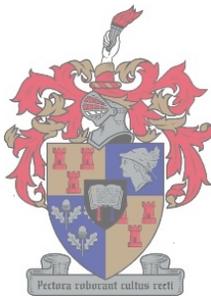


*The development of an antibacterial assay and its use in the  
investigation of the combined effect of tetracycline and  
synthetic antimicrobial peptides on strains of*  
**Escherichia coli**

by

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## **Declaration**

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

## Summary

Magainin 2 is a 23-residue antimicrobial peptide secreted on the skin of *Xenopus laevis*. In our study on antimicrobial peptides, this cationic,  $\alpha$ -helical peptide and two N-terminal deletion analogues were synthesised. The first of the deletion peptides differed from the full length peptide by the omission of two amino acid residues from the N-terminus of magainin 2, while the second deletion peptide had four residues omitted from the N-terminus (termed magainin 2 N<sup>21</sup>, and magainin 2 N<sup>19</sup> respectively). Peptides were purified by gel permeation chromatography and high performance liquid chromatography (HPLC). The synthetic peptides were of high chemical purity as verified with electrospray ionisation mass spectrometry (ESI-MS) and analytical HPLC.

To further evaluate the antimicrobial activity of the peptides in this study, a highly sensitive micro-gel well diffusion assay was developed. This assay was compared to a radial diffusion assay and a microtiter broth dilution method, using gramicidin S as model antibiotic, and *Micrococcus luteus* as the indicator organism. The micro-gel well diffusion assay was as sensitive as the microtiter broth dilution method, and approximately twice as sensitive as the radial diffusion method. Data analysis to calculate minimum inhibitory concentration (MIC), 50% microbial growth inhibition (IC<sub>50</sub>), and maximum bactericidal concentration (MBC) was refined by generating dose-response curves with Prism<sup>®</sup> 2.01 (Graphpad Software Inc.). The MICs, determined by the three methods, were significantly different (P<0.001), highlighting the limitations involved in comparing data obtained from different methods.

Furthermore, the synergistic antimicrobial activity of magainin 2 and tetracycline on tetracycline resistant *Escherichia coli* was investigated. The two N-terminal deletion analogues were also used in the synergism studies. The resistance towards tetracycline is caused by expulsion from resistant cells through efflux pumps. Magainin 2 was selected as antimicrobial peptide because of its ability to form channels in the target membrane, which may help in the circumvention of this form of resistance. All tests were done using the micro-gel well diffusion assay, with the test organism being tetracycline resistant *E. coli*. Magainin 2, at MIC and IC<sub>50</sub> concentrations, improved the activity of tetracycline by  $\pm 12\%$  and  $\pm 29\%$  respectively. Magainin 2 N<sup>21</sup> improved the activity of tetracycline by  $\pm 19\%$  at its IC<sub>50</sub>, and showed no effect at its MIC. Magainin 2 N<sup>19</sup> exhibited no antimicrobial activity on its own,

but still succeeded in improving the activity of tetracycline by approximately  $\pm 9\%$  when used at 138 nmol/mL concentration. In all three cases, this improvement was only observed at tetracycline concentrations below or near its MIC. At higher tetracycline concentrations the improvement of activity, attributed to magainin 2, was not detected. The loss in synergism could be the consequence of competition between tetracycline and the peptide for the magnesium binding sites in the lipopolysaccharide layer of the gram-negative organism. Intensive research is necessary to alleviate the problem of resistance, and our research indicates that the combination of conventional antibiotics and antimicrobial peptides in antibiotic preparations may be a viable option.

## Opsomming

Magainin 2 is 'n 23-residue peptied wat op die vel van *Xenopus laevis* uitgeskei word. In ons studies, wat handel oor antimikrobiëse peptiede, is hierdie kationiese,  $\alpha$ -heliese peptied, sowel as twee N-terminaal delesie analoë gesintetiseer. Twee residue is van die N-terminaal weggelaat om die 21-residu delesie peptied te produseer, terwyl vier residue weggelaat is om die 19-residu peptied te produseer (onderskeidelik magainin 2 N<sup>21</sup> en magainin 2 N<sup>19</sup> genoem). Peptiede is gesuiwer met behulp van gelpermeasie chromatografie en hoë doeltreffendheid vloeistof chromatografie (HPLC). Die hoë chemiese suiwerheid van die sintetiese produkte is deur elektrospoei-ionisasie massaspektrometrie (ESI-MS) en analitiese HPLC bevestig.

Om die antimikrobiëse aktiwiteit van die gesintetiseerde peptiede te evalueer was dit nodig om 'n hoogs sensitiewe mikro-gel dispersie essaï te ontwikkel. Hierdie essaï is met 'n radiale difussie- en 'n mikrotiter-mediumverdunding-essaï vergelyk. Die antimikrobiëse peptied, gramicidin S, as toetspeptied en *Micrococcus luteus* as die indikator organisme, is gebruik in die ontwikkeling van die essaï. Die mikro-gel dispersie-essaï se sensitiwiteit was vergelykbaar met die mikrotiter-mediumverdunding-essaï, terwyl dit ongeveer twee keer so sensitief was as die radiale difussie-essaï. Die berekening van die minimum inhibitoriese konsentrasie (MIC), 50% inhibisie konsentrasie (IC<sub>50</sub>), en maksimum bakterisidale konsentrasie (MBC) is verfyn deur die opstel van dosisrespons-kurwes met behulp van Prism<sup>®</sup>2.01 (Graphpad Software Inc.). Die MICs, wat in al drie metodes bereken is, het betekenisvol van mekaar verskil, wat aandui dat resultate van verskillende metodes nie met mekaar vergelyk kan word nie.

Verder, is die sinergistiese antimikrobiëse aktiwiteit van magainin 2 en tetrasiklien op tetrasiklien-weerstandbiedende *Escherichia coli* ondersoek. Die twee N-terminale delesie analoë is ook in die sinergisme studies gebruik. Die weerstandbiedendheid teenoor tetrasiklien word veroorsaak deur die uitpomp van die antibiotika uit die sel deur middel van efflukspompe. Magainin 2 is gebruik as antimikrobiëse peptied omdat dit die vermoë het om kanale in die teikenmembraan te vorm, en moontlik hierdie vorm van weerstand kan verminder. Alle toetse is gedoen met behulp van die mikro-gel dispersie-essaï, waarin tetrasiklien-weerstandbiedende *Escherichia coli* as toetsorganisme gebruik is. Magainin 2, gebruik by MIC en IC<sub>50</sub> konsentrasies, het die aktiwiteit van tetrasiklien met  $\pm 12\%$  en  $\pm 29\%$  onderskeidelik verbeter. Magainin 2 N<sup>21</sup> by IC<sub>50</sub>, het die aktiwiteit van tetrasiklien met  $\pm 19\%$

verbeter, maar het geen effek by sy MIC getoon. Magainin 2 N<sup>19</sup> het geen antimikrobiese aktiwiteit getoon nie, maar het wel die aktiwiteit van tetrasiklien met  $\pm 9\%$  verbeter as dit by 138 nmol/mL gebruik word. In al drie gevalle is sinergisme net gevind by konsentrasies laer of naby die MIC van tetrasiklien. By hoër tetrasiklienkonsentrasies het die verbetering in aktiwiteit, as gevolg van die teenwoordigheid van magainin 2, verlore gegaan. Die verlies in sinergisme is moontlik as gevolg van kompetisie tussen tetrasiklien en magainin 2 vir magnesiumbindingplekke op die lipopolisakkariedlaag van Gram-negatiewe organismes. Baie navorsing moet egter nog gedoen word om die probleem van weerstand teen antibiotika te oorkom. Hierdie resultate bewys dat die kombinasie van konvensionele antibiotika en antimikrobiese peptiede wel weerstand kan verminder.

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# Table of Contents

## Chapter 1 General Introduction

1.1	THE PROBLEM OF ANTIBIOTIC RESISTANCE .....	1.1
1.2	RESISTANCE PATHWAYS .....	1.2
1.2.1	<i>Introduction of resistant organisms into a previously susceptible population...</i>	1.2
1.2.2	<i>Genetic mutation</i> .....	1.3
1.2.3	<i>Transfer of genetic material</i> .....	1.3
1.2.4	<i>Emergence of strains with inducible resistance</i> .....	1.4
1.2.5	<i>Selection of resistant strains</i> .....	1.4
1.2.6	<i>Circulation of resistant strains</i> .....	1.4
1.3	WHY DOES RESISTANCE DEVELOP? .....	1.4
1.4	MECHANISMS OF ANTIBIOTIC RESISTANCE.....	1.5
1.4.1	<i>Bacterial species surrounded by low-permeability barriers</i> .....	1.5
1.4.2	<i>Active efflux as a mechanism of drug resistance</i> .....	1.8
1.4.3	<i>The efflux system and the outer membrane barrier</i> .....	1.10
1.5	ALTERNATIVE ANTIMICROBIAL AGENTS .....	1.11
1.6	THE ANTIMICROBIAL PEPTIDE MAGAININ .....	1.13
1.6.1	<i>General characteristics of the magainins and their analogues</i> .....	1.13
1.6.2	<i>Antimicrobial and haemolytic activity</i> .....	1.14
1.6.3	<i>Proposed models for the mechanism of action of linear amphipathic <math>\alpha</math>-helical peptides</i> .....	1.16
1.6.4	<i>Classifying the mechanism of action of the magainins</i> .....	1.18
1.6.5	<i>The effect of alterations to the amino acid sequence on the activity of magainin</i> .....	1.20
1.6.6	<i>Magainin as a synergistic agent</i> .....	1.23
1.6.7	<i>Magainin as a therapeutic agent</i> .....	1.24
1.7	DISCUSSION .....	1.25
1.8	REFERENCES .....	1.27

## Chapter 2 Synthesis and purification of magainin 2 and N-terminal deletion analogues

2.1	INTRODUCTION .....	2.1
2.1.1	<i>Solid phase peptide synthesis</i> .....	2.1
2.1.2	<i>Synthetic antimicrobial peptides</i> .....	2.3
2.2	MATERIALS .....	2.4
2.2.1	<i>General reagents and solvents</i> .....	2.4
2.2.2	<i>Reagents for peptide synthesis</i> .....	2.5
2.2.3	<i>Reagents and solvents for amino acid analysis and chromatography</i> .....	2.5
2.2.4	<i>Drying and storage of reagents and products</i> .....	2.6
2.3	METHODS .....	2.6
2.3.1	<i>Preparation of solvents</i> .....	2.6
2.3.2	<i>Preparation and quality control of the amino acid derivatives</i> .....	2.8
2.3.3	<i>Synthesis of the peptides</i> .....	2.9
2.3.4	<i>Purification of the peptides</i> .....	2.14
2.3.5	<i>Analysis of the purified peptides</i> .....	2.15
2.4	RESULTS AND DISCUSSION .....	2.17
2.4.1	<i>Synthesis 1 of magainin 2 and magainin 2 N<sup>21</sup></i> .....	2.17
2.4.2	<i>Synthesis 2 of magainin 2, magainin 2 N<sup>21</sup>, and magainin 2 N<sup>19</sup></i> .....	2.22
2.4.3	<i>Analysis of purified peptides</i> .....	2.27
2.5	CONCLUSIONS .....	2.30
2.6	REFERENCES.....	2.32

## Chapter 3 A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity

3.1	INTRODUCTION .....	3.1
3.2	MATERIALS .....	3.2
3.3	METHODS .....	3.2
3.3.1	<i>Preparation of cells</i> .....	3.2
3.3.2	<i>Radial diffusion assay</i> .....	3.3
3.3.3	<i>Microtiter Broth Dilution Method</i> .....	3.4
3.3.4	<i>Micro-gel well diffusion assay</i> .....	3.4
3.3.5	<i>Data processing</i> .....	3.5

3.4	RESULTS AND DISCUSSION .....	3.6
3.5	CONCLUSIONS .....	3.11
3.6	REFERENCES.....	3.13
Chapter 4	The synergistic antimicrobial effect of tetracycline and magainin 2, and N-terminal deletion analogues, on tetracycline resistant <i>Escherichia coli</i>	
4.1	INTRODUCTION .....	4.1
4.2	MATERIALS .....	4.3
4.3	METHODS .....	4.4
4.3.1	<i>Peptide synthesis and purification</i> .....	4.4
4.3.2	<i>Antimicrobial assay</i> .....	4.4
4.3.3	<i>Data processing</i> .....	4.5
4.3.4	<i>Structure modelling using HyperChem<sup>®</sup> 5.0</i> .....	4.5
4.4	RESULTS AND DISCUSSION .....	4.5
4.4.1	<i>Antimicrobial activities</i> .....	4.5
4.4.2	<i>Determination of synergism between the antimicrobial peptides and tetracycline on tetracycline resistant E. coli</i> .....	4.7
4.4.3	<i>Determination of the combined antimicrobial activity of the antimicrobial peptides and tetracycline on tetracycline resistant E. coli JM109</i> .....	4.11
4.4.4	<i>Determining the effect of ethanol on the antimicrobial activity of magainin 2</i> .....	4.13
4.5	CONCLUSIONS .....	4.14
4.6	REFERENCES.....	4.17
Chapter 5	Conclusions	
	REFERENCES.....	5.7

## List of Abbreviations and Acronyms

ABC	ATP-binding cassette
$\beta$ A	$\beta$ -alanine
BOP	benzotriazol-1-yl-oxy-tris dimethylaminophosphonium hexafluorophosphate
BSA	bovine serum albumin
CFU/mL	colony forming units per milliliter
CV	cone voltage
CC	dicyclohexylcarbodiimide
Dhbt	3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl
DIPCIDI	diisopropylcarbodiimide
DIPEA	N, N'-diisopropylethyl amine
DMF	N, N'-dimethylformamide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ESI	electrospray ionisation
FDNB	1-fluoro-2,4-dinitrobenzene
Fmoc	N <sup>9</sup> -fluorenylmethyloxycarbonyl
HOBt	1-hydroxybenzotriazol
HPLC	high performance liquid chromatography
IC <sub>50</sub>	concentration of antibiotic inhibiting 50% microbial growth
LPS	lipopolysaccharide
M	molar
MBC	maximum bactericidal concentration
MF	major facilitator
MIC	minimum inhibitory concentration
MS	mass spectrometry
<i>m/z</i>	mass to charge ratio
NMR	nuclear magnetic resonance
OCD	orientated circular dichroism
OD	optical density
PBS	phosphate buffered saline
Pfp	pentafluorophenyl

PITC	phenylisothiocyanate
PTC	phenylthiocyanate
PyBOP®	benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate
RDA	radial diffusion assay
$R_f$	distance that analyte moved relative to solvent front
RND	resistance-nodulation-division
SEM	standard error of the mean
Smr	staphylococcal multidrug resistance
SPPS	solid phase peptide synthesis
tBoc	N-t-butyloxycarbonyl
tBu	t-butyl ester
TEA	triethylamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TSB	tryptone soy broth
UV	ultraviolet
$V_e$	elution volume

## Preface

When penicillin became available during the second world war, it was a medical miracle, rapidly vanquishing the biggest wartime killer, namely infected wounds. Discovered initially by a French medical student, Ernest Duchesne, in 1896, and then rediscovered by Scottish physician Alexander Fleming in 1928, the product of the soil mold *Penicillium* crippled many types of disease-causing bacteria. However, only four years later after drug companies began mass-producing penicillin in 1943, microbes began appearing that could resist it. Medical researchers subsequently fought back with the development of novel analogues such as methicillin and oxacillin. By 1953, the antibiotic armamentarium included chloramphenicol, neomycin, terramycin, tetracycline, and cephalosporins. Today, however, it is feared that we are nearing an end to the seemingly endless flow of antimicrobial drugs. Emergence of forms of *Staphylococcus aureus* lacking sensitivity to vancomycin signifies that organisms untreatable by every known antibiotic are on their way. All hope is not, however, lost. In the past 50 years researchers have discovered that the innate immunity in various microorganisms, plants, amphibians, and insects is mediated through the presence of small molecular weight antimicrobial peptides. In the past, researchers have concentrated mainly on the discovery and isolation of these peptides, and it is only recently that their potential as future antibiotics is being recognised.

This thesis describes how the unique mechanism of action of one such peptide, magainin 2, is exploited to alter the membrane viability of resistant organisms and make them more susceptible to the action of conventional antibiotics. In Chapter 1 a brief overview on resistance and the antimicrobial peptide magainin is given. In the following three chapters, the experimental results obtained in this study are reported. In Chapter 2, the synthesis, characterisation, and purification of magainin 2 and two N-terminal deletion analogues are discussed. Chapter 3 describes the development and evaluation of an antimicrobial assay used to test the antimicrobial activities of these peptides. In Chapter 4 we report on the ability of these peptides to make resistant organisms more susceptible to conventional antibiotics. Finally, the general implications and future prospects of this work are discussed in Chapter 5.

# Chapter 1

## *General Introduction*

### **1.1 The problem of antibiotic resistance**

To date, antibiotics have been highly successful in treating various infectious diseases. However, the emergence of numerous strains of resistant bacteria has rapidly changed this situation. The mortality rate in major hospitals, staffed by highly competent personnel, has risen alarmingly as a result of infections by resistant bacteria [1]. Bacterial resistance is not a recent problem, with penicillin resistant *Staphylococcus aureus* having been discovered as far back as the 1950s. The frequency of antibiotic resistant bacteria has increased to such an extent that certain strains have developed resistance to multiple drugs, with some bacteria being resistant to 8-10 antibiotic agents. Up until now, pharmaceutical companies have kept this problem from reaching epic proportions by constantly developing alternative or new antibiotics [2]. Today there are more than 50 penicillins, 75 cephalosporins, 12 tetracyclines, nine aminoglycosides, three carbapenems, and at least 20 fluoroquinolones [3]. The result has been a steady supply of new antibiotics to keep ahead of the problem. The development of new antibiotics, however, has slowed dramatically, with no new antibiotic groups foreseen in the next 10 years [4]. Of major concern is the development of vancomycin resistant *S. aureus*, signifying the emergence of bacteria resistant to every known antibiotic [5].

