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

Alex R. Naidoo

Submitted in partial fulfilment of the degree
Magister Scientiae

In the Faculty of Natural and Agricultural Sciences
University of Pretoria

Pretoria

2014

Submission declaration:

I, (Alex Rolin Naidoo), 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: ……

Plagiarism agreement:

UNIVERSITY OF PRETORIA

FACULTY OF NATURAL AND AGRICULTURAL SCIENCES

DEPARTMENT OF BIOCHEMISTRY

Fullname: _____Alex Rolin Naidoo_____

Student number: _____04319656_____

Title of the work: _____

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

_____

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 _____

Acknowledgements

I would like to thank the following people:

My family for their support, love, patience and encouragement provided throughout this project.

Prof. ARM Gaspar, Department of Biochemistry, University of Pretoria, my supervisor during this project, for her guidance, understanding and support. As well as her time, suggestions and input for this dissertation.

Prof. AWH Neitz, Department of Biochemistry and Prof. M Bester, Department of Anatomy, University of Pretoria, my co-supervisors during this project, for their knowledge, input and guidance in both the project and dissertation.

Ms. S van Wyngaardt, Department of Biochemistry, University of Pretoria, for her assistance and support in problems with both experiments and equipment.

Lezaan Prinsloo and Clerisa Odendaal, for all the laughs, help and support in the laboratory.

And a special thank you to June Serem, whose experience, knowledge, help and support was invaluable in the completion of this project.

Summary

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

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

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

reaction was used to measure the NO scavenging capacity of OsΔC and showed that at a concentration of 12.5 µM, approximately 89% of NO was scavenged.

Overall OsΔC was found to possess potent antimicrobial, antioxidant and NO scavenging activities, while no toxicity of the peptide to mammalian cells was observed. OsΔC thus shows potential as a multifunctional peptide and further research into each of the peptide's bioactivities is needed to determine its full potential.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAPH	2,2’-Azobis (2-methyl-propanimidamide) dihydrochloride
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AMP	Antimicrobial peptides
CD	Circular dichroism
CFU	Colony forming units
CV	Crystal violet
CVS	Crystal violet stain
DCFH-DA	2’,7’-Dichlorofluorescein-diacetate
DIF	Dorsal-related immune factor
GSH	Glutathione
HBD	Human β-defensins
HNP	Human neutrophil peptide
IL-6	Interleukin-6
LB	Luria Bertani broth
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
MBC	Minimum bactericidal concentration
Mel	Melittin
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaP	Sodium phosphate buffer
NED	N-naphthyl-ethylenediamine dihydrochloride
NO	Nitric oxide
NOS	Nitric oxide synthase
OD_{600}	Optical density at 600nm

ORAC	Oxygen radical absorbance capacity
Os-C	C-terminus of *O. savignyi peptide*, cysteines removed
OsΔC	C-terminus of *O. savignyi* defensin peptide, cysteines removed, added tryptophan and C-terminus amidation
PaΔC	C-terminus of *P. apterus* defensin peptide, cysteines removed,added tryptophan and C-terminus amidation
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PGN	Peptidoglycan
PRP	Pattern recognition protein
ROS	Reactive oxygen species
SA	Sulphanilamide
SDS	Sodium dodecyl sulfate
SNP	Sodium nitroprusside
TE	Trolox equivalents
TEAC	Trolox equivalence antioxidant capacity
TFE	Trifluoroethanol
TIR	Toll-interleukin-1 receptor
TNF	Tumour necrosis factor

Chapter 1: Introduction

1.1 Problem statement

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

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

1.2 Innate immunity

The immune system is a defence mechanism to protect the organism from foreign matter or pathogens. A basic requirement of the immune system is the ability to determine which matter is non-self (Steiner, 2004). There are two major parts to immunity, innate and acquired. Innate immunity is the first response and is a fast cellular response to the pathogen, as components of the innate system are always

active within the organism. The acquired immune system is made up of specialised white blood cells and antibodies, requires a few days for its activity to develop due to the complexity of the system. A key distinguishing factor is that acquired immunity includes immunologic memory (Lavine and Strand, 2002).

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

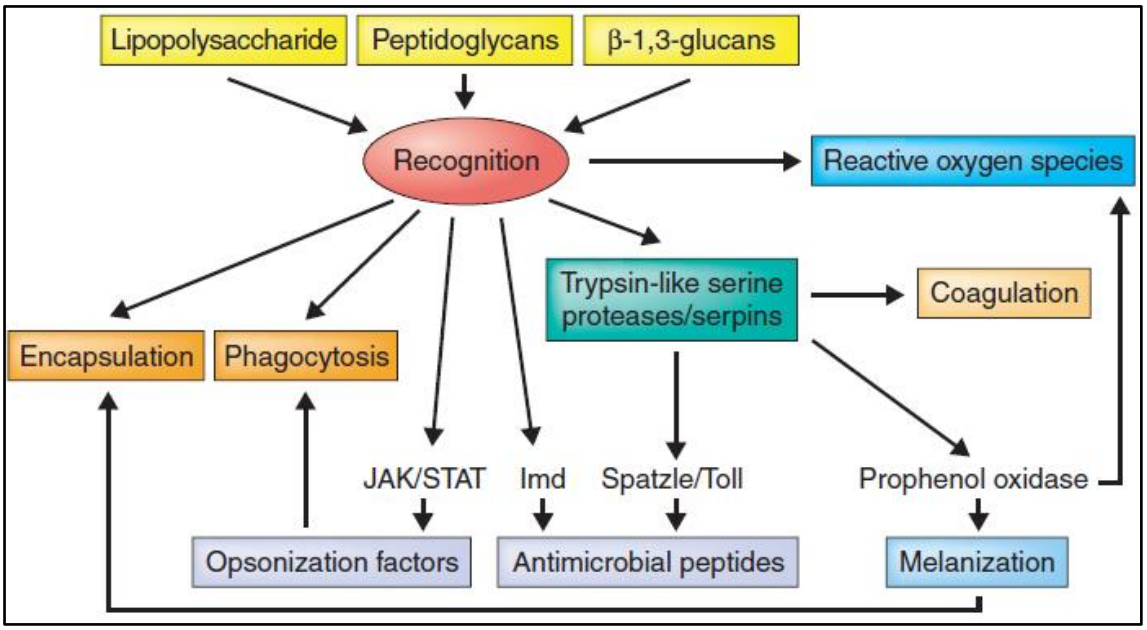


Figure 1.1: Overview of the immune response in *Drosophila melanogaster* (Tzou *et al*., 2002)

In invertebrates the first line of defence is the integument (the outer shell) and the gut, followed by a complex system including cellular and humoral responses. Phagocytosis, melanization, and encapsulation form part of the cellular responses (Figure 1.1). The synthesis and secretion of AMPs by the fat-body constitute the bulk of the humoral response (Kounatidis and Ligoxygakis, 2012). The cell mediated system in insects is a very simplified version of the vertebrate cellular responses, due to the fact that it lacks antigen-antibody complexes and memory cells. Hemocytes are cells involved in the immune response of invertebrates and increase with an increase of foreign matter/infection. There are three basic types of hemocytes; crystal cells, plasmocytes and lamellocytes (Kounatidis and Ligoxygakis, 2012). Plasmocytes are responsible for

removing microorganisms and apoptotic cells by phagocytosis and hence cause their removal from circulation. Production of lamellocytes, is increased when foreign matter is detected, that is too large for the plasmocytes to eliminate (Kounatidis and Ligoxygakis, 2012). Lamellocytes act by encapsulation of foreign matter by surrounding them in layers of cells (Nappi *et al.*, 1995). At the site of injury or within the lamellocyte-capsule, the production of a black pigment is the result of a pathway that converts tyrosine to melanin (Sőderhäll and Cerenius, 1988). Melanization is the isolation of pathogens by the crystal cells, which are suggested to be involved in rapid wound sealing. Larvae lacking crystal cells have a reduced ability for clot formation and decreased wound healing (Kounatidis and Ligoxygakis, 2012; Bidla *et al.*, 2005).

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

1.3 Antimicrobial peptides

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

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

Table 1.1 shows some of the physicochemical properties which are used to differentiate AMPs.

Table 1.1: Physicochemical properties of antimicrobial peptides (Adapted from Brogden, 2005)

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

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

The cationic group, make up the majority of AMPs. These AMPs are also short, less than 40 amino acid residues and typically carry a net positive charge between +2 and +9. This charge is due to a general lack of Glu or Asp (acidic residues) and high numbers of cationic amino acids (Lys and/or Arg). Hydrophobic residues account for 30-50% of the peptide and allow for an amphiphilic structure, when the peptide interacts with membranes. Alterations of the hydrophobicity to charge ratio can result in changes in the selectivity/activity (Zelezetsky *et al*., 2005). These peptides have cysteine residues that form disulphide bridges which can result in the peptide

containing a hinge region (Chrudimska *et al*., 2010). Cationic peptides show broad structural diversity but share the characteristics of being cationic at physiological conditions, contain a similar primary amino acid sequence and allowing the formation of an amphipathic structure (Maloy and Kari, 1995; Saberwal and Nagaraj, 1994).

1.3.1 Linear and amphipathic α-helical peptides

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

1.3.2 Amino acid rich peptides

Proline-rich

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

Glycine-rich

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

Histidine-rich

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

Tryptophan-rich

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

1.3.3 Cysteine-containing peptides

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

Table 1.2: Examples of different cationic peptides, their source and antimicrobial spectrum (Adapted from Vizioli and Salzet, 2002)

Structure and examples	Organism	Antimicrobial spectrum
Linear α-helical peptides		
Cecropins	Insects	Bacteria, fungi, virus, protozoa, metazoa
Magainin	Amphibians	Bacteria, protozoa
Abaecin and apidaecins	Bee	Bacteria
Linear, amino acid rich peptides		
Pro- rich		
Drosocin	Fruit fly	Bacteria
Pyrrhocorisin	Hemipteran	Bacteria, fungi
Gly- rich		
Diptericins	Dipterans	Bacteria
Attacins[1]	Cecropia moth	Bacteria
His- rich		
Histatins	Humans	Bacteria, fungi
Tyr-rich		
Indolicin	Cattle	Bacteria
Cysteine-containing peptides		
Single disulphide bond		
Thanatin	Hemipteran	Bacteria, fungi
Brevinins	Frog	Bacteria
Two disulphide bonds		
Protegrin I	Horseshoe crab	Bacteria, fungi, virus
Androctonin	Scorpion	Bacteria, fungi
Tachyplesin II	Pig	Bacteria, fungi, virus
Three disulphide bonds		
α-defensins	Mammals	Bacteria, fungi
β-defensins	Mammals	Bacteria, fungi
Defensins	Insects	Bacteria, fungi, protozoa
More than three disulphide bonds		
Defensins	Plants	Fungi
Gambicin	Mosquito	Bacteria, fungi, protozoa
Drosomycin	Fruit fly	Fungi

[1] Hultmark (1993)

1.4 Defensins

Defensins are the most conserved class of AMPs, found in a wide range of organisms (some examples shown in Figure 1.2) such as plants, mammals, insects, molluscs and several tick species (Broekaert *et al.,* 1995; Selsted *et al.,* 1985; Matsuyama and Natori, 1988; Lowenberger *et al.,* 1995; Viljakainen and Pamilo, 2005; Charlet *et al.,* 1996; Johns *et al.,* 2001; Tsuji *et al.,* 2007; Chrudimska *et al.,* 2010; Hynes *et al..* 2005; Fogaca *et al*., 2004). They are small cationic peptides of approximately 4 kDa, averaging 34 to 43 residues in length with pI values ranging from 8.0-8.5. These peptides are known to be active against most Gram-positive bacteria and have high affinity for the major phospholipids of bacteria (Gillespie *et al*., 1997).

Defensins have shown bactericidal, antifungal and antiviral activities and are associated with many cells and tissues involved in the host defense (Brandenburg *et al*., 2012). They have been shown to accumulate at a higher rate and to a larger extent at sites of infection compared to inflamed but non-infected tissues, indicating that these peptides are able to distinguish between microorganisms and host tissue with regard to inflammation and to accumulate at the infection sites (Welling *et al*., 2001). Depletion or removal of the AMPs has resulted in higher susceptibility to severe and frequent infections (Ganz *et al*., 1988; Pűtsep *et al*., 2002; Moser *et al*. 2002; Huang *et al*., 2007). HNP-1 (a human α-defensin) inhibits replication of HIV and the influenza virus (Zhang *et al*., 2004; Salvatore *et al*., 2007) and can inactivate papillomavirus, herpes simplex virus, cytomegalovirus, and adenovirus (Buck *et al*., 2006; Daher *et al*., 1986). Human β-defensin can block HIV-1 and influenza virus replication (Bergman *et al*., 2007; Braida *et al*., 2004; Liang *et al*., 2010). Some defensins inhibit HIV entry into the cells by binding/antagonising either the viral proteins or cellular receptors (Gallo *et al*., 2006, Feng *et al*., 2006). The underlying mechanisms of virus inhibition are unknown but antiviral activity seems to be dependent on interaction of the peptides with viral proteins (Brandenburg *et al*., 2012). Mechanisms of antifungal activity by the peptides are similar to antibacterial mechanisms and occur by cell lysis, binding and disruption of outer membrane, interference with cell wall synthesis and depolymerisation of the actin cytoskeleton (Koo *et al*., 2004; De Lucca and Walsh, 1999). Defensins also have shown antiparasitic activity by disrupting cell membrane integrity (McGwire *et al*., 2003).

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

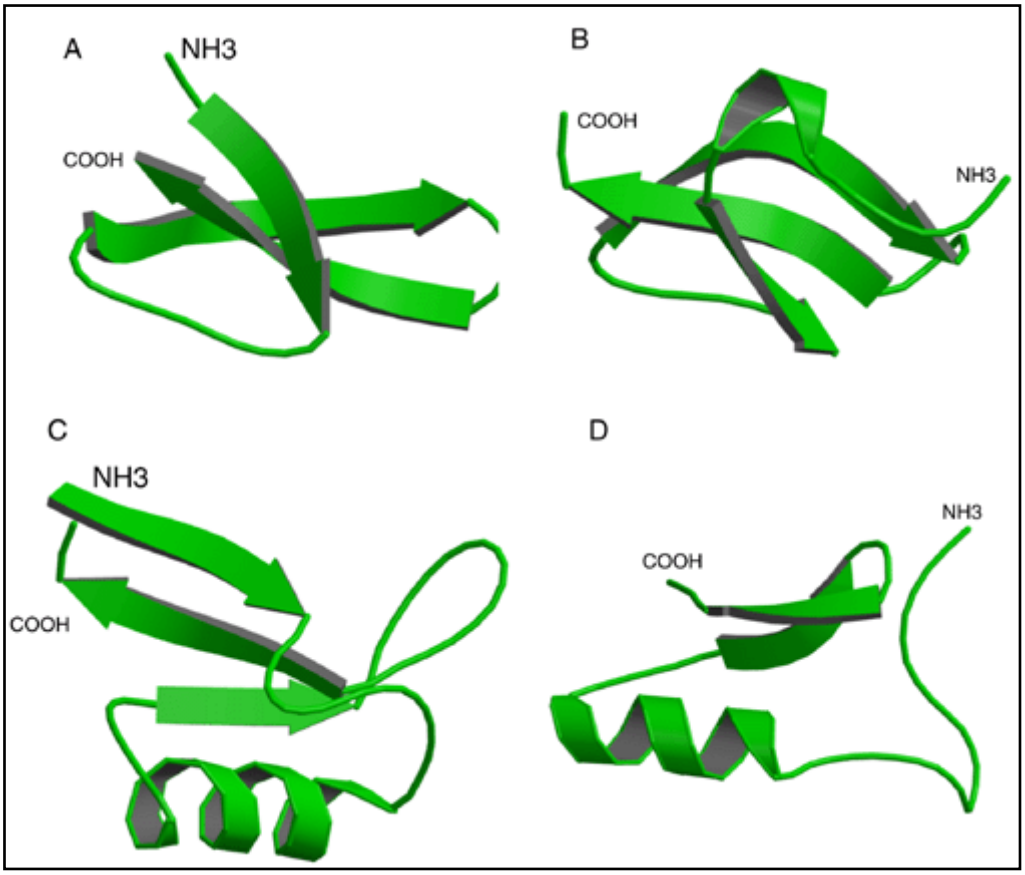


Figure 1.2: Structure of different defensin peptides. (A) Human α-defensin, (B) Human β-defensin, (C) Plant defensin, (D) Insect defensin (Sun and Boyington, 2001)

1.5 Mechanism of action

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

1.5.1 Targeting the microbial cell membrane

This mode of action of cationic AMPs relies upon the interaction with the cell membrane of the microorganism (Figure 1.3). Present in eukaryotic membranes are phosphatidylcholine, phosphatidylethanolamine, sphingomyelin together with sterols, ergosterol and cholesterol which normally have no net charge. Due to the neutral charge of the eukaryotic membrane, no ionic interactions occur between AMPs and the cell membrane (Yeaman and Yount, 2003). Prokaryotic bacteria have negatively charged cytoplasmic membranes and a high electrical potential gradient. These

characteristics are provided in part, by the presence of hydroxylated phospholipids phosphatidylgycerol, phosphatidylserine and cardiolipin, which sustain a net negative charge (Yeaman and Yount, 2003). This negatively charged membrane allows for an electrostatic interaction between the cationic peptide and dense anionic charges of the bacterial membranes, leading to aggregation of AMPs onto the bacterial membrane. An advantage of this mechanism is that it reduces cytotoxicity towards mammalian cells and makes it more difficult for the bacteria to develop resistance as this would require the bacteria to change the properties of their membranes (Laverty *et al*., 2011). The hydrophobic residues of AMPs are important for integration into the bacterial membrane (Wiesner and Vilcinskas, 2010).

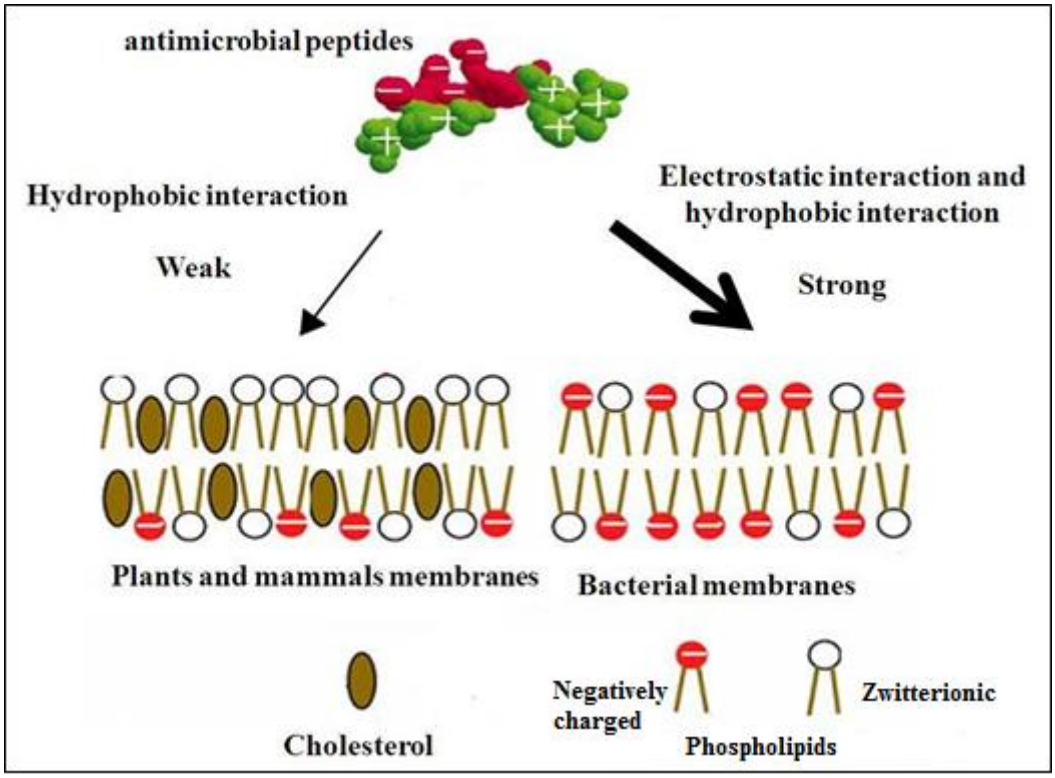


Figure 1.3: Diagram showing attraction of AMPs with the bacterial cell membrane [http://2011.igem.org/Team:St_Andrews/switch]

When comparing the outer membrane structure of a Gram-negative and Gram-positive bacterium, there is an additional outer lipid membrane on Gram-negative bacteria and a self-promoted uptake pathway has been proposed for the action of cationic peptides. The outer membrane is composed of LPS for which peptides have a high affinity. It is proposed that as the AMPs bind, they compete with divalent cations such as

magnesium and calcium ions for their relative binding sites (Chen and Cooper, 2002). As these ions are important for the stability of the cell membrane surface, the removal of them leads to disruption of the outer membrane, resulting in the formation of pores allowing more peptides and other small molecules to pass through. This hypothesis also explains how cationic AMPs work together with conventional antibiotics and this synergy as also been proven with Gram-positive bacteria (Giacometti *et al*., 2000) and fungi (Kumar *et al*., 2005).

