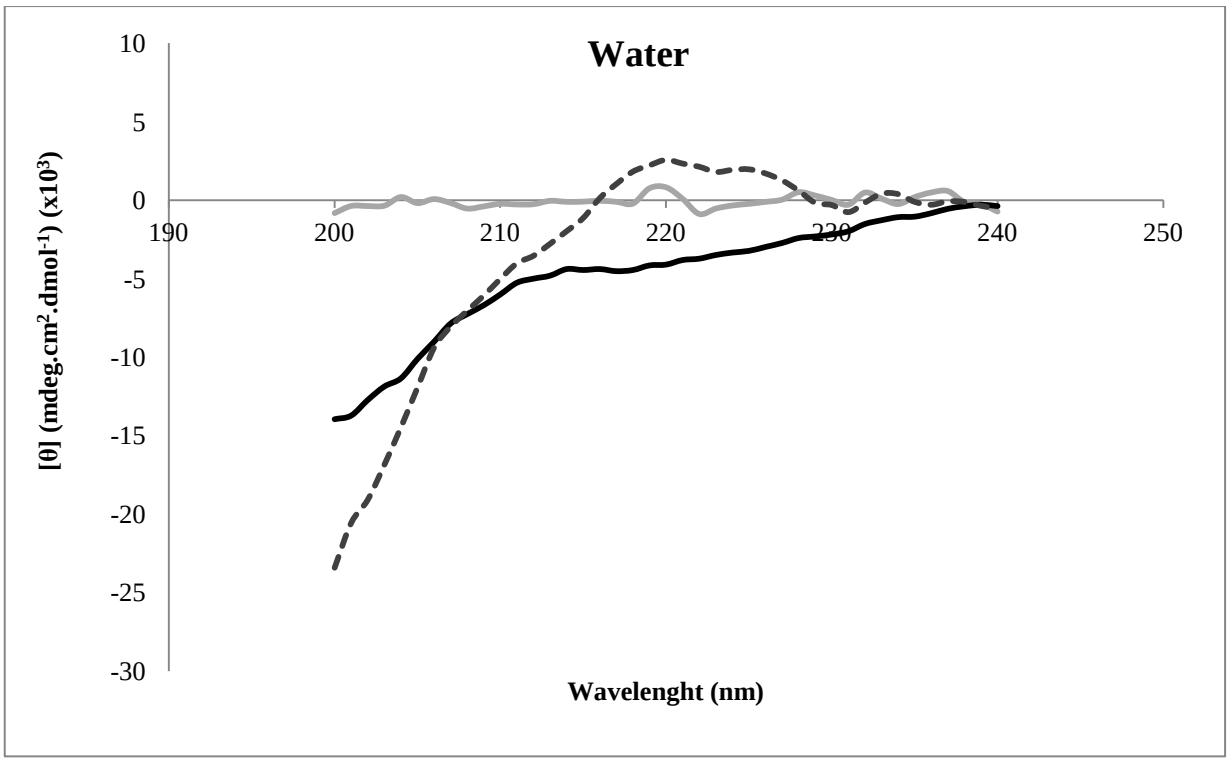


Figure 3.2a: Circular dichroism spectra of three control peptides; melittin (black curve), P4 (solid grey curve) and 9Pbw2 (dashed curve) prepared in 25 mM SDS and water. Data represented as mean ellipicity as a function of wavelength (nm). The spectra spanned from 200 nm to 240 nm because of voltage background noise at wavelengths lower than 200 nm. Data represented here is the average of 10 scans.

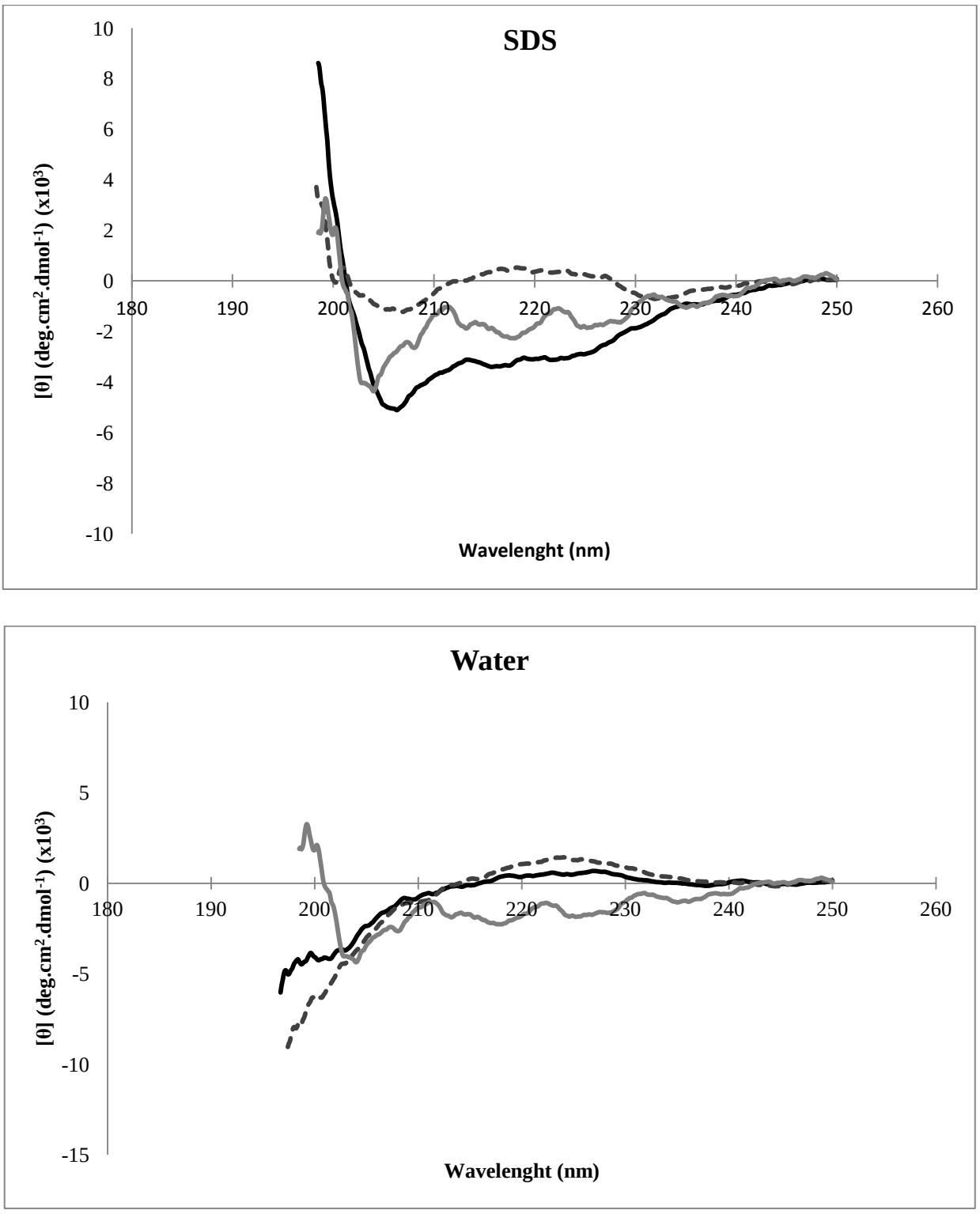
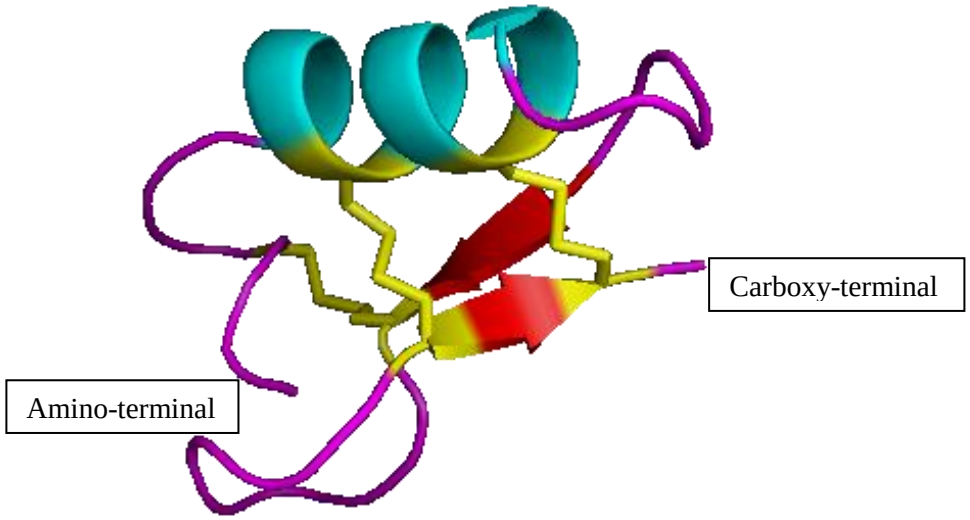


Figure 3.2b: Circular dichroism of spectra synthetic peptides: Os (black curve), Os(red) (solid grey curve) and Os-C (dashed grey curve) prepared in 25 mM SDS and water. Data represented as mean ellipicity as a function of wavelength (nm). The spectra spanned from 200 nm to 240 nm because of voltage background noise at wavelengths lower than 200 nm. Data represented here is the average of 10 scans.

OsDef1



OsDef2

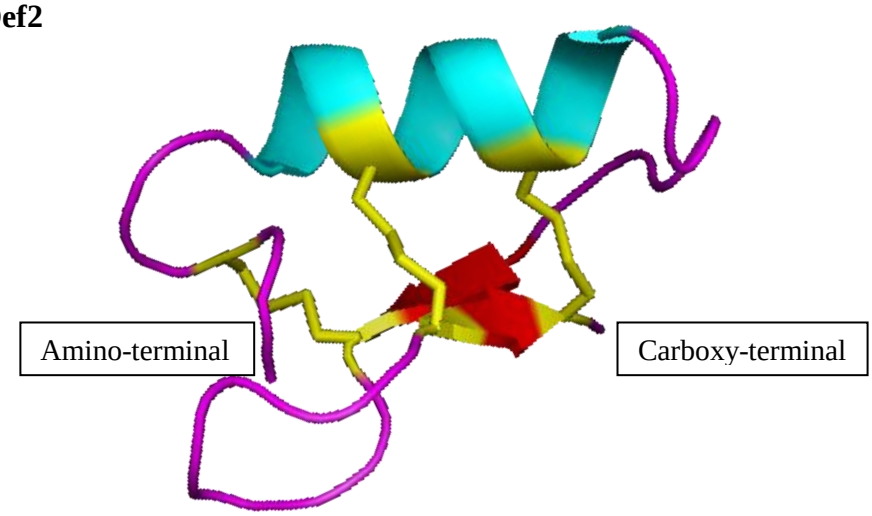


Figure 3.3: Tertiary structures of OsDef1 and OsDef2. Image was obtained by using PyMOL computer based programme. The yellow stick-like structures represent the disulfide linkages found in OsDef1 and OsDef2. The α-helical and β-sheet secondary structures are shown in turquoise and red, respectively. The random coiled structures are indicated in purple.

3.3 Antibacterial activities of synthetic peptides

3.3.1 Determination of minimum inhibitory and minimum bactericidal concentrations

The MICs and MBCs for both defensin isoforms were determined for selected Gram-positive and Gram-negative bacteria. The micro-dilution assay was performed in LB broth (Table 3.3a) and MH broth (Table 3.3b).

Table 3.3a Antibacterial activity of OsDef1 and OsDef2 in Luria-Bertani broth

	Peptide concentration (µg/ml)			
	OsDef1		OsDef2	
Bacterial strains	**MIC**	**MBC**	**MIC**	**MBC**
Gram-positive				
B. subtilis	0.94	0.94	0.24	0.47
	(0.23)	(0.23)	(0.06)	(0.11)
M. luteus	0.94	1.9	0.94	1.9
	(0.23)	(0.46)	(0.22)	(0.45)
S. aureus	>120	>120	>120	>120
	(>28.8)	(>28.8)	(>28.7)	(>28.7)
Gram-negative				
E. coli	>120	>120	>120	>120
	(>28.8)	(>28.8)	(>28.7)	(>28.7)
P. aeruginosa	>120	>120	>120	>120
	(>28.8)	(>28.8)	(>28.7)	(>28.7)

MICs and MBCs represent results from three independent experiments
Values given in parentheses are peptide concentrations expressed in µM

Table 3.3b Minimum inhibitory concentrations of OsDef1 and OsDef2 in Mueller-Hinton broth

	Peptide concentration (µg/ml)	
	OsDef1	OsDef2
Bacterial strains	**MIC**	**MIC**
Gram-positive		
B. subtilis	0.94	0.47
	(0.23)	(0.11)
S. aureus	15.0	0.94
	(3.59)	(0.22)
M. luteus	n.d[a]	n.d[a]
Gram-negative		
E. coli	>120	>120
	(>28.7)	(>28.7)
P. aeruginosa	>120	>120
	(>28.7)	(>28.7)

[a]not determined
MICs represent results from two independent experiments
Values given in parentheses are peptide concentrations expressed in µM

OsDef1 and OsDef2 showed no antibacterial activity against Gram-negative bacteria, even at 120 µg/ml, but were found to be active against *B. subtilis* and *M. luteus* (Gram-positive) at very low peptide concentrations, with MICs ranging from 0.24 µg/ml to 0.94 µg/ml.

In LB broth OsDef1 and OsDef2 showed no antibacterial activity against *S. aureus* with MIC >120 µg/ml. In contrast in MH broth (Table 3.3b), the MIC of OsDef1 and OsDef2 for *S. aureus* decreased to 15 µg/ml and 0.94 µg/ml, respectively. For *B. subtilis*, both OsDef1 and OsDef2 showed similar antibacterial activity in LB broth and MH broth. Antibacterial activity against Gram-negative bacteria did not change when tested in MH broth.

The micro-dilution broth assay was also used to evaluate the antibacterial activity of the carboxy-terminal analogues Os and Os-C. This assay was performed with bacteria prepared in either LB broth or MH broth. Os was only active against *B. subtilis* in MH broth at 60 µg/ml. Os and Os-C showed no further antibacterial activity against either Gram-positive and Gram-negative bacteria, when tested in both broths (Table 3.4).

Table 3.4 Minimum inhibitory concentration of Os and Os-C in broth

	MIC (µg/ml)			
	LB broth		**MH broth**	
Bacterial strains	**Os**	**Os-C**	**Os**	**Os-C**
Gram-positive				
B. subtilis	>120 (>48.8)	>120 (>55.8)	60 (24.4)	>120 (>55.8)
Gram-negative				
E. coli	>120 (>48.8)	>120 (>55.8)	>120 (>48.8)	>120 (>55.8)

MICs represents results of two independent experiments
Values given in parentheses are peptide concentration expressed in µM

3.3.2 Determinations of minimum bactericidal concentrations by the CFU method

Since no or little antibacterial activity for Os and Os-C was observed in the presence of LB or MH broth, the CFU method was performed in order to further investigate the possible bactericidal activities of the synthetic peptides. As explained in the method section, this assay was performed in NaP buffer (10 mM, pH 7.4), thus the effect of additional salts, proteins and other broth components on the bactericidal activity of the peptides was eliminated. P4, previously investigated by Tsuji *et al* (2007) also in NaP buffer, was used as a positive control to evaluate the validity of this method. The MBC values determined for Os, Os-C and P4 against Gram-positive and Gram-negative bacteria are given in Table 3.5.

Potent bactericidal activity was detected for these peptides, with MBC values ranging from 0.94 – 15 µg/ml for Os and 1.88 - 15 µg/ml for Os-C against Gram-positive and Gram-negative bacteria. (Table 3.6). The cysteine-containing peptide, Os, was found to be two-fold more active

than its Os-C counterpart against three of the four tested bacteria, but equally active against *S. aureus*. Both peptides were found to be most active against *P. aeruginosa*, with MBC values of 0.94 µg/ml and 1.88 µg/ml for Os and Os-C, respectively. Os and Os-C were found to be more active than the P4 control.

Table 3.5 Minimum bactericidal concentrations of Os, Os-C and P4 in NaP buffer

	MBC (µg/ml)		
Bacterial strains	**Os**	**Os-C**	**P4**
Gram-positive			
S. aureus	15.0 (6.10)	15.0 (6.98)	120 (52.0)
B. subtilis	1.88 (0.76)	3.75 (1.74)	7.50 (3.25)
Gram-negative			
E. coli	1.88 (0.76)	3.75 (1.74)	7.50 (3.25)
P. aeruginosa	0.94 (0.38)	1.88 (0.87)	15.0 (6.50)

MBCs represents results of three independent experiments using the CFU method
Values given in parentheses are peptide concentration expressed in µM

To summarize, OsDef1 and OsDef2 are only active against Gram-positive bacteria with OsDef2 slightly more active than OsDef1. The carboxy-terminal derivatives of OsDef2 are active against both Gram-positive and Gram-negative bacteria with Os slightly more active than Os-C.