Increased antibiotic resistance has also had enormous cost implications for health care. These result from the use of more costly antibiotics, the need for additional drug testing, prolonged hospital stays, and difficulty in eradicating resistant bacteria. While the health systems of developed countries may be able to cover the additional costs associated with antibiotic resistance, those of developing countries are not. With the lack of cheap alternatives, developing countries are likely to continue to misuse antibiotics and therefore further compound the problem of resistance.

With the unsure future of antibiotics and the rising health care costs associated with the increase in antibiotic resistance, researchers are constantly searching for ways and means to reduce or overcome bacterial resistance. Pharmaceutically, either more effective antibiotics must be developed, or agents based on the mechanism of resistance must be identified and utilised.

The rest of this chapter shall review the following aspects: first, the various pathways by which resistance spreads, and mechanisms used by bacteria to incur resistance; second, antimicrobial peptides from the animal kingdom, specifically the frog peptide magainin and how its antimicrobial characteristics and unique mechanism of action make it ideal for use in future research.

## 1.2 Resistance pathways

It is suspected that there are at least six pathways involved in the appearance or spread of resistance in bacteria [6], which will be discussed separately below.

### 1.2.1 *Introduction of resistant organisms into a previously susceptible population*

Often resistant organisms are able to spread or find their way to new or “unaffected” areas. For example, new strains can enter the hospital setting by way of a patient from the outside, a health-care worker from the outside or another institution, or a contaminated commercial product. This has been illustrated by an episode in which a strain of *Burkholderia cepacia* was introduced into a hospital setting by means of a contaminated iodophor solution which caused only those patients who were treated with the solution to be affected [6]. Research shows that the introduction of resistant strains into hospitals from nursing homes and extended care facilities, as well as transfer in the opposite direction from hospital to nursing home, has become common for certain pathogens [7]. Once resistant strains find their way into various institutions they are extremely difficult to eliminate. Furthermore, the transfer of resistant organisms from the medical environment to the community is also a common occurrence.

### **1.2.2 Genetic mutation**

Changes in only a few base pairs, causing the substitution of one or a few amino acids in a crucial target (enzyme, cell structure, or cell wall) can affect chromosomal structure or control genes leading to new resistant strains [8]. An example of this is shown by certain strains of *Escherichia coli* which have developed resistance to quinolones [9]. Normally, quinolones antagonise bacteria by inhibiting DNA gyrase, an essential bacterial enzyme with A and B subunits. Certain strains of *E. coli* have developed resistance to quinolones by altering the gyrase target site in either the A or B subunit. These changes have resulted in the inactivation of numerous antibiotics that were previously able to control these organisms. Hospitalised patients treated with antibiotics prone to select altered resistance traits can quickly become infected with these resistant strains.

### **1.2.3 Transfer of genetic material**

It is also possible that resistance can be acquired by a previously susceptible strain from another species or genus [10]. It has been shown that many of the antibacterial resistance genes are found on plasmids that can transfer themselves from one genus or species of bacteria to another [11]. Although the ability of microorganisms to exchange genetic material is a known fact, it is evident that researchers have underestimated the extent of this DNA exchange network. There is even evidence to date of the exchange of genetic information between Gram-negative and Gram-positive organisms, although the frequency of such events is unknown [12]. Of particular interest, and concern, is the exchange pathway between staphylococci and enterococci. Already, the genes encoding  $\beta$ -lactamase production and high-level gentamicin resistance have moved from staphylococci into enterococci. This has resulted in resistance to penicillin, ampicillin, and the aminoglycosides [6]. In 1988, it was discovered that *Enterococcus faecalis* was resistant to vancomycin by virtue of a gene (*van A*) carried on a transmissible plasmid [9]. Back then it was thought that the plasmid encoding vancomycin resistance could only be transferred to other streptococci; alarmingly though certain strains of vancomycin resistant *S. aureus* have recently been discovered [5].

### ***1.2.4 Emergence of strains with inducible resistance***

Chromosomal determinants for resistance to a given drug may not be expressed until the organism comes into contact with that drug or similar compounds [6]. When permissive conditions appear, for example the use of new antibiotics or the introduction of new conjugative plasmids, resistance can be manifested rapidly. The trigger for this resistance could be the antimicrobial agent to which the resistance is directed. In some cases, exposure to another antimicrobial agent results in induction or depression of a determinant, such as an enzyme etc., that stimulates resistance to that specific antimicrobial agent.

### ***1.2.5 Selection of resistant strains***

When using a specific antibiotic to kill a certain microorganism the majority of the population is successfully killed; however, often a resistant subset of the strain remains unaffected [6]. Frequently a minority of strains present in a given population may be resistant to a specific antibiotic used, therefore making the antibiotic the selecting factor [13]. It is also found that non-drug factors, such as those causing acetylation or glucuronylation, can change certain strains, thus inadvertently resulting in a selection process.

### ***1.2.6 Circulation of resistant strains***

Resistant strains have been shown to spread in a variety of ways within the health-care setting. For example, organisms can spread from patient to patient, from one patient to another *via* a health-care worker, in contaminated commercial products (for example antiseptics), or widespread transfer of genetic material from the original organism to others [6].

## **1.3 Why does resistance develop?**

The most current outbreaks of resistance involve a number of the above mentioned pathways, each contributing to a greater or lesser degree. For example, in a previous case where a hospital outbreak of multiresistant *Serratia marcescens* took place, it was discovered that the outbreak occurred *via* transmission on the hands of health-care workers who handled contaminated collection basins while emptying the urine catheter collection bags [6]. Two

pathways played a role in this outbreak: the first was the introduction of a new organism into the hospital environment, and the second was the transfer of this organism within the hospital. These pathways, acting alone or in tandem with one another, therefore resulted in the rapid spread of resistant organisms. It is seldom possible to identify which of the pathways was operative in a given abrupt increase in resistance, which implies that the magnitude of each outbreak of resistance in today's settings still remains unclear. Three basic aspects need to be studied in detail if the spread of resistant organisms is to be curbed. First, simple mutations in genes which produce target proteins. Second, the exchange of genetic material among microorganisms which results in them becoming resistant to additional antibiotics. Finally, how the use of large quantities of antibiotics, not just within the hospital environment, but also in community, farm, and aquaculture settings, has increased the selection of new resistant organisms [6].

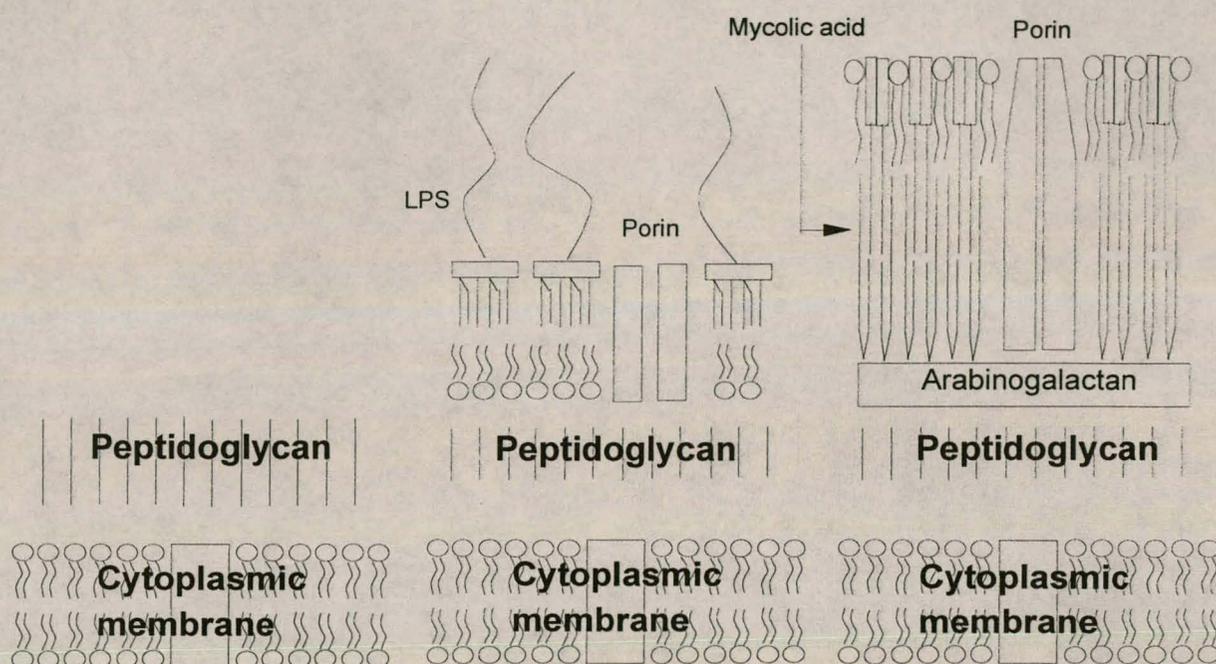
## **1.4 Mechanisms of antibiotic resistance**

Bacteria are known to utilise several mechanisms to develop antibiotic resistance. These mechanisms can either be specific or general in their mode of action. Specific mechanisms include degradation of the drug, inactivation of the drug by enzymatic modification, and alteration of the drug target. The more general mechanisms include cases where, for example, an organism can surround itself with a barrier of low permeability or expel the drug by means of an energy dependent pump [1]. The focus here will be on the general mechanisms of resistance.

### ***1.4.1 Bacterial species surrounded by low-permeability barriers***

Bacteria are unicellular organisms and their cytoplasm is separated from the external environment by the cytoplasmic membrane. The major permeability barrier in any membrane is the lipid bilayer structure, and its barrier property is inversely correlated with its fluidity [1]. It is impossible to make the cytoplasmic membrane much less permeable, because this would require decreasing the membrane fluidity and consequently interfering with the proper functioning of membrane proteins. Therefore some bacteria protect themselves by constructing an additional structure that surrounds the cell outside the cytoplasmic membrane.

Most Gram-positive bacteria are surrounded by a thick peptidoglycan cell wall (Fig. 1.1). This structure, although mechanically strong, appears to offer little resistance to the diffusion of small molecules such as antibiotics. In contrast, Gram-negative bacteria, such as *E. coli*, surround themselves with a second membrane, which functions as an effective barrier. This membrane is composed of lipopolysaccharide (LPS), rather than the usual glycerophospholipid found in most other biological membranes. The fatty acid chains present in LPS are all saturated, which creates a much more tightly packed membrane with a greatly decreased fluidity (Fig. 1.1) [14]. In addition, a LPS molecule contains six or seven covalently linked fatty acid chains, in contrast to the glycerophospholipid that contains two fatty acid residues. The vast majority of clinically important antibiotics do have some hydrophobic properties and can therefore diffuse across the LPS leaflet. The rate at which this occurs though is very slow, indicating that the LPS leaflet does serve as an efficient barrier against the rapid penetration of antibiotics into the cell. Bacteria surrounded by such an effective barrier, however, must develop a separate mechanism to take up essential nutrients from the external medium. For this purpose, the outer membrane contains proteins of a special class, porins, which produce non-specific aqueous diffusion channels across the membrane. It is through these channels that relatively hydrophilic antibiotics can penetrate the cell. A different strategy is adopted by the Gram-negative soil organism, *Pseudomonas aeruginosa*. This organism does not possess the classical high-permeability porins, but instead contains only low-efficiency porins that allow diffusion of small molecules at one-hundredth the rate of the classical porin channels [15]. Thus, hydrophilic antibiotics diffuse across the outer membrane at a very slow rate making this organism resistant to a wide variety of antibiotics. Certain Gram-positive mycobacteria have adopted a similar strategy of surrounding themselves with a barrier of generally low permeability. The mycobacterial barrier appears to consist of a lipid bilayer of unusually high order, and therefore low fluidity, making the mycobacterium resistant to a broad spectrum of antibiotics [16].



*Figure 1.1.* Cell envelopes of bacteria [1]. Most of the Gram-positive bacteria are covered by a porous peptidoglycan layer, which does not exclude most antimicrobial agents (left). Gram-negative bacteria are surrounded by the outer membrane, which functions as an efficient permeability barrier because it contains LPS and porins with narrow, restrictive channels (middle). Mycobacteria produce an unusual bilayer, which functions as an exceptionally efficient barrier, outside the peptidoglycan layer (right) [1].

The main fatty acid in the mycobacterial cell wall is mycolic acid, which contains 70 carbon atoms with only a few double bonds, making its fluidity extremely low (Fig. 1.1). In LPS, six or seven fatty acids were joined to a single head group; here, hundreds of mycolic acid residues are covalently linked to a common head group, an arabinogalactan polysaccharide, which in turn is covalently linked to the underlying peptidoglycan structure [17]. The influx of nutrients is apparently facilitated by the mycobacterial porin, which is present in very small amounts and allows only very slow diffusion of small molecules through its channel [1]. The low permeability of both the lipid matrix and of the porin channel results in the very slow penetration by antibiotics.

Experimentally, it has been shown that reduced permeability of the outer membrane alone does not provide complete resistance to antibiotics, and that significant levels of drug resistance requires a second contributor. This second contributor can have a direct specific

effect on the drug itself (degradation of the drug or enzymatic inactivation of the drug), or it can constitute a general, active efflux of the drug.

#### **1.4.2 Active efflux as a mechanism of drug resistance**

Active drug efflux systems in bacteria can be divided into four families (Table 1.1) on the basis of supramolecular assembly, mechanism, and sequence homology:

1. the “major facilitator (MF) family”, which shows sequence homology to the glucose facilitators of mammalian cells and includes drug efflux proteins of eukaryotic microbes;
2. the resistance-nodulation-division (RND) family, which also includes transporters that pump out cadmium, cobalt and nickel ions;
3. the staphylococcal multidrug resistance (Smr) family, consisting of small transporters that presumably contain only four transmembrane helices; and
4. the ATP-binding cassette (ABC) transporters, which are composed of two transmembrane and two ATP-binding domains [1].

Each member of the first three families is a single cytoplasmic membrane protein that expels drugs using the proton-motive force, or in other words acting as a  $H^+$ -drug antiporter [1]. Of great concern today is the discovery of multidrug efflux systems, confirming the general mechanism of resistance that certain organisms have acquired. Efflux systems such as QacA, Smr, QacE, or MvrC are able to pump out quaternary amine compounds as well as basic dyes [1]. This indicates that the substrates of these systems are at least physically similar, being amphiphilic with positive charges. In contrast, the Bmr transporter, found in a rhodamine-6G-resistant mutant of *Bacillus subtilis*, catalysed the active efflux not only of cationic dyes such as rhodamine-6G and ethidium bromide, antibiotics puromycin (basic) and netropsin (strongly basic), and an organic cation, tetraphenylphosphonium, but also chloramphenicol (uncharged) [1].

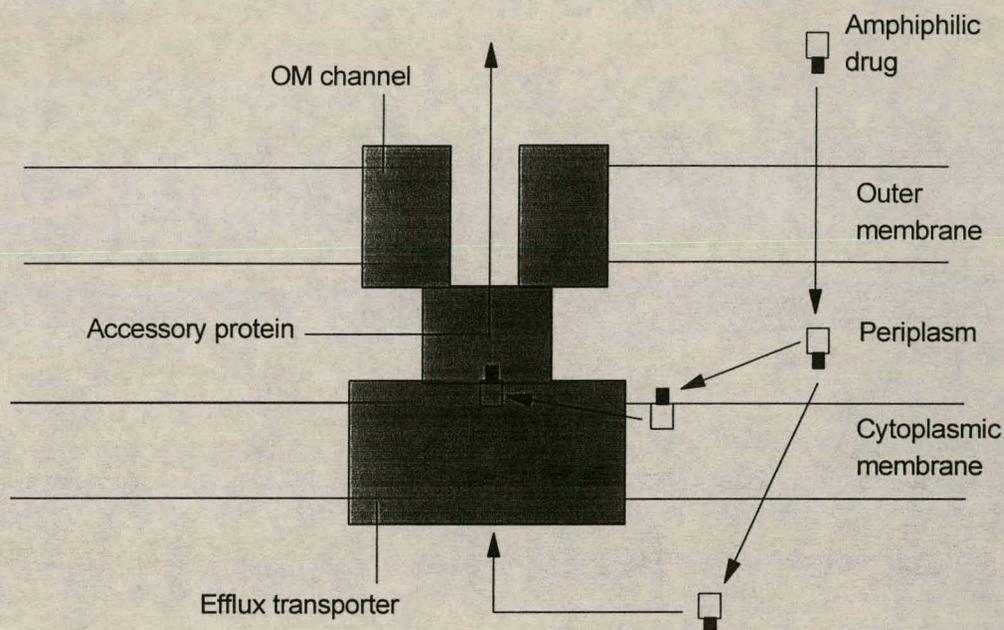
Table 1.1. Examples of active efflux systems in bacteria [1].