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

There are different ways in which AMPs associate on the bacterial cells. Some insert into the membrane and aggregate to form transmembrane pores with their hydrophilic surfaces on the inside and their hydrophobic surfaces interact with the lipids of the membrane surrounding it (Figure 1.4 C). Some sit on the surface of the membrane as monomers or aggregate to form bundles of peptides. Some AMPs can enter the membrane completely and lie within the membrane (Shai, 1995).

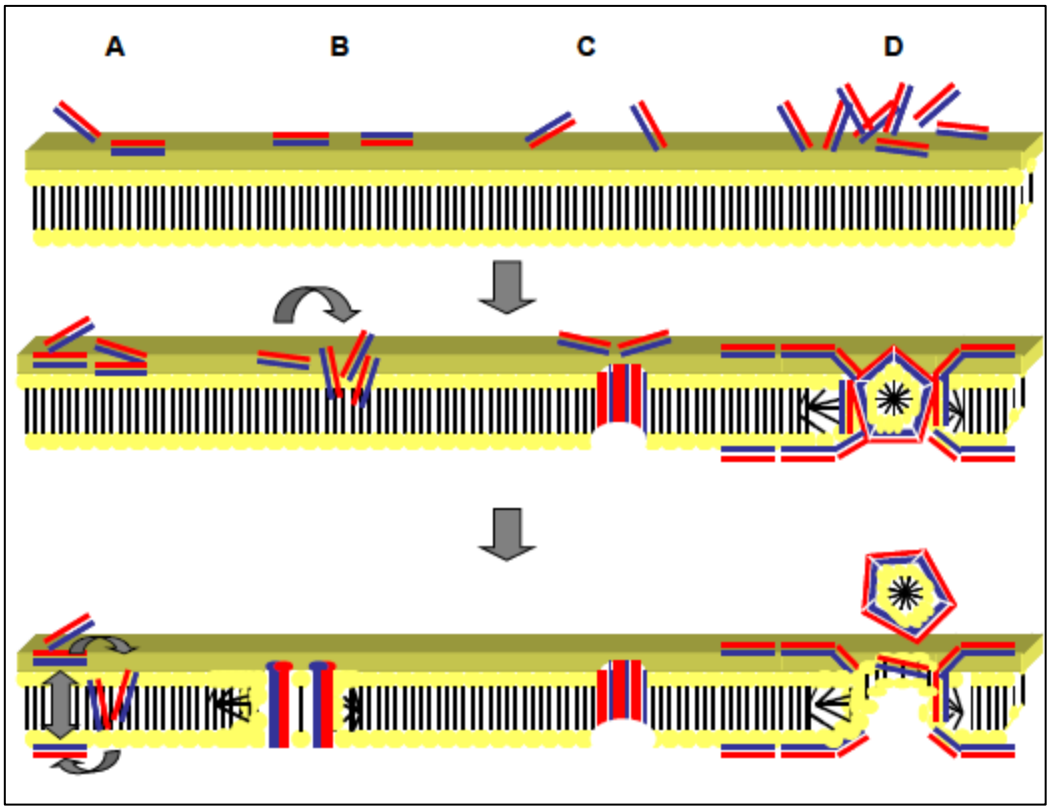


Figure 1.4: Proposed mechanisms for interaction of antimicrobial peptides with bacterial cell membranes with hydrophilic regions (red) and hydrophobic regions (blue). (A) aggregate, (B) toroidal pore, (C) barrel-stave and (D) carpet models (Laverty *et al*., 2011)

In the aggregate model (Figure 1.4 A), AMPs gather on the surface and form an aggregate which spans the membrane, made up of peptide and lipid micelle complexes but have no particular orientation (Laverty *et al*., 2011).

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

In the barrel-stave model (Figure 1.4 C), AMPs aggregate forming dimers and multimers, similar to the carpet model but proposes that the peptides are inserted into the membrane to form a pore. The hydrophobic regions of the AMPs align with the acyl chains of the membrane lipids and the hydrophilic regions form the inner surface of the

pore channel (Wiesner and Vilcinskas, 2010; Laverty *et al*. 2011). As little as 3 peptide molecules can allow for the formation of pores (Shai and Oren, 2001).

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

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

1.5.2 Intracellular targets of AMPs

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

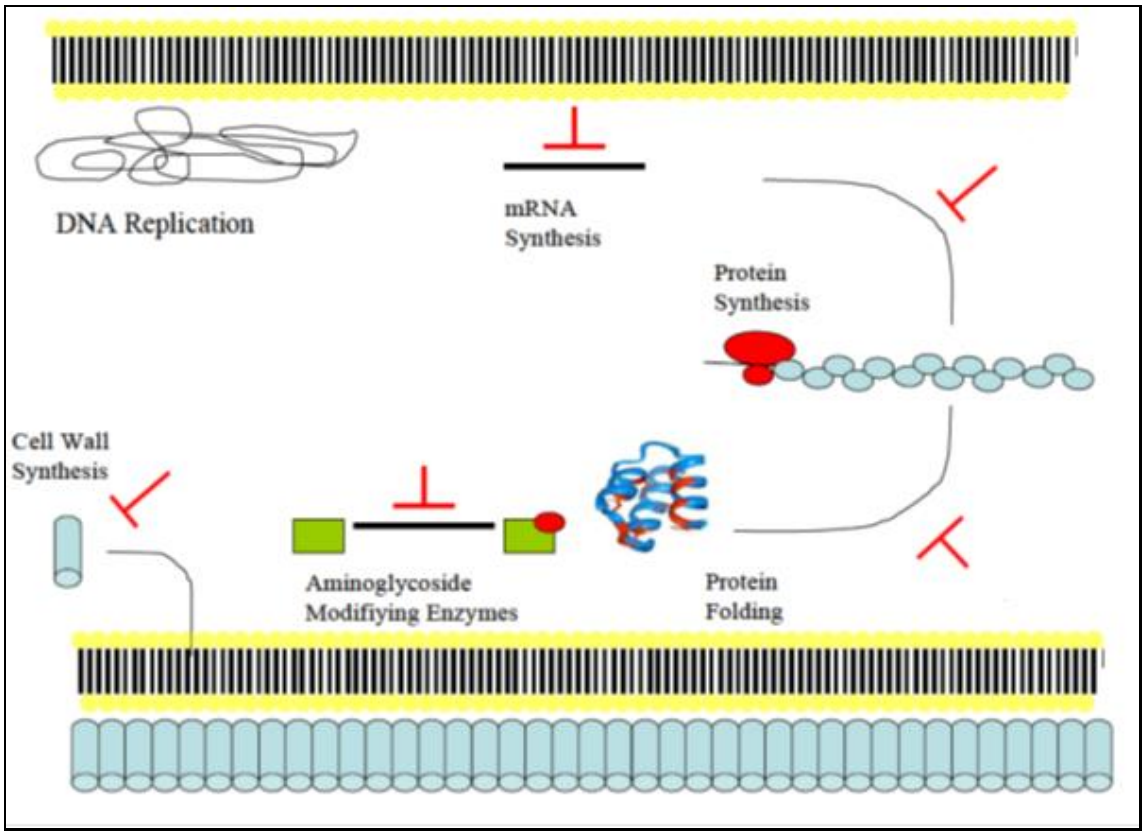


Figure 1.5: Mechanisms of action for antimicrobial peptides and possible targets within the cell (Laverty *et al*., 2011)

1.6 Tick defensins

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

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

1.7 Multiple functions of AMPs

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

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

Research has shown that defensins accumulate at a higher rate and extent at sites of injury, when compared to non-infected but inflamed tissues. This indicates that peptides distinguish between microorganisms and the host tissues and can accumulate at sites of infection (Welling *et al*., 2000, Brandenburg *et al*., 2012). Studies have shown that the antimicrobial activity is affected by cationic concentrations, serum and anionic molecules, however their immunomodulatory activities are less sensitive (Nijnik *et al*., 2010).

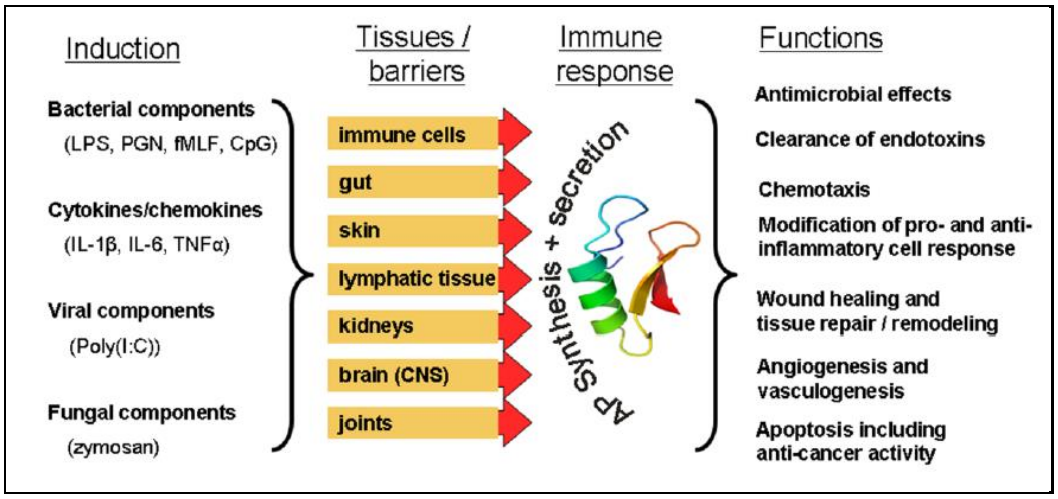


Figure 1.6: Roles of AMPs in the immune response (Brandenburg *et al*., 2012). AP synthesis refers to AMP synthesis

There are a number of papers on the immunomodulatory effects of peptides and also specifically on the multiple functions of defensins in the immune response (Steinstraesser *et al.,* 2009; Ganz 1987 and 2003; Auvynet and Rosenstein 2009; Bowdish *et al*., 2005; Scott and Hancock 2000). Immunomodulation is a complex process and specific processes related to immunomodulation such as, antioxidant and anti-inflammatory effects will be discussed in more detail.

1.7.1 Peptides with antioxidant activity

Oxidation is an important process that occurs in vertebrates and humans and also leads to the formation of free radicals. ROS are important intermediate products of oxidative metabolism (Hipler *et al*., 2000). During respiration there is an unavoidable formation of ROS, free radicals such as super oxide anions (O_2^-) and hydroxide radicals ($OH^\bullet$), and also non-free radicals such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Excess ROS can also be produced by stress to the cell, such as ischemia/reperfusion, exposure to ionizing and ultraviolet radiation and/or inflammation (Hipler *et al*., 2000).

Oxidative stress from ROS causes cellular damage, and affects DNA, proteins, lipids and other small cellular molecules (Lee *et al*., 2006) and has been linked to numerous human diseases such as stroke, diabetes, cancer as well as chronic wounds and septic shock (Najafian and Babji, 2012).

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

1.7.2 Peptides with anti-inflammatory activity

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

complex enzymes in nature (Sun *et al*., 2003). There are three types; neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS).

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

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

1.8 Therapeutic application of AMPs

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

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

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

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

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

AMPs can enhance the potency of current antibiotics by facilitating entry in microorganisms (Giacometti *et al*., 2000). Three basic mechanisms by which AMPs

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

1.9 Background to the study

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

Defensin isoform 1:

PRVRRGYGCPFNQYQCHSHC**SGIRGYRGGYCKGAFKQTCKCY**

Defensin isoform 2:

PRVRRGYGCPFNQYQCHSHC**KGIRGYKGGYCKGAFKQTCKCY**

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

Shorter AMPs would be greatly beneficial because of the high cost of production involved with peptides. A number of tick defensins and defensin derived peptides (discussed in section 1.7) have shown antimicrobial activity. It has also been shown that mammalian defensins have retained their antimicrobial activity, regardless of the

order of disulphide linkages or even the presence of disulphide bonds (Campopiano *et al.*, 2004; Hoover *et al.*, 2003; Klűver *et al.*, 2005; Maemoto *et al.*, 2004; Wu *et al.*, 2003). Varkey *et al.* (2006) showed that peptides derived from the C-terminal region of arthropod defensins from *Pyrrhocoris apterus* (a firebug) and *O. moubata*, in which the cysteines were omitted, were active against both Gram-positive and Gram-negative bacteria. The parent peptide of *O. moubata* defensin A was also only active against Gram-positive bacteria (Nakajima *et al*., 2001). Table 1.4 shows the parent and derived peptide sequences used by Varkey *et al.* (2006). These authors added tryptophan to the N-terminus, to monitor the interaction of the amidated C-terminus with lipid vesicles. Amidation of the C-terminus removes a negative charge and helps stabilize the peptide as it helps mimic natural peptides and protecting the peptide from degradation by enzymes. Fifty percent of mammalian peptide hormones and more than eighty percent of insect hormones have an amidated C-terminus (Eipper and Mains, 1988).

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

Peptide	Sequence	Net charge
Pa	AT**C**DILSFQSQWVTPNHAG**C**ALH**C**VIKGYKGGQ**C**KITV**C**H**C**RR	+5
PaΔC	WVIKGYKGGQKITVHRR-amide	+6
OmC	GYG**C**PFNQYQ**C**HSH**C**SGIRGYKGGY**C**KGLFKQT**C**N**C**Y	+4
OmΔC	WSGIRGYKGGYKGLFKQTNY-amide	+5

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

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

Table 1.5: Original and derived peptide used in this study

Peptide	Sequence	Net charge
OsDef2[A]	GYG**C**PFNQYQ**C**HSH**C**KGIRGYKGGY**C**KGAFKQT**C**K**C**Y	+6
OsΔC[B]	W--KGIRGYKGGY--KGAFKQT--K--Y-amide	+7

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

1.10 Aims of the study

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

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

1.11 Output

Co-author on article: Prinsloo, L., **Naidoo, A.***, Serem, J., Taute, H., Sayed, Y., Bester, M., Neitz, A. & Gaspar, A. (2013). Structural and functional characterization of peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*. *Journal of Peptide Science*, 19, 325–332

*Performed CD analysis as well as interpretation of CD data

Chapter 2: Materials and Methods

2.1 Materials

The peptides Os∆C and Pa∆C were synthesized by GenScript USA. The purity and molecular mass of the peptides were determined by reverse-phase HPLC and mass spectrometry, respectively (see Appendix for data). Na_2HPO_4 and NaH_2PO_4 used in the sodium phosphate buffer (NaP) and melittin were acquired from Sigma Aldrich USA. *Escherichia coli* (ATCC 700923), *Bacillus subtilis* (13933), *Staphylococcus aureus* (U3300) and *Pseudomonas aeruginosa* (ATCC 10145), were used for the antibacterial assays. Chemicals and spectropolarimeter used for circular dichroism (CD) analysis were provided by the School of Molecular and Cell Biology, University of Witwatersrand. Cell lines and chemicals used for the cytotoxicity and antioxidant assays were provided by the Department of Anatomy, University of Pretoria.

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

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

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

2.2 Determination of antibacterial activty

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

The mid-log phase bacteria were diluted to approximately 10^{6} CFU/ml. The bacteria were centrifuged at 14100 g for 90 sec and re-suspended in NaP buffer (10 mM pH 7.4) for Gram-negative bacteria or 1% LB in NaP buffer for Gram-positive bacteria. Washed cultures were diluted to approximately $1x10^{6}$ CFU in either NaP buffer for Gram-negative bacteria or 1% LB in NaP buffer for Gram-positive bacteria, after which 90 µl of bacterial suspension was incubated with 10 µl of peptide (concentration range of 120-0.06 µg/ml), in polypropylene tubes for 2 h at 37^{o}C, in a shaking incubator. The incubated samples were diluted 500 times in either NaP buffer (Gram-negative bacteria) or 1% LB in NaP buffer (Gram-positive bacteria), thereafter 100 µl of the samples were plated out on LB-agar plates and incubated for 16 h at 37^{o}C. The growth control and sterile control contained water and buffer only. The MBC was defined as the concentration at which complete killing was observed.

2.3 Secondary structure determination

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

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

Unordered peptides are usually indicated by a single negative band below 200nm. α-Helical structures have a positive band around 192 nm, with two negative bands around 208 nm and 222 nm. A positive band around 195 nm with a negative band around 217 nm is indicative of β-sheet structures (Gopal *et al*., 2012). Cationic peptides in aqueous solutions usually have unordered conformations but they are amphipathic molecules and can adopt folded states in hydrophobic environments (Gopal *et al*., 2012). Determining the changes in secondary structures may give some insight into the structure-activity relationship and could explain any differences in activity as a result of the modifications made to the peptide.

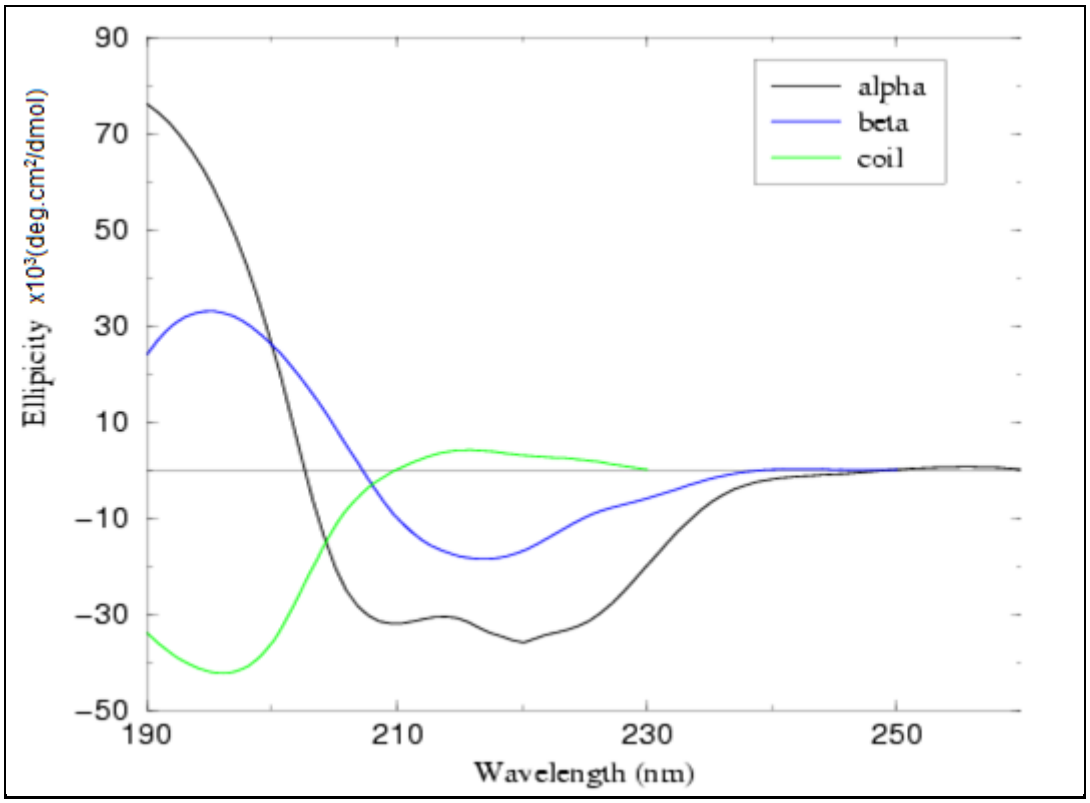


Figure 2.1: Circular dichroism profiles of known secondary structures

[http://besley.chem.nottingham.ac.uk/research/research-prospec.html]

The CD spectroscopy analysis was performed at the University of Witwatersrand with a Jasco J-810 spectropolarimeter, over the 180-250 nm range and a path length of 0.2 cm. The scans were carried out at 20^{o}C with a scan speed of 200 nm/min, using a data pitch of 0.1 nm, at a bandwidth of 0.5 nm and the results are the average of 10 scans. Peptides were prepared in 3 different solutions: 50% trifluoroethanol (TFE), 25 mM sodium dodecyl sulphate (SDS) or in water, at a final peptide concentration of 50 µM. Data from the scans were presented as mean residue ellipticity vs. wavelength. Any data acquired for the CD spectra that were above 800 volts were omitted, due to the unreliability of data beyond that point. The following equation was used to convert the data to mean residue ellipticity which incorporates the concentration and number of amino acid residues:

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

Where $[\theta]\mathrm{mre}$ is the mean residue ellipticity (deg.cm^2.dmol^{-1}), θ is the reading acquired in mdeg, C is the concentration of peptide in mM, n is the number amino acid residues and l is the pathlength (cm).