Since the synthetic peptide, Os, contains three cysteine residues, there is a possibility that the peptide undergoes oxidation once in solution. In order to investigate this, the peptide was reduced by incubating 10 mM (final concentration) DTT with the serially diluted peptide for 30 min prior to performing the antibacterial assay. It was found that pre-incubation of the peptide with DTT decreased the MBC for *E. coli* from 1.94 µg/ml (Table 2.4) to 0.94 µg/ml (Table 3.6).

Table 3.6 Minimum bactericidal concentrations of Os in the presence of DTT

	MBC (µg/ml)			
Bacteria	**Os (red)**	**SD**	**Os**	**SD**
E. coli	0.94 (0.38)	0.42	1.88 (0.76)	0.0

MBCs represents results from at least two independent experiments
Values given in parentheses are peptide concentration expressed in µM
SD is the standard deviation from 2 independent experiments

3.4 Effects of salt and serum on antibacterial activity

The presence of monovalent and divalent cations, as in an *in vivo* environment, has been shown to decrease the antibacterial activities of AMPs (Wu *et al*., 2008). In addition this may also account for the lack of antibacterial activity observed for Os and Os-C in LB and MH broth. The effect of the presence of salts in the incubation mixture on the antibacterial activities of the synthetic peptides, at their respective MBCs, against a typical Gram-positive and Gram-negative bacterial strain was first investigated using the turbidity assay. During this assay the peptides were incubated with bacteria in NaP buffer in the presence of cations. The surviving bacteria were then re-proliferated upon the addition of 2X LB broth and incubation for 5 hrs. Results were expressed relative to the control (no cations added) as percentage antibacterial activity (Fig. 3.4).

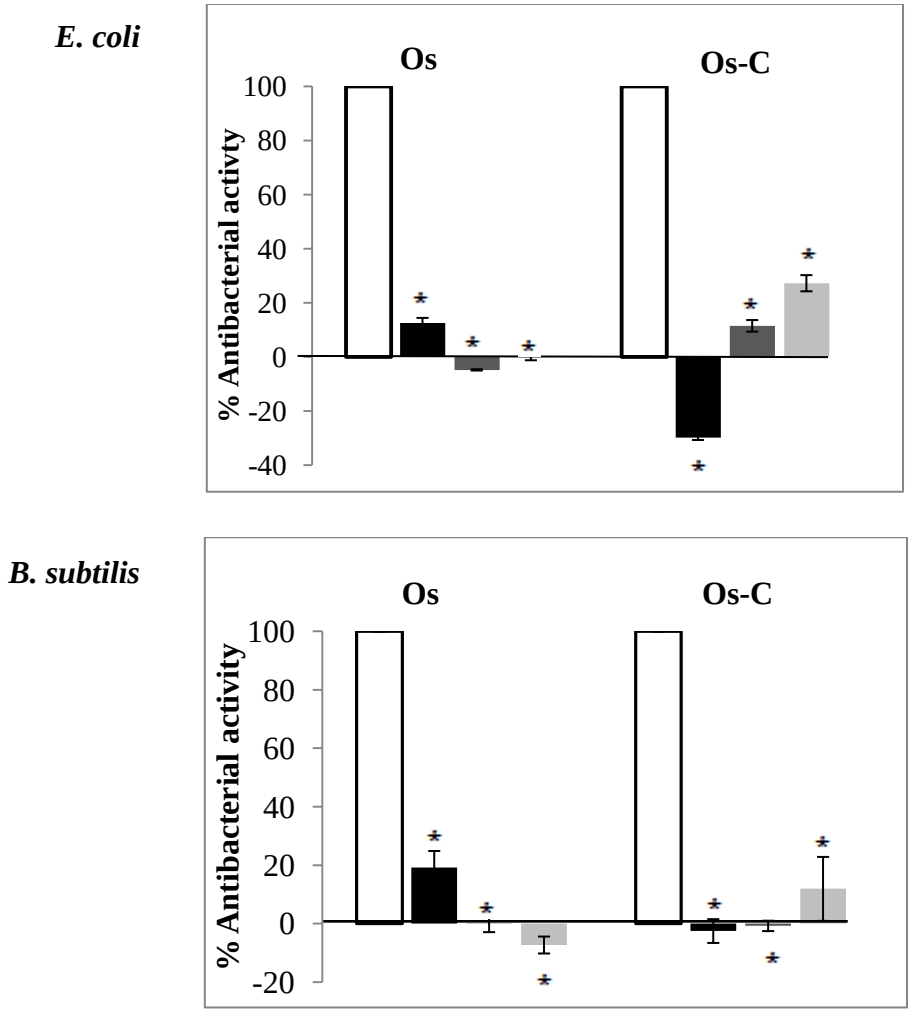


Figure 3.4: Effects of salts on the antibacterial activity of Os and Os-C against *E. coli* and *B. subtilis*. The clear bars represent the activity of the peptides without the salts (control). The black, dark grey and light grey bars represent the antibacterial activity of the peptides in the presence of NaCl (100 mM), $MgCl_2$ (2 mM) and $CaCl_2$ (2 mM), respectively. Antibacterial activities were determined at the MBCs of both peptides. Error bars represent the SEM of three (*A*) and two (*B*) independent experiments. Asterisks indicate statistical significance ($P<0.05$).

For both *E. coli* and *B. subtilis*, both peptides showed significant decrease in antibacterial activity in the presence of cations. The negative % antibacterial activity seen in Fig. 3.4 indicates that there was an increase in bacterial growth in the presence of both salt and the peptide compared to the control where only salt was present. Os retained 10% and 20% antibacterial activity against both *E. coli* and *B. subtilis*, respectively, in the presence of 100 mM NaCl. Os-C showed approximately 30% and 10% activity against *E. coli* in the presence of 2 mM $MgCl_2$ and 2 mM $CaCl_2$, respectively.

In order to confirm the observed salt effects, the CFU method was used to obtain the MBC of the peptides, for a Gram-positive and a Gram-negative bacterial strain, in the presence of 100 mM NaCl (Fig. 3.6). *B. subtilis* was found to be susceptible to 100 mM NaCl, indicating that the salt, in itself, acted as an antibacterial agent. For this reason *S. aureus* was then used to determine the effect of NaCl on the bactericidal activity of Os and Os-C.

Table 3.7 Minimum bactericidal concentrations of peptides in the presence of NaCl

	MBC (µg/ml)			
Bacteria	**Os**		**Os-C**	
	NaP buffer	**NaCl/NaP[a]**	**NaP buffer**	**NaCl/NaP**
Gram-positive				
S. aureus	3.75 (1.52)	120 (48.8)	3.75 (1.74)	>120 (>55.8)
Gram-negative				
E. coli	1.88 (0.77)	1.88 (0.77)	3.75 (1.74)	7.50 (3.49)

[a]100 mM (final concentration) NaCl dissolved in NaP buffer (10 mM, pH 7.4)
MBCs represent results from at least two independent experiments
Values given in parentheses are peptide concentration expressed in µM

Compared to the bactericidal activity in NaP buffer, the MBC of Os for *S. aureus* increased from 3.75 µg/ml to 120 µg/ml whereas for *E. coli* the MBC stayed constant in the presence of 100 mM NaCl. Os-C was found to show no antibacterial activity, even at 120 µg/ml, against *S. aureus* and a two-fold increase in its MBC for *E. coli* in the presence of NaCl (Table 3.7).

Human serum contains various proteins, salts and other components that can affect the activity of AMPs (Ciornei *et al*., 2005). For these peptides to be considered as possible therapeutic agents the effects of the presence of human serum on the bactericidal activity of these peptides must be investigated. Using the CFU method, the bactericidal activity of Os and Os-C in the presence of 30% human serum was investigated (Table 3.8).

The MBC of Os for *E. coli* increased from 1.88 µg/ml to 60 µg/ml in the presence of human serum whereas, against *S. aureus* the MBC of Os stayed the same. Os-C was found to show no antibacterial activity, even at 120 µg/ml, against *S. aureus* and *E. coli* in the presence of human serum.

Table 3.8 Minimum bactericidal concentrations of peptides in the presence of human serum

	MBC (µg/ml)			
	Os		Os-C	
Bacteria	NaP buffer	Serum[a]	NaP buffer	Serum
Gram-positive				
S. aureus	3.75 (1.52)	3.75 (1.52)	3.75 (1.74)	>120 (>55.8)
Gram-negative				
E. coli	1.88 (0.77)	60.0 (24.4)	3.75 (1.74)	>120 (>55.8)

[a]30% (final concentration) human serum dissolved in water
MBCs represent results from at least two independent experiments
Values given in parentheses are peptide concentration expressed in µM

Overall, the bactericidal activity of both Os and Os-C was retained against *E. coli* in the presence of NaCl and Os was found to maintain some, if not all, its activity in the presence of both NaCl and human serum against Gram-positive and Gram-negative bacteria. The bactericidal activity of Os-C was decreased, if not lost, in the presence of 30% human serum and 100 mM NaCl against *E. coli* and *S. aureus*.

3.5 Killing kinetics

The kinetics of bactericidal activity of Os and Os-C, at their respective MBCs, was evaluated using *E. coli* and *B. subtilis*, as representative Gram-negative and Gram-positive strains. The time-course study revealed that Os inactivated 100% of both Gram-positive and Gram-negative bacteria within the first 5 min, whereas Os-C showed 100% bactericidal activity only after 60 min and 120 min against Gram-negative and Gram-positive bacteria, respectively (Fig. 3.5).

The difference in the rate of bactericidal activity of Os and Os-C suggests that the mechanism of action of these two peptides might differ.

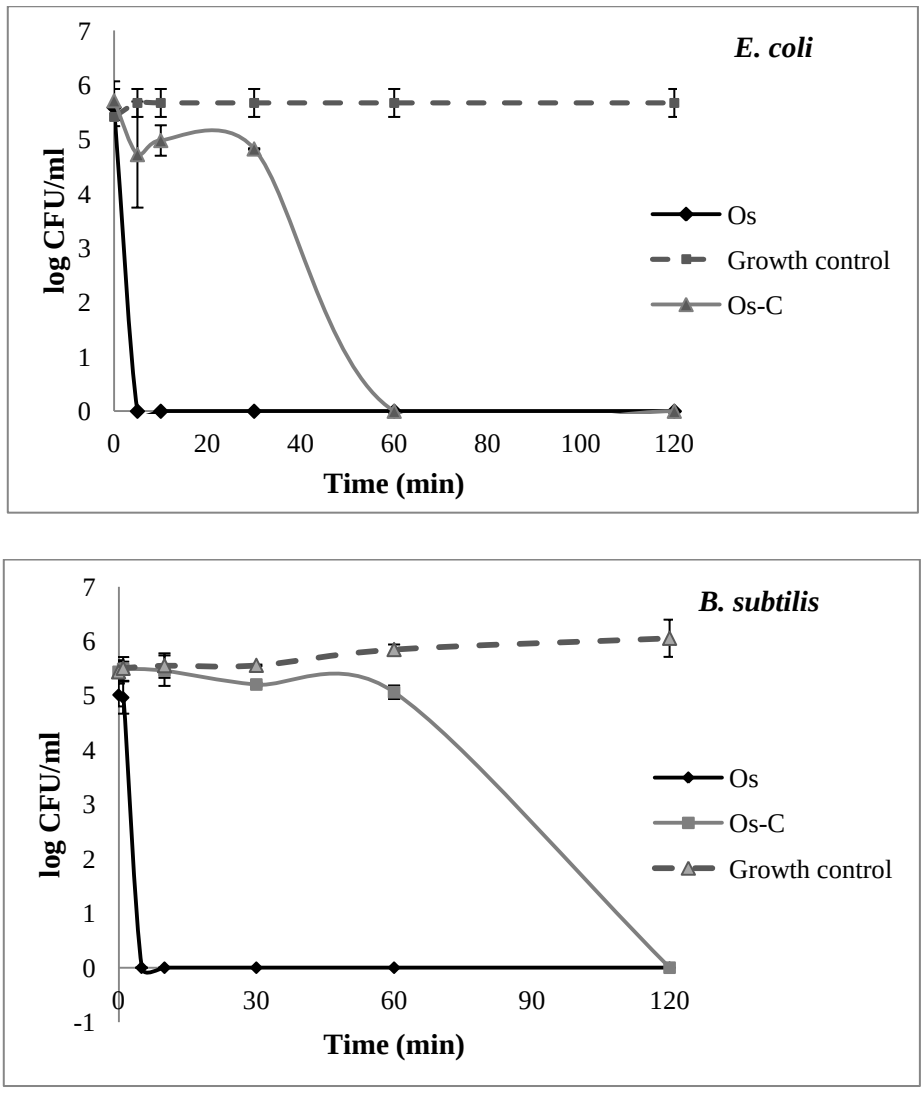


Figure 3.5: Killing kinetics of Os and Os-C against *E. coli* and *B. subtilis*. Results are given as log CFU/ml as a function of incubation time. Os was found to kill *E. coli* and *B. subtilis* after only 5 min, whereas Os-C was effective only after 60 min and 120 min against *E. coli* and *B. subtilis*, respectively. Error bars represents the SEM between at least two independent experiments performed in triplicate.

3.6 Membrane permeabilization studies

Due to the selectivity of Os and Os-C in high salt concentrations and rapid killing kinetics it was decided o further investigate the action related to membrane permeabilization of *E. coli*.

In order to investigate whether or not Os and Os-C cause membrane permeabilization, SYTOX Green nuclear stain was used. SYTOX Green will only bind to nucleic acids, subsequently causing an increase in fluorescence if the bacterial cells are permeable.

Before such assays could be performed, however, it was necessary to confirm the antibacterial activities of the control peptides against the selected bacterial strain, *E. coli* under the same experimental conditions used for Os and Os-C. A turbidity antibacterial assay was performed to

determine the MBCs of melittin and magainin 2 against *E. coli* (Table 3.9). Melittin and magainin 2 were found to be active against *E. coli* with MBCs of 0.47 µg/ml and 7.5 µg/ml, respectively.

Table 3.9 Minimum bactericidal concentration determination of the control peptides, melittin and magainin 2, used in the SYTOX Green permeabilization assay

	Minimum bactericidal concentration (µg/ml)	
Gram-negative	Melittin	Magainin 2
E. coli	0.47 (0.17)	7.50 (2.90)

MBC values obtained from an experiment performed in duplicate using the turbidity assay
Values given in parentheses are concentrations expressed in µM

In a preliminary dose-dependent experiment, melittin, a peptide known to cause *E. coli* membrane permeabilization (Bourbon *et al*., 2008), increased the fluorescence output by 1.8 fold at 120 µg/ml. Magainin 2, previously shown to cause no bacterial membrane permeabilization (Bourbon *et al*., 2008), caused minimal increase in fluorescence. Os-C showed similar results as that observed for magainin 2. Os, however, decreased the fluorescence output (Fig. 3.6a).

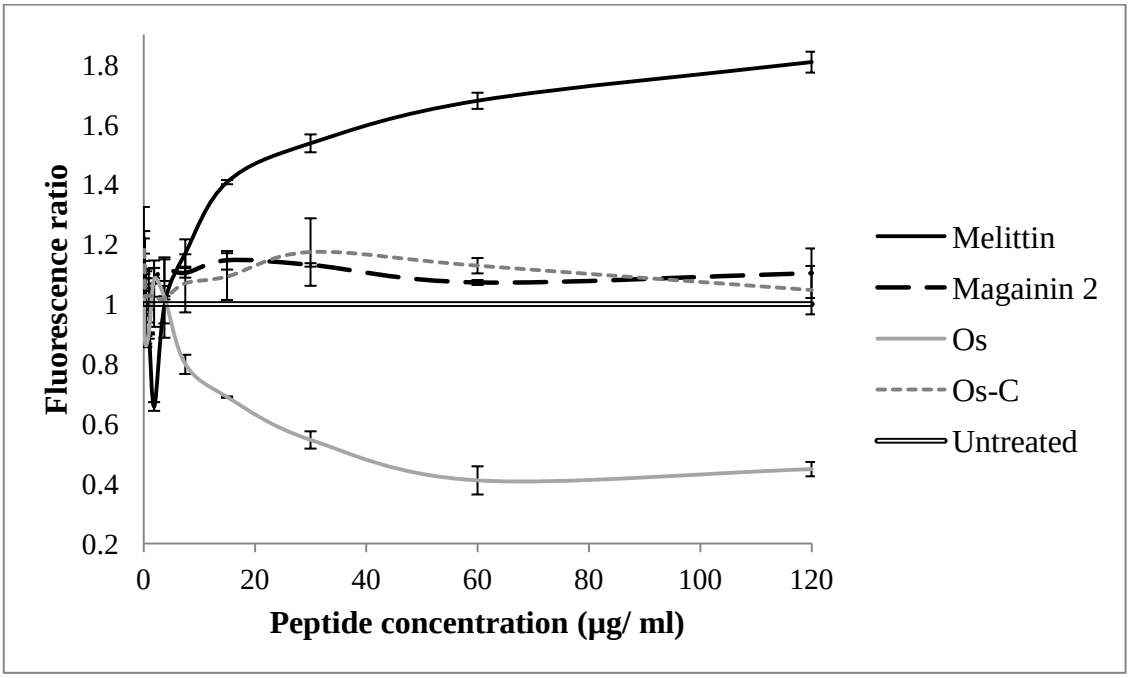


Figure 3.6a: Preliminary SYTOX Green permeabilization assay. Results are given as a ratio between the fluorescence output of the *E. coli* cells treated with peptide compared to that of the untreated cells. Peptide concentrations at 120 µg/ml is equivalent to 52 µM melittin, 46.2 µM Magainin 2, 48.88 µM Os and 55.8 µM Os-C. Error bars represent the SEM of two independent experiments.