Transporter	Organism	Accessory protein	Gene location	Substrate
<b>Major facilitator in Gram-positives</b>				
OtrB	<i>Streptomyces rimosus</i>	None	Chromosome	Oxytetracycline
Tel (L)	Various cocci, <i>Bacillus subtilis</i>	None	Plasmid	Tetracycline
Mmr	<i>Streptomyces coelicolor</i>	None	Chromosome	Methylenomycin
Act II	<i>Streptomyces coelicolor</i>	None	Chromosome	Actinorhodin
TcmA	<i>Streptomyces glaucescens</i>	None	Chromosome	Tetracenomycin
NorA	<i>Staphylococcus aureus</i>	None	Chromosomal	Fluoroquinolones, basic dyes, puromycin, chloramphenicol, tetraphenylphosphonium
QacA	<i>Staphylococcus aureus</i>	None	Plasmid	Quaternary ammonium compounds, basic dyes
Bmr	<i>Bacillus subtilis</i>	None	Chromosome	Basic dyes, chloramphenicol, puromycin, fluoroquinolones
<b>Major facilitator in Gram-negatives</b>				
TetA	<i>Escherichia coli</i>	None	Plasmid	Tetracycline
CmlA	<i>Pseudomonas aeruginosa</i>	None	Plasmid	Chloramphenicol
Bcr	<i>Escherichia coli</i>	None	Chromosome	Bicyclomycin
EmrB	<i>Escherichia coli</i>	EmrA	Chromosome	CCCP, nalidixic acid, tetrachlorosalicylanilide, phenylmercury acetate
EmrD	<i>Escherichia coli</i>	?	Chromosome	CCCP, phenylmercury acetate
<i>RND family</i>				
AcrE(AcrB)	<i>Escherichia coli</i>	AcrA	Chromosome	Basic dyes, SDS, erythromycin, novobiocin, fusidic acid, tetracycline, phenylmercury acetate
EnvD	<i>Escherichia coli</i>	EnvC	Chromosome	Basic dyes, SDS, erythromycin, fusidic acid, tetracycline, mitomycin c
MexB	<i>Pseudomonas aeruginosa</i>	MexA	Chromosome	Tetracycline, chloramphenicol, fluoroquinolones, $\beta$ -lactams, pyoverdine
<i>Smr family</i>				
Smr(QacC)	<i>Staphylococcus aureus</i>	None	Plasmid	Quaternary ammonium compounds, basic dyes
QacE	<i>Klebsiella aerogenes</i>	?	Plasmid	Quaternary ammonium compounds, basic dyes
MvrC(EmrE)	<i>Escherichia coli</i>	?	Chromosome	Methyltriphenylphosphonium, basic dyes
<i>ABC transporters</i>				
MsrA	<i>Staphylococcus epidermidis</i>	?	Plasmid	14- and 15-membered macrolides
DrrA, DrrB	<i>Streptomyces peucetius</i>	?	Chromosome	Daunorubicin, doxorubicin
TlrC, TlrB	<i>Streptomyces fradiae</i>	?	Chromosome	Tylosin

Note: QacB is very similar to QacA; CCCP, carbonyl cyanide m-chlorophenylhydrazone; SDS, sodium dodecylsulfate

### 1.4.3 The efflux system and the outer membrane barrier

Efflux transporters are located in the cytoplasmic membrane, and therefore in Gram-negative cells it may be assumed that antibiotics are pumped out into the periplasm (Fig. 1.1). If this is indeed the case, the efflux is unlikely to make these bacteria more resistant, because the antibiotic will in all likelihood be unable to leave the cell easily because of the outer membrane barrier.



**Figure 1.2** Proposed structure and mechanism of action of most efflux complexes in Gram-negative bacteria [1]. The efflux transporter is usually of the MF or RND family. This is connected to the accessory protein, such as EmrA, AcrA, EnvC or MexA, which in turn is associated with an outer membrane channel protein, OprK in the MexAB system of *P. aeruginosa* and probably TolC in *E. coli*. Amphiphilic drugs (with their hydrophilic ends shown as black rectangles) traverse the outer membrane (often *via* porin channels), and become partially inserted into the bilayer of the cytoplasmic membrane. The transporter captures the drug molecules in the bilayer and pumps them out, bypassing the outer membrane barrier [1].

One way to overcome this barrier was suggested by the presence of accessory proteins that occur together with many efflux transporters of both MF and RND families in Gram-negative cells (Table 1.1). These proteins are thought to bridge the cytoplasmic transporter and an outer membrane channel, so that the drugs can be pumped directly into the surrounding medium rather than the periplasm (Fig. 1.2). As mentioned, the low permeability of the outer membrane is unlikely to produce clinically significant levels of resistance, and a second

contributor is needed for this purpose. In many systems, the active efflux system appears to be this second factor. This, however, does not mean that the outer membrane barrier is unimportant. Since the intracellular concentration of any drug is the result of a balance between influx and efflux, it is likely that the slow influx of various agents through the low permeability outer membrane makes efflux an especially effective mechanism for resistance in many bacteria.

## 1.5 Alternative antimicrobial agents

Plants and animals have to survive in a world laden with pathogenic bacteria and fungi. Perhaps the most interesting aspect of this ecosystem is not the expected staggering losses from susceptibility to infection, as much as the endurance displayed by the diverse organisms in spite of infection [18]. This resilience may be attributed to the presence of a repertoire of host defence mechanisms. In animals (including humans), defence may be mediated by events such as immune response, complement activation, phagocytosis, and release of small molecular weight antimicrobial peptides [19]. However, in insects, amphibians, and other lower organisms—although comparable immune responses are less well characterised—it is clear that small molecular weight peptides play a major role in warding off infections [20].

From the perspective of a microbe, the tissues of living multicellular organisms are rich sources of nutrients. Challenged by the remarkable plasticity of such microbes, vertebrates have evolved multiple and varied molecular defences. Amongst these defences are an array of constitutive or inducible antimicrobial proteins or peptides [21]. Examples of vertebrate antimicrobial peptides include defensins and cathelicidins, both of which exhibit broad-spectrum activity [22]. A host of antimicrobial peptides have also been discovered in plants and insects [23]. The plant defensin family includes the cysteine-rich  $\alpha$ - and  $\beta$ -thionins [24]. Of the insect peptides, the most well studied examples are the cecropins and melittin. Cecropins are a family of homologous antibacterial peptides derived from the *Cecropia* moth, *Hyalophora cecropia* [25]. Cecropin-like peptides have also been found in lepidoptera (butterflies and moths), diptera (flies etc.), and the porcine intestine and blood cells of marine protochordate [26]. Melittin is another insect peptide comprised of 26 amino acid residues, and is isolated from the venom of honeybees [27]. Many more peptides have been isolated from insects such as fruit flies, silkworms, etc. [18, 22, 23]. Certain bacteria are also known

secrete various antimicrobial peptides. Because bacteria are prolific, they continually interact with almost all other cell types. Therefore, many non-bacterial cells, as well as many bacteria, produce specific antimicrobial peptides to combat a bacterial “take-over” of their environment [28]. Bacterial peptides produced by non-ribosomal biosynthesis include gramicidin S from *Bacillus brevis*, and alamethicin from the fungus, *Trichoderma viride*. As opposed to these peptides, the group of peptides termed bacteriocins are produced on the ribosome as a precursor peptide, which is later processed to release the active moiety [29]. Bacteriocins can be divided into several classes: class I are the lantibiotics, which are small membrane active peptides containing post-translationally modified lanthionine residues, while class II-IV are the non-lantibiotics which contain no lanthionine residues (subdivisions to class II bacteriocins have also been suggested) [30, 31]. These, together with numerous other peptides, have been reviewed extensively elsewhere [18, 22, 23, 28].

Perhaps the richest source of antimicrobial peptides is frog skins. The abundance of peptides varies between species; particularly rich sources have been discovered within the genera *Phylomedusa* and *Xenopus*. Peptides secreted by *Xenopus laevis* are produced in the granular glands, which are made up of numerous specialised secretory cells that store large amounts of biologically active peptides and neurotransmitters. These glands are believed to serve physiological roles in defence against macroscopic predators, and in microbial control following wounding. Isolation of cDNAs encoding these peptides has revealed that they are initially synthesised as large polyproteins in the secretory cells. The precursors share a highly conserved signal sequence, approximately 20 amino acids in length, with several clusters of sequence identity and an overall amino acid identity of about 55% [32]. Prior to secretion, proteolytic events cleave the precursor peptides, liberating the active peptide fragments [31]. Peptides secreted by *Xenopus laevis* include caerulein precursor fragments, xenopsin precursor fragments, PGLa, and magainin 1 and 2 [32]. Experimental determination of protein yield showed the magainin peptides to be the major component of the exudate [33]. Following discharge, the peptides undergo proteolytic cleavage at the N-terminal side of a lysine residue (at Xaa-Lys bonds where Xaa is Leu, Gly, Ala or Lys) by the action of an endopeptidase, resulting in half peptides that no longer retain antibiotic activity [34]. This may represent a mechanism by which the secretions are rendered harmless to the frog itself, since prolonged exposure could possibly result in toxic effects [35].

The magainin peptides exhibit antimicrobial activity against a wide range of target organisms including protozoa, fungi, Gram-positive and Gram-negative bacteria, and certain cancer cell lines [36]. They were also noted to be both non-hemolytic and non-cytotoxic [37]. Unique structural features of these peptides are their cationic nature and amphipathic  $\alpha$ -helical conformation on membrane binding (Fig. 1.3) [38]. The above mentioned characteristics indicate that these peptides have excellent potential as antibiotics, and with the steady decline in effective conventional antibiotics, antimicrobial peptides could well be the antibiotics of the future. Magainin 2, possessing a broad-spectrum of activity and being non-cytotoxic, is also one of the most well documented antimicrobial peptides making it ideal for future research. In the following, the characteristics of the peptide, its mechanism of action, and research showing its overall versatility as antimicrobial compound will be discussed.

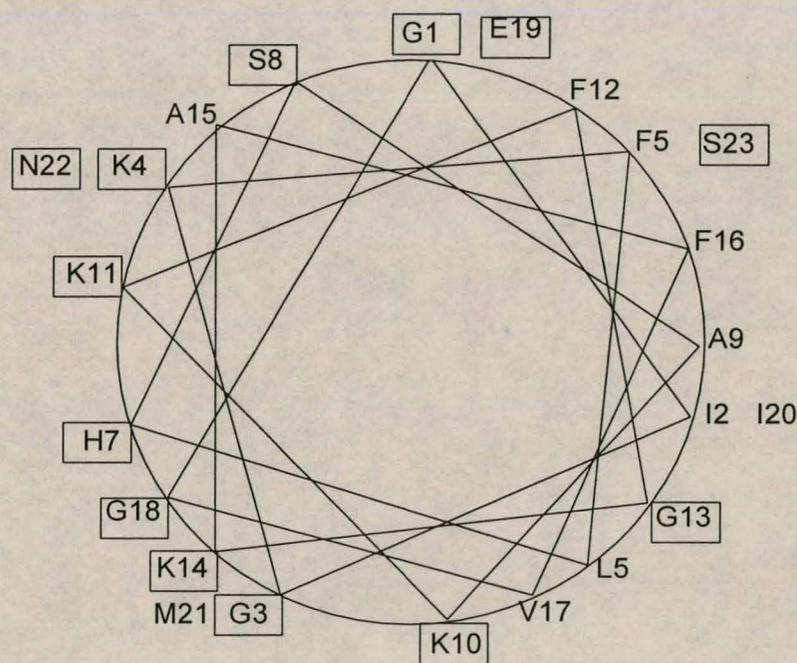


Figure 1.3 Helical wheel projection of magainin 2. Polar residues are boxed [39].

## 1.6 The antimicrobial peptide magainin

### 1.6.1 General characteristics of the magainins and their analogues

As stated previously, a number of magainins have been isolated from the dermal secretions of *Xenopus laevis*. These have led to the development of numerous other synthetic magainin

analogues with increased antimicrobial activity. Both the natural and synthetic peptides were indistinguishable with respect to both biological and chromatographic properties [36]. Some of these magainins together with their amino acid sequences are listed in Table 1.2.

*Table 1.2* Amino acid sequences of magainin and its analogues. Magainin 1 and 2 are the only naturally occurring peptides [40].

Peptide	Sequence
Magainin 1	G I G K F L H S A G K F G K A F V G E I M K S
Magainin 2	G I G K F L H S A K K F G K A F V G E I M N S
Magainin A	$\beta$ A I G K F L H A A K K F A K A F V A E I M N S NH <sub>2</sub>
Magainin B	A I G K F L H A A K K F A K A F V A E I M N S NH <sub>2</sub>
Magainin C	<sup>N-Acetyl</sup> A I G K F L H A A K K F A K A F V A E I M N S NH <sub>2</sub>
Magainin D	$\beta$ AG I G K F L H S A K K F A K A F V A E I M N S NH <sub>2</sub>
Magainin E	$\beta$ A I G K F L H S A K K F A K A F V A E I M N S NH <sub>2</sub>
Magainin F	G I G K F L H S A K K F A K A F V A E I M N S NH <sub>2</sub>
Magainin G	$\beta$ AG I G K F L H S A K K F A K A F V A E I M N S NH <sub>2</sub>
Magainin H	G I G K F L H S $\partial$ K K F $\partial$ K A F V $\partial$ E I M N S NH <sub>2</sub>

Note: All sequence modifications are based on magainin 2. One-letter amino acid abbreviations are used.  $\beta$ A,  $\beta$ -alanine;  $\partial$ , D-alanine.

### 1.6.2 Antimicrobial and haemolytic activity

Table 1.3 lists the antimicrobial activities of the magainin peptides and their analogues after being assayed against Gram-positive and Gram-negative bacteria. Analogues A, B, D, E, F, and G had increased antimicrobial activity, between one and two orders of magnitude higher than magainin 1 or 2, while still maintaining the same spectrum of activity [40]. The H analogue, which contains D-alanine instead of L-alanine at three modification sites, showed no signs of increased antimicrobial activity against any of the microorganisms tested, indicating that a specific conformation was required for antimicrobial activity. To determine

the haemolytic activity of magainin 1 and analogues A, B, C, F, G, and H, experiments were performed using human erythrocytes. The control peptide used to denote 100% haemolysis was honey bee venom, or melittin. Neither magainin 1 nor analogue H showed haemolytic activity at 250 µg/ml of heparinised blood. However, the other analogues exhibited appreciable haemolysis at 100 µg/ml which was approximately equivalent to 1/100 of the potency of melittin [40]. Analogue G showed less than 1% haemolytic activity at 200 µg/ml. These results indicate that the haemolytic activities generally increased in parallel to growth inhibitory activity, except in the case of analogue G, which showed high antimicrobial activity but exceptionally low haemolytic activity [40].

Table 1.3 Antimicrobial activity of magainin and its analogues [40].

Organism	50% and 100% minimal inhibitory concentrations (µg/mL)									
	Magainins					Analogues				
	1	2	A	B	C	D	E	F	G	H
<i>Escherichia coli</i>	100	50	1.2	1.2	10	2.5	1.2	1.2	2.5	70
	250	100	2.5	2.5	25	5	5	5	5	>100
<i>Klebsiella pneumoniae</i>	100	100	5	5	60	10	5	5	10	100
	250	100	25	10	100	25	25	25	25	>100
<i>Pseudomonas aeruginosa</i>	430	>100	15	15	>100	25	25	25	25	100
	>500	>100	25	25	>100	50	50	50	100	>100
<i>Streptococcus agalactiae</i>	60	50	0.25	0.25	0.5	0.25	0.25	0.25	0.5	100
	75	100	1	1	1	1	1	1	2.5	>100
<i>Streptococcus faecalis</i>	360	>100	5	5	5	5	10	10	10	>100
	>500	>100	10	10	25	25	25	25	50	>100
<i>Staphylococcus aureus</i> β-lactamase [+]	500	>100	5	5	7.5	2.5	5	5	5	>100
	>500	>100	10	10	2.5	5	10	10	10	>100
<i>Staphylococcus aureus</i> β-lactamase [-]	360	>100	5	5	10	10	10	10	10	>100
	>500	>100	10	10	25	25	25	25	25	>100

Note: > denotes no activity detected at the concentration indicated.

### ***1.6.3 Proposed models for the mechanism of action of linear amphipathic $\alpha$ -helical peptides***

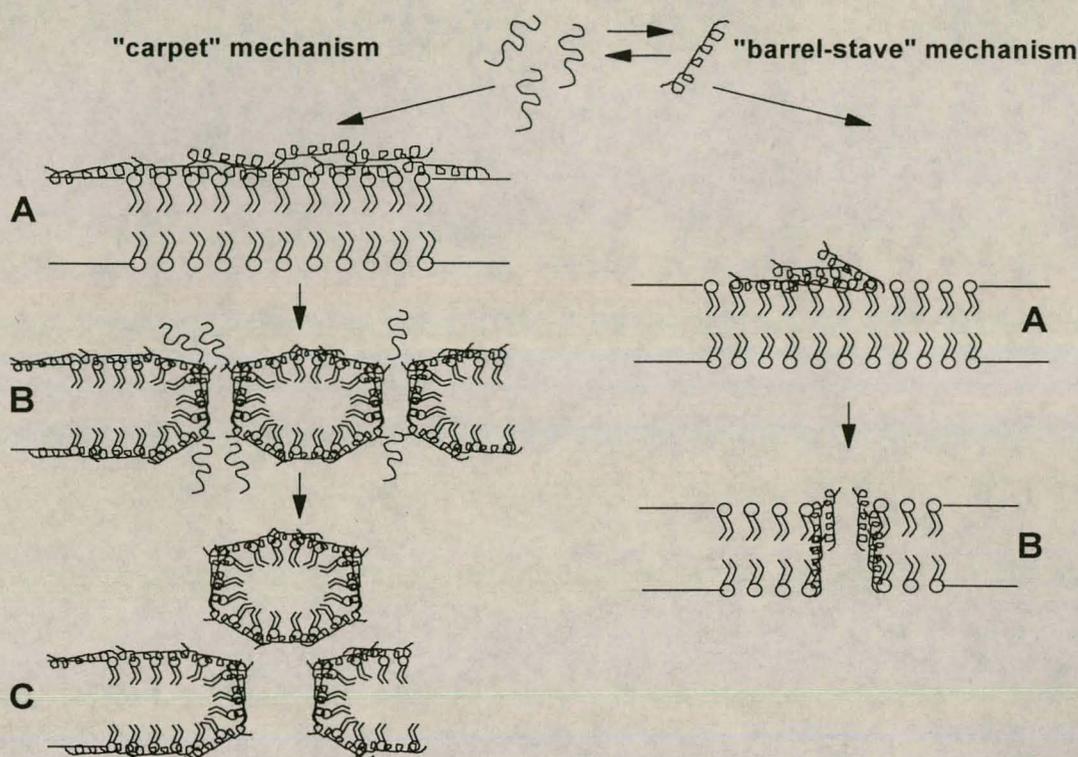
Although the exact mechanism of action of these peptides is still not clearly understood, membrane permeation by amphipathic  $\alpha$ -helical peptides has been proposed to proceed via one of two mechanisms: (a) transmembrane pore formation via a “barrel-stave” mechanism, or (b) membrane destruction/solubilisation via a “carpet-like” mechanism [41].