2.4 Determination of the effect of the peptides on mammalian cells

2.4.1 Erythrocyte hemolytic assay

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

Blood was collected from healthy, consenting donors (ethical clearance obtained from the Research Ethics Committee (Protocol no. 61/201), Faculty of Health Sciences, University of Pretoria). Using a sterile needle connected to a 5 ml EDTA vacuum extraction tube, 5 ml of blood was collected from a vein. Erythrocytes were collected by centrifugation at 2500 g and the plasma and buffy coat were removed. The erythrocytes were washed with NaP buffer (100 mM, pH 7.4). A 10 µl volume of erythrocytes diluted in 80 µl buffer were exposed to 10 µl of the peptides OsΔC or PaΔC (final concentration range 0.25-100 µM) or 10 µl buffer (negative control) or 2% SDS (positive control), and incubated for 30 min at 37^{o}C. SDS is an anionic surfactant commonly used for the disruption of cell walls. The samples were then removed from the incubator and centrifuged at 2500 g for 3 min. A 75 µl volume of the supernatant was collected and placed into a 96 well plate and the absorbance was measured at 570 nm. The results were calculated and expressed as % hemolysis, relative to the SDS control, using the following formula:

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

2.4.2 Cell culture based determination of cytotoxicity

The effects of the peptides were investigated on SC-1 (embryo, *Mus musculus* fibroblast) and Caco-2 (adult, *Homo sapiens*, epithelial, colorectal adenocarcinoma) cell lines using the crystal violet (CV) assay. CV is positively charged (Figure 2.2) and binds to negatively charged molecules such as nucleic acids and the amino acid

residues of proteins such as Glu and Asp. Following exposure to the peptide for 24 h, the cells are fixed and stained with CV. Toxicity can inhibit cell division, causes detachment of the cell or causes cell lysis and decreases the cell content, each of which will result in the decrease of CV staining. The CV is extracted and colour intensity quantified.

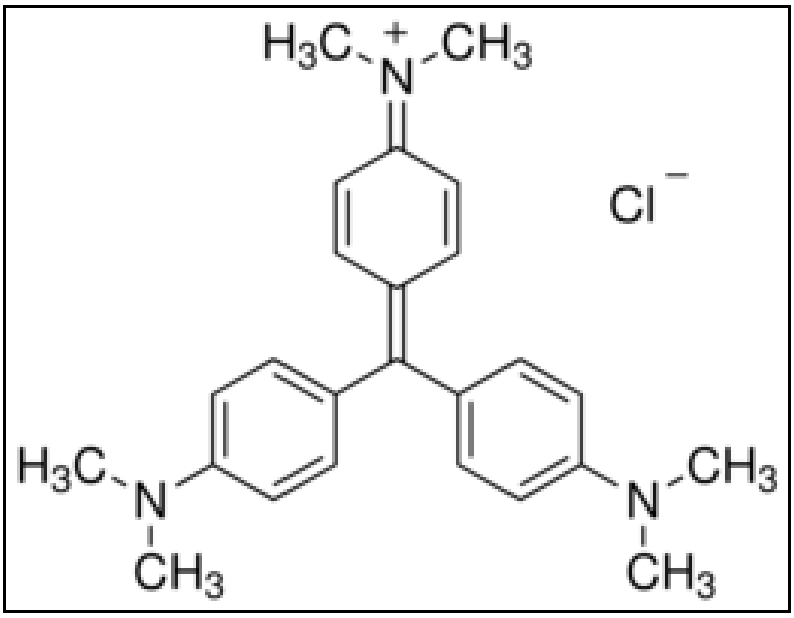


Figure 2.2: The chemical structure of crystal violet compound [http://www.sigmaaldrich.com/catalog/product/sigma/ht90132?lang=en&region=ZA#]

The maintenance and plating of the SC-1 and Caco-2 cell cultures (in DMEM 10% fetal calf serum and 1% antibiotics) used in the experiments were maintained by Ms. JC Serem of the Department of Anatomy, University of Pretoria. In this study cells were plated at a cell concentration of $3x10^4$ per 200 µl in 96 - well flat bottom plates and were incubated for 24 h at 37°C and 5% CO_2 to allow the cells to attach to the tissue culture surface before conducting experiments. Cells were incubated with the synthetic peptides OsΔC and PaΔC at 10, 25 and 50 µM concentrations. Melittin (25 µM) was used as a positive control, for 24 h at 37^{o}C and 5% CO_2. Following exposure, 10 µl of a 20% paraformaldehyde solution was added to the cell culture medium, as a fixative. The fixative and medium were removed and the plates were dried well until the dried layer of cells could be observed. The cells attached to the bottom of the plate were stained by adding 200 µl of a 0.1% (w/v) CV solution prepared in 200 mM of formic acid (pH 3.5) to each well for 30 min. The plate was washed with ddH_2O and dried, the

bound dye was extracted with 50 µl of a 10% acetic acid solution. The absorbance was determined at 630 nm and the data were expressed as percentage compared to the control (no peptide added).

2.5 Antioxidant and nitric oxide scavenging activity

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

2.5.1 Trolox equivalence antioxidant capacity assay

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

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

The oxidant used in the TEAC assay is 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and is generated by reacting a strong oxidizing agent (eg potassium persulfate) with the ABTS salt. The assay measures the relative ability of the antioxidant to scavenge the $ABTS^{\bullet+}$ generated, as compared with a trolox (water soluble vitamin E analogue) standard. The method used here is a based on the

methods of Awika *et al*. (2003), modified for multiple sample analysis by Serem and Bester, (2012). The sample is again quantified in comparison to a known antioxidant, trolox. The assay is inexpensive, easy to use and is a fast reaction (Awika *et al*., 2003).

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

2.5.2 Oxygen radical absorbance capacity assay

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

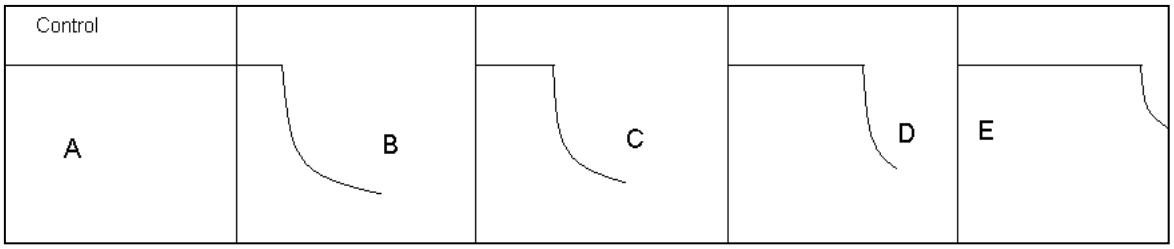


Figure 2.3: Schematic diagram of the ORAC assay (A) control, only fluorescein. (B-E) are increasing trolox concentrations

Procedures were based on a modified method of Ou *et al.* (2002). AAPH was used as a peroxyl radical generator, trolox as standard (0 – 1000 µM) and fluorescein as a fluorescent probe. Phosphate buffered saline (PBS) was used as a blank. To 160 µl 0.139 nM fluorescein working solution, 40 µl of PBS (0.2 M Na_2HPO_4, 0.2 M $NaH_2PO_4 \cdot H_2O$, 0.15 M NaCl, pH 7.4), or trolox standard or peptide (12.5, 25, 50 and 100 µM) were added. GSH and melittin (at the same concentrations as the peptide) were used as reference samples for comparison. This was followed by the addition of 40 µl 0.11 µM AAPH. Samples were mixed and the microplate was placed into the plate reader and incubated at 37°C. The fluorescence was measured every 5 min for 4 h. The assay protocol included: measurement start time of 0.0 sec, 10 flashes per cycle, 300 sec cycle time, 485 nm for the excitation filter and 520 nm for the emission filter. The results were expressed as µM TE per gram sample.

2.5.3 Cell based antioxidant assay

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

Total protective effects against oxidative damage caused by AAPH were determined, as described by Serem and Bester (2012). GSH is not able to cross the cell membrane and therefore is not used as a control (Wellner *et al.*, 1984). A stock solution of 750 µM

DCFH-DA was prepared in PBS and diluted 10x to the working solution of 75 µM. Caco-2 cells were plated at a concentration of $2x10^4$ cells/ml and 100 µl in 96 well plates. DCFH-DA working solution (50 µl) was added to each well, to a final concentration of 25 µM, and cell culture plates were maintained for a further 1 h at 37°C. The medium containing the DCFH-DA solution was carefully removed. Cell culture plates were washed once with PBS and were blotted dry. Peptide (25 µl) (final concentration of 12.5, 25 and 100 µM) was added to each well of the cell culture plates followed by 25 µl AAPH (final concentration of 7.5 mM). Change in fluorescence was measured immediately over 0 – 60 min, every 2 min. The effect of the peptides alone on the cells was also determined. The gradient of the change in fluorescence was calculated, and the data were expressed as % cellular damage where AAPH alone causes 100% damage.

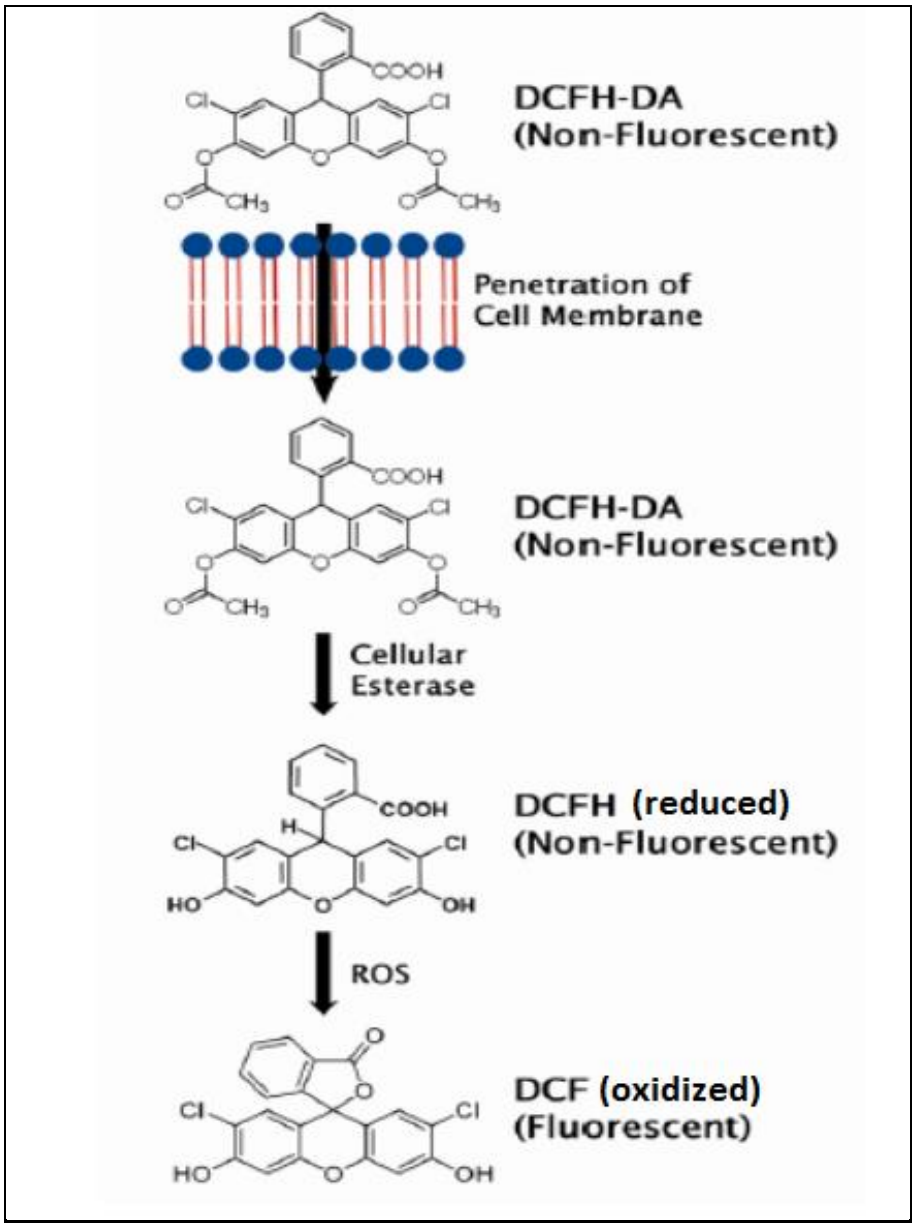


Figure 2.4: DCFH-DA reaction used to measure cellular oxidative damage
(Cell Biolabs, 2012)

2.5.4 Nitric oxide scavenging assay

The ability of a peptide to scavenge NO is beneficial and provides a first level of evaluation related to possible anti-inflammatory activity. NO scavengers may decrease the level of NO at sites of inflammation without affecting the production of NO elsewhere in the body. Determination of the NO scavenging capacity of the peptides was achieved by using sodium nitroprusside (SNP), which spontaneously degrades to produces NO and acts as a NO generator. The NO, in aqueous solutions, is converted

to nitrite (NO_2^-) (Wink *et al.,* 1993). The levels of nitrite can be quantified with the Griess reaction (Figure 2.5). In the assay SNP is incubated with the peptide and generates the highly unstable NO, which converts to nitrite. Sulphanilamide (SA, a diazotizing agent) in an acidic medium is added to the nitrite to form a diazonium salt. A coupling reagent, N-naphthyl-ethylenediamine dihydrochloride (NED), is added to form a stable azo compound (Sun *et al*., 2003). The product is purple in colour and the absorbance is linearly proportional to the concentration of nitrite in the sample. GSH and trolox were used as controls and have shown NO scavenging activity in previous studies (Vriesman *et al.*, 1997; Naik and Vaidya, 2011). PBS is used as the 100% control and the other samples are compared to it. Any scavenging of NO by peptides will result in less nitrite available hence less for conversion to the azo compound and less intense colour.

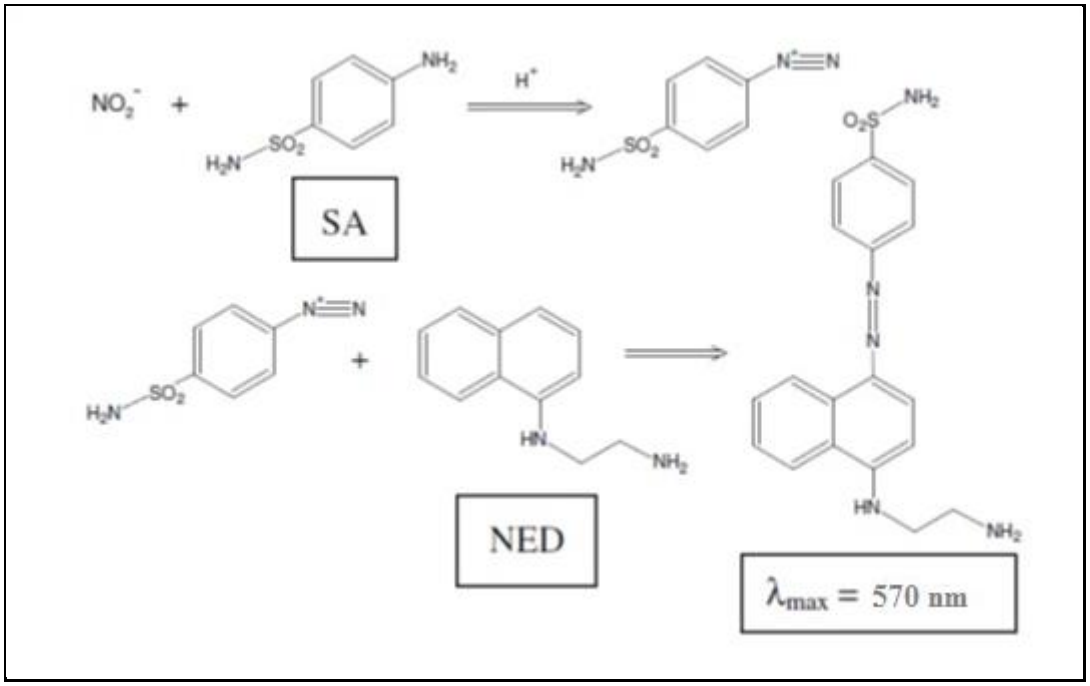


Figure 2.5: Overview of Griess reaction assay (Sun *et al*., 2003)

SNP (5 mM, 80 µl) diluted in PBS, 0.2 M, pH 7.2, is added to 20 µl peptide (final concentrations of 12.5 µM and 25 µM, diluted in dddH_2O) and incubated for 1 h at room temperature in the dark. Thereafter 50 µl 0.33% sulphanilamide (w/v) (diluted in 20% glacial acetic acid) was added and incubated for a further 10 min. NED (0.1% w/v, 50 µl) solution (diluted in 20% glacial acetic acid) was added and the absorbance was read at 570 nm. $NaNO_2$ was used to prepare a standard curve over a range of 0 to 0.1 mM (R^2=0.9998).

Chapter 3: Results

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

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

Peptide	Sequence	Length	Net charge	Molecular Mass (Da)	pI[a]	<H>[b]
PaΔC	WVIKGYKGGQKITVHRR-amide	17	+6	2025.42	12.44	0.261
OsΔC	WKGIRGYKGGYKGAFKQTKY-amide	20	+7	2335.72	10.84	0.155
Os-C[c]	KGIRGYKGGYKGAFKQTKY	19	+6	2150.50	10.58	0.045
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	26	+5	2847.40	12.43	0.511

[a] Iso-electric point.

[b] Hydrophobicity. Data obtained from HeliQuest [heliquest.ipmc.cnrs.fr/].

[c] Analogue of OsΔC, non-amidated and without the N-terminal tryptophan, used as a control in the determination of secondary structure.

3.1 Determination of antibacterial activity

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

Table 3.2: Minimum bactericidal concentration of peptides

Bacteria	MBC (µg/ml)		
	PaΔC[a]	OsΔC	Os-C[b]
Gram-positive			
B. subtilis	0.94 (0.46 µM)	1.90 (0.81 µM)	3.75 (1.74 µM)
S. aureus	ND	30 (12.8 µM)	15 (6.98 µM)
Gram-negative			
E. coli	0.94 (0.46 µM)	0.94 (0.40 µM)	3.75 (1.74 µM)
P. aeruginosa	ND	0.94 (0.40 µM)	1.90 (0.88 µM)

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

3.2 Secondary structure determination

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

The Far-UV CD profiles obtained for PaΔC, OsΔC and Os-C, and the effects of three solvents SDS (a membrane mimicking solvent), TFE (a secondary structure promoting solvent) and water on the structures of the peptides, are shown in Figure 3.1. Evaluation of the spectra is based on the shape and values at specific wavelengths. The PaΔC peptide retained a large degree of random coil content (unordered) in all the solutions. The amount of random coil content of PaΔC decreased slightly (seen by the decreases in the negativity of the peak) in 50% TFE (Figure 3.1 B) and 25 mM SDS

(Figure 3.1 C) as compared to water (Figure 3.1 A), and may have adopted more α-helical content. The negative peaks for PaΔC might indicate the presence of some β-sheet content when in TFE and water and possibly the presence of some α-helical content in the SDS.