A possible explanation for the decrease in fluorescence output when bacteria were treated with increasing concentrations of Os may be that Os binds to the bacterial DNA thereby inhibiting the binding of SYTOX Green to the DNA. To test this theory, melittin in combination with Os was incubated with the bacteria (Fig. 3.6b). Under these conditions melittin would cause permeabilization allowing Os to enter the bacteria and if Os binds DNA it would result in decreased fluorescence.

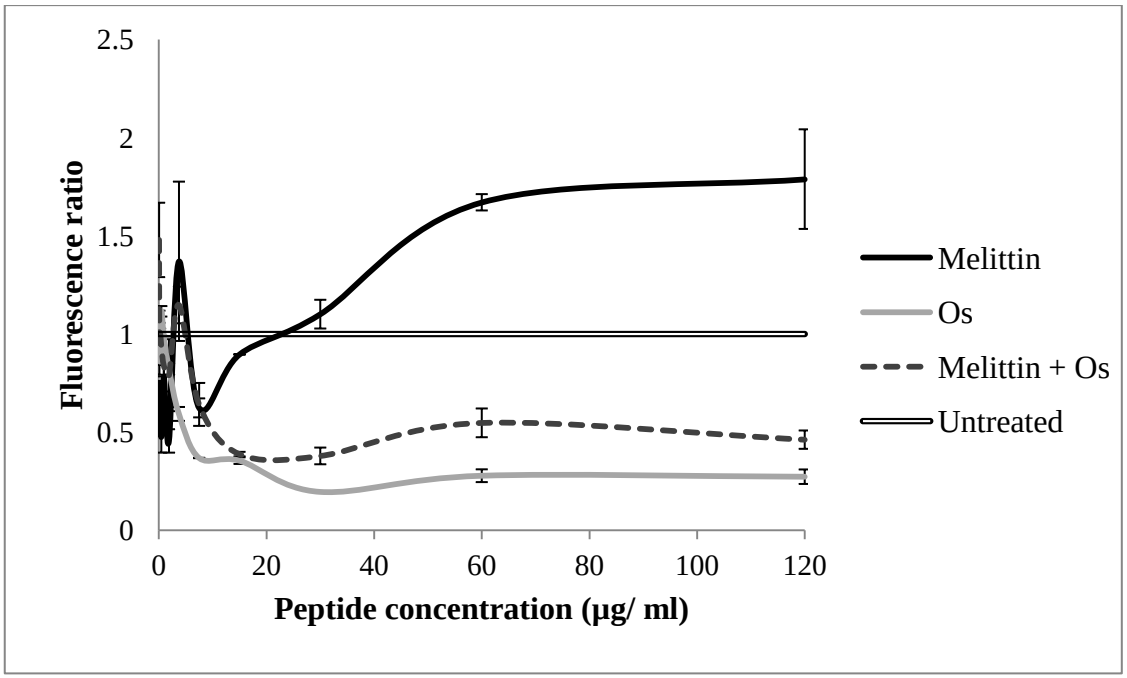


Figure 3.6b: SYTOX Green permeabilization assay when melittin and Os were incubated in combination. Results are given as a ratio between the fluorescence output of the bacterial cells treated with peptide compare to that of the untreated cells. Error bars represent the SEM of two independent experiments performed in triplicate.

A noticeable decrease in fluorescence output of the melittin treated sample when treated in combination with Os was seen (fig 3.7b). This indicates that Os may bind DNA.

Magainin 2 had been reported to cause membrane permeabilization in another study (Imura *et al.*, 2008). It was therefore deemed necessary to use a different negative control for the membrane permeabilization studies. 9Pbw2 was reported to not cause membrane permeabilization (Shin *et al.*, 2009) and was used as the negative control for further permeabilization assays.

The fluorescence output observed for melittin when using 1 x 10^6 CFU/ml of bacteria in the assay was approximately two-fold more than that of the untreated samples. In an attempt to

increase the fluorescence signal, bacterial samples of various concentrations were incubated with control peptides at their respective MBCs and SYTOX Green. For this experiment the peptide concentrations were increased with the same ratio as to what the bacterial concentration was increased. The resulting fluorescence was obtained (Fig. 3.7) and the optimal bacterial concentration for SYTOX Green permeability assay determined.

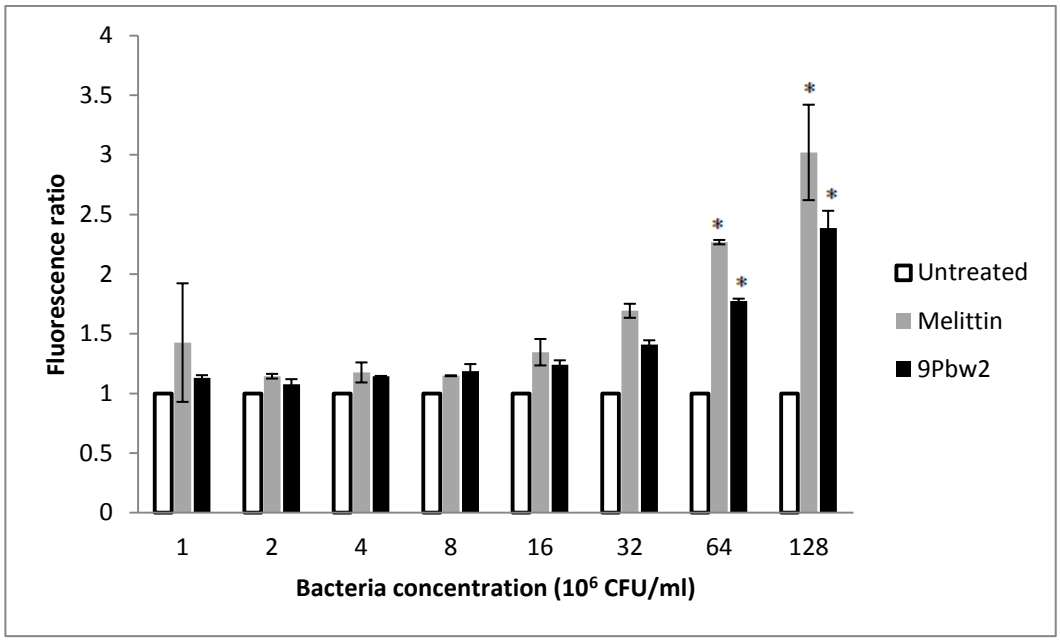


Figure 3.7: Effect of bacterial concentration on fluorescence ratio when compared to the untreated samples at the respective bacterial concentrations. Bacterial samples of varying concentrations were incubated with peptides and SYTOX Green nucleic acid stain. Fluorescence was measured at excitation/emission wavelengths of 483/538 nm and expressed relative to the untreated cells (control). Error bars represent the SEM of two independent experiments performed in triplicate. Asterisks indicate statistical significance ($P<0.05$).

It was decided to use a bacterial concentration of 6.4 x 10^7 CFU/ml for subsequent experiments. At this bacterial concentration a clear distinguishable difference between the fluorescence obtained when incubated with melittin or 9Pbw2 was observed.

However, Hartmann *et al.* (2010) showed that the increase in MBC is not linear with the increase in the amount of bacteria. Thus, in order to use a higher concentration of bacteria in the SYTOX Green assay, the new antibacterial concentrations of the peptides had to be determined under these conditions. The turbidity assay was used to obtain the MBCs of the synthetic peptides when using 6.4 x 10^7 CFU/ml bacteria. Table 3.10 summarises the change in the MBCs of the peptides as the bacterial concentration increased and indicate that the MBC of all the peptides increased 4-fold when the bacterial concentration was increased 64-fold.

Using these newly determined MBCs, another SYTOX Green assay was performed in order to investigate membrane permeabilization at specific time intervals (Fig. 3.8).

Table 3.10 Minimum bactericidal concentrations against increased bacterial concentrations.

	MBC (µg/ml)	
Bacteria concentration	**1 x 10^6 CFU/ml**	**6.4 x 10^7 CFU/ml**
Melittin	0.47	1.88
	(0.17)	(0.66)
9Pbw2	0.94	3.75
	(0.78)	(3.10)
Os	1.88	7.50
	(0.76)	(3.05)
Os-C	3.75	15
	(1.74)	(6.98)

MBCs were obtained from two independent experiments performed in triplicate using the turbidity assay
Values given in parentheses are MBCs expressed in µM

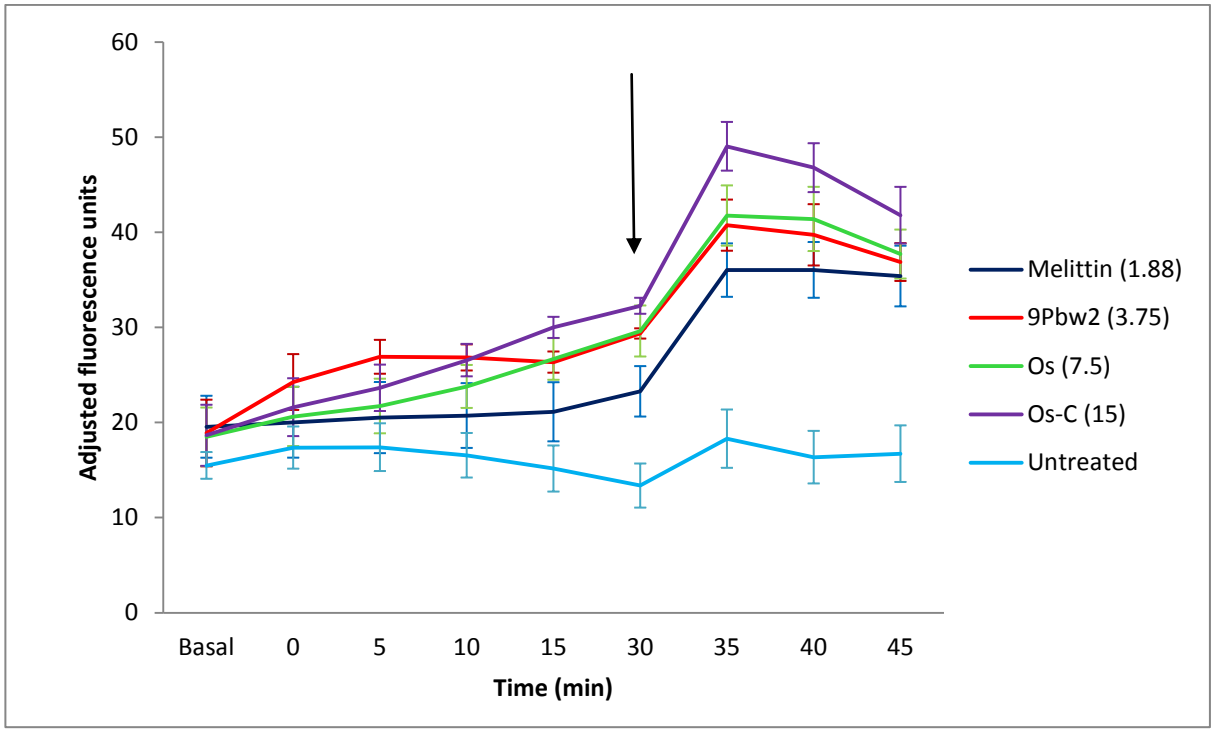


Figure 3.8: Change in SYTOX Green fluorescence over time. *E. coli* were incubated with SYTOX Green and the basal fluorescence measured. The addition of the peptides (at 0 min) initiated the reaction and the resulting SYTOX Green fluorescence was measured over specific incubation time intervals. At 30 min, 1 mM EDTA and 0.5% Triton X-100 were added (indicated by arrow) and the resulting fluorescence measured for another 15 min. Data were represented as the adjusted fluorescence obtained when the blank (sample without bacteria) was subtracted from each fluorescence. The peptides' concentration, in µg/ml, is given in the parentheses. Error bars are representative of SEM of five independent experiments performed in quadruplicate.

The results in Fig. 3.8 showed that all the peptides, at their respective MBCs, caused an increase in SYTOX Green fluorescence, compared to the untreated samples, especially after 30 min incubation time interval. From 0-30 min Os-C, melittin and Os showed a time dependent increase in fluorescence with most rapid increase for Os-C. The negative control, 9Pbw2, also showed increased fluorescence indicating that 9Pbw2 does cause permeabilization and this increase was more rapid than observed for Os, Os-C and melittin, especially at 0-10 min. Treatment with EDTA and Triton X-100 caused increased fluorescence for all peptide treated *E. coli* samples. Addition of EDTA and Triton X-100 to untreated *E. coli* cells did not result in an increase in fluorescence.

Since the untreated *E. coli* samples did not increase in fluorescence after the addition of EDTA and Triton X-100 (Fig. 3.8), the validity of the assay was questionable. Therefore, it was deemed necessary to investigate whether this lack of increase in fluorescence in the control was an effect of the type of bacterial strain used. In order to investigate this, two Gram-positive bacterial strains, *S. aureus* and *B. subtilis*, were used. Both of these strains were incubated with SYTOX Green, under the same conditions as for *E. coli*, and the fluorescence measured over the same time intervals (Fig. 3.9).

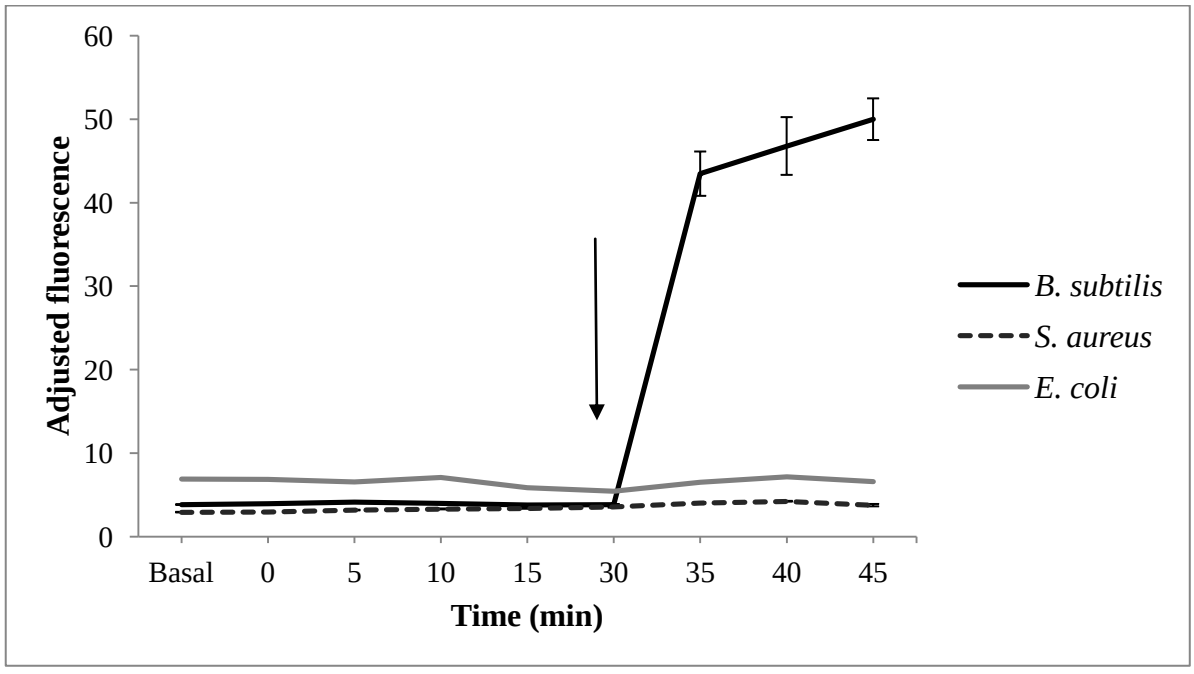


Figure 3.9: SYTOX Green fluorescence of untreated bacterial samples. Two Gram-positive bacterial strains were incubated with SYTOX Green and the fluorescence measured over a specific time interval as well as after the addition of 1 mM EDTA and 0.5% Triton X-100 (indicated by the arrow). Error bars represent the SEM of two independent experiments performed in triplicate.