#### **1.6.3.1 Model of membrane disruption by “barrel-stave” transmembrane pore formation**

A classical transmembrane pore can be described by a barrel-stave model in which transmembrane amphipathic  $\alpha$ -helices form bundles, with their hydrophobic surfaces interacting with the lipid core of the membrane, and their hydrophilic surfaces pointing inwards producing a pore (right panel Fig. 1.4) [42]. As these peptides can insert into the hydrophobic core of the membrane, their binding to the target membrane is predominantly driven by hydrophobic interaction. As a consequence, they can bind to both zwitterionic and charged phospholipid membranes. Furthermore, because the hydrophobic surfaces of several amphipathic  $\alpha$ -helical peptides are facing each other while forming a transmembrane pore, it is unlikely that such helices will be highly homogeneously charged. The barrel-stave mechanism involves four major steps: (a) binding of the monomers to the membrane in an  $\alpha$ -helical structure, (b) molecular recognition between membrane-bound monomers that leads to their assembly already at low surface density of bound peptide, (c) insertion of at least two assembled monomers into the membrane to initiate the formation of a pore, and (d) progressive recruitment of additional monomers to increase the pore size. A prerequisite condition for such a mechanism is that initial assembly of monomers on the surface of the membrane must occur before the peptide is inserted [41]. It is energetically unfavourable for a single amphipathic  $\alpha$ -helix to traverse the membrane as a monomer owing to the fact that the low dielectric constant and inability to establish hydrogen bonds will not allow the fatty acyl region of a lipid bilayer to be in a direct contact with a polar surface of a single amphipathic  $\alpha$ -helix [41].

### 1.6.3.2 Model of membrane disruption via a “carpet-like” mechanism

The carpet mechanism describes a situation in which amphipathic  $\alpha$ -helical peptides initially bind onto the surface of the target membrane and cover it in a carpet-like manner [43, 44]. The target membrane can be permeated only after a threshold concentration has been reached. In contrast to the barrel-stave mechanism, the peptide does not insert into the hydrophobic core of the membrane, but rather binds to the phospholipid headgroups. Initial interaction with the negatively charged target membrane is electrostatically driven, and therefore the active peptides are positively charged. The four steps possibly involved in this model are (a) preferential binding of positively charged peptide monomers to the negatively charged phospholipids, (b) laying of amphipathic  $\alpha$ -helical monomers on the surface of the membrane so that the positive charges of the basic amino acid residues interact with the negatively charged phospholipid headgroups or water molecules, (c) rotation of the molecule leading to reorientation of the hydrophobic residues toward the hydrophobic core of the membrane, and (d) disintegration of the membrane by disrupting the bilayer curvature leading to micellisation. An initial step before the collapse of the membrane packing may include transient holes in the membrane. Transient holes allow the non-specific influx of various ions into the cell, and therefore should also have a cytostatic, or even cytotoxic effect on the cell, owing to disruption of the membrane potential. Transient holes are explained by the toroidal (or wormhole) model [45, 46]: initial adsorption of the peptides to the headgroups expands the membrane laterally, consequently decreasing the bilayer thickness in proportion to the peptide concentration. Because the energy of membrane deformation is proportional to the square of the thickness change, the energy increases quadratically with the peptide concentration. The energy of membrane deformation is part of the free energy of peptide adsorption. Thus at high peptide concentrations, the energy of adsorption may become so high that it drives the lipid-peptide system to configurations of lower energy, causing the membrane to fold back on itself like the inside of a torus (step B in left panel of Fig. 1.4). The strain of membrane expansion is reduced by the incorporation of peptide monomers in the headgroup region, thus stabilising the transient holes. These holes may also allow for the passage of peptide molecules from the outer membrane into the inner membrane of, for example Gram-negative bacteria, in a process referred to as “self-promoting uptake” [47].



*Figure 1.4* In the carpet-like model (left panel) cationic peptides bind the negatively charged phospholipid headgroups of the membrane and form an  $\alpha$ -helix (step A). When a threshold concentration of peptide monomers is reached, the membrane folds in on itself forming a transient pore (described by a toroidal model) (step B). Once a maximum concentration is reached, the membrane solubilises because of micellisation causing cell lysis (step C). In the barrel-stave model (right panel) peptide monomers bind the lipid bilayer as  $\alpha$ -helices due to hydrophobic interaction (step A). Monomer recognition and assembly, even at low surface density of bound peptide, then takes place. Insertion of at least two assembled monomers into the membrane initiates pore formation (step B). Progressive recruitment of monomers increases the pore size [41].

#### 1.6.4 *Classifying the mechanism of action of the magainins*

There is a great deal of literature describing various aspects of magainin's mechanism of action. The majority of results show magainin to permeabilise membranes via a carpet-like mechanism. The following results support this theory.

The conformation of magainin 2 in water has shown to be random, but on addition of trifluoroethanol an  $\alpha$ -helix is induced [48]. Raman and Fourier transform infrared spectroscopy results were also consistent formation of an  $\alpha$ -helix upon binding to lipids, as long as the lipids were negatively charged [49]. Furthermore, Raman, fluorescence, and

differential scanning calorimetry measurements all indicated that at high and moderate lipid to peptide ratios, magainin was orientated parallel to the membrane surface and did not significantly disturb the aliphatic chain region [41]. Orientated circular dichroism (OCD) measurements, however, showed that peptide orientation depended on the amount of peptide bound to the membrane [45]. At low peptide concentrations (expressed as the peptide to lipid molar ratio,  $P/L$ ), the helices were seen to lie parallel to the membrane surface. This has also been shown with NMR [39]. However, for  $P/L$  above  $\sim 1/30$  a substantial fraction of the bound peptide reoriented itself perpendicularly to the plane of the lipid bilayer [45]. Therefore, at low peptide concentrations it can be concluded that magainin imbeds itself in the head groups of negatively charged acidic lipids and lies parallel to the membrane surface. At higher peptide concentrations, neutron in-plane scattering showed that a substantial fraction of the peptide reoriented itself perpendicularly to the membrane [41]. Further, neutron in-plane scattering experiments, comparing pore formation of alamethicin and magainin 2, indicated that magainin 2 formed transient holes which could only be explained by a toroidal type model (see section 1.6.3.2) [45, 50]. These experiments therefore indicate that at high peptide concentrations, magainin orientates itself perpendicularly to the membrane, forming a toroidal channel or pore in the membrane. Occasionally pores were observed at lower concentrations because of fluctuation phenomena—they were, however, unstable and short-lived [51]. It can be assumed that these pores are similar to transient holes observed at higher peptide concentrations. These transient holes would require a lateral expansion in the head group region of the bilayer. Using lamellar X-ray diffraction experiments at concentrations below the critical concentration for lysis, the adsorption of magainin 2 in the headgroup region was noted to expand the membrane laterally, inducing membrane thinning [52]. When a pore is closed the participating peptide monomers remain adsorbed to the headgroup region and would therefore be expected to surface on either side of the membrane [46]. This was supported by experiments showing that the presence of peptide dramatically accelerated the flip-flop half-lives of fluorescent lipids [53]. Finally, at high enough concentrations, these amphipathic  $\alpha$ -helical peptides may cause large-scale membrane permeabilisation due to micellisation (Step C in left panel of fig. 1.4).

Certain aspects of the above mentioned research do not distinguish conclusively between the barrel-stave model and the carpet mechanism regarding the mechanism of action of magainin 2. For instance, in both models it would be expected to find the peptides in an

$\alpha$ -helical conformation, and orientated perpendicularly when interacting with the membrane at high concentrations. On the other hand, aspects like limited interaction of the peptide with the aliphatic chain region, thinning of the membrane, and peptide translocation can all only be described by toroidal holes in the membrane, which occur subsequent to membrane carpeting by the peptide. Although this research supports membrane lysis *via* a carpet-like mechanism, it is still not clear whether disruption of the membrane potential due to transmembrane holes or micellisation is the cause of cell death. It is also possible that cell lysis is a combination of the two, with transmembrane holes initially having a cytostatic effect, and micellisation ultimately causing cell lysis. The formation of transmembrane pores by magainin 2 makes this peptide ideal for use in conjunction with a conventional antibiotic on cells resistant to the antibiotic owing to active efflux pumps. It is postulated that the transmembrane pores will provide an additional means of entry into the cell for the conventional antibiotic, and in this way overcome the active efflux pumps.

### ***1.6.5 The effect of alterations to the amino acid sequence on the activity of magainin***

Numerous attempts have been made to improve the activity of magainin so as to enhance its prospects as an antibiotic. With the primary and secondary structure of magainin well documented, numerous experiments have been performed whereby alterations are made to the primary structure so as to enhance the antimicrobial activity of the peptide.

#### **1.6.5.1 The effect of changing hydrophobicity on the activity of magainin**

The hypothesis that increased hydrophobicity would result in an increased attraction between the peptide and the bacterial membrane was tested in the following way. Four peptides were synthesised where up to four amino acids were substituted under the following guidelines: (1) the four glycine residues of the peptide chain were not replaced because substitution of glycine by most other amino acids is expected to enhance helical propensity; (2) the number and position of the charged amino acids were maintained to avoid modifications of the electrostatic interaction properties; (3) if possible, hydrophobic amino acids were substituted by hydrophilic ones, and hydrophilic residues were replaced by hydrophobic amino acids—the angles of the hydrophobic and hydrophilic helix domains remained constant; and, (4) the

hydrophobic moments of the peptides were preserved by simultaneous amino acid substitutions in the hydrophobic and hydrophilic cores of the amphiphilic helix [54]. The results of this experiment showed that with an increase in hydrophobicity there was a definite increase in specificity for a specific target organism. Haemolytic activity, however, increased with the same factor as the antimicrobial activity. It has been suggested that the prokaryotic specificity of the magainins is the result of the difference in the lipid composition of eukaryotic and prokaryotic membranes [55]. Bacterial membranes are rich in negatively charged lipids such as phosphatidylglycerol and lipopolysaccharide, while the outer leaflet of eukaryotic cell membranes consists predominantly of zwitterionic lipids such as phosphatidylcholine and sphingomyelin [56]. Eukaryotic cell membranes also contain cholesterol while bacterial membranes do not. It is on this basis of these differences that magainins have a high specificity for prokaryotic cell membranes under normal conditions. However, once the hydrophobicity of the peptide was increased, the specificity of the peptide diminished and the sheer hydrophobic strength of the peptide caused it to bind and lyse prokaryotic as well as eukaryotic cell membranes.

In other experiments, researchers increased the hydrophobicity of the magainin analogue, ESF1, by substituting the arginine residue in position seven for a glycine residue. This modification increased the antimicrobial activity of the peptide without affecting haemolysis [57]. Besides the increased attraction between the peptide and the bacterial membrane due to the increased hydrophobicity, increased activity was also related to stronger  $\alpha$ -helix formation in buffer [57].

#### **1.6.5.2 The effect of changing peptide charge on the activity of magainin**

A series of magainin analogues were synthesised with the number of positive charges varying from zero to six (peptides termed  $MG^0$ – $MG^{6+}$ ) [58]. In testing the activity of these peptides it was noted that the affinity of the peptide for the negatively charged membrane correlated with an increase in positive charge of the peptide. In spite of the six positive charges of  $MG^{6+}$  it showed even less lytic activity than  $MG^{2+}$ , suggesting that an increased positive charge perhaps destabilised pore formation [58]. Investigation of pore lifetimes showed them to be in the order  $MG^{2+} \geq MG^{4+} \gg MG^{6+}$ . Therefore, an increase in positive charges enhanced the binding properties of the peptide, but shortened the pore lifetime. The charge distribution of

wild-type magainin 2 seems therefore optimally designed to maximise the lytic activity as well as pore lifetime.

### **1.6.5.3 Augmentation antibacterial activity of magainin by positive-charge chain extension**

Novel magainin 2 analogues were developed by the extension of its chain through the addition of segments of positively charged amino acids to either its N or its C terminus and therefore increasing its helicity [59]. The activity of magainin 2 towards *E. coli*, *P. aeruginosa*, and *S. aureus* was considerably enhanced by these modifications. Its haemolytic activity was only moderately affected.

### **1.6.5.4 Magainin hybrid peptides**

In order to obtain peptides with improved antimicrobial activities and low haemolytic effects, researchers utilised certain properties of separate peptides to develop hybrid peptides. Peptides used in the development of hybrid peptides share the common structural feature of amphipathic  $\alpha$ -helical conformations. One such peptide is the cecropin A-magainin 2 hybrid peptide. Cecropin A (isolated from the haemolymph of *H. cecropia* pupae) is composed of three different regions: an N-terminal end possessing positively charged lysine residues, a central hinge area formed by a Gly-Pro sequence, and a C-terminal hydrophobic stretch [25]. Therefore, cecropin A adopts an  $\alpha$ -helix-hinge- $\alpha$ -helix conformation in a non-polar environment. Because the N-terminal sequence 1 to 12 of magainin 2 is homologous to cecropin A (1-12), a novel hybrid was developed by linking cecropin A (1-8) and magainin 2 (1-12) [60]. The hybrid peptide had no haemolytic activity at a concentration of 100  $\mu\text{g/mL}$ . When compared to the hybrid peptide cecropin A-melittin, it showed similar antimicrobial activity against *B. subtilis*, but showed a two-fold increase in activity against *E. coli* [60]. Further studies have shown the cecropin A-magainin 2 hybrid peptide to possess potent antimalarial and antitumor activities [61].

## **1.6.6 Magainin as a synergistic agent**

### **1.6.6.1 Functional synergism of the magainins PGLa and magainin 2**

Theories regarding the mechanism of action of these peptides suggest that a peptide oligomer is responsible for membrane potential dissipation. The possibility that complexes of a number of magainin 2 molecules, or a number of PGLa molecules, are responsible for the action of these peptides, suggests that mixed complexes of magainin 2 and PGLa may also form and disrupt the membrane potential [62]. In nature *X. laevis* secretes a number of peptides from its dermal glands as protection against harmful organisms, and because of the sheer number of harmful organisms it seems logical that a degree of synergism exists between some of these peptides. Studies using *E. coli* showed that the combination of PGLa and magainin 2 had a much stronger activity than either peptide alone [62]. Synergism was also observed between PGLa and magainin 1. Synergism, however, was not a common occurrence between membrane active peptides, and not all magainin combinations exhibited synergism [62].

Using fluorescence experiments to elucidate the mechanism of synergism between these two peptides, researchers found that both peptides form a stoichiometric 1:1 complex in the membrane phase [63]. Single amino acid mutations in magainin 2 significantly altered the synergistic activity, suggesting that precise molecular recognition is involved in complex formation.

### **1.6.6.2 $\beta$ -Lactam antibiotics potentiate magainin 2 antimicrobial activity**

Magainin 2 was examined against *E. coli* grown with and without antibiotics. Bacteria grown with sub-lethal concentrations of cefepime, a  $\beta$ -Lactam antibiotic, were more susceptible to the antimicrobial activity of magainin 2 both in buffer and in serum [64]. In addition, in a mouse model of *E. coli* infection, magainin 2 was completely inactive as a therapeutic agent alone. However, it significantly increased the survival of mice when it was administered with sub-lethal concentrations of cefepime for the treatment of this systemic bacterial infection [64]. It has been shown that a variety of cationic peptides are inhibited by divalent cations [65]. In contrast, the antagonistic effect of divalent cations on the antimicrobial activity of magainin 2 was significantly lessened when cefepime treated bacteria were examined. It has been proposed that the site of divalent cation inhibition of other cationic agents is located in

the outer membrane [65]. If this is correct, then it is possible that  $\beta$ -lactam antibiotics alter existing or create new sites in the outer membrane for peptide entry that are less susceptible to cation inhibition. The combination of antibiotics and antimicrobial peptides could therefore be a feasible approach to eradicating antibiotic-injured bacteria.

## ***1.6.7 Magainin as a therapeutic agent***

### **1.6.7.1 Spectrum of tested therapeutic activity**

The broad spectrum of activity of magainin has been recognised by researchers and used in a diversity of fields as a potential therapeutic agent. Experiments testing the activity of numerous peptides against herpes simplex virus types 1 and 2, showed that magainin exhibited no notable activity [66]. Magainin peptides were, however, successful in treating melanomas established in athymic nude mice, where melanomas disappeared in six out of nine animals tested [67]. The selectivity of magainins for these tumour cells is thought to be the result of the relatively large amount of anionic substances such as phosphatidyl serine in the tumor cells. These peptides have furthermore shown antitumour activity against ovarian and lung cancer cell lines [68, 69]. The magainin peptides also exhibited potential as food preservatives, showing significant antimicrobial activity against a variety of foodborne pathogens [70]. In other studies magainin was successfully tested as a potential contraceptive agent. Experiments showed that when magainin A was applied intravaginally to rats before mating on the proestrusestrus day, complete arrest of sperm motility with 200  $\mu\text{g}$  concentration was observed, and none of the treated females conceived after treatment [71]. Furthermore, magainin A had the advantage over current spermicides in that it had no irritating effects on the vagina and provided additional protection against acquired infections caused during sexual intercourse [71].

### **1.6.7.2 Magainins as broad-spectrum topical and systemically administered antibiotic agents**

MSI-78 is a 22 amino acid analogue of magainin 2, which evolved through extensive structure-activity studies of the natural peptide. This molecule exhibits potent antibiotic activity against a wide variety of Gram-positive, Gram-negative, anaerobic, and fungal pathogens [72]. In October 1992, MSI-78, formulated as a topical preparation, entered Phase I

clinical trials, a phase of evaluation in which human safety is established. A good safety profile emerged and in February 1993 Phase II studies were initiated. These studies were designed to determine whether the formulated preparation of MSI-78 could effectively reduce dense bacterial flora indigenous to the perineal area from healthy human volunteers. In a study involving 45 subjects, application of the peptide at concentrations ranging from 0.5% to 2.0% significantly reduced all species of bacteria and yeast by several orders of magnitude [72]. The antibacterial effect of a single application persisted through 24 hours. On the basis of these findings, MSI-78 entered Phase IIb/III clinical trials in June 1993, to be evaluated as a topical preparation for the treatment of impetigo, a serious skin infection commonly seen in children. Current available information only dates to March 18, 1997, which announced the successful results of the second, pivotal Phase III clinical trials of MSI-78 for the treatment of infection in diabetic foot ulcers [73].