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

	Secondary structure content (%)		
Peptide	**α-Helical**	**β-Strand**	**Random coil**
PaΔC	0	65	35
OsΔC	0	20	80
Os-C	0	21	79

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

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

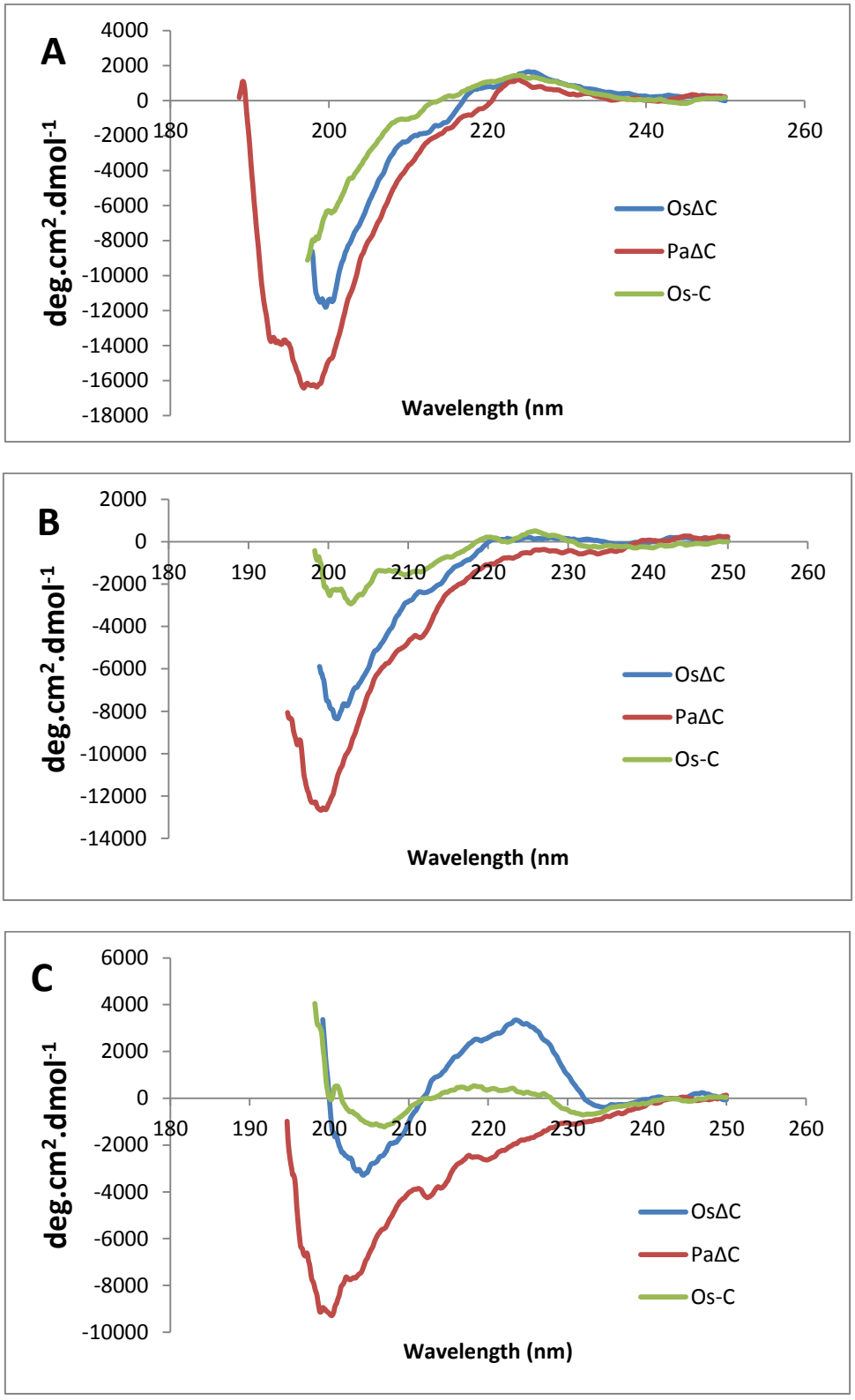


Figure 3.1: Far-UV CD spectra of synthetic peptides OsΔC, PaΔC and Os-C in (A) Water (B) 50% TFE and (C) 25 mM SDS.

3.3 Determination of the effect of the peptides on mammalian cells

3.3.1 Erythrocyte hemolytic assay

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

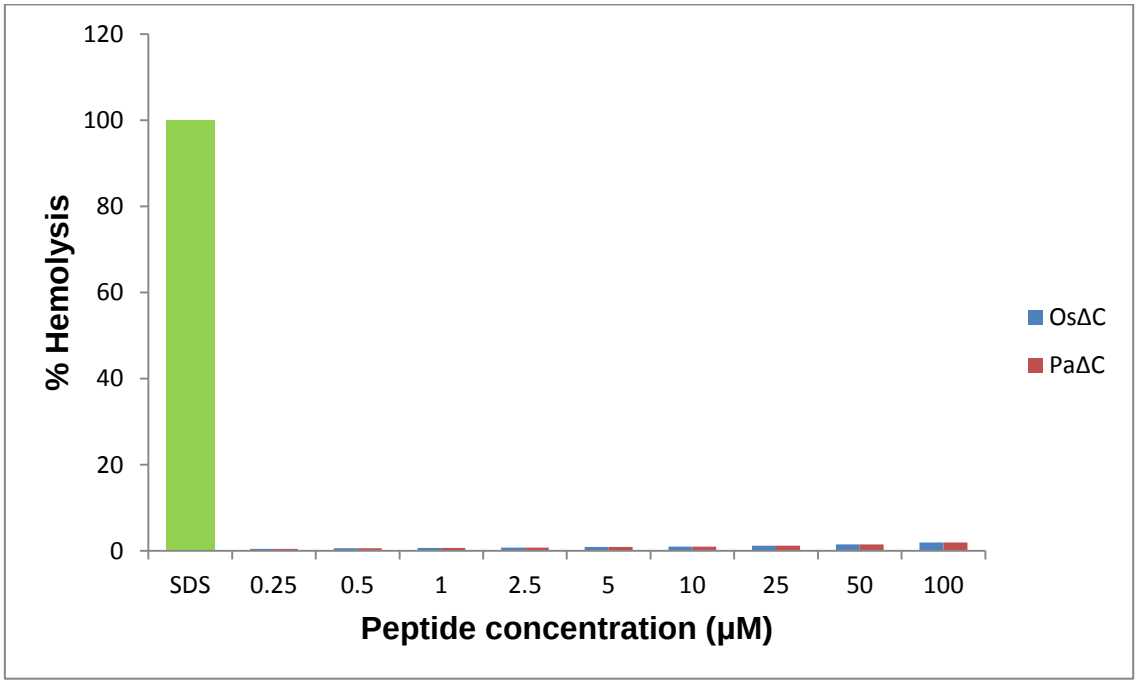


Figure 3.2: Hemolysis of human erythrocytes by synthetic peptides. Erythrocytes were incubated with the peptides (0.25-100 μM). Values are expressed as means ±SEM obtained from two independent experiments performed in triplicate.

3.3.2 Cell culture-based determination of cytotoxicty

The cytotoxicity of the peptides towards mammalian cells was further investigated with the CV staining assay in the SC-1 and Caco-2 cell lines (Figure 3.3 A and B). Both cell lines were exposed for 24 h to 10-50 μM OsΔC and PaΔC, with the control, melittin, at a concentration of 25 μM.

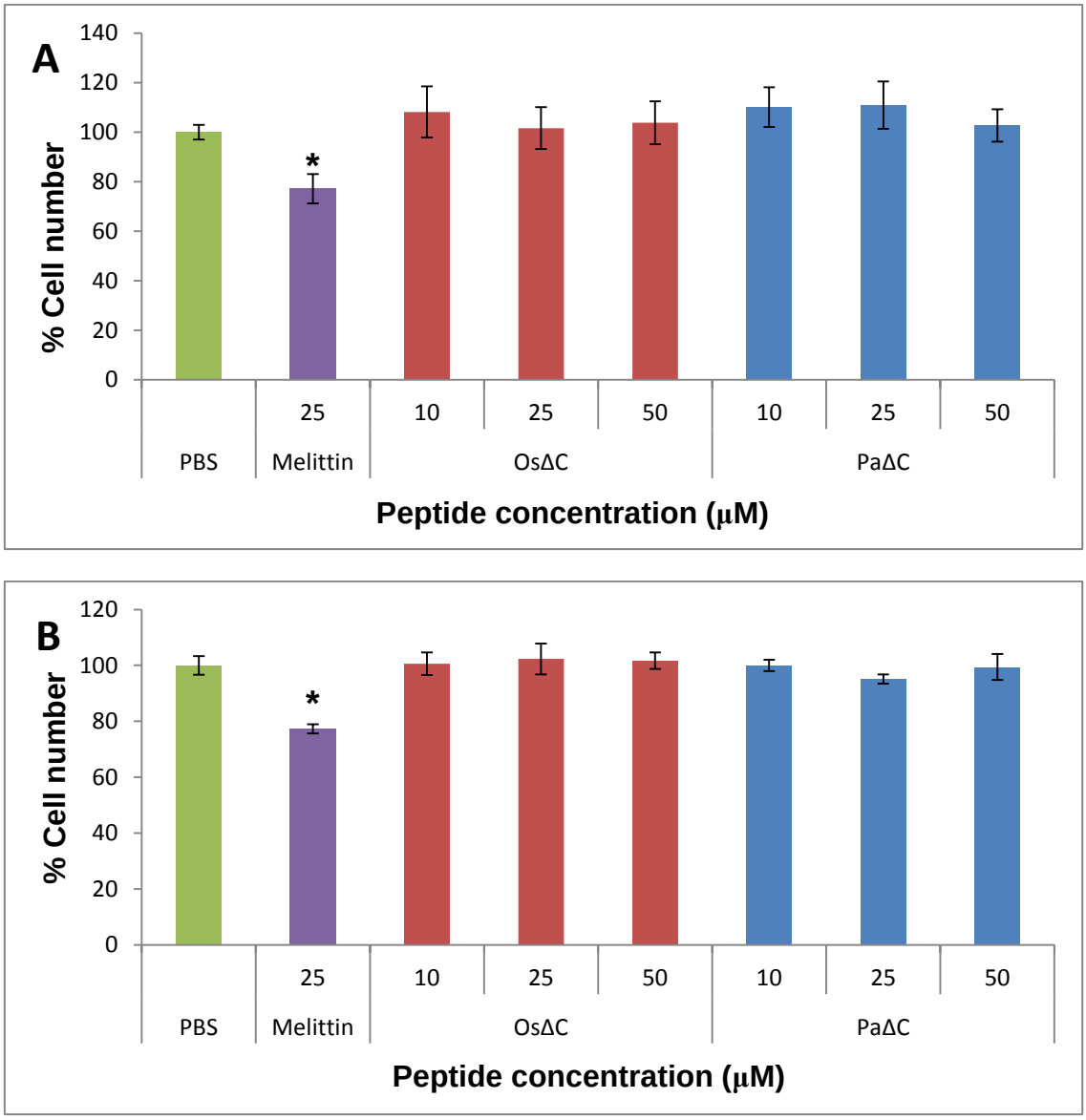


Figure 3.3: Cytotoxicity of synthetic peptides PaΔC and OsΔC on SC-1 cells (A) and Caco-2 cells (B), exposed for 24 h. Melittin was used as the positive control. Values are expressed as means ±SEM obtained from two independent experiments performed in triplicate. * Compared to the control (PBS) significant different (p≤0.001).

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

3.4 Antioxidant and nitric oxide scavenging activity

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

3.4.1 Trolox equivalence antioxidant capacity assay

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

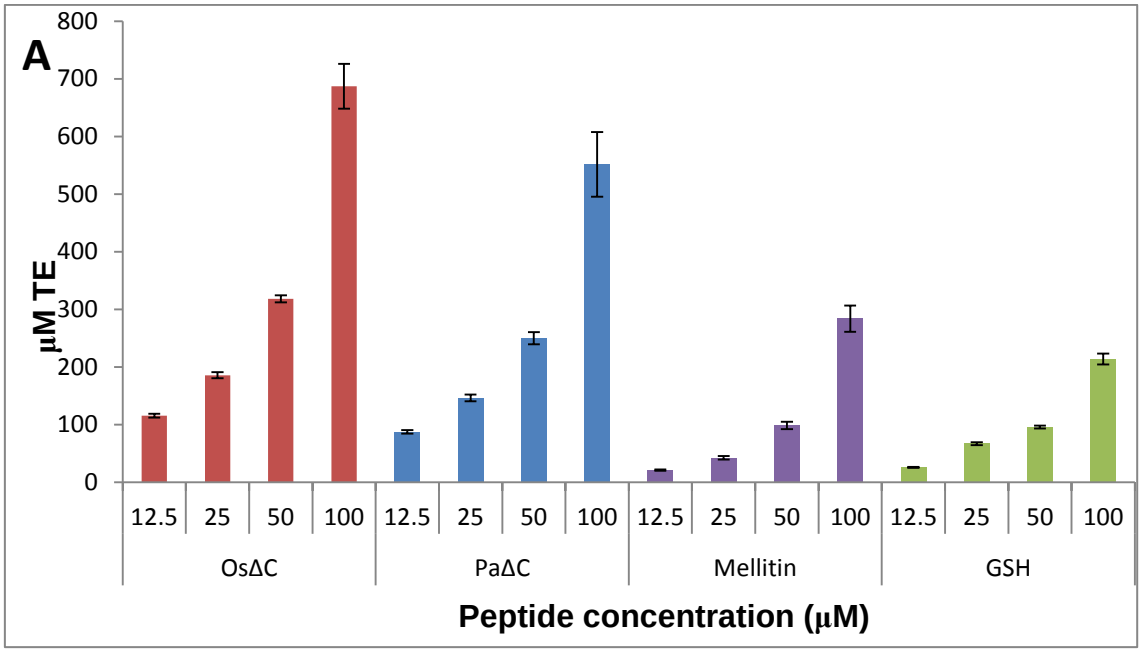


Figure 3.4: Antioxidant activity of synthetic peptides OsΔC and PaΔC measured with the TEAC assay compared with melittin and GSH. Peptides were tested over a concentration range of 12.5-100 µM. Values are expressed as means ±SEM obtained from three independent experiments performed in triplicate

3.4.2 Oxygen radical absorbance capacity assay

The ORAC assay is a competitive reaction system as the antioxidant and sample both compete for generated radicals. To determine the TE of the samples, a 0-800 µM trolox range was used for the standard curve (R^2=0.9943). PaΔC, OsΔC, GSH and melittin all revealed antioxidant activity with PaΔC and GSH displaying a dose dependant response (Figure 3.5). The strongest antioxidant was the OsΔC peptide, which at 25 µM had already reached the maximum capacity of the trolox standard range. Melittin performed poorly as an antioxidant at 12.5 and 25 µM concentrations, having negative values. This resulted in a poor correlation between µM peptide and µM TE. Therefore, the ratio of antioxidant activity to peptide concentration (µM TE/µM peptide) could not be determined accurately. An estimate of the µM TE/µM peptide values (from this data) for OsΔC, PaΔC, melittin and GSH were approximately 20.63, 13.63, 8.61 and 11.42 µM TE/µM peptide, respectively. Using these values, the antioxidant activity of OsΔC and PaΔC are 1.81 and 1.19 times larger than that of GSH, respectively, and was 2.40 and 1.58 times larger than that of melittin, respectively.

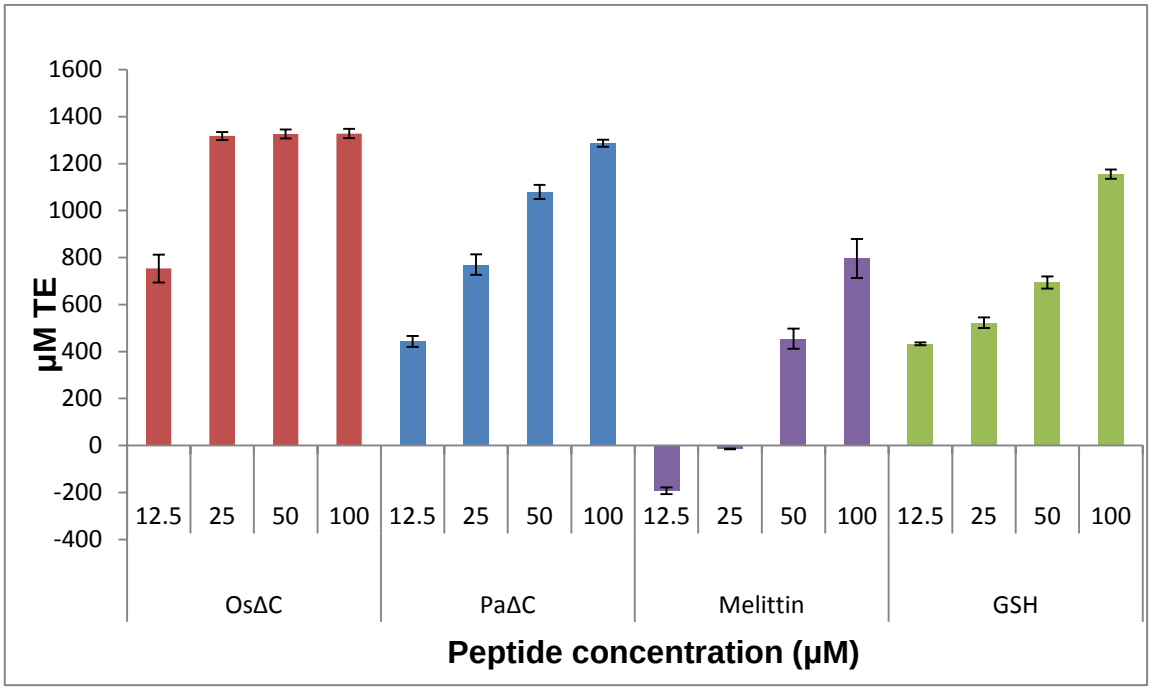


Figure 3.5: Antioxidant activity of synthetic peptides OsΔC and PaΔC measured with the ORAC assay compared with melittin and GSH. Peptides were tested over a concentration range of 12.5-100 µM. Values are expressed as means ±SEM obtained from three independent experiments performed in triplicate.

3.4.3 Cell-based antioxidant assay

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

oxidative protection (negative gradient). An increase in oxidative protection is observed with PaΔC (positive gradient).

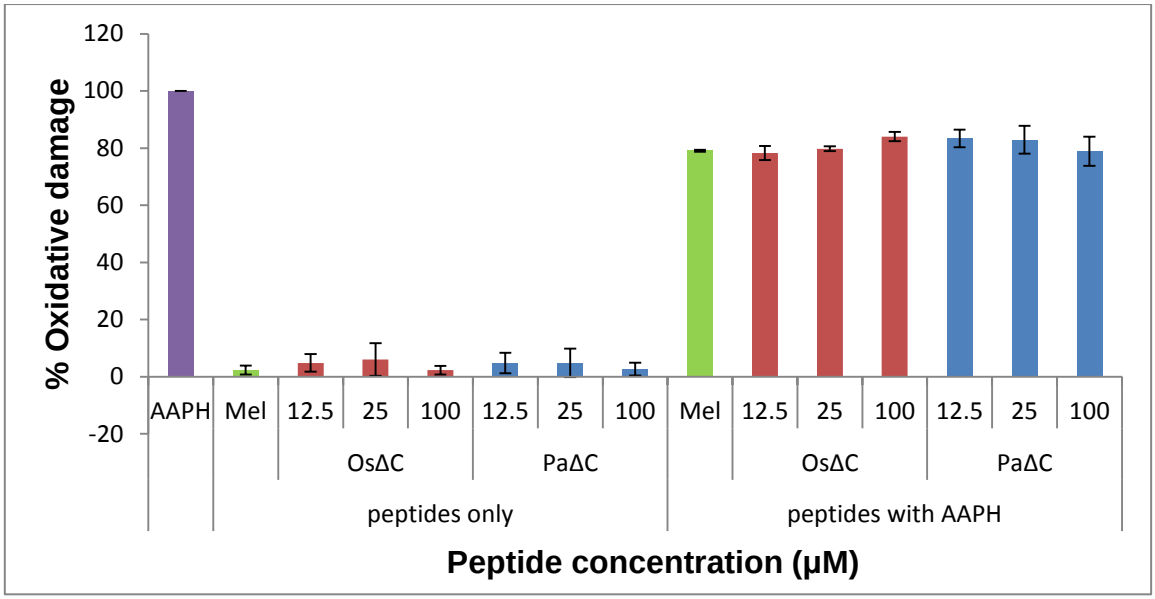


Figure 3.6: Antioxidant activity of the synthetic peptides melittin (Mel), PaΔC and OsΔC, measured with the DCFH-DA assay. Activity was determined over a concentration range of 12.5-100 μM. Melittin was tested at 100 μM. The effect of the peptides without AAPH was also determined. Values are expressed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

3.4.4 Nitric oxide scavenging assay

Due to the fact that the peptides showed strong antioxidant activity in the chemical assays and some activity in the cellular antioxidant assay, the peptides were also evaluated for their NO scavenging ability. Production of RNS may also lead to oxidative damage and NO scavenging activity may aid in protection from this. NO scavengers are also a proposed method of protection in inflammatory disorders (Shah and Billiar, 1998). In this assay SNP is used to produce NO which can be scavenged by molecules with NO scavenging activity. The samples are compared to the NO present in the PBS control, taken as 100% (Figure 3.7). $NaNO_2$ was used to prepare a standard curve over a range of 0 to 0.1 mM (R^2=0.9998).