After the addition of EDTA and Triton X-100, *B. subtilis* showed an increase in fluorescence indicating membrane permeabilization (Fig. 3.9). However, similar to *E. coli*, *S. aureus* did not show an increase in fluorescence after the addition of EDTA and Triton X-100 (Fig. 3.9).

3.7 Plasmid binding properties of Os and Os-C

The preliminary SYTOX Green assay showed a decrease in fluorescence at MBC and lower concentrations of Os (Fig 3.7a). One explanation for this observation is that Os may bind to DNA thus shielding it from SYTOX Green binding. In order to verify whether Os and Os-C are able to bind to bacterial DNA, a plasmid binding assay was performed.

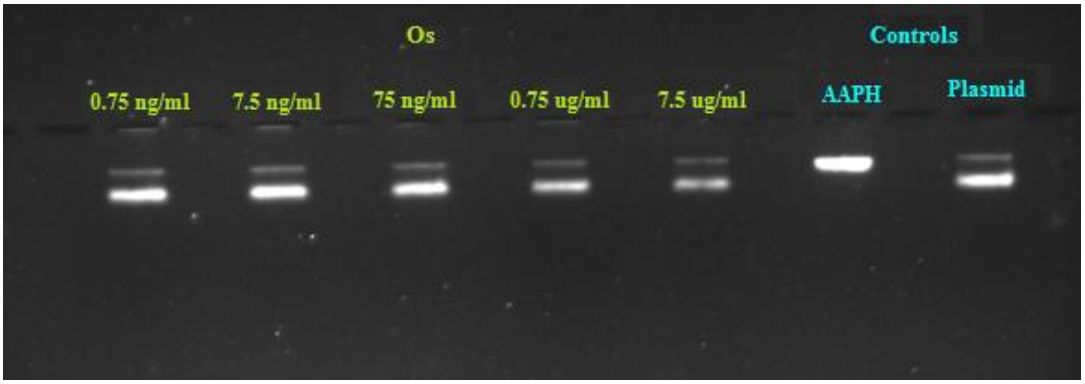


Figure 3.10a: Plasmid binding assay of Os at the MBC. The two controls used were plasmid alone (plasmid) and plasmid incubated with AAPH (AAPH). Peptide (Os), at concentrations ranging from 7.5 µg/ml to 0.75 ng/ ml, incubated with plasmid DNA shown.

A change in the migration pattern of the DNA is indicative of the interaction between peptides and DNA. From Fig. 3.10a it was observed that some plasmid binding might occur when Os was incubated at a concentration equal to its highest MBC (7.5 µg/ml), since the lowest band at this concentration was fainter than that observed at lower Os concentrations. To confirm binding, the experiment was performed for both Os and Os-C using a peptide concentration range of 60 ng/ ml to 0.6 mg/ml (Fig. 3.10b). This corresponds to a concentration range of 0.02 µM to 244 µM for Os and 0.03 µM to 279 µM for Os-C.

From Fig. 3.10b it can be seen that when plasmid is incubated with peptide at high concentrations, the plasmid seems to disappear. At high concentrations of Os (0.6 and 0.06 mg/ml) and of Os-C (0.6 mg/ml) there are no bands visible. The reason for this could be that when the peptide binds to the plasmid DNA, the charge of the DNA is altered and that it, therefore, will not migrate towards the positive pole.

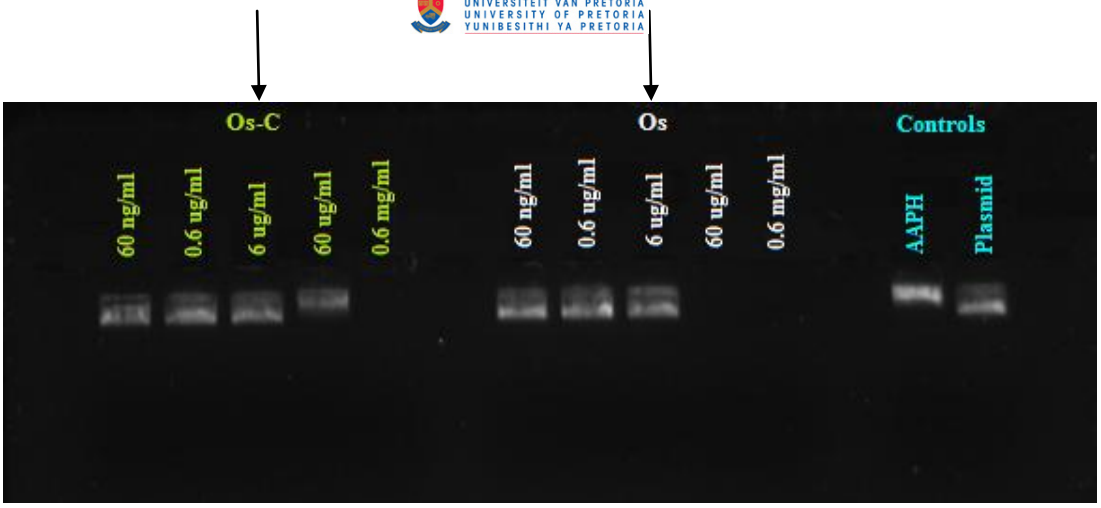


Figure 3.10b: Agarose gel of plasmid binding assay of Os and Os-C at higher peptide concentrations. AAPH and plasmid (without peptide, final concentration of 50 µg/ml) were used as the positive and negative controls, respectively. Arrows indicate peptide concentration closest to MBC.

3.8 Cytotoxicity

Os and Os-C kill both Gram-positive and Gram-negative bacteria. To evaluate whether these peptides are selective with regard to bacterial cells, the effect of Os and Os-C on mammalian cells was investigated using SC-1 and Caco-2 cell lines and the CV assay. CV binds to cellular DNA and protein. A decrease in cell number due to detachment of dead cells will cause a decrease in the amount of bound CV. Thus, the degree of staining is indicative of the number of remaining cells.

The light microscopy images clearly depict the effects of the peptides on mammalian cells where a decrease in stain indicates a decrease in cell number. Melittin caused significant decrease in the number of SC-1 cells (Fig. 3.11) whereas Os and Os-C looked similar to the untreated cells. At high concentrations of Os and of Os-C the SC-1 cell number either stayed constant or appears to be slightly increased (Fig. 3.11). Light microscopy photos of Caco-2 cells treated with peptide showed similar results (Fig. 3.11). By extracting the bound CV this effect can be quantified by measuring the absorbance of the sample at 630 nm. The absorbance of the extracted CV is directly proportional to the number of attached cells (Fig. 3.12).

Fig. 3.12 depicts the quantitative analysis of the cytotoxic effects of the peptides against two eukaryotic cell lines. Melittin, the positive control, caused 40% decrease in cell number when tested at 7.5 µg/ml (2.6 µM) and 30 µg/ml (10.5 µM) and a 50% decrease at 120 µg/ml (52 µM) on SC-1 cells (Fig. 3.12). When tested on Caco-2 cells, melittin caused a decrease of 20% in the cell number at 30 µg/ml and 120 µg/ml (Fig. 3.12). Os and Os-C did not cause any significant decrease in SC-1 and Caco-2 cell number, even at 120 µg/ml.

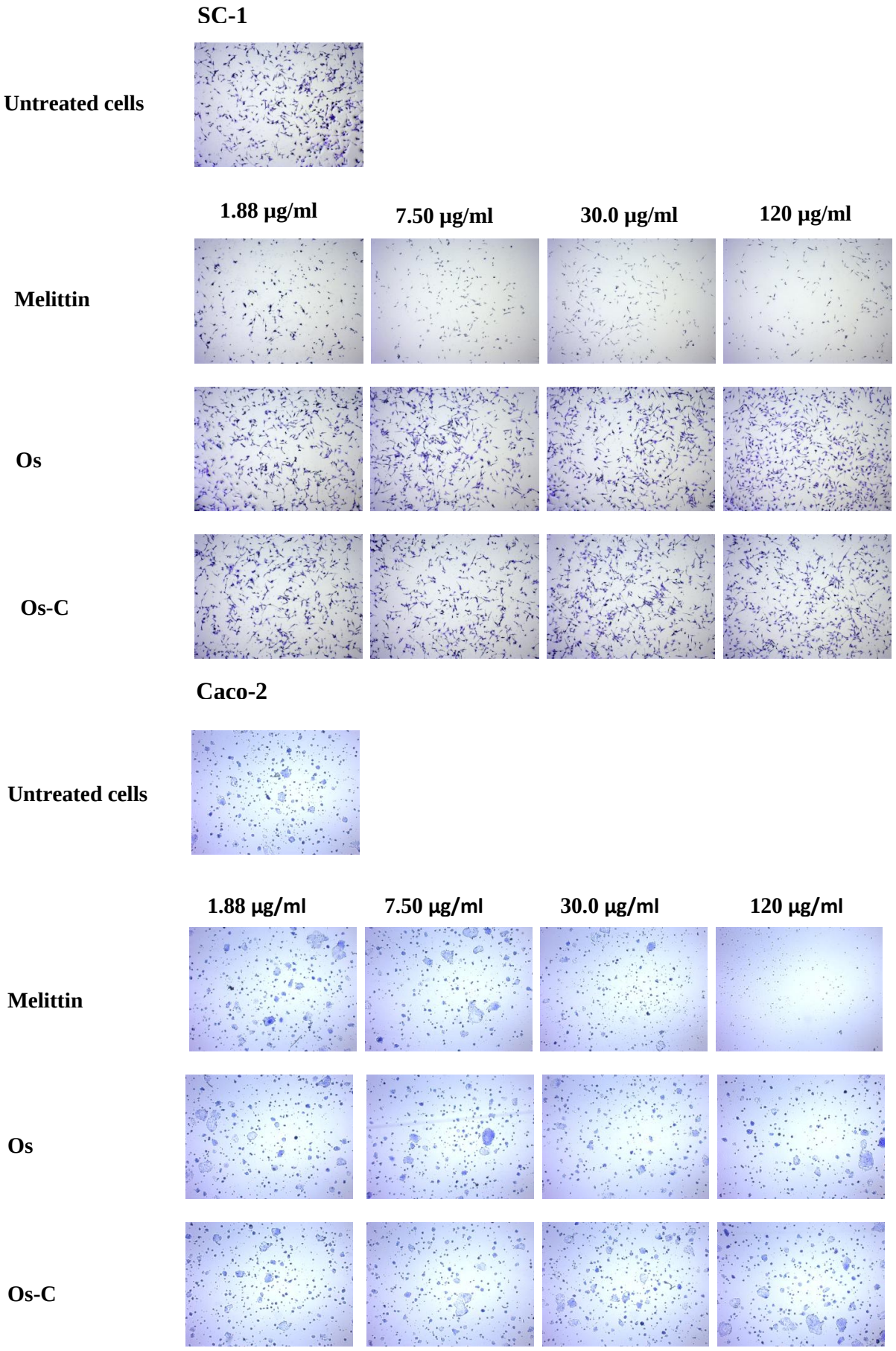


Figure 3.11: Light microscopy images of SC-1 and Caco-2 CV stained cells. The images above are a visual depiction of the cytotoxicity results. Each column represents an increase in peptide concentration (1.90 - 120 µg/ml).

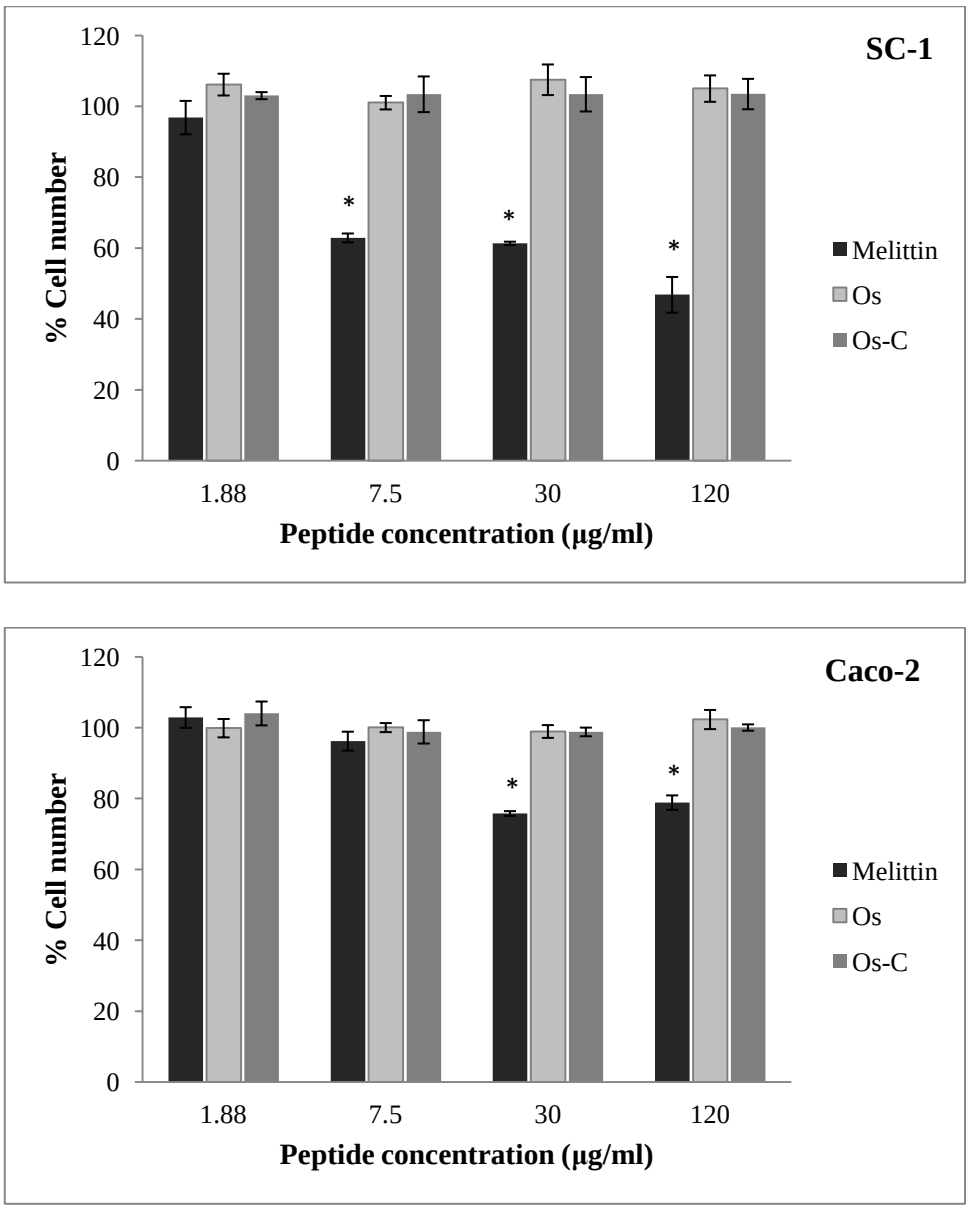


Figure 3.12: Cytotoxicity results of Os, Os-C and melittin against SC-1 and Caco-2 cells. Results are given as a percentage of viable cells in comparison with the untreated cells (control, 100%). Peptide concentration of 120 µg/ml is equivalent to 52 µM for melittin, 49 µM for Os and 55.8 µM for Os-C. Error bars represent the SEM of two independent experiments performed in triplicate. *Statistically significant ($P<0.05$) compared to control.

CHAPTER 4: DISCUSSION

Defensins form a large part of the innate immune response of various organisms, including lower invertebrates (Reddy *et al.*, 2004). Invertebrate defensins display antibacterial activity against Gram-positive bacteria and no or little activity against Gram-negative bacteria (Nakajima *et al.*, 2003) and fungi (Bulet *et al.*, 1999). Two defensin isoforms, OsDef1 and OsDef2, were previously identified from the midgut of *O. savignyi* [N. Olivier, manuscript in preparation]. During this study the structural characteristics and antibacterial properties of these two isoforms, as well as the carboxy-terminal analogue peptides of OsDef2, were investigated. The amino acid sequences of the two carboxy-terminal analogue peptides differed with respect to the presence (Os) and absence (Os-C) of cysteine residues.

All the peptides examined during this study are positively charged (+4 to +6 at pH 7), short (9 – 37 residues), contain both hydrophobic and hydrophilic residues. It has been noted that the structure of an AMP will affect its antimicrobial activity (Sitaram & Nagaraj, 1999). For example, a study conducted by Giangaspero *et al*. (2001) showed that the antimicrobial activity of a peptide might increase with an increase in α-helical properties. Therefore, the secondary structures of the synthetic peptides were elucidated using a prediction tool (PSIPRED) and CD spectroscopy.