Many researchers have also started to explore antimicrobial peptides as systemically administered agents. In these experiments, bacterial infections are introduced in mice, which are then subsequently treated with intravenously administered antibiotics. Several antibiotic peptides did in fact exhibit activity in the *in vivo* infection model [72]. The development of peptide antibiotics as systemic therapeutics will naturally lag behind their development as topical agents because of the extensive evaluation of toxicity required for this human application.

## 1.7 Discussion

Multidrug resistance has clearly become a global crisis, and new and unique methods will be imperative in combatting this problem. One of the most ingenious mechanisms of resistance utilised by resistant bacteria is the general mechanism of an outer membrane barrier functioning synergistically with an active efflux pump. To overcome this form of resistance it would be necessary to remove the functional synergism that exists between these two components.

We propose to use the antimicrobial peptide magainin 2 in conjunction with a conventional antibiotic, tetracycline, against tetracycline resistant *E. coli* in attempt to overcome the tetracycline resistance that is incurred through active efflux pumps. The mode of action, broad spectrum of activity, structural versatility, and therapeutic qualities make the magainin

peptides ideal candidates for this study. The synergistic qualities of magainin with other peptides and antibiotics has already been described. Of major interest was the ability of  $\beta$ -lactam antibiotics to potentiate the antimicrobial activity of magainin 2 (section 1.6.6.2). Synergy between these two antibiotic agents was hypothesised to result from the permeabilising effect of the peptide, which disturbed the integrity of the bacterial outer membrane and facilitated entry of the antibiotic into the bacterial cell [72]. Therefore, we intend to use the same synergistic approach on bacteria that expel the antibiotic as a mechanism of resistance. It is hoped that the permeabilising effect of magainin 2 on the bacterial outer membrane will facilitate the entry of tetracycline into the cell to such a degree that the active efflux pumps will be neutralised and the bacteria will once again become susceptible to the tetracycline.

The following chapters shall describe the synthesis of the antimicrobial peptides (Chapter 2), the development and evaluation of an antimicrobial assay (Chapter 3), and the investigation of the synergy between the antimicrobial peptides and a conventional antibiotic on resistant organisms (Chapter 4). Finally, general implications and future prospects of this work are discussed in Chapter 5.

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## Chapter 2

### *Synthesis and purification of magainin 2 and N-terminal deletion analogues*

#### 2.1 Introduction

##### 2.1.1 *Solid phase peptide synthesis*

The synthesis of peptides has been a challenge to organic chemists since the turn of the century. Numerous breakthroughs have been made this century, with the most notable and earliest being the synthesis of the lactogenic nonapeptide amide hormone oxytocin by Du Vigenaud and his co-workers in the early 1950s, initiating a new era in both biology and chemistry [1]. New biologically active compounds were being isolated regularly, requiring new and improved methods for their synthesis. At this stage the classical method of solution phase peptide synthesis was hard pressed to meet the explosive increase in demand. Although successful, this method of synthesis provided only modest yields owing to a high rate of side chain modifications. It was R.B. Merrifield who subsequently developed solid phase peptide synthesis (SPPS) in 1959 [2], which has since proven to be the method of choice for synthesising peptides and small proteins.

Since the conception of SPPS, the chemistry developed by Merrifield has remained fairly standardised. The protocol uses a chemical scheme based on graduate acidolysis [3]. The Merrifield peptide synthesis proceeds as follows:

1. Covalent attachment of an *N*- $\alpha$ -*tert*-butyloxycarbonyl (tBoc) amino acid to functionalised crosslinked polystyrene resin beads. The tBoc group protects the N-terminus of the amino acid being attached, so as to prevent polymerisation;

2. Removal of the acid labile tBoc group with trifluoroacetic acid (TFA) and neutralisation of the free amino terminus with triethylamine (TEA);
3. Coupling the second tBoc-amino acid to the free amino terminus of the resin bound amino acid *via* a carbodiimide activating agent. Throughout this procedure, side-chain protection of the coupled amino acids is conferred by ether, ester and urethane derivatives based on benzyl alcohol with electron-withdrawing halogens for greater acid stability. Other side-chain protection schemes are based on ether and ester derivatives of cyclopentyl and cyclohexyl alcohol;
4. Cleavage of the peptide and removal of the side-chain protection groups is carried out with hydrogen fluoride (HF) in the presence of suitable scavengers that trap reactive carbonium ions and thereby prevent them from undergoing deleterious side-reactions with sensitive amino acids [4].

Although successful in synthesising peptides of high purity, repetitive TFA acidolysis, required for the deprotection of the tBoc amino blocking group, often leads to the alteration of sensitive peptide bonds as well as acid catalysed side-reactions [4]. Furthermore, HF used for cleavage of the peptide from the resin is not only costly, but also extremely dangerous, and requires the use of expensive protective laboratory apparatus.

It was from these concerns that fluoronylmethoxycarbonyl (Fmoc) chemistry was developed by Atherton, Sheppard and Dryland [5]. This protocol substituted the acid-labile tBoc-amino protective group, used in the Merrifield synthesis protocol, with a base-labile N<sup>9</sup>-Fmoc-group, which required only mild basic removal conditions. This base labile Fmoc-group could rapidly be removed in a  $\beta$ -elimination reaction brought about by secondary amines such as piperidine, which also scavenged the dibenzofulvene intermediate, preventing back-addition to the peptide chain. Peptide synthesis according to the Fmoc protocol proceeds as follows:

1. Covalent attachment of an Fmoc-amino acid to a rigid solid phase consisting of functionalised dimethylacrylamide polymerised within Kieselguhr (marketed as Pepsyn K or Novasyn <sup>®</sup>K);
2. Removal of the base labile Fmoc group using piperidine;

3. Coupling of the second amino acid to the resin bound amino acid after activation. Activation can take place in a number of ways: First, activation can take place by the formation of an active anhydride. Carbodiimides such as dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIPICIDI) are often used in this type of *in situ* activation. During the coupling reaction 1-hydroxybenzotriazole (HOBt) can be used to prevent racemisation and dehydration of the acid amide side-chains of asparagine and glutamine. Second, pre-activated amino acid esters can be used. Examples of active esters include p-nitrophenyl [6], 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt) [4, 6], and pentafluorophenyl (Pfp) esters [4, 6, 7]. Amino acid esters are generally used with HOBt as acylation catalyst [4, 8]. Third, another type of *in situ* activation can be performed using benzotriazol-1-yl-oxy-tris-dimethylaminophosphonium hexafluorophosphate (BOP) [9, 10], or its lesser toxic analogue, benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP<sup>®</sup>). These reagents react with the *N*<sup>α</sup>-protected amino acids in the presence of a base to form highly active oxybenzotriazolyl esters [8]. During coupling, side-chain protection is conferred by tertiary butanol based ether, ester and urethane derivatives.
4. Cleavage of the peptide, and removal of the side-chain protection groups, is carried out with TFA in the presence of suitable scavengers that trap reactive carbonium ions and thereby prevent them from undergoing deleterious side-reactions with sensitive amino acids [4].

The Fmoc-polyamide protocol was used for synthesis purposes because of its milder chemical approach, and because it is a well-established method in our laboratory.

### **2.1.2 Synthetic antimicrobial peptides**

Synthetic antimicrobial peptides have played a major role in developing the antibiotic potential of antimicrobial peptides [11, 12, 13]. A vast amount of information regarding the structure-function of these peptides has been deduced from synthetic antimicrobial peptide analogues and deletion peptides. Because of its unique and well documented mechanism of action, as well as its broad-spectrum of activity and lack of toxicity, magainin 2 (Table 2.1) was synthesised for future research (see Chapter 1). Two N-terminal deletion analogues (Table 2.1) were also synthesised. The first of the deletion peptides differed from the full

length peptide by the omission of two amino acid residues from the N-terminal of magainin 2, while the second deletion peptide had four residues omitted from the N-terminal (termed magainin 2 N<sup>21</sup>, and magainin 2 N<sup>19</sup> respectively). The N-terminal deletion analogues were synthesised to determine the importance of the N-terminal of the peptide with regards to antimicrobial and membrane activity. Two syntheses were performed, with the second synthesis being an optimisation of the overall synthesis and purification procedure of the peptides.

*Table 2.1* Primary structures of magainin 2 and the two N-terminal deletion peptides.

Peptide	Amino acid sequence	M <sub>r</sub>
magainin 2	G I G K F L H S A K K F G K A F V G E I M N S	2466.93
magainin 2 N <sup>21</sup>	G K F L H S A K K F G K A F V G E I M N S	2296.72
magainin 2 N <sup>19</sup>	F L H S A K K F G K A F V G E I M N S	2111.49

## 2.2 Materials

### 2.2.1 General reagents and solvents

*N, N'*-dimethylformamide (DMF; 99.5%), potassium hydroxide (KOH), sodium carbonate (anhydrous) and self-indicating silica gel were from Saarchem (Krugersdorp, South Africa). Trifluoroacetic acid (TFA; >98% and 99.5%), glacial acetic acid, chloroform (>99%), diethyl ether (99.5%), ethanol (99.8%), butan-1-ol (99.5%), pyridine (99.5%), n-hexane (99.8%), potassium cyanide (KCN), *di*-phosphorous-pentoxide, 2',7'-dichlorofluorescein, ninhydrin, molecular sieve (3Å), aluminium oxide 90 and Kieselgel 60-F<sub>254</sub> thin layer plates were from Merck (Darmstadt, Germany). Piperidine (98%), ethyl acetate (99.8%), 1,4-dioxan (99.8%), and 2-methylbutan-2-ol (t-amyl alcohol; 98%) were from BDH Chemicals (Poole, UK). TFA (>98%) and piperidine (98%) were also purchased from Sigma Chemicals Co. (St. Louis, USA). Fluka Chemicals (Buchs, Switzerland) supplied 1-fluoro-2,4-dinitrobenzene (FDNB),

1,2-ethanedithiol (>98%), and thioanisole (>99%). High purity dry nitrogen gas was from Afrox (South Africa).

### **2.2.2 Reagents for peptide synthesis**

Pepsyn KA resin (0.2 meq/g), Fmoc-L-Ser(tBu)-Odhbt, Fmoc-L-Asn-OPfp, Fmoc-L-Asn-OH, Fmoc-L-Ile-OPfp, Fmoc-L-Met-OPfp, Fmoc-L-Glu(OtBu)-OPfp, Fmoc-L-Gly-OPfp, Fmoc-L-Val-OH, Fmoc-L-Phe-OPfp, Fmoc-L-Ala-OPfp, Fmoc-L-Lys(Boc)-OH, Fmoc-L-His(Boc)-OPfp were from Milligen-Millipore (Milford, USA). Fmoc-L-Ser(tBu)-OH, Fmoc-L-Leu-OH and pentafluorophenol were from Sigma Chemical Co. (St. Louis, USA). Benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP<sup>®</sup>) was from Calbiochem-Novabiochem Co. (La Jolla, USA). N, N'-dicyclohexylcarbodiimide (DCC) was from Merck (Darmstadt, Germany). Fluka Chemicals (Buchs, Switzerland) supplied 1-hydroxybenzotriazole (HOBt) and N, N'-diisopropylethyl amine (DIPEA).

### **2.2.3 Reagents and solvents for amino acid analysis and chromatography**

Constant boiling hydrochloric acid (HCl, 30%), sodium acetate (99.5%) and phenol (99.5%) were from Merck (Darmstadt, Germany). Phenylisothiocyanate (PITC) and amino acid standards were from Pierce Chemicals (Rockford, USA). Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA, >98%) was from Fluka Chemicals (Buchs, Switzerland). Methanol (HPLC-grade, UV cut-off 205nm) and acetonitrile (HPLC-grade, UV cut-off 190 nm) were from Romil LTD (Cambridge, UK). Pico-Tag<sup>®</sup> sample diluent, Nova-Pak C<sub>18</sub> analytical HPLC columns, 0.45 mikron HV membrane filters were from Waters-Millipore (Milford, USA). Polygosil (C<sub>18</sub>, 60 Å, irregular particle size) packing material, used to prepare the semi-preparative HPLC column, was from Macherey-Nagel (Düren, Germany). High quality triethylamine (TEA) was from Aldrich Chemical Co. (Gillingham, UK). CM Sepharose CL-6B cation exchange resin was from Sigma Chemical Co. (St. Louis, USA). Sephadex G-10 resin was from Pharmacia Fine Chemicals (Uppsala, Sweden). Analytical grade water was prepared by filtering glass distilled water through a Millipore Milli Q<sup>®</sup> water purification system.

### 2.2.4 *Drying and storage of reagents and products*

Fmoc-amino acids and peptide synthesis resins were stored at 4°C in desiccators with silica gel as drying agent. The FDNB was stored at <10°C, while PYBOP® and HOBt were stored at -20°C, with silica gel as drying agent. Before use, amino acid derivatives, resin, PYBOP®, and HOBt were dried overnight under high vacuum, with *di*-phosphorous-pentoxide as drying agent. The DMF was stored on molecular sieve (3Å) at room temperature in amber bottles. PITC, DIPEA and amino acid standards were stored at -20°C, while TEA (high quality for amino acid analysis) was stored at <10°C, all under N<sub>2</sub>-atmosphere. Lyophilised peptides were stored under vacuum in a desiccator with silica gel as drying agent at 4°C. All other reagents were stored at room temperature.

Detergents, such as regular cleaning agents, can influence the activity of antimicrobial peptides. Therefore, glassware returned from the initial wash, was rinsed three times with distilled water, three times with 60% ethanol, three times with analytical grade water, and finally placed in an oven to dry at temperatures ranging between 110 and 140°C. Vials used in peptide synthesis were pyrolysed at 565- 570°C.

## 2.3 Methods

### 2.3.1 *Preparation of solvents*

#### 2.3.1.1 *Distillation of N, N'-dimethylformamide*

It is important that freshly distilled, highly pure DMF, which is the principal solvent used in Fmoc-polyamide solid phase peptide synthesis, be used at all times. Impure DMF can be contaminated with secondary amines and H<sub>2</sub>O, which in turn cause the loss of the Fmoc- and active ester groups of the peptide. DMF reacts with water to form dimethylamine and formic acid [14, 15].

Undistilled DMF was shaken up with 10-20 g/L dry potassium hydroxide pellets to remove residual water, or the DMF was stored for at least one week on activated molecular sieve (3Å). Impurities such as volatile secondary amines were removed by fractional distillation under vacuum (high vacuum pump, 5-10 mm Hg) and a dry nitrogen bleed. The first 10-15%

of the distillate was discarded and the constant boiling fraction (45°C at 10 mm Hg) was collected. Only DMF which passed Sanger's test for amines was used (see below), otherwise it was redistilled.

### 2.3.1.2 Sanger's test for amines

Secondary amines present in DMF can cleave the base labile Fmoc-group from the N-terminus of the peptide. Sanger's test for amines was used to determine the quality of the DMF prior to use [16]. It was performed by mixing equal volumes FDNB (1.0 mg/mL in 95% ethanol) and DMF, incubating the mixture at room temperature for 30 minutes, and then determining the absorbance of the reaction mixture at 381 nm. A 0.5 mg/mL FDNB in 95% ethanol solution was used as blank. An acceptable blank's absorbance was in the region of 0.2 absorbance units. For synthesis purposes, DMF with an absorbance of between 0.025 and 0.05 units higher than the blank was used in the coupling procedure as well as the washing steps prior to the coupling procedure. Lower quality DMF with an absorbance of between 0.05 and 0.08 units higher than the blank was used for the other washing steps. To ensure the removal of all traces of piperidine (used in the deblocking steps during the peptide synthesis protocol), the Sanger test was performed on DMF samples from the final washing step. The samples were in most cases only incubated for 2 to 3 minutes and compared to a blank solution. Clean samples were deemed to be no more than 0.01 units higher than the blank solution.

### 2.3.1.3 Distillation of piperidine and pyridine

For the same reasons as DMF, piperidine was required to be of high purity. Piperidine was distilled over dry potassium hydroxide (10 to 20 g/L) and dry nitrogen bleed at atmospheric pressure. The first 10-15% of the distillate was discarded and the constant boiling fraction (105°C) was collected. The distilled piperidine was stored in dark bottles and used within three months [14]. Pyridine for use in Kaiser-ninhydrin test was purified in the same manner.

### 2.3.1.4 Distillation of *N, N'*-diisopropylethyl amine

*N, N'*-diisopropylethyl amine was initially distilled over ninhydrin (1-2 g/L) and then redistilled over dry potassium hydroxide pellets (5-10 g/L). Distillation was performed at atmospheric pressure under a dry nitrogen bleed. A small forerun was discarded, and the fraction with boiling point between 125-127°C was collected. Small volumes (4 mL) were

aliquoted in amber vials, flushed with dry N<sub>2</sub>, sealed tightly and stored at -20°C. Only small volumes were distilled because of the instability of the mixture [6].

### **2.3.2 Preparation and quality control of the amino acid derivatives**

#### **2.3.2.1 Thin layer chromatography (TLC)**

Thin layer chromatography was performed on aluminium backed Kieselguhr 60-F<sub>254</sub> TLC-plates. A mobile phase containing chloroform:methanol:acetic acid (85:10:5) was used for the Fmoc amino acids, and chloroform:methanol (85:10) was used for the Fmoc amino acid Opfp esters. Acetic acid was not used with Fmoc amino acid Opfp esters as it cleaved the Opfp group [17]. Initially the plates were visualised under UV-light (268 nm). Once dry they were sprayed with 0.2% ninhydrin in 95% ethanol and developed at 110°C for 10 minutes to detect residual amino groups. Derivatives were used for synthesis if they exhibited a single spot with the correct R<sub>f</sub> value.

#### **2.3.2.2 Melting point determination**

Before a synthesis the melting points of the derivatives and catalysts (PyBOP<sup>®</sup>, HOBt) were determined. A small amount of derivative was placed in the bottom of a capillary tube and the melting range was determined using a Gallenkamp melting point apparatus. Amino acid derivatives used for synthesis had to be within 5°C of their literature values [18].