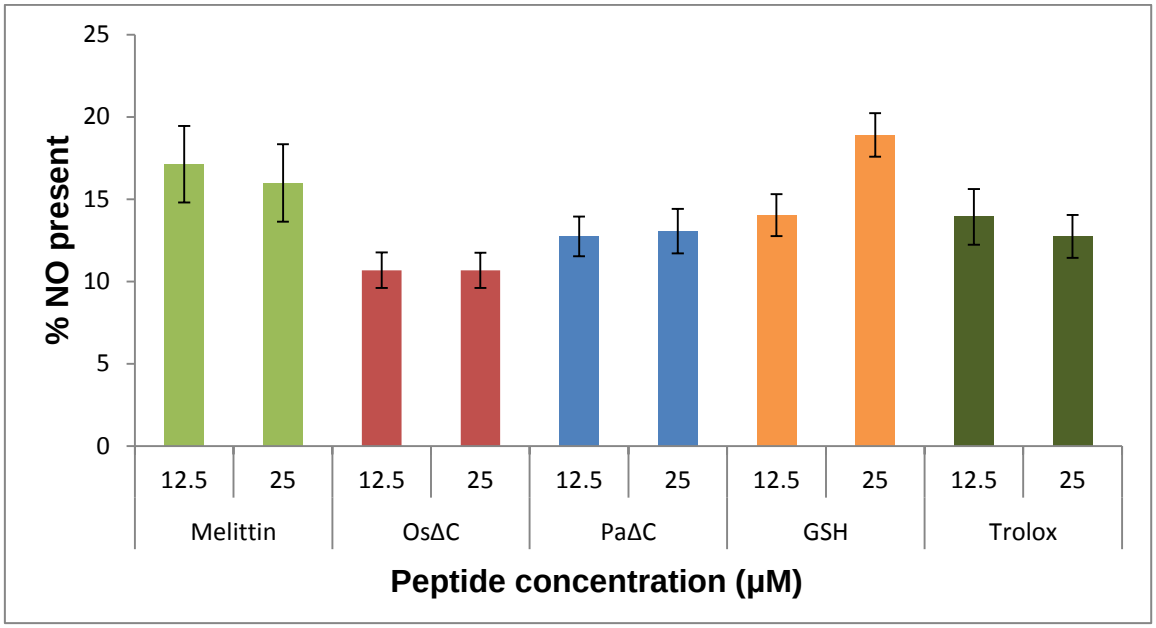


Figure 3.7: Nitric oxide scavenging activity of the synthetic peptides PaΔC and OsΔC, GSH, trolox and melittin. Values are expressed as means ±SEM obtained from three experiments performed in triplicate.

Strong NO scavenging activity was found for all the samples tested, showing more than 80% scavenging activity. OsΔC at 25 μM showed the strongest NO scavenging activity (89%). The activity of PaΔC was comparable to that of trolox.

Chapter 4: Discussion

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

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

If the MBC values of OsΔC are compared to the MBC values of its analogue Os-C (Table 3.2), as determined by Prinsloo *et al.* (2013), OsΔC was found to be 2 times more active against *P. aeruginosa* and *B. subtilis* compared to Os-C. OsΔC was also 4 times more active against *E. coli* (0.40 vs. 1.74 μM), whereas Os-C was 2 times more active (15 vs. 30 μM) against *S. aureus* respectively. Comparing the sequence of

OsΔC to that of Os-C, the addition of Trp to the N-terminus, increased the hydrophobicity and amidation at the C-terminus added an additional positive charge. It has been shown that the C-terminal fragment of OsDef2 with cysteines removed, is a viable strategy for shortening peptides. This strategy has actually broadened the antibacterial spectrum of the peptide. The parent peptide, OsDef2, is only active against Gram-positive bacteria whereas the OsΔC peptide is active against Gram-positive and Gram-negative bacteria. One of the limitations associated with the use of AMPs is the high cost of synthesis. Shorter peptides which retain their activity are important in the development of AMPs as therapeutic agents, however, some shorter peptides have been shown to be cytotoxic (Leontiadou *et al.*, 2006; Makovitzki *et al.*, 2006).

One of the strategies used in an attempt to improve cell selectivity is optimization of physicochemical parameters (Matusuzaki, 2009). The disulphide bonds of β-sheet AMPs which stabilize both the β-hairpin and β-sheet structures may not be necessary for their antimicrobial activity as linear derivatives of tachyplesin, lactoferrin and defensins have retained their antimicrobial activity, and also lost their hemolytic activity (Gifford *et al*., 2005; Ramamoorthy *et al*., 2006; Varkey *et al*., 2006). Human β-defensin 3 contains an α-helical turn and a three stranded antiparallel β-sheet, stabilized by three disulphide bonds. Reducing the disulphide bonds did not alter the antimicrobial activity nor affect the overall formation of the native secondary structures (Wu *et al*., 2003; Klűver *et al*., 2005) but it did affect the chemotactic activities. Reduction of the disulphide bonds in human β-defensin 1 created an unstructured, flexible peptide which showed improved activity against *Candida albicans* and some Gram-positive bacteria (Schroeder *et al*., 2011). An increase in charge in some cases has been shown to increase antimicrobial activity without significantly affecting hemolytic activity (Zelezetsky and Tossi, 2006). A similar charge to activity relationship was seen in a study by Nguyen *et al.* (2011), with C-terminal analogues of macrophage inflammatory protein-3α. The analogue with greater charge showed more antibacterial activity against Gram-positive and Gram-negative bacteria. Charge is important for activity against Gram-negative bacteria, while hydrophobicity and hydrophobic moment increase activity against Gram-positive bacteria but also hemolytic activity (Dathe and Wieprecht, 1999; Wieprecht *et al*., 1996, 1997). End-tagging of peptides with oligo-tryptophan increases the hydrophobicity, which resulted in a decrease in toxicity to human cells and promoted peptide-induced lysis of phospholipid liposomes and killing of bacteria and fungi (Schmidtchen *et al*., 2011).

In this study the increase in charge together with the increase in hydrophobicity resulted in a more active peptide. This is not always the case as there is a fine balance between the amount of charge and the amount of hydrophobicity of a peptide that allows for attraction to bacterial membranes and insertion into it. Makovitski *et al*. (2006) reported that the balance between charge and hydrophobicity plays an important role just as in larger AMPs and showed that altering both the amino acid residues and chain length of a peptide to increase the hydrophobicity and/or charge, did not necessarily result in higher antimicrobial activity. It is difficult to predict or estimate what effect a physicochemical change will cause as many factors are involved in the activity of the peptide and they do not always act independently (Matsuzaki, 2009).

Many linear peptides (which lack cysteine residues) are unstructured in aqueous environments and adopt stable amphipathic conformations in membranous or membrane mimicking environments (Hwang and Vogel, 1998). CD is a commonly used method for investigating the secondary structures of proteins and peptides. The OsΔC and Os-C peptides presented with different activities, OsΔC showed lower MBCs with three of the four bacteria tested. A change in secondary structure, as a result of the addition of a Trp and amidation, might be the cause of the increased activity. The CD spectra of the peptides were studied in water, TFE and SDS solutions (Figure 3.1). TFE is a secondary structure promoting solution (Waring *et al.*, 1995, Roccatano *et al.*, 2002), while SDS is a membrane mimicking solution. The PaΔC peptide maintained a largely random coil structure conformation in all the media tested, with the amount of random coil structure decreasing slightly in TFE and slightly more in SDS. PaΔC may contain some β-sheet content in TFE and SDS media and some α-helical content in the SDS. Varkey *et al.* (2006) reported that the spectrum of PaΔC in water was characteristic of an unordered conformation. In SDS, the authors reported a mixed population of random coil and α-helical structure. In TFE, the spectrum suggested the presence of some β-hairpin and α-helical structure. The OsΔC peptide adopted a largely random coil structure in both the water and TFE media. Some β-sheet content may be present in water, the amount of which increased in TFE. In SDS, OsΔC adopted a more α-helical structure. Comparing the spectra of peptides OsΔC and Os-C, there is a clear difference. The Os-C peptide showed a much greater change in structure when in TFE and SDS solutions. The change to adopt less random coil and more α-helical content is seen in TFE and in SDS the peptide adopted a largely α-helical secondary structure (Figure 3.1).

The positive peaks seen may be the presence of turn formations but also may be caused by interference from aromatic residues. Os-C has a smaller positive peak than Os∆C and this may be a result of interference by the added Trp residue to Os∆C (Figure 3.1). Khan *et al.* (1989) found that the peaks for Tyr, Trp and Phe were present at 225, 224 and 214 nm, respectively. Although the position of each peak differs, Khan *et al.* (1989) found that together these residues resulted in a peak around 222 nm. The authors also noted that the CD spectra were influenced by the amount of Phe contained in the peptide, where the higher Phe content results in a shift of the peaks to shorter wavelengths. Pa∆C has one Trp and one Tyr residue, whereas Os∆C has one Trp, three Tyr and one Phe residues and the Os-C peptide has one Phe and three Tyr residues. The resulting peaks could mask some of the spectra and the presence of Phe could account for the slight shift in the peaks when compared to the standard peaks for α-helix and β-sheet formations.

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

The peptides have shown strong antibacterial activity, but to be considered for further development as a therapeutic agent, they must also be non-toxic to mammalian cells. The cytotoxicity of the peptides were investigated by testing the effect of the peptides on human erythrocytes, mouse fibroblast cells (Sc-1 cell line) and human colon cancer cells (Caco-2 cell line). With the hemolytic assay the erythrocyte membrane represents a typical bilayer membrane and damage to the membrane will result in the release of

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

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

The assays with Sc-1 mouse fibroblast cells and Caco-2 human colon cells, showed no cytotoxic activity of the peptides (Figure 3.3). The positive control used was melittin which presented with approximately a 20% decrease in cells, with its activity being slightly higher with Sc-1 cells. The decrease is significant and Prinsloo *et al.* (2013) showed that it is a dosage dependant response. Nishimura *et al*. (2004) investigated the effect of human defensins, α-defensin (HNP-1) and β-defensins (hBD-1, -2, -3) on eukaryotic cell lines. At low concentrations the α-defensin showed enhanced cell proliferation with retinal epithelial cells and lung epithelial cells, but no proliferation with fibroblasts. The β-defensin showed very little effect at low and high concentrations. Proliferation of fibroblasts by a defensin has been reported before by Murphy *et al*. (1993). Wong *et al*. (2006) also showed that a plant defensin induced a mitogenic response from mouse splenocytes. In this study, a slight increase in the growth of

Caco-2 cells was observed. Statistically, the proliferation was not significant and may be due to non-specific staining of dead cells by the dye but the possibility of the peptides having a mitogenic response, should be further investigated. Allowing the cells to grow for a longer period of time in the presence of the peptides may show larger differences between control and exposed cells.

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

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

closer look is needed into the reaction mechanisms to fully explain the reasons for the observed differences.

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

The DCFH-DA assay shows the antioxidant capacity of the peptides within a cell, which in this study was the Caco-2 cell line. The conversion of non-fluorescent DCFH to the fluoresecent DCF, occurs by the action of ROS within the cell. To affect the production of DCF the peptides should scavenge available ROS inside the cell. AAPH is a free radical generator which is added to the cells and the assay measures each peptide's ability to scavenge the radicals within the cell. The effect of the peptides without the addition of AAPH was also determined and a small increase in radicals was noted (Figure 3.6). A concentration dependant increase in oxidative stress was found when Caco-2 cells were incubated with a Trp-rich peptide fraction of milk protein, despite the fraction having shown strong antioxidant activity in an ORAC assay (Elisia *et al.* 2011).

The authors explained the observation being a result of the production of ROS from exposure of Trp-containing milk peptides, primarily generated by mitochondria as by-products of cellular metabolism. Even though Trp-containing peptides contributed to an increase in pro-oxidant activity, no cytotoxicity was observed. Elisia *et al.* (2011) attributed this to the activation of the Nrf-2 pathway, a cellular adaptive pathway which responds to cellular stress by activation of an antioxidant response.

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

There are two ways the production of NO may become detrimental to the body. Firstly, the over production of NO during inflammation and, secondly, it may lead to the generation of RNS. The role of NO in immune responses and inflammation is well known (Stuehr and Marletta, 1985; Karupiah *et al.,* 1993). The inhibition of NO would be beneficial in chronic inflammatory diseases. Two strategies exist for inhibiting NO, the first is to inhibit the production of NO by inhibiting the enzymes responsible for its production (eNOS and iNOS). This however has presented with adverse effects (for both nonselective and selective inhibition of the enzymes) due to the beneficial role that NO also plays in the body (Hutcherson *et al*., 1990; Petros *et al*., 1994; Spain *et al*., 1994; Wolfe and Dasta, 1995; Kim and Greenburg, 2002; Lopez *et al*., 2004). The second option is to decrease the amount of NO available by making use of NO scavengers, which would not affect the functioning of eNOS or iNOS. The NO scavenging capacity of the synthetic peptides in this study was investigated (Figure 3.7). The results in this study demonstrated that all the samples offer some NO scavenging capacity. OsΔC and PaΔC performed only slightly better than melittin and GSH. Chen *et al.* (2005) showed that GSH depletion in hepatocytes resulted in the cells having higher levels of ROS, in response to NO exposure and suggested that it could be attributed to the loss of GSH scavenging activity for NO. There are a number of research articles documenting the anti-inflammatory activity of melittin by inhibition of

NOS and the LPS-induced accumulation of NO (Srivastava *et al.*, 2012; Moon *et al.*, 2007), but to our knowledge none on the direct NO scavenging capacity of melittin. Naik and Vaidya (2011) found high NO scavenging capacity (42%) for trolox (0.2 mg/ml). In comparison to melittin and trolox, strong NO scavenging capacity for both OsΔC and PaΔC peptides was found (Figure 3.7). Further research into the mechanism by which the peptides scavenge NO is required.

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

Chapter 5: Conclusion

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

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

Other immunomodulatory activities need to be further investigated to determine the full potential of OsΔC. Some peptides have been shown to assist in wound healing by their effect on the production and control of other mediators involved in the process (Mookherjee *et al.,* 2006; Hartman and Meisel, 2007; Yang *et al*., 2009; Zhang *et al*., 2008; van der Does *et al*., 2010). Notably the LPS-binding activity in reducing inflammation and the effect of the peptides on regulation of chemokines and cytokines should be investigated. Other aspects which should be investigated are the effects of salts on antibacterial activity, killing kinetics and the peptides' mechanism of action. In the case of acne legions and sepsis, peptides with antibacterial and anti-inflammatory

properties would be beneficial and the antioxidant activity would control the production of ROS and prevent oxidative damage associated with infections (McInturff *et al*., 2005). Another use for peptides with this activity could be the coating of prosthetic implants to reduce infections while also creating an environment which promotes wound healing, e.g. by reduction of ROS

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

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

Chapter 6: References

Aarbiou, J., Verhoosel, R. M., van Wetering, S., de Boer, W. I., Van Krieken, J. H. J. M., Litvinov, S. V., Rabe, K. F. & Hiemstra, P. S. (2004). Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression in vitro. *American Journal of Respiratory Cell and Molecular Biology*, *30*, 193-201.

Ahn, C. B., Je, J. Y., & Cho, Y. S. (2012). Antioxidant and anti-inflammatory peptide fraction from salmon byproduct protein hydrolysates by peptic hydrolysis. *Food Research International*, *49*, 92-98.

Alemán, A., Giménez, B., Pérez-Santin, E., Gómez-Guillén, M. C., & Montero, P. (2011). Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate. *Food Chemistry*, *125*, 334-341.

Alisi, C. S., & Onyeze, G. O. C. (2008). Nitric oxide scavenging ability of ethyl acetate fraction of methanolic leaf extracts of *Chromolaena odorata (Linn.)*. *African Journal of Biochemistry Research*, *2*, 145-150.

Auvynet, C., & Rosenstein, Y. (2009). Multifunctional host defense peptides: antimicrobial peptides, the small yet big players in innate and adaptive immunity. *Federation of European Biochemical Societies Journal*, *276*, 6497-6508.

Awika, J. M., Rooney, L. W., Wu, X., Prior, R. L., & Cisneros-Zevallos, L. (2003). Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. *Journal of Agricultural and Food Chemistry*, *51*, 6657-6662.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., & Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences*, *87*, 1620-1624.

Bergman, P., Johansson, L., Asp, V., Plant, L., Gudmundsson, G. H., Jonsson, A. B., & Agerberth, B. (2005). *Neisseria gonorrhoeae* downregulates expression of the human antimicrobial peptide LL-37. *Cellular Microbiology*, *7*, 1009-1017.

Bergman, P., Walter-Jallow, L., Broliden, K., Agerberth, B., & Soderlund, J. (2007). The antimicrobial peptide LL-37 inhibits HIV-1 replication. *Current HIV Research*, *5*, 410-415.

Bertolini, A., Ottani, A., & Sandrini, M. (2001). Dual acting anti-inflammatory drugs: a reappraisal. *Pharmacological Research: The Official Journal of the Italian Pharmacological Society*, *44*, 437.

Bidla, G., Lindgren, M., Theopold, U., & Dushay, M. S. (2005). Hemolymph coagulation and phenoloxidase in *Drosophila* larvae. *Developmental & Comparative Immunology*, *29*, 669-679.

Blasa, M., Gennari, L., Angelino, D., & Ninfali, P. (2010). Fruit and vegetable antioxidants in health. *Bioactive foods in promoting health: fruits and vegetables*, 37-58.

Boman, H. G., Agerberth, B., & Boman, A. (1993). Mechanisms of action on *Escherichia coli* of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. *Infection and Immunity*, *61*, 2978-2984.

Boehr, D. D., Draker, K. A., Koteva, K., Bains, M., Hancock, R. E., & Wright, G. D. (2003). Broad-spectrum peptide inhibitors of aminoglycoside antibiotic resistance enzymes. *Chemistry & Biology*, *10*, 189-196.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., ... & Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clinical Infectious Diseases*, *48*, 1-12.

Bougatef, A., Nedjar-Arroume, N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D., & Nasri, M. (2010). Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle *Sardinella aurita* by-products proteins. *Food Chemistry*, *118*, 559-565.

Bowdish, D. M., Davidson, D. J., Scott, M. G., & Hancock, R. E. (2005). Immunomodulatory activities of small host defense peptides. *Antimicrobial Agents and Chemotherapy*, *49*, 1727-1732.

Braida, L., Boniotto, M., Pontillo, A., Tovo, P. A., Amoroso, A., & Crovella, S. (2004). A single-nucleotide polymorphism in the human β-defensin 1 gene is associated with HIV-1 infection in Italian children. *AIDS*, *18*, 1598-1600.