The secondary structures of the peptides were predicted using PSIPRED, a computer-based program (http://bioinf.cs.ucl.uk/psipred/). P4, the carboxy-terminal derivative of longicornis, was used as a control in the antibacterial assays, was predicted to consist mostly of β-strands (Table 3.2). Melittin, known to contain an α-helix and a random-coil (Vogel & Jähnig, 1986), was predicted correctly by PSIPRED to be mostly α-helical (88%) and random coiled (Table 3.2). Both OsDef1 and OsDef2 were predicted to consist predominantly of random coils (62%) with the rest of the secondary structures consisting of roughly equal amounts of α-helical and β-sheeted secondary structures. The secondary structure predictions obtained from PSIPRED (Table 3.2) showed that Os is mostly random coiled (68%) and α-helical (32%). Os-C, in comparison, was predicted to consist of β-strands (21%) and predominantly random coils (79%). The confidence levels of the PSIPRED predictions (Fig. 3.1) between the different peptide varied with the α-helical properties of melittin having the highest confidence. The confidence level of the secondary structure prediction of Os and Os-C were low and can, therefore, not be seen as a reliable prediction.

These PSIPRED predictions are only valid if confirmed by experimental data. Therefore, the secondary structures of the synthetic peptides were obtained using CD spectroscopy. The

peptides were dissolved in four different solvents for CD spectroscopy: NaP buffer (10 mM, pH 7.4); water; 50% (*v/v*) TFE and 25 mM SDS. NaP buffer was the medium used to test the bactericidal activity of the peptides (section 2.6.2) and water the medium in which the peptides were prepared initially (section 2.1), therefore it was deemed important to investigate the secondary structure characteristics of the peptides in these two solutions. SDS is a solution that, when prepared in the correct peptide to SDS ratio (1:500) or above the critical micelle concentration of 9.5 mM, will form micelles thereby creating a membrane-mimicking environment (Tulumello & Deber, 2009 and Khan & Shah, 2007). The concentration of SDS used during this study, 25 mM, was therefore sufficient to create a membrane-mimicking environment in the presence of 50 µM peptide. TFE is an α-helix stabilizing solvent and will; therefore, induce secondary structures of the peptides (Luidens *et al*., 1995). The CD spectra of all the peptides used in this study were determined in all four of the solvents mentioned above but only the spectra performed in water and SDS are shown in the results section. Although the reference CD spectra (Fig. 2.1) are plotted with different units ($\Delta\varepsilon$) than that used to plot the results of this study ([θ]), the overall shapes of the reference spectra can be used for comparative purposes.

Melittin, P4 and 9Pbw2 were used as positive controls since the CD spectra and secondary structures of these peptides have been reported. Melittin, an AMP isolated from honey bee venom, is known to consist of an α-helix and a random-coil (Vogel & Jähnig, 1986). Near perfect α-helical CD spectra were obtained when melittin was prepared in SDS (Fig. 3.2a) and TFE (result not shown). In water and NaP buffer (result not shown) melittin had no defined structure. Melittin, observed to consist mostly out of α-helical properties (Fig. 3.2a), has, as predicted by its helical wheel (results not shown), a hydrophobic face (amino acid sequence of LLILL) and therefore, an amphipathic nature. This enhances the ability of melittin to insert into a bacterial cell membrane, thereby causing permeabilization of the plasma membrane. Thus, the secondary structure prediction and the experimentally obtained CD data confirm the antibacterial characteristics previously described for melittin (Vogel & Jähnig, 1986; van den Bogaart *et al*., 2008). This α-helical and amphipathic nature of melittin not only enhances its antimicrobial activity but also increases its cytotoxic and hemolytic characteristics (Pérez-Paya *et al*., 1994). The CD spectrum of P4 in water (Fig. 3.2a) was similar as observed by Rahman *et al*. (2009) with no defined secondary structure. When dissolved in TFE, a secondary structure promoting solution, the spectrum of P4 was also similar to what was previously reported consisting mostly of random coils and equal amounts of α-helices and β-sheets (results not shown). Furthermore, P4 showed α-helical properties when prepared in SDS (Fig. 3.2a). 9Pbw2 is a 9 amino acid

peptide derived from a defensin-like peptide isolated from beetle larvae. The peptide was previously shown to have an α-helical secondary structure when prepared in SDS (Shin *et al*., 2009). During this study when 9Pbw2 was prepared in SDS the CD spectra showed α-helical properties, whereas it had no defined secondary structure when prepared in NaP buffer (results not shown) and water (Fig. 3.2a).

Os contains three cysteine residues which might form intramolecular disulfide bonds. The presence of these bonds will influence the secondary structure of the peptide. It was, therefore, deemed necessary to investigate the difference between the secondary structures of Os and the reduced from of Os. By reducing Os, with the addition of 10 mM DTT, the possible effect of disulfide bonds on the secondary structure of the peptide, was eliminated. The CD spectra of Os and Os(red) differed only slightly when prepared in NaP buffer (result not shown). Furthermore, Os(red) showed slightly more α-helical properties than Os when prepared in both SDS (Fig. 3.2b) and TFE (results not shown). Both Os and Os(red) were found to be unstructured in water. Os-C differs in amino acid composition compared to Os in that the three cysteine residues (present in Os) were omitted in Os-C. A difference between the secondary structures of Os and Os-C was, therefore, expected. In both water and NaP buffer, Os-C was found to be unstructured. When prepared in SDS, Os-C had some β-sheet characteristics whereas Os had more α-helical properties. Os-C was found to consist mostly of β-sheet and random-coiled secondary structures (Fig. 3.2b) with very little α-helical properties in comparison to Os and Os(red).

The secondary structures of invertebrate defensins are conserved among all insect species and consist of an α-helical structure at the amino-terminal followed by a β-sheet segment at the carboxy-terminal (Sitaram & Nagaraj, 1999). This was also evident in the tertiary structure of OsDef1 and OsDef2 obtained by PyMOL modelling (Fig. 3.3). Therefore, a mixture of α-helical and β-sheet secondary structures for OsDef1 and OsDef2 was expected and were accurately predicted by PSIPRED predictions (Table 3.2).

The antibacterial activity of invertebrate defensins may depend on the amphipathic nature of the peptides (Sitaram & Nagaraj, 1999). When combining the PSIPRED predictions (Table 3.1) and the PyMOL modelling (Fig 3.4) with the helical wheels (not given) of OsDef1 and OsDef2, the conclusion can be drawn that, since both OsDef1 and OsDef2 contain some α-helical properties, both these peptides will have a slight hydrophobic face (amino acid sequence of CCCI) at the amino-terminal (α-helical) segment and positive, hydrophilic residues at the carboxy-terminal

region. Thus, both OsDef1 and OsDef2 will have amphipathic segments and can, therefore, be predicted to be able to interact with microbial membranes.

Invertebrate defensins have been reported to be active mainly against Gram-positive bacterial strains (Li *et al*., 2012; Nakajima *et al*., 2003 and Chrudimská *et al*., 2011). Charge is essential for the activity of cationic peptides as the electrostatic interaction between the anionic bacterial cell envelope and the cationic peptide will give rise to its antibacterial activity. It was, therefore, predicted that OsDef2, having a slightly higher positive charge (+6) than OsDef1 (+5), will have a higher antibacterial activity. Furthermore, it was expected that the defensin peptides would show less or no antibacterial activity against Gram-negative bacteria. The next aim of the study was to investigate the antibacterial properties of the two defensin isoforms, OsDef1 and OsDef2. Both these isoforms were found to be active against Gram-positive bacteria, with MBCs ranging from 0.94 µg/ml to 1.9 µg/ml, but not against Gram-negative bacteria. The MICs obtained for these peptides (Table 3.3a and b) gave a clear indication that at very low peptide concentration (0.24 µg/ml) the defensin peptides can inhibit the growth of the bacteria cells. Both peptides were, however, not active against *S. aureus* when tested in LB broth (Table 3.3a). This result was unexpected since the other tested Gram-positive bacterial strains were susceptible towards the defensin peptides. A possible explanation for this can be that some strains of *S. aureus* are notoriously known to be highly resistant toward antibacterial agents (Lowy, 2001) and that the tested *S. aureus* strain used during this experiment might be resistant towards the defensin peptides. However, both OsDef1 and OsDef2 were found to be active against *S. aureus* when tested in MH broth (Table 3.3b). This indicated that the activity of the defensin peptides was dependent on the environment in which the assay was performed.

The study conducted by Nakajima *et al*. (2003) showed defensin isoform A from *O. moubata* was also active against only Gram-positive bacteria with MICs of 0.1 µg/ml against both *M. luteus* and *S. aureus* when tested in MH broth, comparable to that of OsDef1 and OsDef2. It should be noted that the defensin studied by Nakajima *et al*. (2003) was air oxidized thereby ensuring the formation of disulfide bonds as the cysteine pairing between Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6 in invertebrate defensins has been reported to be essential for the function of the peptide (Lehrer & Ganz, 1994). Chrudimská *et al*. (2011), however, showed that when the cysteine residues of two defensin isoforms of *Ixodes ricinus* were reduced, by the addition of β-mercaptoethanol, the peptides were still active against Gram-positive bacteria. Furthermore, these authors showed that the defensin isoform with the arginine residue at the 8th position (net charge of +7) was more active than the other isoform (Phe8, +6) with MICs of 0.75 µg/ml, 0.37 µg/ml and 25 µg/ml for *B. subtilis*, *M. luteus* and *S. aureus*, respectively. Overall their findings

suggest that the disulfide bonds are not necessary for antibacterial activity but that net charge does influence activity and that *S. aureus* is slightly resistant towards these defensins (Chrudimská *et al*., 2011). During this study, the cysteine residues in OsDef1 and OsDef2 were reduced, by the addition of DTT, prior to the antibacterial assays. As mentioned, both OsDef1 and OsDef2 were still active against Gram-positive bacteria even without disulfide bonds. Defensins with higher net positive charges have been shown to display higher antibacterial activities than defensins that are less positively charged (Isogai *et al*., 2010). OsDef2 (net charge of +6) showed slightly more antibacterial activity than OsDef1 (+5) against *B. subtilis*. The similarities between the hydrophobicity (Table 3.1) and structures (Fig. 3.3) of OsDef1 and OsDef2 explain the similarities in their antibacterial activities.

The chemical synthesis of peptides is an expensive process. For this reason research is focussed on finding the smallest but most active fragment of the AMP. The positive charges of the two defensin isoforms are carried on the carboxy-terminal region of the peptide and previous studies showed that antimicrobial activity is retained if only the carboxy-terminal fragment of arthropod defensins is used (Tsuji *et al*., 2007 and Varkey *et al*, 2006). For this reason the carboxy-terminal fragments of the most active defensin isoform, OsDef2, were synthesized and tested for antibacterial activity. When determined in LB broth, both Os and Os-C were found to have no antibacterial activities, at peptide concentrations as high as 120 µg/ml, against both Gram-positive and Gram-negative bacteria (Table 3.4). When tested in MH broth, Os-C displayed no antibacterial activity, whereas Os was active against *B. subtilis* (MIC of 60 µg/ml). This indicated that the antibacterial activities of the peptides were possibly influenced by the presence of salts and proteins in the broths. This dependency of antibacterial activity AMPs, on the amount of salts in the environment, has been previously observed (Dorschner *et al*., 2006; Wu *et al*., 2008 and Maisetta *et al*., 2008). This is a disadvantage that can hamper the development of AMPs as therapeutic agents since the environment in which AMPs will be applied in patients will have salts and proteins present.

It was, therefore, necessary to test the antibacterial activities of Os and Os-C without the presence of salts and proteins. Thus, the antibacterial activities of Os and Os-C as well as of P4, which was used as a control, were determined in NaP buffer (10 mM, pH 7.4) using the CFU method. No DTT were added to the peptides prior to performing the antibacterial assays since this was not done for the control peptide, P4, by Tsuji *et al*. (2007). Therefore, the cysteine residues were not reduced for comparative reasons. The activity of P4 against *E. coli* was found to be similar (MBC of 3.25 µM) than that previously reported (2 µM) by Tsuji *et al*. (2007). Os was found to be two-fold more active than Os-C against three of the four targeted bacterial

strains, with the bactericidal concentration the same for Os and Os-C against *S. aureus* (Table 3.5). Both Os and Os-C were more active than P4 against all the targeted bacterial strains. *P. aeruginosa*, a Gram-negative bacterial strain, was the most sensitive of the targeted bacteria with the lowest MBC of 0.94 µg/ml.

Os contains three cysteine residues that may become oxidized prior to experimental assays. In order to determine if the oxidation state of the peptide plays a significant role in the antibacterial activity, the antibacterial activity of the reduced peptide was investigated against a model Gram-negative bacterial strain, *E. coli*. Although 10 nmol DTT was added to the synthetic peptide prior to lyophilisation, the amount of dilution steps, during the peptide preparation steps and antibacterial assays, may have rendered the amount of DTT ineffective. Therefore, additional DTT was added to the peptides prior to conducting the antibacterial assay. It was found that when DTT was added to Os prior to antibacterial assay, the MBC of Os for *E. coli* changed. The MBC of Os pre-treated with DTT for *E. coli* decreased from 1.9 µg/ml to 0.94 µg/ml (Table 3.6). The decrease in the MBC value of Os indicated that the peptide may have undergone oxidation and furthermore that the reduced state of the peptide is two-fold more active against the selected bacterial strain. Additionally, the reduced state of Os showed slightly higher α-helical structure composition than Os and Os-C in SDS (Fig. 3.2b), proving that the structures of these peptides influence the antibacterial activity.

It has been shown that the antibacterial activities of AMPs are decreased in the presence of cations (Wu *et al*., 2008). The electrostatic interaction between cationic AMPs and negatively charged bacterial cell membranes are the primary mode of action for these peptides (Bulet *et al*., 1999). Monovalent and divalent cations will cause competitive disruption of this electrostatic interaction, thereby decreasing the antibacterial activities of cationic AMPs. Since the ionic environment of physiological systems differs vastly to that in which the MBCs of the peptides were determined, it was necessary to investigate the effect of cations on the antibacterial activity of Os and Os-C. The turbidity assay was used to preliminarily determine whether the presence of cations will affect the antibacterial activity of the peptides. Fig. 3.4 summarises the effect observed when Os and Os-C were incubated with *E. coli* and *B. subtilis* in the absence (positive control) and the presence (test samples) of cations: Na^{+}, Mg^{2+} and Ca^{2+} at physiological concentrations. The negative percentages observed in some solutions, for example the antibacterial activity of Os-C against *E. coli* in the presence of NaCl, indicated that the growth of the bacteria increased in the presence of peptides in comparison with the growth control (bacteria incubated with respective salts and without peptides). The reason for the decrease in growth when bacteria were incubated with just cations (growth controls) might be that the salts

displayed antibacterial activities. When the peptides were incubated with the bacteria in the presence of salts the antibacterial activities of the peptides were decreased thereby increasing the bacterial growth compared to the growth controls.

Both peptides showed noticeable decrease in antibacterial activity against *E. coli* and *B. subtilis* in the presences of all the tested cations. Some antibacterial activity was observed when Os-C was incubated with *E. coli* in the presence of Mg^{2+} and Ca^{2+}. Os retained 20% of its activity against *E. coli* and *B. subtilis* in the presence of Na^{+}. These results were expected as it was found that the antibacterial activities of Os and Os-C decreased in the presence of LB and MH broth when the turbidity assay (Table 3.4) was used. From these results it could be concluded that the presence of salts affects the antibacterial activity of both Os and Os-C.

B. subtilis was found to be sensitive towards NaCl, when the CFU method was used, not being able to survive in the presence of this salt. This confirms the hypothesis of the decrease in growth when the turbidity assay was used (Fig. 3.4). This phenomenon was not unexpected, seeing that salt is known to inhibit bacterial growth, especially when used in food preservation (Shee *et al*., 2010). Due to the sensitivity of *B. subtilis* towards NaCl, another Gram-positive bacterial strain, *S. aureus*, was used to investigate the salt and serum effects on the antibacterial activities of Os and Os-C.