#### **2.3.2.3 Purification of Fmoc amino acid derivatives**

Kaiser positive samples (section 2.4.3.4), or samples with an incorrect melting point were purified and re-evaluated prior to a synthesis. Fmoc-amino acids without acid labile side chain protection were suspended in 1% acetic acid (or 0.1% acetic acid if an acid labile side chain protection group is present) and the precipitate collected by filtration. The precipitate was then washed with water, ice-cold methanol, ethyl acetate, and diethyl ether and dried thoroughly under vacuum. If the Kaiser test remained positive, the derivative was recrystallised from hexane/ethyl acetate. The Pfp esters were recrystallised from hexane and/or washed with ice-cold ethyl acetate and n-hexane.

#### 2.3.2.4 Synthesis of Fmoc amino acid pentafluorophenyl esters

The pre-activation of Fmoc-Asn-OH and Fmoc-Gln-OH to form their pentafluorophenyl (Pfp) ester was necessary, because other activation procedures, such as with PyBOP<sup>®</sup> and DCC can lead to the formation of aspartimides and glutamides [4]. A method described by Atherton *et al* [6], and adapted by Rautenbach [15] was used for the synthesis of Fmoc-Asn-OPfp. Prior to the synthesis, the Fmoc-L-Asn-OH showed a positive Kaiser test, and was therefore washed as described in section 2.4.2.3. A 96% yield of the derivative was obtained after washing, and a negative Kaiser test indicated that the majority of the impurities had been removed. The synthesis then proceeded as follows: Fmoc-L-Asn-OH (3.42 g; 9.6 mmole) was dissolved in 13 mL dioxan, and the pentafluorophenol (2.09 g; 11 mmole) in 4 mL dioxan. The two were combined and allowed to cool in an ice-bath. Dicyclohexylcarbodiimide (2.18 g; 11 mmole) in 8 mL dioxan was added drop-wise to the stirring slurry. After reacting for 75 minutes, the white creamy slurry was further diluted with 27 mL dioxan. The dilute slurry was then allowed to react for a further 23 hours at room temperature. The solution was filtered to remove the white dicyclohexylurea precipitate. The precipitate was washed with 3x10 mL dioxan and the combined filtrate was evaporated under vacuum. The addition of n-hexane led to the formation of slightly yellow crystals, which were washed with ethyl acetate and methanol and dried under vacuum. Melting point determination (154-156°C) and TLC ( $R_f=0.7$ ) showed that the amino acid pentafluorophenyl ester had successfully been synthesised. A 52% yield of the derivative was obtained. Both the commercial and the synthesised product contained a small amount of Fmoc-L-Asn-OH.

#### 2.3.3 Synthesis of the peptides

The peptides were synthesised at room temperature (20-25°C) according to the Fmoc-polyamide protocol using a shake flask procedure [15]. Two syntheses were done, with the second being an optimisation of the synthesis and purification procedures of magainin 2 and the deletion peptides. The protocol for a complete cycle of operations including washing, coupling, deblocking, and sampling steps is set out in Table 2.2.

### 2.3.3.1 Coupling of the first amino acid

Pepsyn KA-resin (capacity of 0.2 milli-equivalent per gram resin) was used in the syntheses, to which the first amino acid was coupled *via* the PyBOP<sup>®</sup> method (described hereafter). The coupling efficiency was evaluated using the Fmoc-test (section 2.4.3.5), and the coupling step was repeated until the resin was completely saturated. Prior to synthesis the resin was swollen for approximately 20 minutes in high purity DMF (20 mL/g).

### 2.3.3.2 Elongation of the peptide chain

For the elongation steps, the required reagent quantities were calculated from the resin capacity (0.2 milli-equivalent per gram of dry resin). Fmoc-amino acid OPfp esters were coupled with HOBt as catalyst and trapping agent [4, 8], each at a three fold molar excess. Fmoc-amino acids were coupled using the PyBOP<sup>®</sup> reagent in the following manner: a three fold molar excess of the protected amino acid and HOBt, were each dissolved in a minimum volume of DMF (0.5-1 mL), combined, and initially added to the resin. Following this, PyBOP<sup>®</sup>, at a 3 fold molar excess, was mixed with a 6 fold molar excess of DIPEA, both in a minimum volume DMF (0.5-1 mL), and the combination also added to the resin [15, 19]. Complete acylation varied from 60 to 90 minutes, as determined with the Kaiser test and/or the Fmoc test.

*Table 2.2* Operations for one cycle of the peptide synthesis procedure [Table reproduced with permission from 15].

Synthesis step	Time	Volume	Monitoring	Comment
1. Swelling of resin	20 min.	4 bed volumes		bedvolume = 5mL/gram resin
2. Anchoring of first amino acid <i>via</i> activated Fmoc-amino acid to resin	2 hours	<1/2 bed volume	Fmoc-test	Refer to sections 2.3.3.1 and 2.3.3.5
3. DMF wash		6 X 3 bed volumes	Absorbance at 310nm	Absorbance must be zero
4. Resin sample A			i. Kaiser test (2.3.3.4)  (ii. Amino acid analysis; 2.3.5.3)	i. If test not negative, extend or repeat coupling step in elongation.  ii. Amino acid analysis is used in monitoring anchoring step
5. Removal of Fmoc group (deblocking) with 20 % piperidine in DMF	30 min.	3 bed volumes	Absorbance at 290nm	Refer to sections 2.3.3.3 and 2.3.3.5
6. DMF wash		10X3 bed volumes		
7. DMF wash sample		0.5 ml	Sanger test	Wash until test is negative (2.3.1.2)
8. Resin sample B				i. If test not positive, repeat deblocking  ii. Post synthesis evaluation
9. Coupling of next amino acid <i>via</i> activated Fmoc-amino acid	60 min.	<1/2 bed volume		Refer to 2.3.3.2. Repeat steps 3 and 9 if necessary
10. Repeat steps 3 to 8				
11. Repeat steps 9 and 10 for $\alpha$ -amino acids in sequence				
12. Washing and drying of resin				Refer to 2.3.3.3
13. Liberation of peptide acid from resin				Refer to 2.3.3.3

### 2.3.3.3 Removal of the Fmoc-group from the attached amino acid

Once coupling was completed (as shown by a negative Kaiser test) the resin was washed thoroughly to remove the active amino acid derivative and catalyst. To remove the Fmoc-group from the coupled amino acid, three bedvolumes of piperidine (20% in DMF) was added to the resin for a period of 30 minutes. After each deblocking step, the absorbance of the piperidine mixture was recorded at 290 nm. Absorbance readings at 290 nm were compared to readings obtained from previous deblocking steps to show the extent of deblocking. On removal of the piperidine, the resin was washed thoroughly with DMF until an acceptable Sanger test was obtained (section 2.3.1.2).

### 2.3.3.4 The Kaiser test

The Kaiser test is specific for primary amino groups [20]. Three solutions make up the test: (a) 500 mg ninhydrin in 10 ml 95% ethanol, (b) 40 g phenol in 10 ml 95% ethanol and (c) 2 ml 0.001 M KCN solution diluted to 100 ml with distilled pyridine. The Kaiser test was used during peptide synthesis to evaluate the deblocking, as well as the coupling of an amino acid. The test proceeds as follows: a few resin beads (4 to 5) were dried using diethyl ether, after which three to five drops of each solution were added to the beads. The reaction mixture was then incubated for 5 minutes in a water bath at 80-90°C. The colour of the resin beads and reaction mixture was evaluated using a magnified eyepiece. A positive result, as expected after deblocking, usually entailed the beads becoming dark blue. A negative result, as expected after coupling, showed no change in the colour of the beads.

Quality control of the Fmoc-amino acid derivatives was also done using the Kaiser test. Five milligrams of the dried derivative was dissolved in 100  $\mu$ L DMF, after which 100  $\mu$ L of each of the Kaiser test solutions were added. Colours were evaluated after 5 minutes incubation at 90°C. To determine the extent of contamination of a derivative, its Kaiser test result was compared to a standardised colour chart.

### 2.3.3.5 The Fmoc-test

To evaluate the coupling of a Fmoc amino acid, and specifically the coupling of the first amino acid, the UV absorbance character of the liberated Fmoc-fulveen group was exploited [4, 21]. A test was devised whereby the concentration of Fmoc-groups released subsequent to

the deblocking step could be used to calculate the percentage coupling. This test proved to be very useful in replacing the other alternative, amino acid analysis, a time consuming procedure.

In developing the Fmoc-test a 0.1 mmole concentration of Fmoc-leucine was treated with 10 mL of 20% piperidine in DMF for 15 minutes to release the Fmoc-group. The 20% piperidine in DMF was removed, diluted a 100 times with DMF, and used in a dilution series (triplicate determinations per concentration value). The absorbance of sample in the dilution series was determined at 290 nm using DMF as a blank. The linear relationship between the percentage liberated Fmoc and absorbance at 290 nm was as follows:

$$A_{290} = 0.002848 \times \% \text{ liberated Fmoc-groups} - 0.009975$$

To evaluate a coupling step, 10-15 mg of resin was removed and dried for an hour. The resin was weighed analytically and then deblocked with a volume of 20% piperidine in DMF; equivalent to 20 mL per 0.1 mmole of expected Fmoc groups. After deblocking, a 100 times dilution (triplicate determinations) was made of the deblocking mixture and the absorbance determined at 290 nm. The absorbance values were entered into the previously determined standard curve equation and the percentage coupling determined.

### 2.3.3.6 Removal of the completed peptide from the resin

After the deprotection and DMF wash of the final amino acid, the resin was washed with *t*-amyl alcohol, glacial acetic acid, again *t*-amyl alcohol, and finally dried with diethyl ether [4, 6, 17]. For the first synthesis, the peptides were cleaved from the resin using 95% TFA, 5% phenol, and methionine (1 g/50 mL). The resin was treated with this cleavage cocktail for three hours<sup>1</sup> at room temperature with occasional stirring. For the second synthesis, the peptides were cleaved from the resin using reagent K (82.5% TFA, 5% phenol, 5% H<sub>2</sub>O, 5% thioanisol, and 2.5% 1,2-ethanedithiol) [22]. After treatment with the respective cleavage cocktails, the resin was removed by filtration and washed with TFA, glacial acetic acid, and analytical grade water. The resin was then washed with ether, dried and analysed for amino acids to determine the extent of peptide cleavage. The filtrate containing the peptide was

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<sup>1</sup> The reaction time depends mainly on the sequence of the peptide and the type of side chain protection [4].

concentrated under vacuum on a Buchi Rotavapor at 45°C. The peptide concentrate was dissolved in 50 mL analytical grade water and by-products were extracted three times with 50 mL diethyl ether. The combined ether fractions were then washed with 50 mL analytical grade water to recover any peptide in the ether. The combined aqueous fractions were freeze-dried.

### **2.3.4 Purification of the peptides**

#### **2.3.4.1 Gel permeation chromatography**

Peptides were initially purified by Sephadex G10 gel permeation chromatography using analytical grade water was used as eluant. Freeze-dried crude peptides were dissolved in an amount of water corresponding to 10% of the column bedvolume, their absorbance measured at 230 nm and 257 nm<sup>2</sup> and then loaded onto the Sephadex G10 column. Flow rates were 10 to 14 mL/hour, and the eluates were monitored at 230 nm or 257 nm. The major absorbing fractions were collected and freeze-dried.

#### **2.3.4.2 Cation exchange chromatography**

A CM Sepharose CL-6B column (25 × 1.5 cm) was used for further purification. A 0.35 - 0.45M ammonium acetate (pH 6.00) gradient successfully eluted the peptides and was therefore used for further purifications.

Before use, the column was regenerated by running a linear 0.35-0.45M ammonium acetate (pH 6.00) gradient, and then equilibrated with three bedvolumes of 0.35M ammonium acetate (pH 6.00). Freeze-dried cationic peptide preparations were dissolved in the equilibration buffer and then loaded onto the column. The flow rate was 14 mL/hour. Elution was started with the equilibration buffer, and after an hour (14 mL) a linear gradient was introduced, with the highest concentration ammonium acetate, 0.45 M (pH 6.00), as the final concentration. The eluted fractions were monitored at 230 nm and 257 nm and the major absorbing fractions were collected and freeze-dried.

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<sup>2</sup> Phenylalanine, an amino acid in the magainin 2 sequence, absorbs maximally at 257 nm.

### 2.3.4.3 High performance liquid chromatography

As peptides at a purity of >96% were required, semi-preparative HPLC was used as the final purification step. Peptide samples (5 mg/mL) were dissolved in 50% acetonitrile in analytical grade water. Prior to use, all samples were centrifuged for 5 minutes at -1000 g to remove undissolved particles. The HPLC system was comprised of the following components: two Waters 510 pumps, a Waters Model 440 detector (monitoring at 254 nm), a WISP 712 sample processor, a MAXIMA software controller system, and a semi-preparative C<sub>18</sub> Polygosil HPLC column (irregular particle size, 60 Å pore size, 250 mm x 10 mm). A linear gradient (3 mL/min flow rate) was created using eluant A (0.1% TFA) and eluant B (90% acetonitrile and 10% A) as shown in Table 2.3 [14, 17]. Peptide fractions were collected, and freeze-dried for further analysis.

Table 2.3 Gradient program used for the chromatography of the peptides.

Time (min)	Flow rate; preparative HPLC (mL/min)	Flow rate; analytical HPLC (mL/min)	% A	% B
0	3.0	1.0	80	20
1	3.0	1.0	80	20
13	3.0	1.0	0	100
14	3.0	1.0	0	100
20	3.0	1.0	80	20
25	3.0	1.0	80	20

## 2.3.5 Analysis of the purified peptides

### 2.3.5.1 Analytical high performance liquid chromatography

Analytical reverse phase high performance liquid chromatography was used to determine the purity of the synthetic peptides. A C<sub>18</sub> Nova-Pak HPLC column was used with the system described under section 2.3.4.3. The chromatography was monitored at 254 nm. The same

gradient was employed as for preparative HPLC, but the flow rate was reduced to 1.0 mL/minute (Table 2.3).

### 2.3.5.3 Amino acid analysis

Amino acid analyses were done using a pre-column derivatisation with phenylisothiocyanate (PITC), according to the Pico-Tag<sup>®</sup> method [23, 24]. Samples to be analysed were thoroughly dried under vacuum. Gaseous phase hydrolysis was done under a nitrogen atmosphere using 6 N HCl containing 1% phenol. Gaseous phase hydrolysis took place for 24 hours at 110°C. A shortened hydrolysis procedure (150°C, 1 hour) could also be used if necessary. Hydrolysed samples were dried under vacuum to remove all traces of the acid. Following this, the hydrolysed samples were “re-dried” by adding ethanol:water:TEA (1:2:1) (10  $\mu$ L) to each sample to obtain an optimal pH for derivatisation. The re-drying solution was removed under vacuum, and 20  $\mu$ L of a derivatisation solution containing methanol:TEA:water:PITC (7:1:1:1) was added to each of the samples. Samples were derivatised for 10-20 minutes at room temperature before being dried under vacuum. Dried samples were dissolved in an appropriate amount (200  $\mu$ L) of Pico-Tag<sup>®</sup> diluent and filtered through HV 0.45  $\mu$ m Millipore filters.

Samples were chromatographed at 44°C using a Nova-Pak C<sub>18</sub> HPLC column (150 mm x 3.9 mm) using the previously described HPLC system (section 2.3.4.3). Derivatised amino acids and a Pierce standard amino acid mixture (hydrolysis standard) were separated with a binary non-linear gradient using eluant A (0.14 M sodium acetate, 10 mM EDTA and 0.5 mL TEA per litre, titrated to pH 6.40 with acetic acid and then mixed with 6% acetonitrile) and eluant B (60% acetonitrile containing 10 mM EDTA) [23, 24]. The standard amino acid mixture (hydrolysis standard) was used to calibrate the analysis.

### 2.3.5.4 Mass spectrometry

A Micromass Quattro triple quadrupole mass spectrometer fitted with an electrospray ionisation source was used for electrospray ionisation mass spectrometry. Peptide samples were dissolved in 50% acetonitrile in water. Ten  $\mu$ L (2 ng peptide in 50% acetonitrile/water containing 0.05% TFA) of the sample was introduced into the ESI-MS at 20  $\mu$ L/minute, with applied capillary voltage of 3.5 kV using a Rheodyne injector valve. The cone voltage was 70 V with the skimmer lens offset at 5 V. The source temperature was 80°C. Data were acquired

in the positive mode for these cationic peptides, scanning the first analyser ( $MS_1$ ) through  $m/z$  between 200 and 1500 at a scan rate of 5 sec/scan atomic mass units/second ( $m/z$  is the molecular mass to charge ratio). Representative spectra were produced by combining the scans across the elution peak and subtracting the background.

## 2.4 Results and Discussion

### 2.4.1 *Synthesis 1 of magainin 2 and magainin 2 N<sup>21</sup>*

#### 2.4.1.1 Coupling of the first amino acid

The first amino acid ( $Ser_{23}$ ) is attached to the resin with an ester bond<sup>3</sup>. The PyBOP<sup>®</sup> method (section 2.4.3.2), with Fmoc-L-Ser(tBu)-Odhbt as active amino acid derivative, was used for this coupling. The initial coupling was evaluated after four and eight hours using the Fmoc-test (section 2.4.3.5). After four hours, 55% coupling was obtained, while after eight hours, 72% coupling was obtained. The coupling mixture was then removed and replaced with fresh mixture and allowed to couple for a further four hours. An evaluation of the coupling showed 85% coupling of serine to the resin, which was confirmed with amino acid analysis (results not shown). Further attempts at increasing the percentage coupling failed and it was assumed that the resin had a 0.17 milli-equivalents/gram resin.