Brandenburg, L. O., Merres, J., Albrecht, L. J., Varoga, D., & Pufe, T. (2012). Antimicrobial peptides: multifunctional drugs for different applications. *Polymers*, *4*, 539-560.

Brogden, K. A., De Lucca, A. J., Bland, J., & Elliott, S. (1996). Isolation of an ovine pulmonary surfactant-associated anionic peptide bactericidal for *Pasteurella haemolytica*. *Proceedings of the National Academy of Sciences*, *93*, 412-416.

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

Brogden, N. K., & Brogden, K. A. (2011). Will new generations of modified antimicrobial peptides improve their potential as pharmaceuticals? *International Journal of Antimicrobial Agents*, *38*, 217-225.

Broekaert, W. F., Terras, F. R., Cammue, B. P., & Osborn, R. W. (1995). Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant Physiology*, *108*, 1353.

Buck, C. B., Day, P. M., Thompson, C. D., Lubkowski, J., Lu, W., Lowy, D. R., & Schiller, J. T. (2006). Human α-defensins block papillomavirus infection. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 1516-1521.

Bulet, P., & Stocklin, R. (2005). Insect antimicrobial peptides: structures, properties and gene regulation. *Protein and Peptide Letters*, *12*, 3-11.

Campopiano, D. J., Clarke, D. J., Polfer, N. C., Barran, P. E., Langley, R. J., Govan, J. R., Maxwell, A. & Dorin, J. R. (2004). Structure-activity relationships in defensin dimers, a novel link between β-defensin tertiary structure and antimicrobial activity. *Journal of Biological Chemistry*, *279*, 48671-48679.

Cao, G., Alessio, H. M., & Cutler, R. G. (1993). Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine*, *14*, 303-311.

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

Charlet, M., Chernysh, S., Philippe, H., Hetru, C., Hoffmann, J. A., & Bulet, P. (1996). Innate immunity Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis*. *Journal of Biological Chemistry*, *271*, 21808-21813.

Chen, T., Pearce, L. L., Peterson, J., Stoyanovsky, D., & Billiar, T. R. (2005). Glutathione depletion renders rat hepatocytes sensitive to nitric oxide donor–mediated toxicity. *Hepatology*, *42*, 598-607.

Chen, H. M., Muramoto, K., & Yamauchi, F. (1995). Structural analysis of antioxidative peptides from soybean. β-Conglycinin. *Journal of Agricultural and Food Chemistry*, *43*, 574-578.

Chen, H. M., Muramoto, K., Yamauchi, F., & Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry*, *44*, 2619-2623.

Chen, H. M., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998). Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. *Journal of Agricultural and Food Chemistry*, *46*, 49-53.

Chen, C. Z., & Cooper, S. L. (2002). Interactions between dendrimer biocides and bacterial membranes. *Biomaterials*, *23*, 3359-3368.

Chen, Y., Mant, C. T., Farmer, S. W., Hancock, R. E., Vasil, M. L., & Hodges, R. S. (2005). Rational design of α-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. *Journal of Biological Chemistry*, *280*, 12316-12329.

Chen, Y., Guarnieri, M. T., Vasil, A. I., Vasil, M. L., Mant, C. T., & Hodges, R. S. (2007). Role of peptide hydrophobicity in the mechanism of action of α-helical antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*, *51*, 1398-1406.

Chen, X., Niyonsaba, F., Ushio, H., Hara, M., Yokoi, H., Matsumoto, K. & Ogawa, H. (2007). Antimicrobial peptides human β-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability. *European Journal of Immunology*, *37*, 434-444

Cho, Y. S., Lee, S. H., Kim, S. K., Ahn, C. B., & Je, J. Y. (2011). Aminoethyl-chitosan inhibits LPS-induced inflammatory mediators, iNOS and COX-2 expression in RAW264. 7 mouse macrophages. *Process Biochemistry*, *46*, 465-470.

Chrudimská, T., Chrudimský, T., Golovchenko, M., Rudenko, N., & Grubhoffer, L. (2010). New defensins from hard and soft ticks: Similarities, differences and phylogenetic analyses. *Veterinary Parasitology*, *167*, 298-303.

Chrudimská, T., Slaninová, J., Rudenko, N., Růžek, D., & Grubhoffer, L. (2011). Functional characterization of two defensin isoforms of the hard tick Ixodes ricinus. *Parasitolog Vectors*, *4*, 63.

Condé, R., Argüello, M., Izquierdo, J., Noguez, R., Moreno, M., & Lanz, H. Natural Antimicrobial Peptides from Eukaryotic Organisms. edited by Varaprasad Bobbarala, Published: September, 2012.

Daher, K. A., Selsted, M. E., & Lehrer, R. I. (1986). Direct inactivation of viruses by human granulocyte defensins. *Journal of Virology*, *60*, 1068-1074.

Davidson, D. J., Currie, A. J., Reid, G. S., Bowdish, D. M., MacDonald, K. L., Ma, R. C., Hancock, R. E. W. & Speert, D. P. (2004). The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *The Journal of Immunology*, *172*, 1146-1156.

Dathe, M., & Wieprecht, T. (1999). Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1462*, 71-87.

Dawson, R. M., & Liu, C. Q. (2008). Properties and applications of antimicrobial peptides in biodefense against biological warfare threat agents. *Critical Reviews in Microbiology*, *34*, 89-107.

De Lucca, A. J., & Walsh, T. J. (1999). Antifungal peptides: novel therapeutic compounds against emerging pathogens. *Antimicrobial Agents and Chemotherapy*, *43*, 1-11.

De Smet, K., & Contreras, R. (2005). Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnology Letters*, *27*, 1337-1347.

Eipper, B. A., & Mains, R. E. (1988). Peptide α-amidation. *Annual Review of Physiology*, *50*, 333-344.

Elisia, I., Tsopmo, A., Friel, J. K., Diehl-Jones, W., & Kitts, D. D. (2011). Tryptophan from human milk induces oxidative stress and uregulates the Nrf-2–mediated stress response in human intestinal cell lines. *The Journal of Nutrition*, *141*, 1417-1423.

Feng, Z., Dubyak, G. R., Lederman, M. M., & Weinberg, A. (2006). Cutting Edge: Human β Defensin 3—A Novel Antagonist of the HIV-1 Coreceptor CXCR4. *The Journal of Immunology*, *177*, 782-786.

Fogaça, A. C., Lorenzini, D. M., Kaku, L. M., Esteves, E., Bulet, P., & Daffre, S. (2004). Cysteine-rich antimicrobial peptides of the cattle tick *Boophilus microplus*: isolation, structural characterization and tissue expression profile. *Developmental & Comparative Immunology*, *28*, 191-200.

Galli, F., Battistoni, A., Gambari, R., Pompella, A., Bragonzi, A., Pilolli, F., Luigi, L., Marta, P., Maria, C.D. & Cabrini, G. (2012). Oxidative stress and antioxidant therapy in cystic fibrosis. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1822*, 690-713.

Gallo, S. A., Wang, W., Rawat, S. S., Jung, G., Waring, A. J., Cole, A. M. & Blumenthal, R. (2006). θ-Defensins prevent HIV-1 Env-mediated fusion by binding gp41 and blocking 6-helix bundle formation. *Journal of Biological Chemistry*, *281*, 18787-18792.

Ganz, T. (1987). Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. *Infection and Immunity*, *55*, 568-571.

Ganz, T., Metcalf, J. A., Gallin, J. I., Boxer, L. A., & Lehrer, R. I. (1988). Microbicidal/cytotoxic proteins of neutrophils are deficient in two disorders: Chediak-Higashi syndrome and “specific" granule deficiency. *Journal of Clinical Investigation*, *82*, 552.

Ganz, T., & Lehrer, R. I. (1995). Defensins. *Pharmacology & Therapeutics*, *66*, 191-205.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nature Reviews Immunology*, *3*, 710-720.

Gao, G., Lange, D., Hilpert, K., Kindrachuk, J., Zou, Y., Cheng, J. T., & Kizhakkedathu, J. N. (2011). The biocompatibility and biofilm resistance of implant coatings based on hydrophilic polymer brushes conjugated with antimicrobial peptides. *Biomaterials*, *32*, 3899-3909.

Gennaro, R., Zanetti, M., Benincasa, M., Podda, E., & Miani, M. (2002). Pro-rich antimicrobial peptides from animals: structure, biological functions and mechanism of action. *Current Pharmaceutical Design*, *8*, 763-778.

Giacometti, A., Cirioni, O., Del Prete, M. S., Paggi, A. M., D’Errico, M. M., & Scalise, G. (2000). Combination studies between polycationic peptides and clinically used antibiotics against Gram-positive and Gram-negative bacteria. *Peptides*, *21*, 1155-1160.

Giacometti, A., Cirioni, O., Barchiesi, F., & Scalise, G. (2000). In-vitro activity and killing effect of polycationic peptides on methicillin-resistant *Staphylococcus aureus* and interactions with clinically used antibiotics. *Diagnostic Microbiology and Infectious Disease*, *38*, 115-118.

Gifford, J. L., Hunter, H. N., & Vogel, H. J. (2005). Lactoferricin. *Cellular and Molecular Life Sciences*, *62*, 2588-2598.

Gillespie, J. P., Kanost, M. R., & Trenczek, T. (1997). Biological mediators of insect immunity. *Annual Review of Entomology*, *42*, 611-643.

Ginsburg, I. (2004). Bactericidal cationic peptides can also function as bacteriolysis-inducing agents mimicking β-lactam antibiotics? It is enigmatic why this concept is consistently disregarded. *Medical Hypotheses*, *62*, 367-374.

Ginsburg, I., & Koren, E. (2008). Are cationic antimicrobial peptides also 'double-edged swords'?. *Expert Review of Anti-infective Therapy*, *6*, 453-462.

Giuliani, A., Pirri, G., & Nicoletto, S. F. (2007). Antimicrobial peptides: an overview of a promising class of therapeutics. *Central European Journal of Biology*, *2*, 1-33.

Gómez-Guillén, M. C., Turnay, J., Fernández-Dıaz, M. D., Ulmo, N., Lizarbe, M. A., & Montero, P. (2002). Structural and physical properties of gelatin extracted from different marine species: a comparative study. *Food Hydrocolloids*, *16*, 25-34.

Gopal, R., Park, J. S., Seo, C. H., & Park, Y. (2012). Applications of circular dichroism for structural analysis of gelatin and antimicrobial peptides. *International Journal of Molecular Sciences*, *13*, 3229-3244.

Guaní-Guerra, E., Santos-Mendoza, T., Lugo-Reyes, S. O., & Terán, L. M. (2010). Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clinical Immunology*, *135*, 1-11.

Haddad, I. Y., Pataki, G., Hu, P. I. N. G., Galliani, C., Beckman, J. S., & Matalon, S. (1994). Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. *Journal of Clinical Investigation*, *94*, 2407.

Hancock, R. E. (2001). Cationic peptides: effectors in innate immunity and novel antimicrobials. *The Lancet Infectious Diseases*, *1*, 156-164.

Hancock, R. E., & Sahl, H. G. (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology*, *24*, 1551-1557.

Handcock, R. E. W., Wiegand, I., Hilpert, K (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*, 163-175.

Hancock, R. E., Brown, K. L., & Mookherjee, N. (2006). Host defence peptides from invertebrates–emerging antimicrobial strategies. *Immunobiology*, *211*, 315-322.

Haney, E. F., Hunter, H. N., Matsuzaki, K., & Vogel, H. J. (2009). Solution NMR studies of amphibian antimicrobial peptides: linking structure to function? *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1788*, 1639-1655.

Harris, F., Dennison, S. R., & Phoenix, D. A. (2009). Anionic antimicrobial peptides from eukaryotic organisms. *Current Protein and Peptide Science*, *10*, 585-606.

Hartman, R., & Meisel, H. (2007). Food-derived peptides with biological activity: from research to food applications. *Current Opinion in Biotechnology*, *18*, 163-169.

Hawrani, A., Howe, R. A., Walsh, T. R., & Dempsey, C. E. (2008). Origin of low mammalian cell toxicity in a class of highly active antimicrobial amphipathic helical peptides. *Journal of Biological Chemistry*, *283*, 18636-18645.

Hipler, U. C., Wollina, U., Denning, D., & Hipler, B. (2000). Fluorescence analysis of reactive oxygen species (ROS) generated by six isolates of *Aspergillus fumigatus*. *Bioluminescence and Chemiluminescence*. 407-414.

Hirsch, T., Spielmann, M., Zuhaili, B., Fossum, M., Metzig, M., Koehler, T., Steinau, H., Yao, F., Onderdonk, A. B., Steinstraesser, L. & Eriksson, E. (2009). Human β-defensin-3 promotes wound healing in infected diabetic wounds. *The Journal of Gene Medicine*, *11*, 220-228.

Hoffmann, J. A., Reichhart, J. M., & Hetru, C. (1996). Innate immunity in higher insects. *Current Opinion in Immunology*, *8*, 8-13.

Hoffmann, J. A., & Reichhart, J. M. (2002). *Drosophila* innate immunity: an evolutionary perspective. *Nature Immunology*, *3*, 121-126.

Hoogstraal, H. (1985). Argasid and nuttalliellid ticks as parasites and vectors. *Advances in Parasitology*, *24*, 135-238.

Hoover, D. M., Wu, Z., Tucker, K., Lu, W., & Lubkowski, J. (2003). Antimicrobial characterization of human β-defensin 3 derivatives. *Antimicrobial Agents and Chemotherapy*, *47*, 2804-2809.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, *53*, 1841-1856.

Huang, L. C., Reins, R. Y., Gallo, R. L., & McDermott, A. M. (2007). Cathelicidin-deficient (Cnlp−/−) mice show increased susceptibility to *Pseudomonas aeruginosa* keratitis. *Investigative Ophthalmology & Visual Science*, *48*, 4498-4508.

Huang, G. J., Deng, J. S., Chen, H. J., Huang, S. S., Liao, J. C., Hou, W. C., & Lin, Y. H. (2012). Defensin protein from sweet potato *Ipomoea batatas* [L.] Lam.'Tainong 57') storage roots exhibits antioxidant activities *in vitro* and *ex vivo*. *Food Chemistry*, *56*, 2989-2995.

Hultmark, D., Steiner, H., Rasmuson, T., & Boman, H. G. (1980). Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. *European Journal of Biochemistry*, *106*, 7-16.

Hultmark, D. (1993). Immune reactions in *Drosophila* and other insects: a model for innate immunity. *Trends in Genetics*, *9*, 178-183.

Hutcheson, I. R., Whittle, B. J., & Boughton-Smith, N. K. (1990). Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. *British Journal of Pharmacology*, *101*, 815-820.

Huther, A., & Dietrich, U. (2007). The emergence of peptides as therapeutic drugs for the inhibition of HIV-1. *AIDS Review*, *9*, 208-17.

Hwang, P. M., & Vogel, H. J. (1998). Structure-function relationships of antimicrobial peptides. *Biochemistry and Cell Biology*, *76*, 235-246.

Hynes, W. L., Ceraul, S. M., Todd, S. M., Seguin, K. C., & Sonenshine, D. E. (2005). A defensin-like gene expressed in the black-legged tick, *Ixodes scapularis*. *Medical and Veterinary Entomology*, *19*, 339-344.

Ichinose, F., Hataishi, R., Wu, J. C., Kawai, N., Rodrigues, A. C. T., Mallari, C., Post, C. M. & Zapol, W. M. (2003). A selective inducible NOS dimerization inhibitor prevents systemic, cardiac, and pulmonary hemodynamic dysfunction in endotoxemic mice. *American Journal of Physiology-Heart and Circulatory Physiology*, *285*, 2524-2530.

Isogai, E., Isogai, H., Okumura, K., Hori, H., Tsuruta, H., & Kurebayashi, Y. (2011). Tertiary structure-related activity of tick defensin (persulcatusin) in the taiga tick, *Ixodes persulcatus*. *Experimental and Applied Acarology*, *53*, 71-77.

Je, J. Y., Park, P. J., & Kim, S. K. (2005). Antioxidant activity of a peptide isolated from Alaska pollack *Theragra chalcogramma* frame protein hydrolysate. *Food Research International*, *38*, 45-50.

Je, J. Y., & Kim, S. K. (2012). 7 Chitosan as potential marine nutraceutical. *Advances in Food and Nutrition Research*, *65*, 121.

Johns, R., Sonenshine, D. E., & Hynes, W. L. (2001). Identification of a defensin from the hemolymph of the American dog tick, *Dermacentor variabilis*. *Insect Biochemistry and Molecular Biology*, *31*, 857-865.

Khan, M. Y., Villanueva, G., & Newman, S. A. (1989). On the origin of the positive band in the far-ultraviolet circular dichroic spectrum of fibronectin. *Journal of Biological Chemistry*, *264*, 2139-2142.

Karupiah, G., Xie, Q. W., Buller, R. M., Nathan, C., Duarte, C., & Macmicking, J. D. (1993). Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. *Science*, *261*, 1445-1448.

Kelly, S. M., Jess, T. J., & Price, N. C. (2005). How to study proteins by circular dichroism. *Biochimica et Biophysica Acta-Proteins and Proteomics*, *1751*, 119-139.

Kim, H. W., & Greenburg, A. G. (2002). Nitric oxide scavenging, alone or with nitric oxide synthesis inhibition, modulates vascular hyporeactivity in rats with intraperitoneal sepsis. *Shock*, *17*, 423-426.

Kim, C., & Kaufmann, S. H. (2006). Defensin: a multifunctional molecule lives up to its versatile name. *Trends in Microbiology*, *14*, 428-431.

Klüver, E., Schulz-Maronde, S., Scheid, S., Meyer, B., Forssmann, W. G., & Adermann, K. (2005). Structure-activity relation of human β-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity. *Biochemistry*, *44*, 9804-9816.

Koczulla, R., von Degenfeld, G., Kupatt, C., Krötz, F., Zahler, S., Gloe, T., Issbrucker, K. & Bals, R. (2003). An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *Journal of Clinical Investigation*, *111*, 1665-1672.

Koo, J. C., Lee, B., Young, M. E., Koo, S. C., Cooper, J. A., Baek, D., Lim, C. O., Lee, S. Y., Yun, D. & Cho, M. J. (2004). Pn-AMP1, a plant defense protein, induces actin depolarization in yeasts. *Plant and Cell Physiology*, *45*, 1669-1680.

Kopáček, P., Hajdušek, O., Burešová, V., & Daffre, S. (2010). Tick innate immunity. In *Invertebrate Immunity*, *708*, 137-162

Korhonen, R., Lahti, A., Kankaanranta, H., & Moilanen, E. (2005). Nitric oxide production and signaling in inflammation. *Current Drug Targets-Inflammation & Allergy*, *4*, 471-479.

Kounatidis, I., & Ligoxygakis, P. (2012). *Drosophila* as a model system to unravel the layers of innate immunity to infection. *Open Biology*, *2*, 120075

Kragol, G., Lovas, S., Varadi, G., Condie, B. A., Hoffmann, R., & Otvos, L. (2001). The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. *Biochemistry*, *40*, 3016-3026.

Kumar, M., Chaturvedi, A. K., Kavishwar, A., Shukla, P. K., Kesarwani, A. P., & Kundu, B. (2005). Identification of a novel antifungal nonapeptide generated by combinatorial approach. *International Journal of Antimicrobial Agents*, *25*, 313-320.

Lai, Y., & Gallo, R. L. (2009). AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends in Immunology*, *30*, 131-141.

Lai, R., Lomas, L., Jonczy, J., Turner, P., & Rees, H. (2004). Two novel non-cationic defensin-like antimicrobial peptides from haemolymph of the female tick, *Amblyomma hebraeum*. *Biochemistry Journal*, *379*, 681-685.

Laverty, G., Gorman, S. P., & Gilmore, B. F. (2011). The potential of antimicrobial peptides as biocides. *International Journal of Molecular Sciences*, *12*, 6566-6596.

Lavine, M. D., & Strand, M. R. (2002). Insect hemocytes and their role in immunity. *Insect Biochemistry and Molecular Biology*, *32*, 1295-1309.

Lee, S. O., Kim, S. Y., Han, S. M., Kim, H. M., Ham, S. S., & Kang, I. J. (2006). *Corni fructus* scavenges hydroxy radicals and decreases oxidative stress in endothelial cells. *Journal of Medicinal Food*, *9*, 594-598.