In literature the effect of salts on the antibacterial activity of peptides is mostly evaluated using the CFU method. To quantify the effects of cations on the antibacterial activity of Os and Os-C, the MBCs of the peptides were, therefore, determined in the presence of 100 mM NaCl, using the CFU method. The same method was used to quantify the effect of the presence of human serum on the antibacterial activities of Os and Os-C. Table 3.7 summarises the effect of 100 mM Na^{+} on the bactericidal activity of Os and Os-C, compared to that found when tested in NaP buffer (10 mM, pH 7.4). Using this method, the presence of Na^{+} did not affect the bactericidal activity of Os against *E. coli* and only a 2-fold increase in the MBC of Os-C against the same bacteria was observed. In the presence of NaCl, both Os and Os-C showed a decrease in bactericidal activity against *S. aureus*. The MBC of Os increased 32-fold and Os-C was found to be inactive against *S. aureus* at a peptide concentration of 120 µg/ml (Table 3.7). Overall, Os retained some, if not all, of its bactericidal activity against *E. coli* and *S. aureus*, in the presence of NaCl. In a study conducted by Friedrich *et al*. (1999) the authors found that by increasing the hydrophobicity and α-helical structural characteristics of cationic peptides, derived from the amino-terminal of cecropins A and the carboxy-terminal of melittin, the antibacterial activities of the peptides were retained in the presence of NaCl. Since Os has more α-helical properties than

Os-C when prepared in SDS (Fig. 3.2b), this difference in the antibacterial activities of Os and Os-C in the presence of NaCl can be explained by the difference in the secondary structures of the peptides.

In most studies, serum is heat inactivated thereby only investigating the effect of binding of serum proteins to peptides on the antibacterial activity of the peptides. Marcellini *et al*. (2009) studied the effect of heat inactivated human serum (70% *v/v*) on the antibacterial activity of esculentin, an AMP isolated from frog skin. The authors found that the antibacterial activity of the peptide decreased in the presence of human serum indicating possible interaction of the serum proteins with the peptide. In a study conducted by Maisetta *et al*. (2008) it was shown that human serum albumin decreased and abolished the antibacterial activity of human β-defensin 3 against *S. aureus* and *Acinetobacter baumannii* (Gram-negative bacteria), respectively. These results indicate that serum proteins, alone, can affect the antibacterial activities of AMPs. The effect of non-heat inactivated 30% human serum on the bactericidal activity of Os and Os-C is summarised in Table 3.8. Os retained some, if not all, of its bactericidal activity in the presence of human serum against both Gram-positive and Gram-negative bacteria. *S. aureus* was the most susceptible against Os in the presence of human serum. Os-C was inactive at concentrations of 120 µg/ml and lower against both Gram-positive and Gram-negative bacteria. Ciornei *et al*. (2005) studied the effects of non-heat inactivated human serum on the antibacterial activities of analogues of the human AMP, LL-37. The authors synthesized these analogues so that these peptides contained less hydrophobic residues than the parent, LL-37, peptide. It was observed that by decreasing the amount of hydrophobic amino acids the peptides retained their antibacterial properties in the presence of 40% human serum. Furthermore, the authors found that the LL-37 fragments were active against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), but not Gram-positive bacteria (*S. aureus*), in the presence of 150 mM NaCl.

The MBCs of Os and Os-C against *S. aureus* were repeated for the salt and serum effect assays. A difference in these MBCs, determined in NaP buffer, was observed when comparing Table 3.5 with Table 3.8. This 4-fold decrease in MBC of both Os and Os-C against *S. aureus* was a result of the conditions in which the bacteria were prepared. When the initial MBC determination assays were performed the overnight bacterial cultures were stored at 4 ºC and used repeatedly for the sub-culturing of the bacterial samples. When the salt and serum effects assays were performed the overnight samples were used once and then discarded, thus “fresh” bacterial stocks were used for each independent experiment. This change in bacterial preparation had no effect on the antibacterial susceptibility of *E. coli*, *P. aeruginosa* or *B. subtilis*. The tendency of

S. aureus to become resistant towards antibacterial agents might explain the sensitivity of this bacterial strain towards the change in preparation procedure.

When comparing the percentage hydrophobic residues of Os and Os-C (Table 3.1) it can be seen that Os has a lower percentage hydrophobic amino acid composition than Os-C. By incorporating the findings of Ciornei *et al*. (2005), it can be assumed that the ability of Os and the inability of Os-C to resist the effects of the presence of salt and serum on the antibacterial activity are linked to the hydrophobicities of the peptides. Furthermore, it is interesting to note that the antibacterial activity of Os against *E. coli* was retained but the activity against *S. aureus* was lost in the in the presence of NaCl, the same finding made by Ciornei *et al*. (2005).

Since the susceptibility of *E. coli* and *B. subtilis* to Os and Os-C were the same, when tested in NaP buffer (Table 3.5), these bacterial strains were used to investigate the time that is required for each of the synthetic peptides to kill targeted bacteria at the respective MBCs of the peptides. Os killed the targeted bacteria (*E. coli* and *B. subtilis*) effectively after only 5 min, whereas, with Os-C, required 60 and 120 min of incubation to completely kill *E. coli* and *B. subtilis*, respectively (Fig. 3.5). This major difference in the killing kinetics of the Os and Os-C is indicative of a possible difference in mode of action. *B. subtilis* is known to form spores under stress (Grossman & Losick, 1988). This can explain the delayed killing when targeted by Os-C (120 min) and the occurrence of additional colonies (growth control increased from 180 at 5 min to 360 after 120 min). If this is true, then Os which killed *B. subtilis* within 5 min, shows potential as an antimicrobial agent against bacterial spores. Fast killing kinetics, as was observed with Os, have been previously reported for other AMPs. Varkey *et al*. (2006) found that, at its MBC, OM∆C killed *E. coli* within 10 min. The other arthropod defensin analogue investigated by these authors, PA∆C, took only 5 min to completely kill *E. coli*. Nakajima *et al*. (2003) showed that *O. moubata* defensin took up to 60 min to kill *M. luteus* whereas magainin 2 analogue killed the same bacterial strain after 1 min.

As stated previously, the difference in the rate of killing of Os and Os-C might suggest a difference in their mode of action. Furthermore, the differences in their secondary structures may also cause a difference in their mode of action. Sitaram and Nagaraj (1999) discussed in great lengths the importance of charge and secondary structure on the antibacterial activity and mode of action of AMPs. Generally an AMP, or a segment thereof, must be α-helical in order to form pores or toroidal pores which will lead to the permeabilization of the bacterial cell (Sitaram & Nagaraj, 1999). Melittin and magainin 2 were used as the positive and negative controls, respectively, during the preliminary SYTOX Green permeabilization assays. SYTOX Green will

fluoresce upon the binding of nucleic acids, thus an increase in fluorescence is indicative of damaged cellular membranes, enabling the SYTOX Green to enter the cell or the DNA to leak out of the cell. Sitaram and Nagaraj (1999) showed that melittin, when incubated with bacteria and SYTOX Green, caused an increase in the fluorescence of the SYTOX Green nucleic acid stain. This indicated that melittin causes permeabilization or leakage of bacterial membranes. Bourbon *et al*. (2008) further showed that magainin 2 did not increase the fluorescence of SYTOX Green indicating that the antibacterial mode of action of magainin 2 does not involve the permeabilization of bacterial cells. However, Matsuzaki (1998) suggested that magainin 2 does cause membrane permeabilization of bacterial cells by forming toroidal pores of finite size. Imura *et al*. (2008) confirmed this by showing that an equipotent magainin 2 analogue formed pores in bacterial membranes which allowed the bacterial membranes to be permeable to molecules smaller than 6.6 kDa. In another study, melittin caused lysis of membranes at high peptide concentrations but was reported to form pores at low concentrations (van den Bogaart *et al*., 2008). Pore formation may be a function of peptide concentration and incubation time.

Both magainin 2 and melittin were found to be active against *E. coli* in the concentration range used for the assay (Table 3.9). When incubated with melittin, the fluorescence output of SYTOX Green was higher than that of the untreated bacterial cells (control) whereas when treated with magainin 2, no noteworthy difference of SYTOX fluorescence was seen in comparison with the untreated cells (Fig. 3.6a). From these results one may conclude that the mode of action of magainin 2 does not involve the permeabilization of the bacterial membrane. When incubated with Os-C, similar results to that of magainin 2 were observed (Fig. 3.6a). This indicated that the mode of action of Os-C might not involve permeabilization of bacterial cell membranes. It is however, not possible to elucidate the mode of action of Os-C on this data alone. The slow killing kinetics of Os-C, observed in Fig. 3.5, suggested that the mode of action of Os-C might involve intracellular targets. When combining this observation to that of the SYTOX Green assay results, it may be concluded that the possible mode of action of Os-C involves the formation of pores, as reported for magainin 2 (Matsuzaki, 1998), allowing Os-C to enter the cell targeting intracellular components.

When incubated with Os, the fluorescence of SYTOX Green decreased dramatically in comparison with the untreated cells (Fig. 3.6a). This was unexpected as these results indicate that the bacterial cells were more permeable when not treated with Os. Although some bacterial cell death was expected, in the untreated samples, this conclusion is false since Os causes complete killing of *E. coli* at concentrations lower than the highest concentration used in this assay (Table 3.6). There are two possible explanations for this observation: i) Os caused complete lysis of

bacterial cells resulting in intracellular contents quenching the fluorescence signal, or ii) that Os bound to the bacterial DNA shielding it from SYTOX Green stain. The first explanation seems unlikely, for then the same decrease in fluorescence would have been seen when the bacteria were treated with melittin. The second explanation seems more plausible; but, it does not explain the dramatic decrease in SYTOX Green fluorescence. It is possible that both these explanations are true and that the combination of quenching and binding to the bacterial DNA is what was observed.

To test whether the observed effect could be as a result of Os binding to the bacterial DNA, the assay was repeated where melittin was incubated, with the bacterial cells and SYTOX Green stain, in combination with Os. It was hypothesised that if melittin causes lysis of the bacterial membrane and Os binds to the exposed DNA, a decrease in fluorescence of SYTOX Green, in comparison with that observed when incubated with melittin alone, would be observed. Indeed the fluorescence signal of the melittin-Os combination was lower than that of melittin alone (Fig. 3.6b). This indicated that Os did bind to bacterial DNA and thereby shielded it from the nucleic acid stain.

From these preliminary SYTOX Green permeabilization assays, no absolute conclusion on the antibacterial mode of action of Os and Os-C could be drawn. However, two possible problematic parameters were observed. The first being that the bacterial concentration that was used produced a low fluorescence output. At 1 x 10^6 CFU/ml the fluorescence output signal for the samples treated with all the peptides was smaller than 1, whereas the fluorescence of SYTOX Green when incubated with plasmid DNA (data not shown), was higher than 50 when using the Fluoroskan Ascent FL fluorometer. This indicated that the DNA concentration in the bacterial samples was too low to be accurately detected. Luca *et al*. (2012) reported that a higher bacterial concentration (in the range of 1 x 10^7 CFU/ml) was needed for SYTOX Green fluorescence detection. For this reason, the SYTOX Green fluorescence of samples with increasing bacterial concentrations was determined. 9Pbw2, used here as a negative control, was observed by Shin *et al*. (2009) to not cause membrane permeabilization. It should be noted, however, that the authors measured the leakage of a fluorescent dye, calcein, out of synthetic large unilamellar vesicles after only 3 min of exposure. Fig. 3.7 summarises the relative SYTOX Green fluorescence with increasing bacteria concentration compared to that of bacteria not treated with peptide. The peptide concentration was increased with the same factor as the bacteria concentration. From Fig. 3.7 it was found that the bacteria concentration that was high enough to obtain sufficient fluorescence when treated with melittin, as well as a significant difference in the fluorescence

caused by melittin and 9Pbw2, was 64 x 10^6 CFU/ml. Thus, the bacterial concentration used for the SYTOX Green permeabilization time studies that followed was 6.4 x 10^7 CFU/ml.

The second troubling factor was that the peptide concentration range used in the preliminary studies was too broad and the highest peptide concentration was too high. Thevissen *et al.* (1999) reported that the SYTOX Green fluorescence of a bacterial sample treated with plant defensins decreased at high peptide concentrations. The authors speculated that the reason for this decrease was that, at high concentrations, the peptides interacted with the bacterial DNA, thereby decreasing the DNA available for binding by SYTOX Green. This may explain the decrease in the SYTOX Green fluorescence when *E. coli* was incubated with Os at high peptide concentrations (Fig. 3.6a and Fig. 3.6b). Therefore, the SYTOX Green permeabilization assays that followed were conducted at the MBCs of the respective peptides for *E. coli*.

Hartmann *et al*. (2010) observed that the MIC of AMPs against bacteria differs at higher bacterial concentration and that the increase in MICs is not linear to the increase in bacterial concentration. It was therefore, necessary to determine the MBC of each peptide against the higher bacteria concentration. Table 3.10 summarises the MBCs when tested against two different bacterial concentrations using the turbidity assay. It was found that the antibacterial concentrations of the synthetic peptides increased 4-fold when the bacterial concentration increased 64-fold. These new MBCs were then used in the SYTOX Green permeabilization assays that followed.

The previous experiments measured the SYTOX Green permeabilization of bacterial cells after incubating *E. coli* with peptides for 1 hr or 2 hrs. The antibacterial effect of the peptides measured after that period could, however, have been a secondary effect. The increase in fluorescence observed in Fig. 3.6a could have been a result of damage caused to the bacterial cell membrane by a secondary effect since it is possible that the peptides bound to intracellular targets resulting in the weakening or degradation of the cell membrane, thereby resulting in permeabilization. The increase in SYTOX Green fluorescence will indicate possible membrane permeabilization, incorrectly, as the peptides' mode of action. For this reason, it was deemed necessary to investigate the SYTOX Green fluorescence over a shorter time interval.

Fig. 3.8 depicts the change in SYTOX Green fluorescence as a function of incubation time. When the bacteria were incubated with melittin, used here as a positive control, there was an increase in fluorescence only after 30 min incubation, whereas with 9Pbw2, an increase in fluorescence was seen directly after the addition of the peptide (0 min). The fluorescence caused by 9Pbw2 stayed constant after 5 min, increasing by 5 units after 30 min. Incubating *E. coli* with

Os caused a steady increase in SYTOX Green fluorescence over time reaching the same fluorescence as with 9Pbw2 after 15 min. Comparing the possible mode of action of 9Pbw2 with that of Os, 9Pbw2 caused permeabilization upon addition to *E. coli* cells, whereas Os caused a gradual linear increase in permeability of the bacterial cell membrane over time. Os-C showed a similar effect but more rapid than Os, indicating membrane permeabilization possibly as a secondary effect. What is apparent is that melittin, 9Pbw2, Os and Os-C caused permeabilization of *E. coli* cell membranes after 30 min of incubation. Scanning electron microscopy of *E. coli* treated with melittin, Os and Os-C at the respective MBCs showed that melittin caused membrane disruption forming micellization of the membrane. Furthermore, Os and Os-C treated *E. coli* cells were empty, raisin-like cells (Prinsloo *et al*., 2013). These results indicated that melittin, Os and Os-C affect *E. coli* cell membranes using different modes of action. Overall, it can be concluded that both Os and Os-C cause membrane permeabilization of *E. coli* cells resulting in the leakage of intracellular components. Differences in the mode of action may be due to differences in charge, α-helical structure and hydrophobic natures of the peptides.

EDTA (1 mM), a chelating agent, and Triton X-100 (0.5%), a non-ionic detergent, were added to the incubated bacteria-peptide samples in order to obtain maximum SYTOX Green fluorescence. Upon the addition of EDTA and Triton X-100 (Fig. 3.8, 35 min), the bacterial cells treated with peptides increased in SYTOX Green fluorescence. The untreated samples, however, did not show an increase in fluorescence when EDTA and Triton X-100 were added. The increase in fluorescence observed with the peptide-treated samples could be due a synergistic effect between the peptide and the EDTA and Triton X-100. Another possible explanation is that the peptides caused the bacterial cellular membranes to become destabilized, which was confirmed by the initial increase in SYTOX Green fluorescence, thereby making the *E. coli* cells susceptible to the effects of EDTA and Triton X-100.

The lack of increase in the fluorescence of the untreated cells, upon the addition of EDTA and Triton X-100, was unexpected. It was possible that the bacterial strain that was used was not susceptible to EDTA and Triton X-100. In order to validate this explanation, another two, Gram-positive bacterial strains were used. The SYTOX Green fluorescence of two untreated bacterial strains was measured before and after the addition of EDTA and Triton X-100 (Fig. 3.9). *S. aureus* displayed the same characteristics as *E. coli*, whereas with *B. subtilis* the SYTOX Green fluorescence increased upon the addition of EDTA and Triton X-100. These findings illustrated that there is a difference in the susceptibility of different bacterial strains to EDTA and Triton X-100. Furthermore, this finding validated the methodology that was used. Alternatively, to determine the maximum fluorescence other detergents, such as SDS, can be used.