#### 2.4.1.2 Elongation of the peptide chain

Following the initial coupling of  $Ser_{23}$  to the resin, the next amino acid in the sequence to be coupled was  $Asp_{22}$ . After coupling for 60 minutes the Kaiser test was still positive, which implied that coupling was not yet complete. Amino acid analysis showed that only 62% coupling had taken place. The coupling reaction was repeated and allowed to proceed for four hours. The Kaiser test, however, remained positive, and amino acid analysis showed that no further coupling had taken place. At this stage we thought that owing to the prolonged

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<sup>3</sup> Coupling the first amino acid is often a difficult process which, unless carried out under controlled conditions, can lead to low substitution, racemisation or dipeptide formation [4].

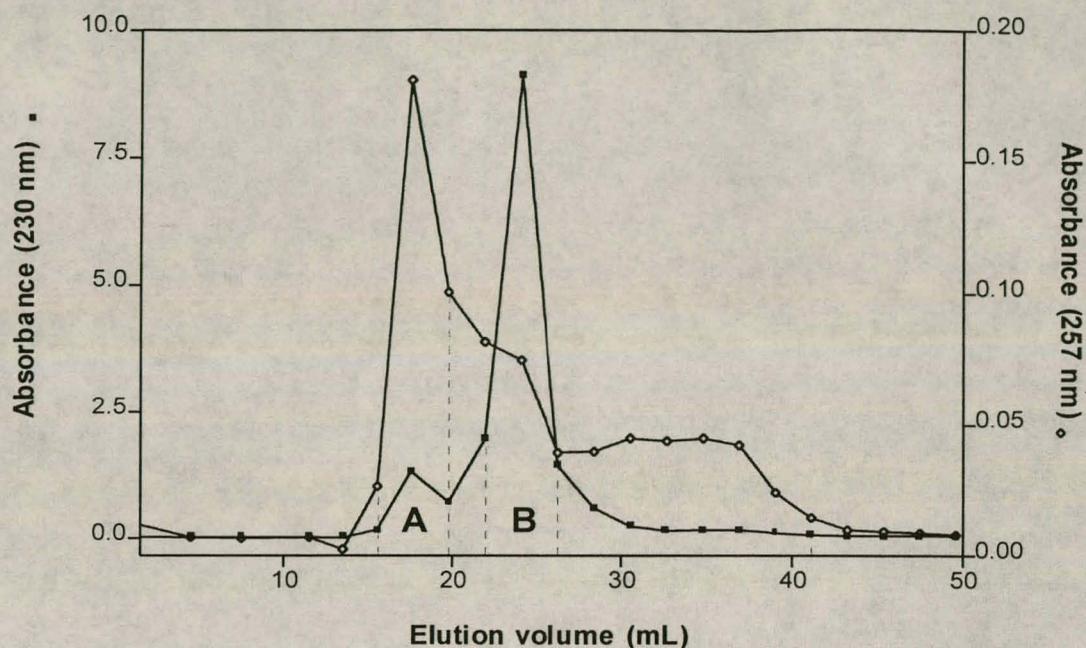
exposure to the coupling mixture, the Fmoc groups protecting the N-terminus of the coupled asparagine might have been liberated, and synthesis was therefore continued. Subsequent quality control analysis of the Fmoc-L-asparagine-OPfp derivative revealed its degradation. TLC analysis showed that the derivative was contaminated with L-asparagine as well as breakdown products of the Pfp group. The melting point was also considerably lower than expected (132-140°C versus 154-156°C).

Although the yield from this synthesis was already compromised at this early stage, we decided to continue to optimise the synthesis and purification of this peptide. For the rest of the synthesis satisfactory Kaiser tests were obtained. After coupling of 21 residues, half of the resin was removed for the liberation of the shortened peptide magainin 2 N<sup>21</sup>. The full-length magainin 2 peptide was synthesised on the remaining resin. Because of the expected low yield of full length of peptide ( $\pm 38\%$  of the peptide product lacked Asn<sub>22</sub>) from the synthesis at this point, we decided to synthesise the shortened peptide magainin 2 N<sup>19</sup> at a later stage.

After cleavage of the peptides, the yields (dry mass) of the crude freeze-dried peptides were not determined because of the large amount of methionine, used as scavenger (section 2.3.3.6), still present in the samples. Amino acid analysis of the resins after cleavage of the peptides showed that all of the peptide had been liberated from the resin (results not shown).

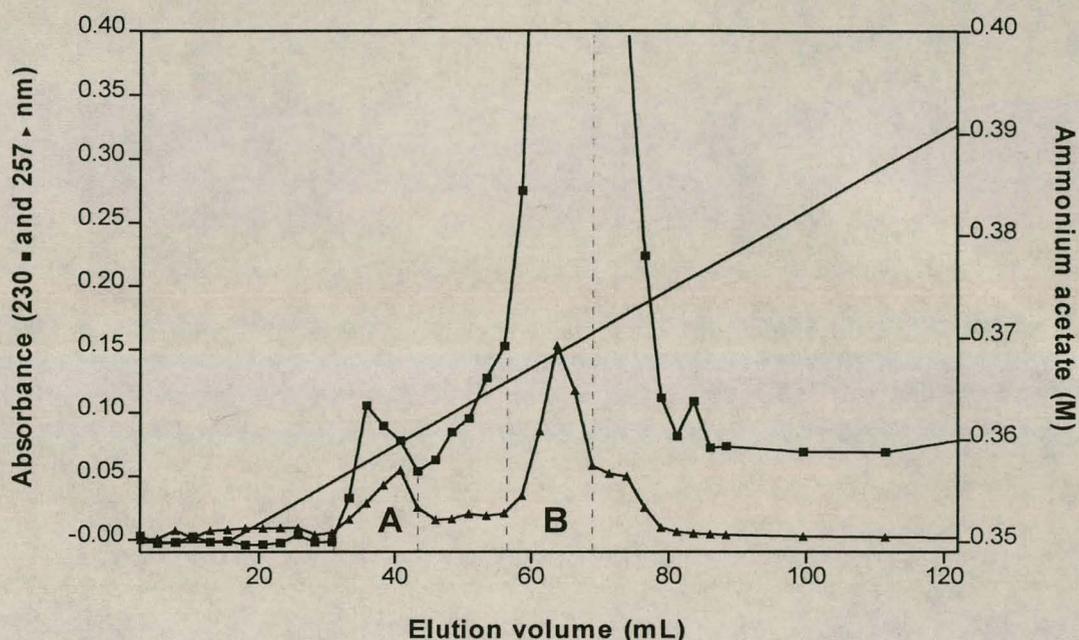
#### 2.4.1.3 Purification and analysis of peptides products from synthesis 1

Low molecular mass impurities and the methionine/scavenger mixture were removed from the crude peptides using gel-permeation chromatography (Fig. 2.1). The large amount of methionine (400 mg at 1 g/50 mL) used in the cleavage mixture, and the limitations imposed by the column size (1.5 cm × 25 cm) necessitated numerous chromatography runs per peptide. Two major fractions were obtained in the case of each peptide, with the peptide fraction eluting first ( $V_e = 17$  mL;  $V_e$  is the elution volume) and the methionine containing fraction second ( $V_e = 24$  mL). Conservative pooling to ensure the removal of all methionine, as well as low percentage coupling values for the first two amino acids, resulted in low yields and coupling efficiencies for the two peptides. ESI-MS analysis of the purified peptide products confirmed that gel permeation had successfully removed most of the methionine from both peptides, however, the deletion peptides lacking Asn<sub>22</sub> were present in each of the peptide preparations (results not shown).



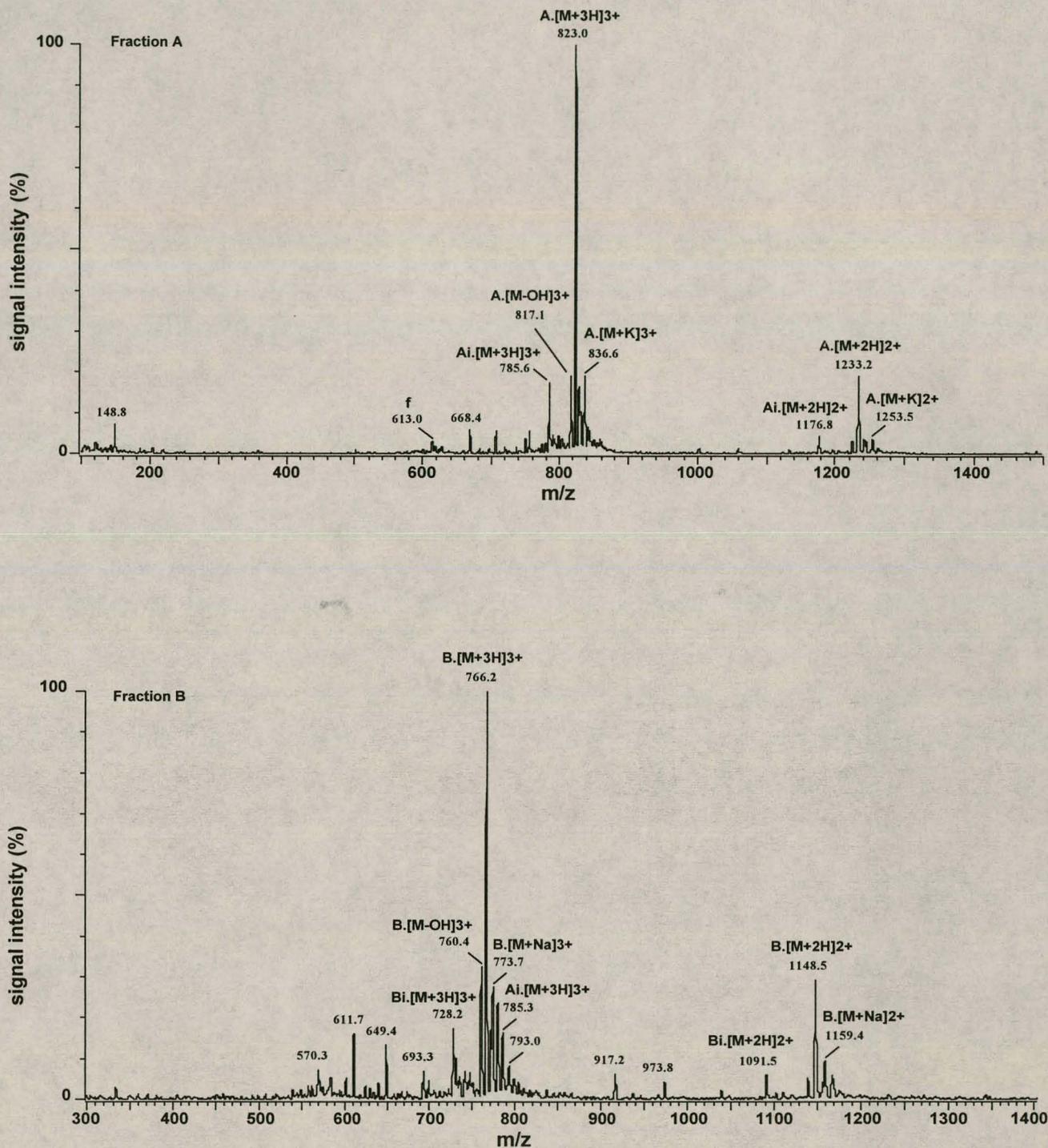
*Figure 2.1* An example of the elution profile exhibited by the crude magainin 2 peptide when chromatographed on Sephadex G10. The crude peptide mass was 50 mg. Peak **A** contained the peptide fraction, and peak **B** the methionine and scavenger fraction.

The peptide products from synthesis 1 were further purified with cation exchange chromatography. Identical chromatograms were obtained during the chromatography of the magainin 2 and magainin 2 N<sup>21</sup> peptide preparations. Three fractions eluted using a linear 0.01M to 0.15 M ammonium acetate (pH 6) gradient (results not shown). The ESI-MS analysis of the three fractions indicated that no peptide material was present in any of the peaks (results not shown). Subsequent regeneration of the column with 0.5M ammonium acetate eluted both peptides showing that the cationic nature of both peptides had been largely underestimated. The optimal gradient for the purification of these cationic peptides was found to be a linear gradient starting with 0.35M ammonium acetate (pH 6) as the equilibration buffer and ending with 0.45M ammonium acetate (pH 6) as the final concentration (Fig. 2.2).



*Figure 2.2* CM Sepharose CL-6B cation exchange chromatography of the retained peptide preparations of magainin 2 and magainin 2 N<sup>21</sup>, and the deletion peptides magainin 2 and magainin 2 N<sup>21</sup>, each lacking Asn<sub>22</sub>. Fraction A represents magainin 2 and the deletion peptide lacking Asn<sub>22</sub>. Fraction B represents magainin 2 N<sup>21</sup> and the deletion peptide lacking Asn<sub>22</sub>.

With this gradient it was possible to separate the peptide mixture eluted from the column during regeneration; however, it was impossible to remove the deletion peptides, which were retained with their full-length counterparts. In Fig.2.3 the ESI-MS spectra of the two peptide preparations (fractions A and B in Fig 2.2) are shown. TLC of the peptides together with the deletion peptides showed single ninhydrin-positive components with identical  $R_f$  values ( $R_f=0.68$ ), confirming the similarity of all four peptides (results not shown). The final option in purifying the peptides would have been HPLC, but because of the product loss during the repetitive purification steps, the final yields for both peptides were very low. We therefore decided to re-synthesise the peptide using the experience gained from this first synthesis.



**Figure 2.3** Positive mode ESI-MS of fraction A and B collected after CM Sepharose CL-6B cation exchange chromatography of the peptide mixture. Fraction A contains the following peptides: **A** representing magainin 2, and **Ai** representing magainin 2 excluding asparagine 22. Fraction B contains the following peptides: **B** representing magainin 2 N<sup>21</sup>, and **Bi** representing magainin 2 N<sup>21</sup> excluding asparagine 22. Fraction B showed minor contamination with peptide Ai.

## 2.4.2 *Synthesis 2 of magainin 2, magainin 2 N<sup>21</sup>, and magainin 2 N<sup>19</sup>*

### 2.4.2.1 Coupling of the first amino acid

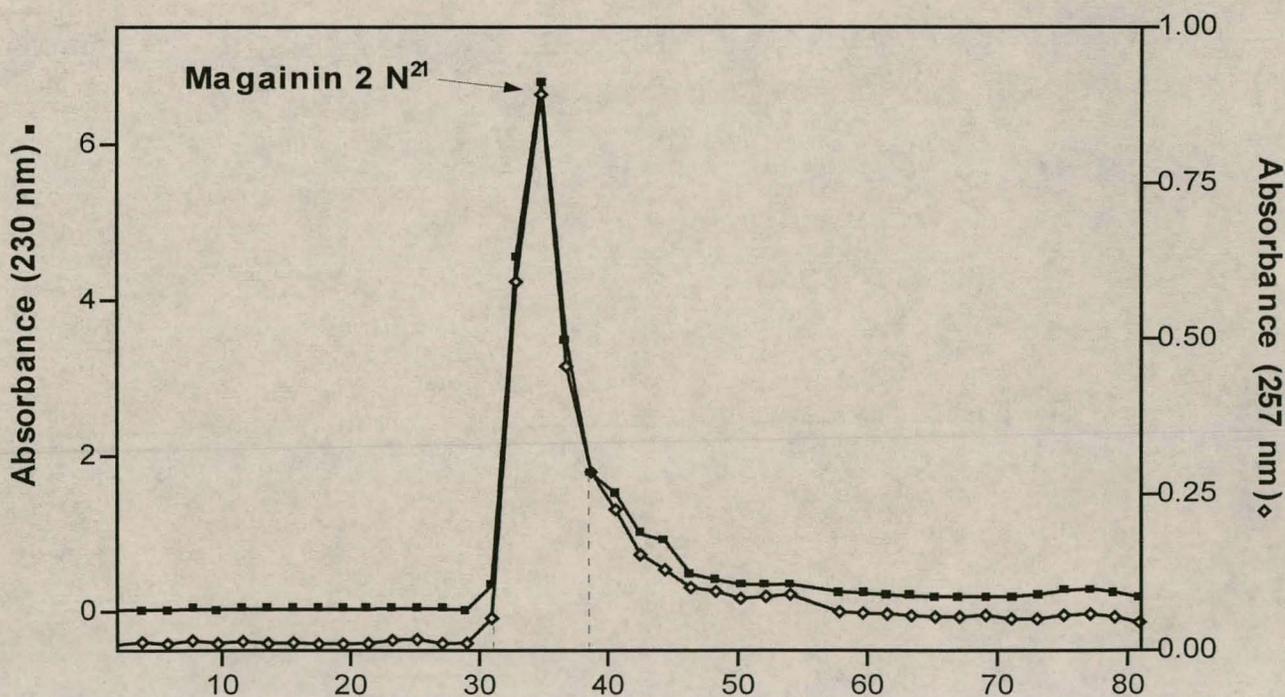
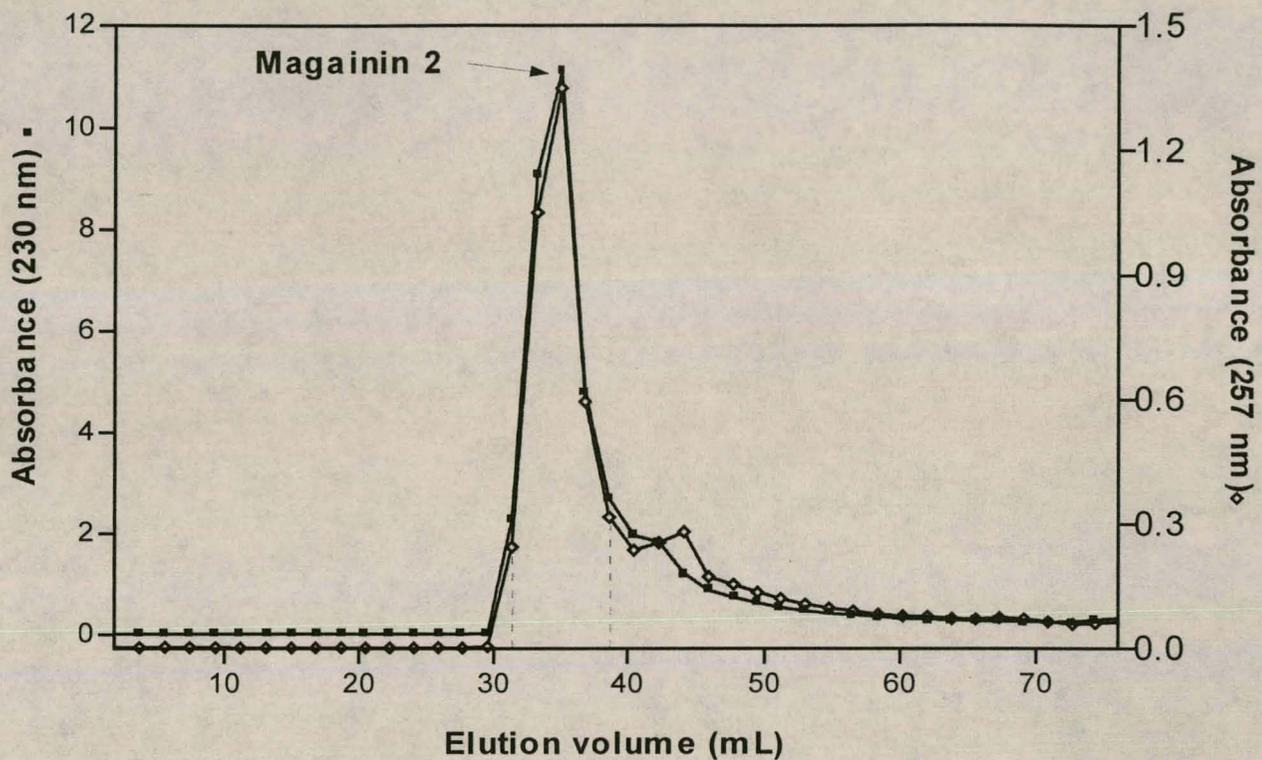
Pure Fmoc-L-Asn-Opfp was synthesised (section 2.3.2.4) and stringent quality control tests were performed on the other derivatives. Fmoc-Gly-Opfp did not pass quality control testing and was re-crystallised as described in section 2.3.2.3. The PYBOP<sup>®</sup> method, with another Ser-derivative, Fmoc-L-Ser(tBu)-OH, was again used to couple Ser<sub>23</sub> to the resin. After an initial four-hour coupling period the Fmoc test showed the resin to be 74% saturated. After a second two-hour coupling the Fmoc test showed the resin to be 100% saturated with the first Fmoc amino acid. This indicated that the initial assumption of the resin capacity being 0.17 milli-equivalent/gram was incorrect. The successful coupling was attributed to the use of Fmoc-L-Ser(tBu)-OH in place of the Fmoc-L-Ser(tBu)-Odhbt derivative.