Lee, H. S., Park, C. B., Kim, J. M., Jang, S. A., Park, I. Y., Kim, M. S., Cho, J. H. & Kim, S. C. (2008). Mechanism of anticancer activity of buforin IIb, a histone H2A-derived peptide. *Cancer Letters*, *271*, 47-55.

Leontiadou, H., Mark, A. E., & Marrink, S. J. (2006). Antimicrobial peptides in action. *Journal of the American Chemical Society*, *128*, 12156-12161.

Liang, Q. L., Zhou, K., & He, H. X. (2010). Retrocyclin 2: a new therapy against avian influenza H5N1 virus *in vivo* and *vitro*. *Biotechnology Letters*, *32*, 387-392.

López, A., Lorente, J. A., Steingrub, J., Bakker, J., McLuckie, A., Willatts, S. & Grover, R. (2004). Multiple-center, randomized, placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: effect on survival in patients with septic shock*. *Critical care medicine*, *32*, 21-30.

Lowenberger, C., Bulet, P., Charlet, M., Hetru, C., Hodgeman, B., Christensen, B. M., & Hoffmann, J. A. (1995). Insect immunity: Isolation of three novel inducible antibacterial defensins from the vector mosquito, *Aedes aegypti*. *Insect Biochemistry and Molecular Biology*, *25*, 867-873.

Lundy, F. T., Nelson, J., Lockhart, D., Greer, B., Harriott, P., & Marley, J. J. (2008). Antimicrobial activity of truncated α-defensin (human neutrophil peptide (HNP)-1) analogues without disulphide bridges. *Molecular Immunology*, *45*, 190-193.

Maemoto, A., Qu, X., Rosengren, K. J., Tanabe, H., Henschen-Edman, A., Craik, D. J., & Ouellette, A. J. (2004). Functional analysis of the α-defensin disulfide array in mouse cryptdin-4. *Journal of Biological Chemistry*, *279*, 44188-44196.

Maher, S., & McClean, S. (2006). Investigation of the cytotoxicity of eukaryotic and prokaryotic antimicrobial peptides in intestinal epithelial cells *in vitro*. *Biochemical Pharmacology*, *71*, 1289-1298.

Makovitzki, A., Avrahami, D., & Shai, Y. (2006). Ultrashort antibacterial and antifungal lipopeptides. *Proceedings of the National Academy of Sciences*, *103*, 15997-16002.

Maloy, W. L., & Kari, U. P. (1995). Structure–activity studies on magainins and other host defense peptides. *Biopolymers*, *37*, 105-122.

Marcuse, R. (1960). Antioxidative effect of amino-acids. *Nature, 186*, 886 - 887

Maritz-Olivier, C., Stutzer, C., Jongejan, F., Neitz, A. W., & Gaspar, A. R. (2007). Tick anti-hemostatics: targets for future vaccines and therapeutics. *Trends in Parasitology*, *23*, 397-407.

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

Matsuzaki, K. (1999). Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochimica et Biophysica Acta - Biomembranes*, *1462*, 10.

Matsuzaki, K. (2009). Control of cell selectivity of antimicrobial peptides. *Biochimica et Biophysica Acta-Biomembranes*, *1788*, 1687-1692.

Matsuyama, K., & Natori, S. (1988). Molecular cloning of cDNA for sapecin and unique expression of the sapecin gene during the development of *Sarcophaga peregrina*. *Journal of Biological Chemistry*, *263*, 17117-17121.

Matsuyama, K., & Natori, S. (1988). Purification of three antibacterial proteins from the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina*. *Journal of Biological Chemistry*, *263*, 17112-17116.

Medzhitov, R., & Janeway, C. (1997). Innate immunity: Minireview the virtues of a nonclonal system of recognition. *Cell*, *91*, 295-298.

Memarpoor-Yazdi, M., Asoodeh, A., & Chamani, J. (2012). A novel antioxidant and antimicrobial peptide from hen egg white lysozyme hydrolysates. *Journal of Functional Foods*, *4*, 278-286.

Metz-Boutigue, M. H., Shooshtarizadeh, P., Prevost, G., Haikel, Y., & Chich, J. F. (2010). Antimicrobial peptides present in mammalian skin and gut are multifunctional defence molecules. *Current pharmaceutical design*, *16*, 1024-1039.

McDermott, A. M., Bioactive peptides (2009), Howl, J., Jones, S. (eds). CRC Press, Boca Raton, Florida, 357-401.

McGwire, B. S., Olson, C. L., Tack, B. F., & Engman, D. M. (2003). Killing of African trypanosomes by antimicrobial peptides. *Journal of Infectious Diseases*, *188*, 146-152.

McInturff, J. E., Wang, S. J., Machleidt, T., Lin, T. R., Oren, A., Hertz, C. J., & Kim, J. (2005). Granulysin-derived peptides demonstrate antimicrobial and anti-inflammatory effects against *Propionibacterium acnes*. *Journal of Investigative Dermatology*, *125*, 256-263.

Miller, N. J., Rice-Evans, C., Davies, M. J., Gopinathan, V., & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical science*, *84*, 407-407.

Miteva, M., Andersson, M., Karshikoff, A., & Otting, G. (1999). Molecular electroporation: a unifying concept for the description of membrane pore formation by antibacterial peptides, exemplified with NK-lysin. *Federation of European Biochemical Societies Letters*, *462*, 155-158.

Mookherjee, N., Brown, K. L., Bowdish, D. M., Doria, S., Falsafi, R., Hokamp, K., Roche, F. M. & Hancock, R. E. (2006). Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. *The Journal of Immunology*, *176*, 2455-2464.

Mookherjee, N., & Hancock, R. E. W. (2007). Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. *Cellular and Molecular Life Sciences*, *64*, 922-933.

Moon, D. O., Park, S. Y., Lee, K. J., Heo, M. S., Kim, K. C., Kim, M. O., ... & Kim, G. Y. (2007). Bee venom and melittin reduce proinflammatory mediators in lipopolysaccharide-stimulated BV2 microglia. *International Immunopharmacology*, *7*, 1092-1101.

Moser, C., Weiner, D. J., Lysenko, E., Bals, R., Weiser, J. N., & Wilson, J. M. (2002). β-Defensin 1 contributes to pulmonary innate immunity in mice. *Infection and Immunity*, *70*, 3068-3072.

Murphy, C. J., Foster, B. A., Mannis, M. J., Selsted, M. E., & Reid, T. W. (1993). Defensins are mitogenic for epithelial cells and fibroblasts. *Journal of Cellular Physiology*, *155*, 408-413.

Naik, D. G., & Vaidya, H. S. (2011). Antioxidant properties of volatile oil of Indian propolis. *Journal of ApiProduct and ApiMedical Science, 3*, 89-93.

Najafian, L., & Babji, A. S. (2012). A review of fish-derived antioxidant and antimicrobial peptides: their production, assessment, and applications. *Peptides*, *33*, 178-185.

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

Nakajima, Y., Taylor, D., & Yamakawa, M. (2002). Involvement of antibacterial peptide defensin in tick midgut defense. *Experimental & Applied Acarology*, *28*, 135-140.

Nakajima, Y., 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-General Subjects*, *1624*, 125-130.

Nakajima, Y., Saido-Sakanaka, H., Ogihara, K., Taylor, D., & Yamakawa, M. (2005). Antibacterial peptides are secreted into the midgut lumen to provide antibacterial midgut defense in the soft tick, *Ornithodoros moubata* (*Acari Argasidae*). *Applied Entomology and Zoology*, *40*, 391-397.

Nappi, A. J., Vass, E., Frey, F., & Carton, Y. (1995). Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. *European Journal of Cell Biology*, *68*, 450-456.

Nguyen, L. T., Haney, E. F., & Vogel, H. J. (2011). The expanding scope of antimicrobial peptide structures and their modes of action. *Trends in Biotechnology*, *29*, 464-472.

Nijnik, A., Madera, L., Ma, S., Waldbrook, M., Elliott, M. R., Easton, D. M., Mayer, M. L., Mullaly, S. C., Kindrachuk, J., Jenssen, H. & Hancock, R. E. (2010). Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *The Journal of Immunology*, *184*, 2539-2550.

Nishimura, M., Abiko, Y., Kurashige, Y., Takeshima, M., Yamazaki, M., Kusano, K., Saitoh, M., Nakashima, K., Inoue, I. & Kaku, T. (2004). Effect of defensin peptides on eukaryotic cells: primary epithelial cells, fibroblasts and squamous cell carcinoma cell lines. *Journal of Dermatological Science*, *36*, 87-95.

Niyonsaba, F., Ushio, H., Nakano, N., Ng, W., Sayama, K., Hashimoto, K. & Ogawa, H. (2006). Antimicrobial peptides human β-defensins stimulate epidermal keratinocyte migration, proliferation and production of proinflammatory cytokines and chemokines. *Journal of Investigative Dermatology*, *127*, 594-604.

Nuttall, P. A. (1998). Displaced tick-parasite interactions at the host interface. *Parasitology*, *116*, 65-72.

Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J. A., & Deemer, E. K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *Journal of Agricultural and Food Chemistry*, *50*, 3122-3128.

Pacher, P., Beckman, J. S., & Liaudet, L. (2007). Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews*, *87*, 315-424.

Park, P. J., Jung, W. K., Nam, K. S., Shahidi, F., & Kim, S. K. (2001). Purification and characterization of antioxidative peptides from protein hydrolysate of lecithin-free egg yolk. *Journal of the American Oil Chemists' Society*, *78*, 651-656.

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

Petros, A., Lamb, G., Leone, A., Moncada, S., Bennett, D., & Vallance, P. (1994). Effects of a nitric oxide synthase inhibitor in humans with septic shock. *Cardiovascular research*, *28*, 34-39.

Pkorny, A., & Almeida, P. F. F. (2004). Kinetics of dye efflux and lipid flip-flop induced by delta-lysin in phosphatidylcholine vesicle and the mechanism of graded release by amphipathic, β-helical peptides. *Biochemistry*, *43*, 8846-8857.

Pompella, A., Visvikis, A., Paolicchi, A., Tata, V. D., & Casini, A. F. (2003). The changing faces of glutathione, a cellular protagonist. *Biochemical Pharmacology*, *66*, 1499-1503.

Prinsloo, L., Naidoo, A., Serem, J., Taute, H., Sayed, Y., Bester, M., Neitz, A. & Gaspar, A. (2013). Structural and functional characterization of peptides derived from the carboxy-terminal region of a defensin from the tick *Ornithodoros savignyi*. *Journal of Peptide Science*, *19*, 325–332.

Pütsep, K., Carlsson, G., Boman, H. G., & Andersson, M. (2002). Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *The Lancet*, *360*, 1144-1149.

Raghuraman, H., & Chattopadhyay, A. (2007). Melittin: a membrane-active peptide with diverse functions. *Bioscience Reports*, *27*, 189-223.

Rahman, M., Tsuji, N., Boldbaatar, D., Battur, B., Liao, M., Umemiya-Shirafuji, R., You, M., Tanaka, T. & Fujisaki, K. (2010). Structural characterization and cytolytic activity of a potent antimicrobial motif in longicin, a defensin-like peptide in the tick *Haemaphysalis longicornis*. *The Journal of veterinary medical science/the Japanese Society of Veterinary Science*, *72*, 149.

Ratcliffe, N. A., & Rowley, A. F. (1987). Insect responses to parasites and other pathogens. *Immune Responses in Parasitic Infections*, *4*, 271-332.

Rajapakse, N., Mendis, E., Byun, H. G., & Kim, S. K. (2005). Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *The Journal of Nutritional Biochemistry*, *16*, 562-569.

Ramamoorthy, A., Thennarasu, S., Tan, A., Gottipati, K., Sreekumar, S., Heyl, D. L., An, F. Y. P., & Shelburne, C. E. (2006). Deletion of all cysteines in tachyplesin I abolishes hemolytic activity and retains antimicrobial activity and lipopolysaccharide selective binding. *Biochemistry*, *45*, 6529-6540.

Ribeiro, J. C., Weis, J. J., & Telford, S. R. (1990). Saliva of the tick *Ixodes dammini* inhibits neutrophil function. *Experimental Parasitology*, *70*, 382-388.

Ribeiro, J. M., & Francischetti, I. M. (2003). Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annual Review of Entomology*, *48*, 73-88.

Roccatano, D., Colombo, G., Fioroni, M., & Mark, A. E. (2002). Mechanism by which 2, 2, 2-trifluoroethanol/water mixtures stabilize secondary-structure formation in peptides: a molecular dynamics study. *Proceedings of the National Academy of Sciences*, *99*, 12179-12184.

Royet, J. (2004). *Drosophila* melanogaster innate immunity: an emerging role for peptidoglycan recognition proteins in bacteria detection. *Cellular and Molecular Life Sciences CMLS*, *61*, 537-546.

Saberwal, G., & Nagaraj, R. (1994). Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: facets of their conformational features, structure-function correlations and membrane-perturbing abilities. *Biochimica et Biophysica Acta*, *1197*, 109.

Saito, Y., Konnai, S., Yamada, S., Imamura, S., Nishikado, H., Ito, T., Onuma, M. and Ohashi, K. (2009). Identification and characterization of antimicrobial peptide, defensin, in the taiga tick, Ixodes persulcatus. *Insect molecular biology*, *18*, 531-539.

Salvatore, M., García-Sastre, A., Ruchala, P., Lehrer, R. I., Chang, T., & Klotman, M. E. (2007). β-Defensin inhibits influenza virus replication by cell-mediated mechanism. *Journal of Infectious Diseases*, *196*, 835-843.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., & Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology*, *21*, 1-26.

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides*, *31*, 1949-1956.

Sato, H., & Feix, J. B. (2006). Peptide–membrane interactions and mechanisms of membrane destruction by amphipathic α-helical antimicrobial peptides. *Biochimica et Biophysica Acta - Biomembranes*, *1758*, 1245-1256.

Selsted, M. E., Harwig, S. S., Ganz, T., Schilling, J. W., & Lehrer, R. I. (1985). Primary structures of three human neutrophil defensins. *Journal of Clinical Investigation*, *76*, 1436.

Schmidtchen, A., Ringstad, L., Kasetty, G., Mizuno, H., Rutland, M. W., & Malmsten, M. (2011). Membrane selectivity by W-tagging of antimicrobial peptides. *Biochimica et Biophysica Acta-Biomembranes*, *1808*, 1081-1091.

Schroeder, B. O., Wu, Z., Nuding, S., Groscurth, S., Marcinowski, M., Beisner, J., Buchner, J., Schaller, M., Stange, E. F. & Wehkamp, J. (2011). Reduction of disulphide bonds unmasks potent antimicrobial activity of human [bgr]-defensin 1. *Nature*, *469*, 19-423.

Scott, M. G., & Hancock, R. E. (2000). Cationic antimicrobial peptides and their multifunctional role in the immune system. *Critical Reviews in Immunology*, *20*. 407-431

Serem, J. C., & Bester, M. J. (2012). Physicochemical properties, antioxidant activity and cellular protective effects of honeys from Southern Africa. *Food Chemistry*, *133*, 1544-1550.

Shah, N. S., & Billiar, T. R. (1998). Role of nitric oxide in inflammation and tissue injury during endotoxemia and hemorrhagic shock. *Environmental Health Perspectives*, *106*, 1139-1143.

Shah, N. P. (2000). Effects of milk-derived bioactives: an overview. *British Journal of Nutrition*, *84*, 3-10.

Shai, Y., & Oren, Z. (2001). From “carpet” mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides*, *22*, 1629-1641.

Shai, Y. (1995). Molecular recognition between membrane-spanning polypeptides. *Trends in biochemical sciences*, *20*, 460-464.

Sivaperumal, P., Kamala, K., Natarajan, E., & Dilipan, E. (2013). Antimicrobial peptide from crab haemolymph of *Ocypoda macrocera* (Milne Edwards 1852) with reference to an antioxidant: A case study. *International Journal of Pharmacy and Pharmaceutical Sciences*, *5*, 719-727.

Söderhäll, K., & Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology*, *10*, 23-28.

Sørensen, O. E., Cowland, J. B., Theilgaard-Mönch, K., Liu, L., Ganz, T., & Borregaard, N. (2003). Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *The Journal of Immunology*, *170*, 5583-5589.

Sonenshine, D. E. (1993). *Biology of ticks. Volume 2*. Oxford University Press.

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

Spain, D. A., Wilson, M. A., & Garrison, R. N. (1994). Nitric oxide synthase inhibition exacerbates sepsis-induced renal hypoperfusion. *Surgery*, *116*, 322-330.

Srivastava, R. M., Srivastava, S., Singh, M., Bajpai, V. K., & Ghosh, J. K. (2012). Consequences of alteration in leucine zipper sequence of melittin in its neutralization of lipopolysaccharide-induced proinflammatory response in macrophage cells and interaction with lipopolysaccharide. *Journal of Biological Chemistry*, *287*, 1980-1995.

St Georgiev, V. S., Western, K. A., & McGowan, J. J. (2008). *National Institute of Allergy and Infectious Diseases, NIH* (Vol. 2). Springer.

Steiner, H. (2004). Peptidoglycan recognition proteins: on and off switches for innate immunity. *Immunological Reviews*, *198*, 83-96.

Steinstraesser, L., Kraneburg, U. M., Hirsch, T., Kesting, M., Steinau, H. U., Jacobsen, F., & Al-Benna, S. (2009). Host defense peptides as effector molecules of the innate immune response: a sledgehammer for drug resistance?. *International Journal of Molecular Sciences*, *10*, 3951-3970.

Stuehr, D. J., & Marletta, M. A. (1985). Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proceedings of the National Academy of Sciences*, *82*, 7738-7742.

Subbalakshmi, C., & Sitaram, N. (1998). Mechanism of antimicrobial action of indolicidin. *Federation of European Microbiological Studies Microbiology Letters*, *160*, 91-96.

Sun, P. D., & Boyington, J. C. (2001). Overview of protein folds in the immune system. *Current Protocols in Immunology*, Appendix-1N, 44-45

Sun, J., Zhang, X., Broderick, M., & Fein, H. (2003). Measurement of nitric oxide production in biological systems by using Griess reaction assay. *Sensors*, 3, 276-284.

Taha, F. S., Mohamed, S. S., Wagdy, S. M., & Mohamed, G. F. (2013). Antioxidant and antimicrobial activities of enzymatic hydrolysis products from sunflower protein isolate. *World Applied Sciences Journal*, *21*, 651-658.

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

Tosteson, M. T., Holmes, S. J., Razin, M., & Tosteson, D. C. (1985). Melittin lysis of red cells. *The Journal of Membrane Biology*, *87*, 35-44.

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

Tzou, P., De Gregorio, E., & Lemaitre, B. (2002). How *Drosophila* combats microbial infection: a model to study innate immunity and host–pathogen interactions. *Current Opinion in Microbiology*, *5*, 102-110.

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.

Varkey, J., & Nagaraj, R. (2005). Antibacterial activity of human neutrophil defensin HNP-1 analogs without cysteines. *Antimicrobial Agents and Chemotherapy*, *49*, 4561-4566.

van der Does, A. M., Beekhuizen, H., Ravensbergen, B., Vos, T., Ottenhoff, T. H., van Dissel, J. T., Drijfhout, J. W., Hiemstra, P. S., & Nibbering, P. H. (2010). LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. *The Journal of Immunology*, *185*, 1442-1449.

van der Does, A. M., Bogaards, S. J., Ravensbergen, B., Beekhuizen, H., van Dissel, J. T., & Nibbering, P. H. (2010). Antimicrobial peptide hLF1-11 directs granulocyte-macrophage colony-stimulating factor-driven monocyte differentiation toward macrophages with enhanced recognition and clearance of pathogens. *Antimicrobial Agents and Chemotherapy*, *54*, 811-816.

van der Goes van Naters-Yasui, A., Taylor, D., Shono, T., & Yamakawa, M. (2000). Purification and partial amino acid sequence of antibacterial peptides from the hemolymph of the soft tick, *Ornithodoros moubata*, (Acari: Argasidae). In M. Kazimirova, M. Labuda, & P. A. Nutall (Eds.), *Proceedings of the 3rd International Conference Ticks and Tick-Borne Pathogens: Into the 21st Century,* 189-194.