An assay was performed to investigate the DNA binding capabilities of Os and Os-C. Plasmid DNA consists of three forms: super coiled, circular, and linear. When run on an agarose gel, the migration of these three forms differ with open circular being the slowest and super coiled the fastest. In Fig. 3.10a and Fig. 3.10b, the plasmid control (no added peptide) showed only two distinct bands. It was, however, sufficient to use as a comparison to the samples incubated with peptide. AAPH, a radical generator, was used as a positive control. Free radicals are known to interact with DNA (O'Brien, 1985) in such a way that it can create "nicks" in super coiled plasmid DNA resulting in open circular or linear plasmid DNA. In Fig. 3.10a and Fig. 3.10b it is clear that the sample incubated with AAPH has one less band (lowest band) indicating that the super coiled plasmid DNA was nicked, causing the formation of linear plasmid DNA. Some cationic AMPs have been reported to bind to bacterial DNA thereby eliciting their mode of action. If the cationic peptides bind to the negative phosphate backbone of DNA a decrease in the migration of the DNA, towards the positive pole and/or a decrease in EtBr staining, will be observed.

In Fig. 3.10a, at the highest tested Os concentration (7.5 µg/ml) the lower (super coiled) band is fainter than the same bands of the samples incubated with a lower Os concentration. This indicated that there was some interaction between Os and the plasmid DNA. During this preliminary plasmid binding assay, the plasmid to peptide ratio ranged from 1:1.5 x 10^{-5} to 1:0.15. In order to test whether Os interacts with DNA, plasmid was incubated with higher concentrations of Os. For comparison purposes the plasmid-binding ability of Os-C was also investigated (Fig. 3.10b). The concentration range of Os and Os-C (Fig. 3.10b) ranged from 60 ng/ ml to 0.6 mg/ml which corresponds to plasmid to peptide ratio range of 1:0.0012 to 1:12. At the two highest concentrations of Os, 0.6 and 0.06 mg/ml, no bands were observed. The same was true for Os-C at 0.6 mg/ml. This indicated that at high concentrations of Os and Os-C the peptides bind to negatively charged plasmid DNA, thereby decreasing the migration of DNA through the agarose gel towards the positive pole. It is possible that this peptide-DNA interaction is to such a degree that the overall charge of the molecule becomes positive resulting in the migration towards the negative pole. This might explain the complete lack of bands, not even in the wells, in the samples incubated with high peptide content.

Another explanation might be that Os and Os-C binds to the DNA and subsequently shielding the DNA from binding to EtBr. The interaction of EtBr, a DNA interchelator, with DNA causes the fluorescence observed when viewed under UV light. By blocking the binding of EtBr to DNA, the peptides will cause the bands to be invisible. Whatever the explanation, it may be concluded that Os and Os-C, at high concentrations, bind DNA. Thus, this assay confirmed what

was speculated in the discussion of the SYTOX Green assay results. It is, therefore, highly plausible that Os interacts with the bacterial DNA thereby shielding it from SYTOX Green. For this to be possible, Os must cause permeabilization of the bacterial cell or enter the cell by another mechanism.

The potential of an AMP being used pharmaceutically as an antimicrobial agent is largely influenced by the effect of the AMP on mammalian or eukaryotic cells. In a study performed by our group, the hemolytic activity of Os and Os-C on human red blood cells was determined. It was found that both Os and Os-C did not cause hemolysis of RBCs even at peptide concentrations 16 times higher than the highest MBC (Prinsloo *et al.*, 2013). Erythrocytes are highly differentiated cells that are non-dividing and contain no organelles or DNA. Mammalian cells grown in cell culture have a high metabolic rate and undergo mitosis. An ideal AMP for further clinical evaluation should have activity against bacteria but must be non toxic towards mammalian cells. For this purpose 2 cell lines, SC-1 (mouse embryonic fibroblasts) and Caco-2 (human colon carcinoma cells), were used to investigate the effects of Os and Os-C on eukaryotic cells. The CV assay was used to determine the number of attached cells following the exposure of Os and Os-C for 24 hrs. CV binds and stains DNA and cellular proteins. Toxicity can result in cell death and *in vitro* can cause (i) cells to detach or (ii) lysis of attached cells with loss of cellular content. In each instance this will result in decreased CV staining.

During this study SC-1 and Caco-2 cell lines were used to determine the effects of Os and Os-C on mammalian cells. SC-1 cells are mouse fibroblasts that grow more rapidly (doubling time of 48 hrs) than Caco-2 cells which are human colon epithelial cells (doubling time of 62 hrs). Melittin, known to cause lysis of eukaryotic cell membranes, was used as a positive control. Light microscopy photos (Fig. 3.11) depict the effects of the peptides on SC-1 cells and Caco-2 cells. At high melittin concentrations (120 µg/ml), the number of stained cells (in both cases) were markedly decreased as when compared to the controls (no peptide added). Both Os and Os-C did not affect the cell number of either the cell lines even at the highest peptide concentrations (120 µg/ml), whereas melittin caused significant decrease in SC-1 cells (up to 60%) and Caco-2 cells (up to 25%). These differences between SC-1 and Caco-2 sensitivity may be due to differences in doubling times.

Since the cytotoxicity assays were performed in the presence of medium, the lack in cytotoxicity after exposure of Os and Os-C may be due to the binding of the peptides to the albumin present in the cell culture medium. Serum albumin has been reported to bind to various molecules and drugs and can serve as a carrier of these compounds (Varshney *et al.*, 2010). Considering this

property of albumin it is possible that Os and Os-C bound to albumin decreasing the possible cytotoxic effects of these peptides. Melittin, however, was still shown to be cytotoxic under the same conditions; therefore the assay can be seen as a valid indication of cytotoxicity. It is important to note that the antibacterial activity of Os was not affected by the presence of serum albumin (Table 3.8) thereby indicating that the peptide retains activity, including possible cytotoxic activity, in the presence of albumin. Furthermore, hemolytic effects and cellular toxicity effects are due to the presence of α-helical structure such as found in melittin (Zang *et al*., 1999). Os-C lack α-helical properties as was found by CD spectrometry (Fig. 3.2b) in SDS. Both the hemolysis and cytotoxicity assay indicated that Os and Os-C were not toxic to eukaryotic cells and can, therefore, be considered for further investigation as potential antibacterial therapeutic agents.

CHAPTER 5: CONCLUSION AND REFERENCES

Conclusion and future prospects

AMPs show promise as potential therapeutic agents in the battle against the ever-increasing bacterial resistance towards currently available antibiotics. Two defensin isoforms (OsDef1 and OsDef2), naturally occurring AMPs, were previously identified in the midgut of the tick *O. savignyi*. During this study OsDef2 was successfully used as a template for the design of smaller AMPs. Os and Os-C, derived from the carboxy-terminal of OsDef2, were shown to not only be active against Gram-positive bacteria, as was observed for the parent peptide, but also against Gram-negative bacteria. Os, the cysteine containing derivative, showed higher antibacterial activity than the derivative without cysteine residues, Os-C. Future studies will be aimed at broadening the antimicrobial activity spectrum of Os and Os-C. Bactericidal activities of these peptides against MRSA strains will be investigated. Possible fungicidal activity will be investigated using the CFU method described by Tsuji *et al*. (2007) where the authors reported fungicidal activity for P4 against *Pichia pastoris*. The potential activities of Os and Os-C against biofilm formation will be investigated using the CV method described by Pompilio *et al*. (2012).

Os showed promise as a potential therapeutic agent as it exhibited fast killing kinetics against Gram-positive and Gram-negative bacteria, retained bactericidal activity in physiological conditions and were not toxic towards mammalian cells. More in-depth mode of action studies of Os will be performed using a triple stain method described by Mangoni *et al*. (2004) in which CTC (5-cyano-2,3-ditolyl tetrazolium chloride), DAPI (4',6-diamidino-2-phenylindole) and FITC (Fluorecein isothiocyanate) will used to, respectively, stain viable bacteria, all bacterial cells and bacterial cells with permeabilized membranes. Some AMPs exhibit their antibacterial activities by targeting intrecellular components such as DNA. Os and Os-C were found to bind to plasmid DNA at high concentrations. However, before this can be seen as a possible mode of action of these peptides, it must be determined whether these peptides traverse the bacterial membrane. To investigate this effect a fluorescent dye, Alexa Fluor®, will be used to label the peptides as described by Gee *et al*, 2013. The location of these fluorescently labelled peptides will then be viewed using fluorescence microscopy. Furthermore, the effect of the oxidation state of Os on the antibacterial mode of action of the peptide will also be investigated. Hocquellet *et al*. (2010) found that the antibacterial activity as well as the membrane permeabilization and DNA binding capabilities of a human AMP were different between the oxidised and reduced (added DTT) forms of the AMP.

References

Allahverdiyev, A.M., Bagirova, M., Abamor, E.S., Ates, S.C., Koc, R.C., Miraloglu, M., Elcicek, S., Yaman, S. & Unal, G. 2013. The use of platensimycin and platencin to fight antibiotic resistance. *Infection and Drug Resistance*, 6: 99-114.

Barlow, P.G., Svoboda, P., Mackellar, A., Nash, A.A., York, I.A., Pohl, J., Davidson, D.J. & Donis, R.O. 2011. Antiviral activity and increased host defense against Influenza infection elicited by the human cathelicidin LL-37. *PLoS ONE*, 6: 1-9.

Basir, Y.J., Knoop, F.C., Dulka, J. & Conlon, J.M. 2000. Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretions of the pickerel frog, *Rana palustris*. *Biochimica et Biophysica Acta*, 1543: 95-105.

Bourbon, C., Bry, C., Roggemans, C., Soulard, C., Thizon, C. & Garbav, B. 2008. Use of a real-time polymerase chain reaction thermocycler to study bacterial cell permeabilization by antimicrobial peptides. *Analytical Biochemistry*, 1: 279-281.

Brogden, K.A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature Reviews*, 3: 238-250.

Brown, K.L. & Hancock, R.E.W. 2006. Cationic host defense (antimicrobial) peptides. *Current Opinion in Immunology*, 18: 24-30.

Bulet, P., Hetru, C., Dimarcq, J. & Hoffmann, D. 1999. Antimicrobial peptides in insects; structure and function. *Developmental & Comparative Immunology*, 23: 329-344.

Bulet, P., Stöcklin, R. & Menin, L. 2004. Anti-microbial peptides: from invertebrates to vertebrates. *Immunological Reviews*, 198: 169-184.

Campbell, N.A. & Reece, J.B. 2005. *Biology*. 7th ed. Pearson education, Inc.: San Francisco, pp. 898-919.

Chiba, K., Kawakami, K. & Tohyama, K. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. 1998. *Toxicology in Vitro*, 12: 251-258.

Cho, S.H., Lee, B.D., An, H. & Eun, J.B. 2005. Kenojeinin 1, antimicrobial peptide isolated from the skin of the fermented skate, *Raja kenojei*. *Peptides*, 26: 581-587.

Chrudimská, T., Slaninová, J., Rudenko, N., Růžek, D. & Grubhoffer, L. 2011. Functional characterization of two defensin isoforms of the hard tick *Ixodes ricinus*, *Parasites & Vectors*, 4: 1-6.

Ciornei, C., Sigurdardottir, T., Schmidtchen, A. & Bodelsson, M. 2005. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. *Antimicrobial Agents and Chemotherapy*, 49: 2845-2850.

Cociancich, S., Ghazi, A., Hetru, C., Hoffmann, J.A. & Letellier, L. 1993. Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus*. *The Journal of Biological Chemistry*, 268: 19239-19245.

Conlon, J.M., Al-Ghaferi, N., Abraham, B., Jiansheng, H., Cosette, P., Leprince, J., Jouenne, T. & Vaudry, H. 2006. Antimicrobial peptides from diverse families isolated from the skin of the Asian frog, *Rana graham*. *Peptides*, 27: 2111-2117.

Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M. & Bienert, M. 2001. Optimization of the antimicrobial activity of magainin peptides by modification of charge. *FEBS letters*, 501: 146-150.

Dorschner, R.A., Lopez-Garcia, B., Peschel, A., Kraus, D., Morikawa, K., Nizet, V. & Gallo, R.L. 2006. The mammalian ionic environment dictates microbial susceptibility to antimicrobial defence peptides. *The FASEB Journal*, 20: 35-42.

Ericksen, B., Wu, Z., Lu, W. & Lehrer, R.I. 2005. Antibacterial activity and specificity of the six human α-defensins. *Antimicrobial Agents and Chemotherapy*, 49: 269-275.

Fogaça, A.C., da Silva, P.I.J., Miranda, M.T., Bianchi, A.G., Miranda, A., Ribolla, P.E. & Daffre, S. 1999. Antimicrobial activity of the bovine hemoglobin fragment in the tick *Boophilus microplus*. *Journal of Biological Chemistry*, 274: 25330-25334.

Fogaça, A.C., Almeida, I.C., Eberlin, M.N., Tanaka, A.S., Bulet, P. & Daffre, S. 2006. Ixodidin, a novel antimicrobial peptide from the hemocytes of the cattle tick *Boophilus microplus* with inhibitory activity against serine proteinases. *Peptides*, 27: 667-674.

Fox, J.L. 2013. Antimicrobial peptides stage a comeback. *Nature Biotechnology*, 31: 379-382.

Friedrich, C., Scott, M.G., Karunaratne, N., Yan, H. & Hancock, R.E.W. 1999. Salt-resistant α-helical cationic antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*, 43: 1542-1548.

Ganz, T. 2003. Defensins: antimicrobial peptides of innate immunity. *Nature reviews*, 3: 710-720.

Gee, M.L., Burton, M., Grevis-James, A., Hossain, M.A., McArthur, S., Palombo, E.A., Wade, J.D. & Clayton, H.A. 2013. Imaging the action of antimicrobial peptides on living bacterial cells. *Scientific reports*, 3: 1557; DOI: 10.1038/srep01557.

Giangaspero, A., Sandri, L. & Tossi, A. 2001. Amphipathic α helical antimicrobial peptides. *The FEBS Journal*, 268: 5589-5600.

Gordon, Y.J. & Romanowski, E.G. 2005. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Current eye Research*, 30: 505-515.

Grossman, A.D. & Losick, R. 1988. Extracellular control of spore formation in *Bacillus subtilis*. *Proc. Natl. Acad. Sci.*, 85: 4369-4373.

Hancock, R.E.W. 2001. Cationic peptides: effectors in innate immunity and novel antimicrobials. *The LANCET infectious Diseases*, 1: 156-164.

Hancock, R.E.W. & Rozek, A. 2001. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiology Letters*, 1: 143-149.

Harder, J., Bartels, J., Christophers, E. & Schröder, J.M. 2001. Isolation and characterization of human β-defensin-3, a novel human inducible peptide antibiotic. *The Journal of Biological Chemistry*, 276: 5707-5713.

Hartmann, M., Berditsch, M., Hawecker, J., Ardakani, M.F., Gerthsen, D. & Ulrich, A.S. 2010. Damage of the bacterial cell envelope by antimicrobial peptides Gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrobial Agents and Chemotherapy*, 54: 3132-3142.

Hocquellet, A., Odaert, B., Cabanne, C., Noubhani, A., Dieryck, W., Joucla, G., Le Senechal, C., Milenkov, M., Chaignepain, S., Schmitter, J.M., Claverol, S., Santarelli, X., Dufourc, E.J., Bonneu, M., Garbay, B. & Costaglioli, P. 2010. Structure-activity relationship of human liver-expressed antimicrobial peptide 2. *Peptides*, 31: 58-66.

Hoffmann, J.A. 1995. Innate immunity of insects. *Current opinion in immunology*, 7: 4-10.

Hoffmann, J.A. & Hetru, C. 1992, *Immunology today*, vol. 13, pp. 411-415.

Imura, Y., Choda, N., Matsuzaki, K. 2008. Magainin 2 in action: distinct mode of membrane permeabilization in living bacterial and mammalian cells. *Biophysical Journal*, 95: 5757-5765.

Isogai, E., Isogai, H., Takahashi, K., Kobayashi-Sakamoto, M. & Okumura, K. 2009. Antimicrobial activity of three defensins and four mammalian cathelicidin-derived synthetic peptides against Lyme disease spirochetes and bacteria isolated from the midgut. *Experimental Applied Acarology*, 49: 221-228.

Isogai, E., Isogai, H., Okumura, K., Hori, H., Tsuruta, H., Kurebayashi, Y. 2010. Tertiary structure-related activity of tick defensin (persulcatusin) in the taiga tick, *Ixodes persulcatus*. *Experimental Applied Acarology*, DOI: 10.1007/s10493-010-9379-3.

Jakubowski, 2013. Chapter 2 *Protein structure: probing composition, sequencing and conformation*. [Online] Available at: http://employees.csbsju.edu/hjakubowski/classes/ch331/protstructure/olcompseqconform.html.

Janeway, C.A. Jr., Travers, P., Walport, M. *et al*. Immunobiology: The immune system in health and disease. 5th edition. New York: Garland Science, 2001. The components of the immune system. [Online] Available at: http://www.ncbi.nlm.nih.gov/books/NBK27092/

Khan, A.H. & Shah, S.S. 2007. Determination of critical micelle concentration (cmc) of sodium dodecyl sulfate (SDS) and the effect of low concentration of pyrene on its cmc using ORIGIN software. *Journal of the Chemical Society of Pakistan*, 30: 186-191.

Kindt, T.J., Goldsby, R.A. & Osborne, B.A. 2007, *Kuby Immunology*.

Kokryakov, V.N., Harwig, S.S.L., Panyutich, E.A., Shevchenko, A.A., Aleshina, G.M., Shamova, O.V., Korneva, H.A. & Lehrer, R.I. 1993. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. *FEBS Letters*, 327: 231-236.

Lai, R., Lomas, L.O., Joncxy, J., Turner, P.C. & Rees, H.H. 2004. Two novel non-cationic defensin-like antimicrobial peptides from hemolymph of the female tick, *Amblyomma hebraeum*. *Biochemistry Journal*, 379: 681-685.

Lazarev, V.N. & Govorun, V.M. 2010. Antimicrobial peptides and their use in medicine. *Applied Biochemistry and Microbiology*, 46: 803-814.

Lee, J.Y., Boman, A., Chuanxin, S., Andersson, M., Jörnvall, Mutt, V. & Boman, H.G. 1989. Antibacterial peptide from pig intestine: isolation of mammalian cecropins. *Proceedings of the National Academy of Science of the United States of America*, 86: 9159-9162.

Lehrer, R.I. & Ganz, T. 1994. Endogenous vertebrate antibiotics: defensins, protegrins, and other cysteine-rich antimicrobial peptides. *Annals New York Academy of Sciences*, 228-239.

Lehrer, R.I. 2004. Primate defensins. *Nature Reviews*, 2: 727-738.

Li, Y., Xiang, Q., Zhang, Q., Huang, Y. & Su, Z. 2012. Review: overview on the recent study of antimicrobial peptides: origins, functions, relative mechanisms and application. *Peptides*, 37: 207-215.

Lichtenstein, A. 1991. Mechanism of mammalian cell lysis mediated by peptide defensins: evidence for an initial alteration of the plasma membrane. *The Journal of Clinical Investigation*, 1: 93-100.

Liu, Z., Liu, H., Liu, X. & Wu, X. 2008. Purification and cloning of a novel antimicrobial peptide from salivary glands of the hard tick, *Ixodes sinensis*. *Comparative Biochemistry and Physiology part B: Biochemistry and Molecular Biology*, 149: 557-561.

Lodish, H., Berk, A., Kaiser, C.A., Kriege, M., Scott, M.P., Bretscher, A., Ploegh, H. and Matsudaira, P. 2008. *Molecular cell biology*. 6th ed. W.H. Freeman and Company: New York.

Lowry, F.D. 2001. Antimicrobial resistance: the example of *Staphylococcus aureus*. *The Journal of Clinical Investigation*, 111: 1265-1273.

Lu, X., Che, Q., Lu, Y., Wang, M., Lu, Z., Feng, F., Liu, J. & Yu, H. 2010. A novel defensin-like peptide from salivary glands of the hard tick, *Haemaphysalis longicornis*. *Protein Science*, 19: 392-397.

Luca, V., Stringaro, A., Colone, M., Pini, A. & Mangoni, M.L. 2012. Esculentin(1-12), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen *Pseudomonas aeruginosa*. *Cellular and Molecular Life Sciences*, DOI: 10.1007/s00018-013-1291.

Luidens, M.K., Figge, J., Breese, K. & Vajda, S. 1995. Predicted and trifluoroethanol induced α-helicity of polypeptides. *Biopolymers*, 39: 367-376.

Madigan, M.T., Martinko, J.M., Dunlap, P.V. & Clark, D.P. 2009. *Brock biology of microorganisms*. 12th ed. Pearson International Edition: San Francisco.

Maisetta, G., Luca, M.D., Esin, S., Florio, W., Brancatisano, F.L., Bottai, D., Campa, M. & Batoni, G. 2008. Evaluation of the inhibitory effects of human serum components on bactericidal activity of human beta defensin 3. *Peptides*, 29: 1-6.

Makovitzki, A., Avrahami, D. & Shai, Y. Ultrashort antibacterial and antifungal lipopeptides. *Proceedings of the National Academy of Science* , 103: 15997-16002.

Mandal, S.M., Dey, S., Mandal, M., Sarkar, S., Maria-Neto, S. & Franco, O.L. 2009. Identification and structural insights of three novel antimicrobial peptides isolated from the green coconut water. *Peptides*, 30: 633-637.

Mangoni, M.L., Papo, N., Barra, D., Simmaco, M., Bozzi, A., Giulio, A.D. & Rinaldi, A.C. 2004. Effects of the antimicrobial peptide temporin L on cell morphology, membrane permeability and viability of *Escherichia coli*. *Biochemistry Journal*, 380: 859-865.

Marcellini, L., Borro, M., Gentile, G., Rinaldi, A.C., Stella, L., Aimola, P., Barra, D. & Mangoni, M.L. 2009. Esculentin-1b(1-18) – a membrane-active antimicrobial peptide that synergizes with antibiotics and modifies the expression level of a limited number of proteins in *Escherichia coli*. *FEBS Journal*, 276: 5647-5664.

Marr, K.A., Gooderham, W.J. & Hancock, R.E.W. 2006. Antibacterial peptides for therapeutic use: obstacles and realistic outlook. *Current Opinion in Pharmacology*, 6: 468-472.

Matsuzaki, K. 1998. Magainins as paradigm for the mode of action of pore forming polypeptides. *Biochimica et Biophysica Acta*, 1376: 391-400.

Nakajima, Y., Van der Goes van Naters-Yasui, A., Taylor, D. & Yamakawa, M. 2001. Two isoforms of a member of the arthropod defensin family from the soft tick, *Ornithodoros moubata* (Acari: Argasidae). *Insect Biochemistry and Molecular Biology*, 31: 747-751.

Nakajima, Y., Van der Goes van Naters-Yasui, A., Taylor, D. & Yamakawa, M. 2002. Antibacterial peptide defensin is involved in midgut immunity of the soft tick, *Ornithodoros moubata*. *Insect Molecular Biology*, 11: 611-618.

Nakajima, Y., Ishibashi, J., Yukuhiro, F., Asaoka, A., Taylor, D. & Yamakawa, M. 2003. Antibacterial activity and mechanism of action of tick defensin against Gram-positive bacteria. *Biochimica et Biophysica Acta*, 1624: 125-130.

O'Brien, P.J. 1985. Free-radical-mediated DNA binding. *Environmental Health Perspectives*, 64: 219-232.

Ovchinnikova, T.V., Baladin, S.V., Aleshina, G.M., Tagaev, A.A., Leonova, Y.F., Krasnodembsky, E.D., Men'shenin, A.V. & Kokryakov, V.N. 2006. Aurelin, a novel antimicrobial peptide from jellyfish *Aurelia aurita* with structural features of defensins and channel-blocking toxins. *Biochemical and Biophysical Research Communications*, 348: 514-523.

Park, C.B., Kim, H.S. & Kim, S.C. 1998. Mechanism of action of the antimicrobial peptide buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochemical and Biophysical Research Communications*, 244: 253-257.

Peters, B.M., Shirtliff, M.E. & Jabra-Rizk, M.A. 2010. Antimicrobial peptides: primeval molecules or future drugs? *PLoS Pathogens*, 6: 1-4.

Pérez-Payá, E., Houghten, R.A. and Blondelle, S. 1994. Determination of the secondary structure of selected melittin analogues with different haemolytic activities. *Biochemical Journal*, 299: 587-591.

Pichu, S., Ribeirob, J.M.C. & Mathera, T.N. 2009. Purification and characterization of a novel salivary antimicrobial peptide from the tick, *Ixodes scapularis*. *Biochemical and Biophysical Research Communications*, 390: 511-515.

Piers, K.L., Brown, M.H. & Hancock, R.E.W. 1993. Recombinant DNA procedures for producing small antimicrobial cationic peptides in bacteria. *Gene*, 134: 7-13.

Pompilio, A., Crocetta, V., Scocchi, M., Pomponio, S., Vincenzo, V.D., Mardirossian, M., Gherardi, G., Fiscarelli, E., Dicuonzo, G., Gennaro, R. & Bonaventura, G.D. 2012. Potential noveltherapeutic strategies in cystic fibrosis: antimicrobial and anti-biofilm activity of natural and designed α-helical peptides against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*. *BMC Microbiology*, 12: 145-155.

Prinsloo, L., Naidoo, A., Serem. J., Steyn, H., Sayed, Y., Bester, M.J., Neitz, A.W.H. & Gaspar, A.R.M. 2013. Structural and functional characterization of peptides derived from the C- terminal of the soft tick, *Ornithodoros savignyi*, defensin isoform 2. *Journal of Peptide Science*, 19:325-332.

Rahman, M., Tsuji, N., Boldbaatar, D., Battur, B., Liao, M., Umemiya-Shirafuji, R., You, M., Tanaka, T. & Fujisaki, K. 2009. Structural characterization and cytolytic activity of apotent antimicrobial motif in longicin, a defensin-like peptide in the tick *Haemaphysalis longicornis*. *Journal of Veterinary Medical Sciences*, 72: 149-156.

Reddy, K.V.R., Yedery, R.D. & Aranha, C. 2004. Antimicrobial peptides: premises and promises. *International Journal of Antimicrobial Agents*, 24: 536-547.

Roth, B.L., Poot, M., Yue, S.T. & Millard, P.J. 1997. Bacterial viability and antibiotic susceptibility testing with SYTOX Green nucleic acid stain. *Applied and Environmental Microbiology*, 63: 2421-2431.

Rudenko, N., Golovchenko, M. & Grubhoffer, L. 2007. Gene organization of a novel defensin of *Ixodes ricinus*: first annotation of an intron/exon structure in a hard tick defensin gene and first evidence of the occurrence of two isoforms of one member of the arthropod defensin family. *Insect Molecular Biology*, 16: 501-507.

Schnappinger, D. & Hillen, W. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanism. *Archives of Microbiology*, 165: 359-369.

Selsted, M.E., Novotny, M.J., Morri, W.L., Tang, Y.Q., Smith, W. & Culler, J.S. 1992. Indolicidins, a novel bacterial tridecapeptide amide from neutrophils. *Journal of Biological Chemistry*, 267:4292-4295.

Shee, A.K., Raja, R.B., Sethi, D., Kunhambu, A. & Arunachalam, K.D. 2010. Studies on the antibacterial activity potential of commonly used food preservatives. *International Journal of Engineering Science and Technology*, 2: 264-269.

Shin, S., Kim, J., Lee, J., Jung, K., Hwang, J., Lee, J., Lee, D.G., Kim, I., Shin, S.Y. & Kim, Y. 2009. Design of a potent 9-mer antimicrobial peptide analogs of protaetiamycine and investigation of mechanism of antimicrobial action. *Journal of Peptide Science*, 15: 559-568.

Sievert, D.M., Ricks, P., Edwards, J.R., Schneider, A., Patel, J., Srinivasan, A., Kallen, A., Limbago, B. & Fridkin, S. 2013. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centres for Disease Control and Prevention, 2009-2010. *Infection Control Hospital Epidemiology*, 34: 1-14.

Silhavy, T.J., Kahne, D. & Walker, S. 2010. The bacterial cell envelope. *Cold Spring Harbar Perspectives in Biology*, 2:a000414.

Sitaram, N. & Nagaraj, R. 1999. Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity. *Biochimica et Biophysica Acta*, 1462: 29-54.

Sonenshine, D.E., Ceraul, S.M., Hynes, W.E., Macaluso, K.R. & Azad, A.F. 2002. Expression of defensins-like peptides in tick hemolymph and midgut in response to challenge with *Borrelia burgdorferi*, *Escherichia coli* and *Bacillus subtilis*. *Experimental and Applied Acarology*, 28: 127-134.

Sonenshine, D.E. & Hynes, W.L. 2008. Molecular characterization and related aspects of the innate immune response in ticks. *Frontiers in Bioscience*, 13: 7046-7063.

Stensvag, K., Haug, T., Sperstad, S.V., Rekdal, O., Indrevll, B. & Styrvold, O.B. 2008. Arasin 1, a proline-arginine-rich antimicrobial peptide isolated from the spider crab, *Hyas araneus*. *Developmental Comparative Immunology*, 32: 275-285.

Tang, Y.Q., Yuan, J., Ösapay, G., Ösapay, K., Tran, D., Miller, C.J., Ouellette, A.J. & Selsted, M.E. 1999. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated α-defensins. *Science*, 286: 498-502.

Taylor, D. 2006. Innate immunity in ticks: a review. *Journal of Acarology Society Japan*, 15: 109-127.

Thevissen, K., Terras, F.R.G. & Broekaert, W. 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Applied and Environmental Microbiology*, 65: 5451-5458.

Tsuji, N., Battsetseg, B., Boldbaatar, D., Miyoshi, T., Xuan, X., Oliver Jr., J.H. & Fujisaki, K. 2007. Babesial vector tick defensin against *Babesia* sp. parasites. *Infection and Immunity*, 75: 3633-3640.

Tulumello, D.V. & Deber, C.M. 2009. SDS micelles as a membrane-mimetic environment for transmembrane segments. *Biochemistry*, 48: 12096-12103.

Van den Bogaart, G., Guzmán, J.V., Mika, J.T. & Poolman, B. 2008. On the mechanism of pore formation by melittin. *The Journal of Biological Chemistry*, 283: 33854-33857.

Varkey, J., Singh, S. & Nagaraj, R. 2006. Antibacterial activity of linear peptides spanning the carboxy-terminal β-sheet domain of arthropod defensins. *Peptides*, 27: 2614-2623.

Varshney, A., Sen, P., Ahmad, E., Rehan, M., Subbarao, N. & Khan, R.H. 2010. Ligand binding strategies of human serum albumin: how can the cargo be utilized. *Chirality*, 22: 77-87.

Vogel, H. & Jähnig, F. 1986. The structure of melittin in membranes. *Biophysical Journal*, 50: 573-582.

Vollmer, W. 2008. Structural variation in the glycan strands of bacterial peptidoglycan. *FEMS Microbial Reviews*, 32: 287-306.

von Horsten, H.H., Schafer, B. & Kirchhoff, C. 2004. SPAG/isoform HE2C, an atypical anionic β-defensin-like peptide. *Peptides*, 25: 1223-1233.

Wu, M., Maier, E., Benz, R. & Hancock, R.E.W. 1999. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. *Biochemistry*, 38: 7235-7242.

Wu, G., Ding, J., Li, H., Li, L., Zhao, R., Shen, Z., Fan, X. & Xi, T. 2008. Effects of cations and pH on antimicrobial activity of thanatin and s-thanatin against *E. coli* ATCC 25922 and *B. subtilis* ATCC 21332. *Current Microbiology*, 57: 552-557.

Yu, D., Shenga, Z., Xua, X., Lia, J., Yanga, H., Liuc, Z., Rees, H.H. & Lai, R. 2006. A novel antimicrobial peptide from salivary glands of the hard tick, *Ixodes sinensis*. *Peptides*, 27: 31-35.

Zang, L. & Hancock, R.E.W. 2000. Peptide antibiotics. *Antibiotic resistance and antibiotic development*, eds. D. Hughes & D.I. Anderson, Harwood Academic Publishers, pp. 209-232.

Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proceedings of the National Academy of Science of the United States of America*, 84: 5449-5453.

Zhang, L., Benz, R. & Hancock, R.E.W. 1999. Influence of proline residues on the antibacterial and synergistic activities of α-helical peptides. *Biochemistry*, 38: 8102-8111.

Zhang, H., Zang, W., Wamg, X., Zhou, Y., Wang, N. & Zhou, J. 2011. Identification of a cysteine-rich antimicrobial peptide from the salivary glands of the tick *Rhipicephalus haemaphysaloides*. *Peptides*, 32: 441-446.

Zheng, H., Zhou, L., Yang, X., Wang, D. & Liu, J. 2012. Cloning and characterization of a male-specific defensin-like antimicrobial peptide from the tick *Haemaphysalis longicornis*. *Developmental & Comparative Immunology*, 37: 207-211.

Zhong, J., Wang, W., Yang, X., Yan, X. & Liu, R. 2013. A novel cysteine-rich antimicrobial peptide from the mucus of the snail of *Zchatina fulica*. *Peptides*, 39: 1-5.

Zhou, J., Liao, M., Ueda, M., Gong, H., Xuan, X. & Fujisaki, K. 2007. Sequence characterization and expression patterns of two defensin-like antimicrobial peptides from the tick *Haemaphysalis longicornis*. *Peptides*, 28: 1304-1310.