Vilmos, P., & Kurucz, É. (1998). Insect immunity: evolutionary roots of the mammalian innate immune system. *Immunology Letters*, *62*, 59-66.

Viljakainen, L., & Pamilo, P. (2005). Identification and molecular characterization of defensin gene from the ant *Formica aquilonia*. *Insect Molecular Biology*, *14,* 335-338.

Vizioli, J., & Salzet, M. (2002). Antimicrobial peptides from animals: focus on invertebrates. *Trends in Pharmacological Sciences*, *23*, 494-496.

Vriesman, M. F., Haenen, G. R., Westerveld, G. J., Paquay, J. B., Voss, H. P., & Bast, A. (1997). A method for measuring nitric oxide radical scavenging activity. Scavenging properties of sulfur–containing compounds. *Pharmacy World and Science*, *19*, 283-286.

Walsh, E. G., Maher, S., Devocelle, M., O'Brien, P. J., Baird, A. W., & Brayden, D. J. (2011). High content analysis to determine cytotoxicity of the antimicrobial peptide, melittin and selected structural analogs. *Peptides*, *32*, 1764-1773.

Wang, G., Li, X., & Wang, Z. (2009). APD2: the updated antimicrobial peptide database and its application in peptide design. *Nucleic Acids Research*, *37*, 933-937.

Waring, A. J., Faull, K. F., Leung, C., Chang-Chien, A., Mercado, P., Taeusch, H. W., & Gordon, L. M. (1995). Synthesis, secondary structure and folding of the bend region of lung surfactant protein B. *Peptide Research*, *9*, 28-39.

Welling, M. M., Paulusma-Annema, A., Balter, H. S., Pauwels, E. K., & Nibbering, P. H. (2000). Technetium-99m labelled antimicrobial peptides discriminate between bacterial infections and sterile inflammations. *European Journal of Nuclear Medicine*, *27*, 292-301.

Welling, M. M., Lupetti, A., Balter, H. S., Lanzzeri, S., Souto, B., Rey, A. M., Savio, E. O., Paulusma-Annema, A., Pauwels, E. K. J. & Nibbering, P. H. (2001). 99mTc-labeled antimicrobial peptides for detection of bacterial and *Candida albicans* infections. *Journal of Nuclear Medicine*, *42*, 788-794.

Wellner, V. P., Anderson, M. E., Puri, R. N., Jensen, G. L., & Meister, A. (1984). Radioprotection by glutathione ester: transport of glutathione ester into human lymphoid cells and fibroblasts. *Proceedings of the National Academy of Sciences*, *81*, 4732-4735.

Wieprecht, T., Dathe, M., Schümann, M., Krause, E., Beyermann, M., & Bienert, M. (1996). Conformational and functional study of magainin 2 in model membrane environments using the new approach of systematic double-D-amino acid replacement. *Biochemistry*, *35*, 10844-10853.

Wieprecht, T., Dathe, M., Beyermann, M., Krause, E., Maloy, W. L., MacDonald, D. L., & Bienert, M. (1997). Peptide hydrophobicity controls the activity and selectivity of magainin 2 amide in interaction with membranes. *Biochemistry*, *36*, 6124-6132.

Wiesner, J., & Vilcinskas, A. (2010). Antimicrobial peptides: the ancient arm of the human immune system. *Virulence*, *1*, 440-464.

Wink, D. A., Darbyshire, J. F., Nims, R. W., Saavedra, J. E., & Ford, P. C. (1993). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: Determination of the kinetics for oxidation and nitrosation by intermediates generated in the nitric oxide/oxygen reaction. *Chemical research in toxicology*, *6*, 23-27.

Wolfe, T. A., & Dasta, J. F. (1995). Use of nitric oxide synthase inhibitors as a novel treatment for septic shock. *The Annals of pharmacotherapy*, *29*, 36-46.

Wong, J. H., Zhang, X. Q., Wang, H. X., & Ng, T. B. (2006). A mitogenic defensin from white cloud beans (*Phaseolus vulgaris*). *Peptides*, *27*, 2075-2081.

Wu, Z., Hoover, D. M., Yang, D., Boulègue, C., Santamaria, F., Oppenheim, J. J., Lubkowski, J., & Lu, W. (2003). Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human β-defensin 3. *Proceedings of the National Academy of Sciences*, *100*, 8880-8885.

Yang, H., Wang, X., Liu, X., Wu, J., Liu, C., Gong, W., Zhao, Z., & Lai, R. (2009). Antioxidant peptidomics reveals novel skin antioxidant system. *Molecular & Cellular Proteomics*, *8*, 571-583.

Yang, R., Zhang, Z., Pei, X., Han, X., Wang, J., Wang, L., Long, Z., Shen, X. & Li, Y. (2009). Immunomodulatory effects of marine oligopeptide preparation from Chum Salmon (*Oncorhynchus keta*) in mice. *Food Chemistry*, *113*, 464-470.

Yeaman, M. R., & Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacological Reviews*, *55*, 27-55.

Yeung, A. T., Gellatly, S. L., & Hancock, R. E. (2011). Multifunctional cationic host defence peptides and their clinical applications. *Cellular and Molecular Life Sciences*, *68*, 2161-2176.

Zambrowicz, A., Pokora, M., Eckert, E., Szołtysik, M., Dąbrowska, A., Chrzanowska, J., & Trziszka, T. (2012). Antioxidant and antimicrobial activity of lecithin free egg yolk protein preparation hydrolysates obtained with digestive enzymes. *Functional Foods in Health and Disease*, 2, 487-500.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, *415*, 389-395.

Zelezetsky, I., Pag, U., Sahl, H. G., & Tossi, A. (2005). Tuning the biological properties of amphipathic α-helical antimicrobial peptides: Rational use of minimal amino acid substitutions. *Peptides*, *26*, 2368-2376.

Zelezetsky, I., & Tossi, A. (2006). α-helical antimicrobial peptides—Using a sequence template to guide structure–activity relationship studies. *Biochimica et Biophysica Acta-Biomembranes*, *1758*, 1436-1449.

Zhang, K., Lu, Q., Zhang, Q., & Hu, X. (2004). Regulation of activities of NK cells and CD4 expression in T cells by human HNP-1,-2, and-3. *Biochemical and Biophysical Research Communications*, *323*, 437-444.

Zhang, L. T., Yao, Y. M., Lu, J. Q., Yan, X. J., Yu, Y., & Sheng, Z. Y. (2008). Recombinant bactericidal/permeability-increasing protein inhibits endotoxin-induced high-mobility group box 1 protein gene expression in sepsis. *Shock*, *29*, 278-284.

Zheng, L., Su, G., Ren, J., Gu, L., You, L., & Zhao, M. (2012). Isolation and characterization of an oxygen radical absorbance activity peptide from defatted peanut meal hydrolysate and its antioxidant properties. *Journal of agricultural and food chemistry*, *60*, 5431-5437.

Appendix

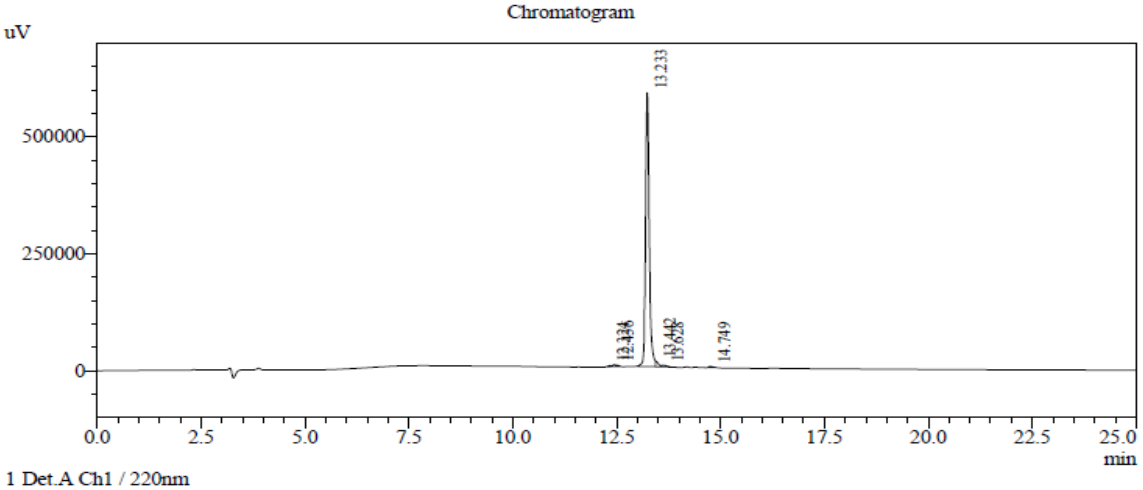
Sample Name: PAdeltaC
Sample ID : 148607-4

Pump A : 0.065% trifluoroacetic in 100% water (v/v)
Pump B : 0.05% trifluoroacetic in 100% acetonitrile (v/v)
Total Flow : 1 ml / min
Wavelength : 220 nm

Time	Unit	Command	Value	Comment
0.01	Pumps	Pump B Conc.	5	
25.00	Pumps	Pump B Conc.	65	
25.01	Pumps	Pump B Conc.	95	
31.00	Pumps	Pump B Conc.	95	
31.01	Pumps	Pump B Conc.	5	
40.00	Pumps	Pump B Conc.	5	
40.01	Controller	Stop		

<<Column Performance>>
<Detector A>
Column : AlltimaTM C18 4.6 x 250 mm

Chromatogram



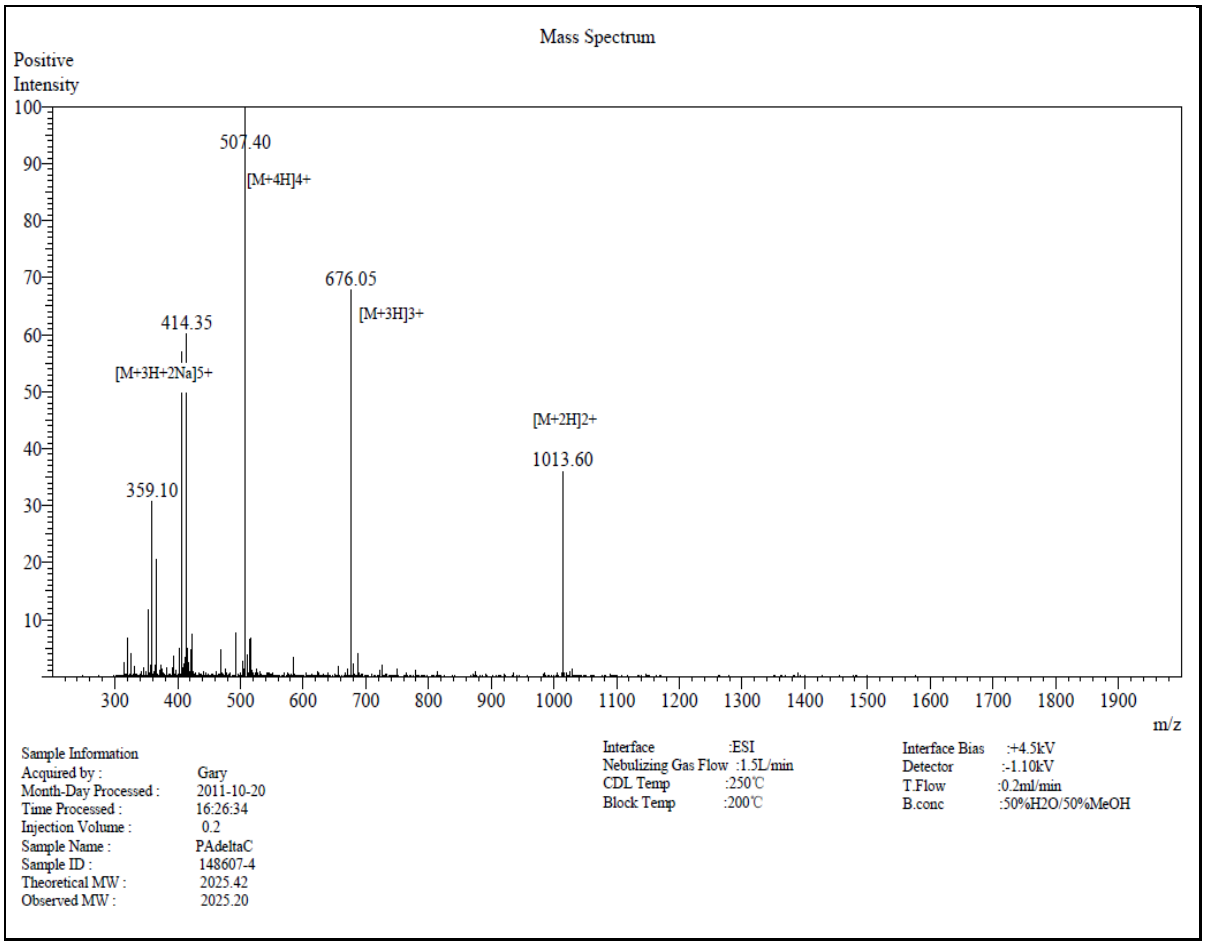
1 Det.A Ch1 / 220nm

PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	12.324	9554	2533	0.253
2	12.436	38835	4251	1.028
3	13.233	3626184	584715	95.958
4	13.442	60521	12135	1.602
5	13.628	28353	3395	0.750
6	14.749	15464	2635	0.409
Total		3778910	609663	100.000

HPLC analysis of PaΔC



Mass spectrum analysis of PaΔC

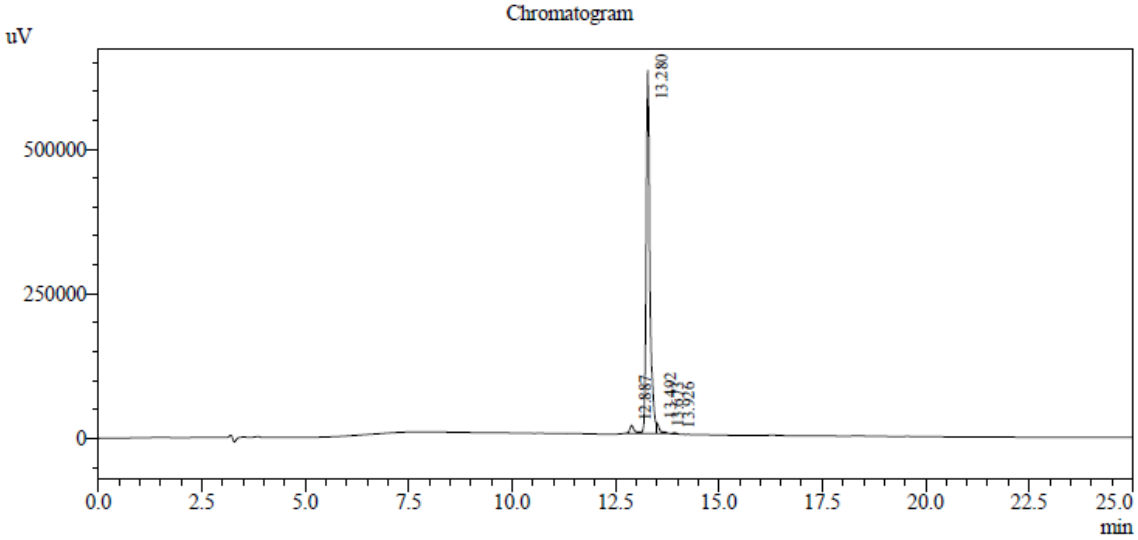
Sample Name: OSdeltaC
Sample ID : 148607-5

Pump A : 0.065% trifluoroacetic in 100% water (v/v)
Pump B : 0.05% trifluoroacetic in 100% acetonitrile (v/v)
Total Flow : 1 ml / min
Wavelength : 220 nm

Time	Unit	Command	Value	Comment
0.01	Pumps	Pump B Conc.	5	
25.00	Pumps	Pump B Conc.	65	
25.01	Pumps	Pump B Conc.	95	
31.00	Pumps	Pump B Conc.	95	
31.01	Pumps	Pump B Conc.	5	
40.00	Pumps	Pump B Conc.	5	
40.01	Controller	Stop		

<<Column Performance>>
<Detector A>
Column : AlltimaTM C18 4.6 x 250 mm

Chromatogram



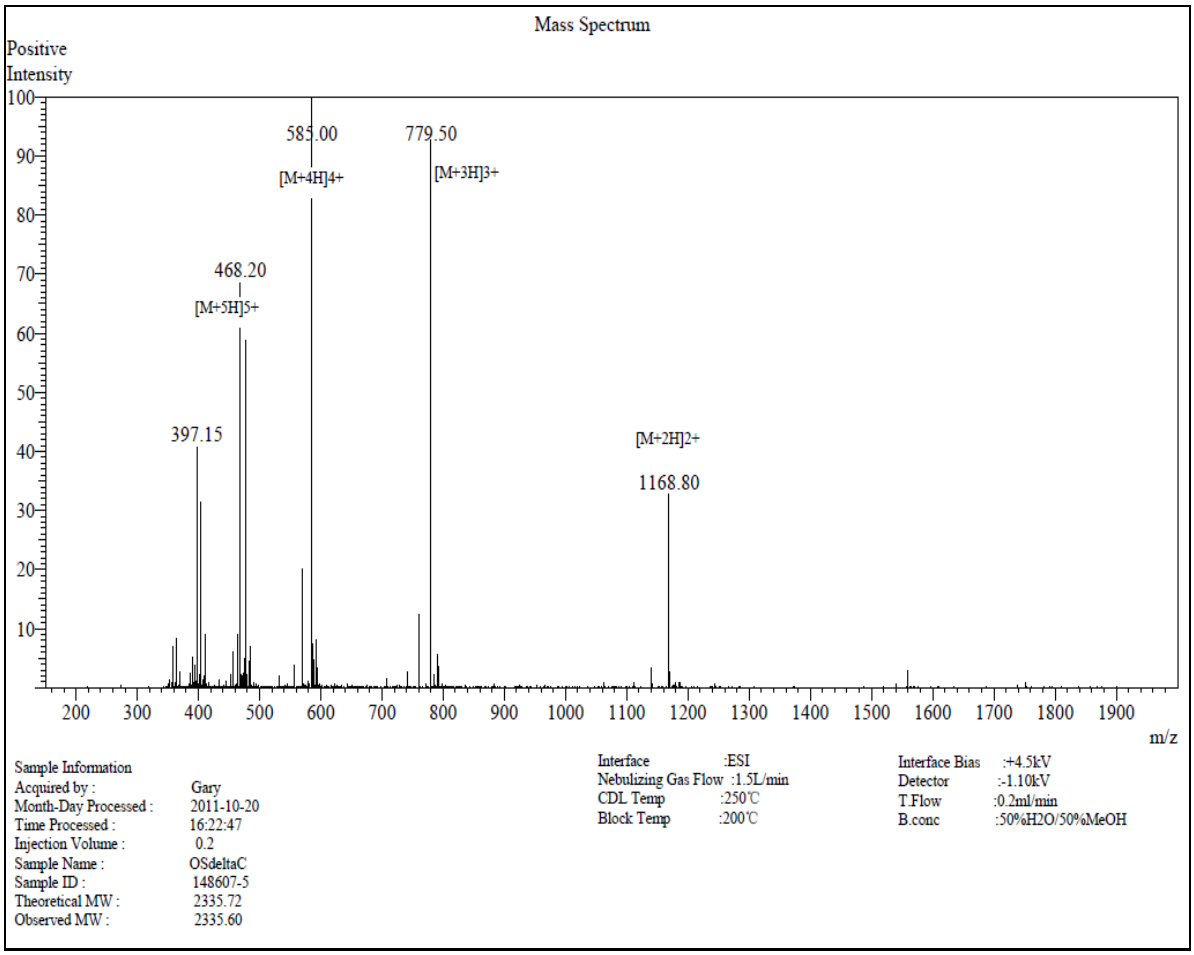
1 Det.A Ch1 / 220nm

PeakTable

Detector A Ch1 220nm

Peak#	Ret. Time	Area	Height	Area %
1	12.887	105886	14074	2.536
2	13.280	3970824	628076	95.101
3	13.492	69766	18372	1.671
4	13.673	18108	2719	0.434
5	13.926	10800	2260	0.259
Total		4175384	665501	100.000

HPLC analysis of OsΔC



Mass Spectrum analysis of OsΔC