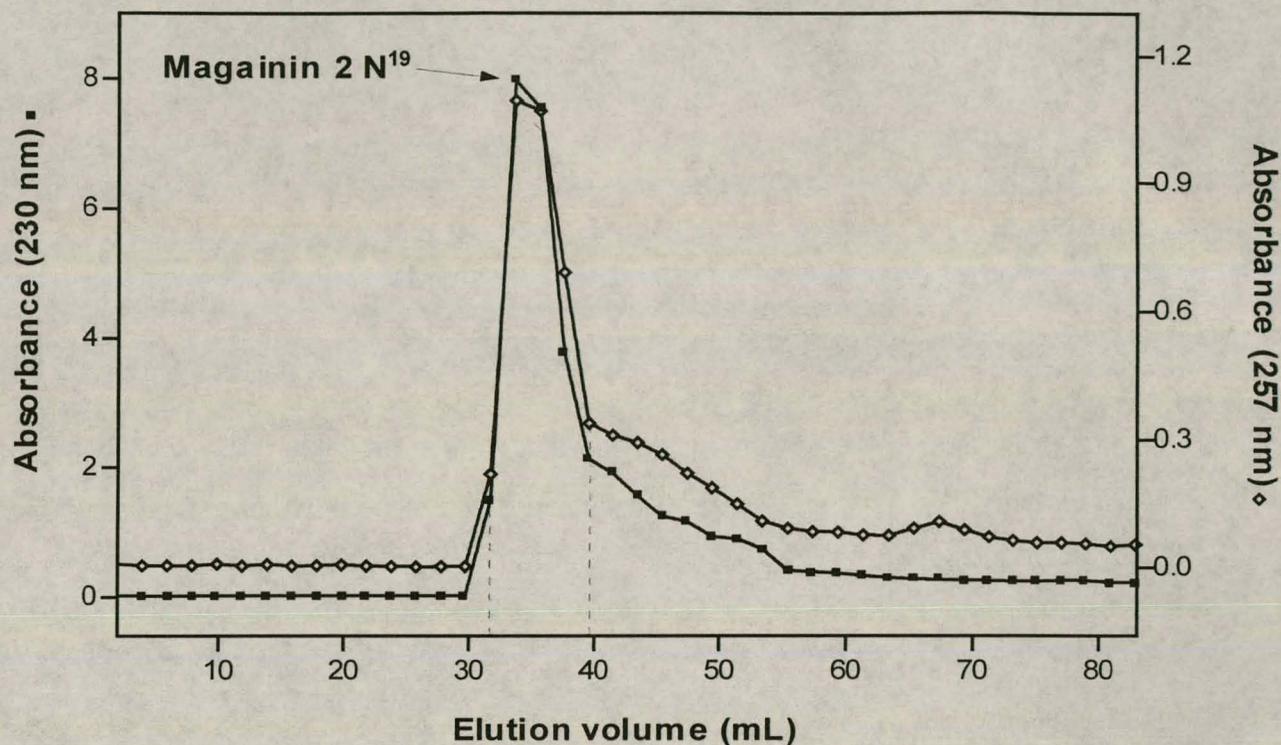
### 2.4.2.2 Elongation of the peptide chain

Satisfactory Kaiser tests were obtained for each of the next 22 coupling/deblocking steps. After coupling 19 residues, a third of the resin was removed for the liberation of the deletion analogue, magainin 2 N<sup>19</sup>. Two more amino acids were coupled to the rest of the resin, and half was removed for the liberation of the deletion analogue, magainin 2 N<sup>21</sup>. The last two N-terminal amino acids were coupled to the rest of the resin for the liberation of the full-length peptide. Peptides were cleaved from the resin as described under section 2.3.3.6. Amino acid analysis of resins samples after cleavage showed that all peptide material was successfully liberated (results not shown).

### 2.4.2.3 Purification of peptides

Initial analytical HPLC of the crude peptides (results not shown) showed the presence of some impurities in all three peptide preparations. The peptides were subsequently chromatographed on Sephadex G10 (Fig. 2.4). A larger column was used (45 cm × 1.5 cm) for this purification step, making it possible to increase the concentration of peptide loaded onto the column, while still retaining suitable resolution. For all three peptide preparations, a single major absorbing fraction was obtained which eluted between 33 and 35 mL.





*Figure 2.4* Examples of the elution profiles exhibited by the crude peptide preparations of magainin 2 and deletion analogues when chromatographed on Sephadex G10. Each of the crude peptides was chromatographed in two separate runs with the dry peptide masses ranging from 30-40 mg per run.

Taking into account chromatographic losses as a result of conservative pooling of fractions and possible interaction between the peptides and the plastic tubing of the chromatographic set-up, peptide yields and coupling efficiencies were calculated for each peptide (Table 2.4). From analytical HPLC of the Sephadex G10 chromatographed peptides it was calculated<sup>4</sup> that magainin 2 N<sup>19</sup> was 73% pure, magainin 2 N<sup>21</sup> was 84% pure and magainin 2 was 60% pure (results not shown).

To obtain peptides at a purity of >96%, the three peptides were subsequently purified using semi-preparative HPLC. Fractions were collected as shown in Fig. 2.5. Peptide yields are listed in Table 2.4.

<sup>4</sup> % Purity = area of peptide peak / total area peaks × 100.

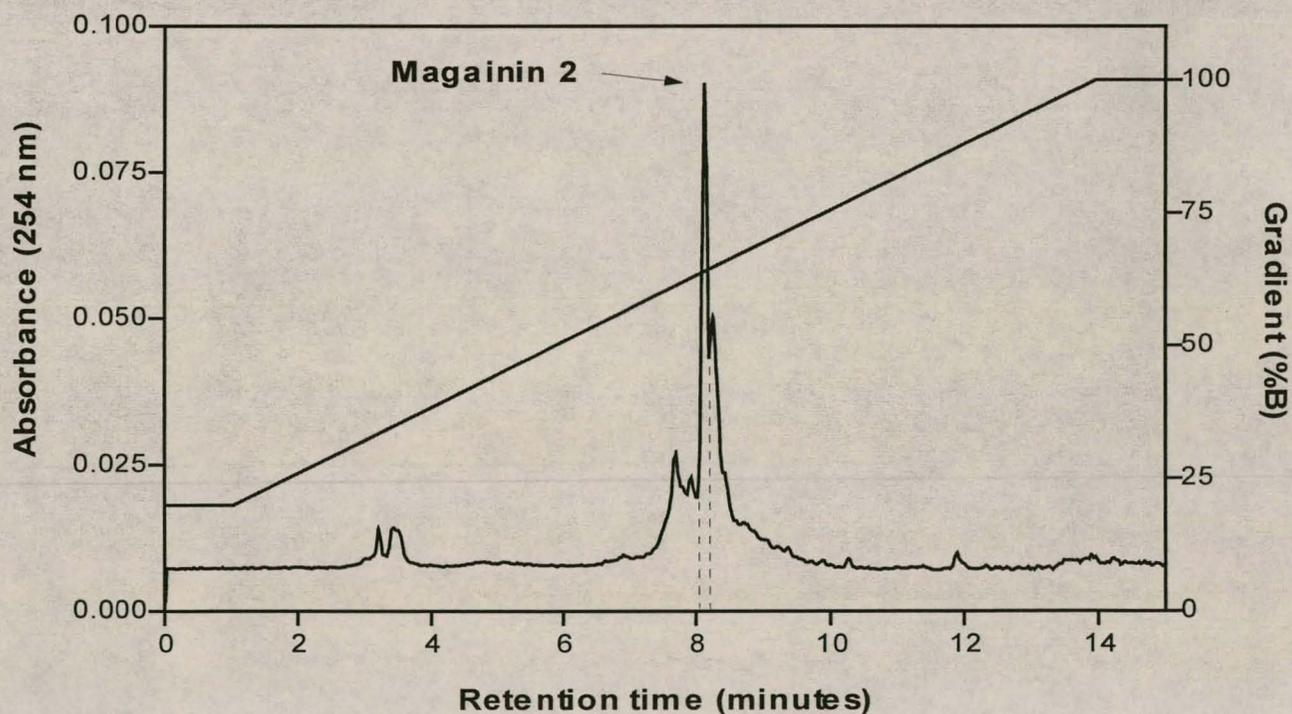
**Table 2.4** A summary of the yields obtained for the synthesis and purification of magainin 2 and the N-terminal deletion peptides. Yields were determined on the dry mass of each peptide.

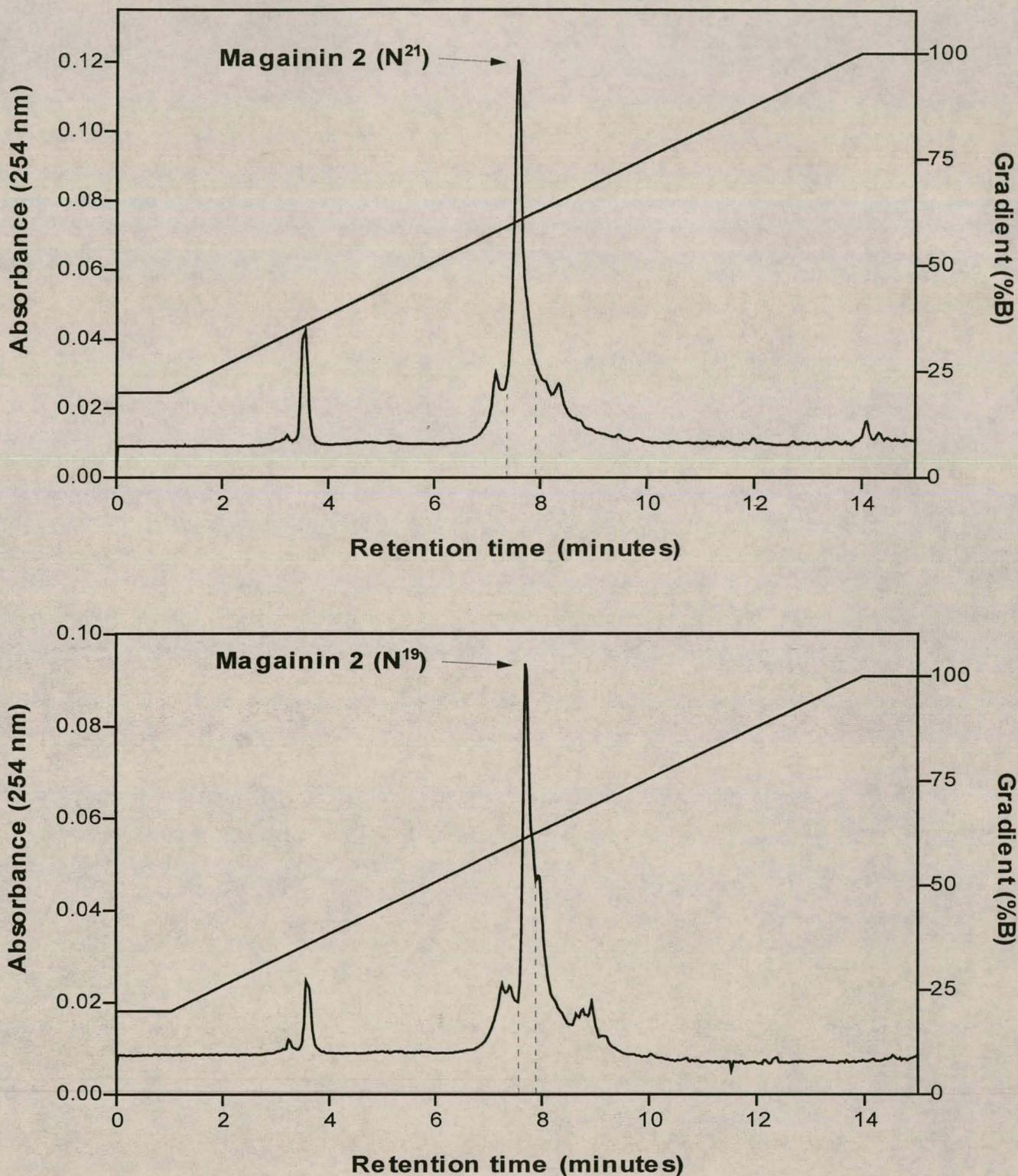
Peptide	% Crude yield <sup>a</sup>	% Average coupling efficiency <sup>b</sup>	% Yield from preparative HPLC <sup>c</sup>
magainin 2	53	97	53
magainin 2 N <sup>21</sup>	52	97	62
magainin 2 N <sup>19</sup>	53	97	55

<sup>a</sup>% Crude yield = mass peptide obtained / mass peptide expected × 100

<sup>b</sup>% Coupling efficiency = Yield <sup>1/n</sup> × 100 (n = number of amino acids in peptide)

<sup>c</sup>% HPLC yield = mass HPLC injected peptide / mass purified peptide × 100



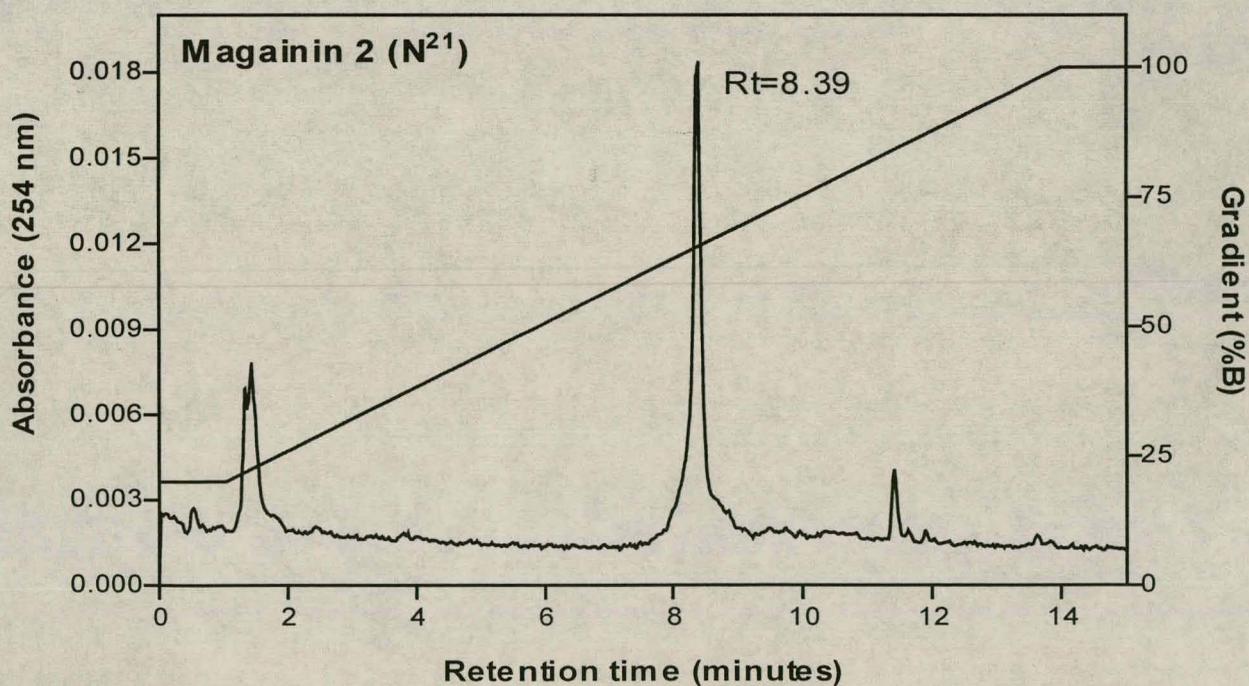
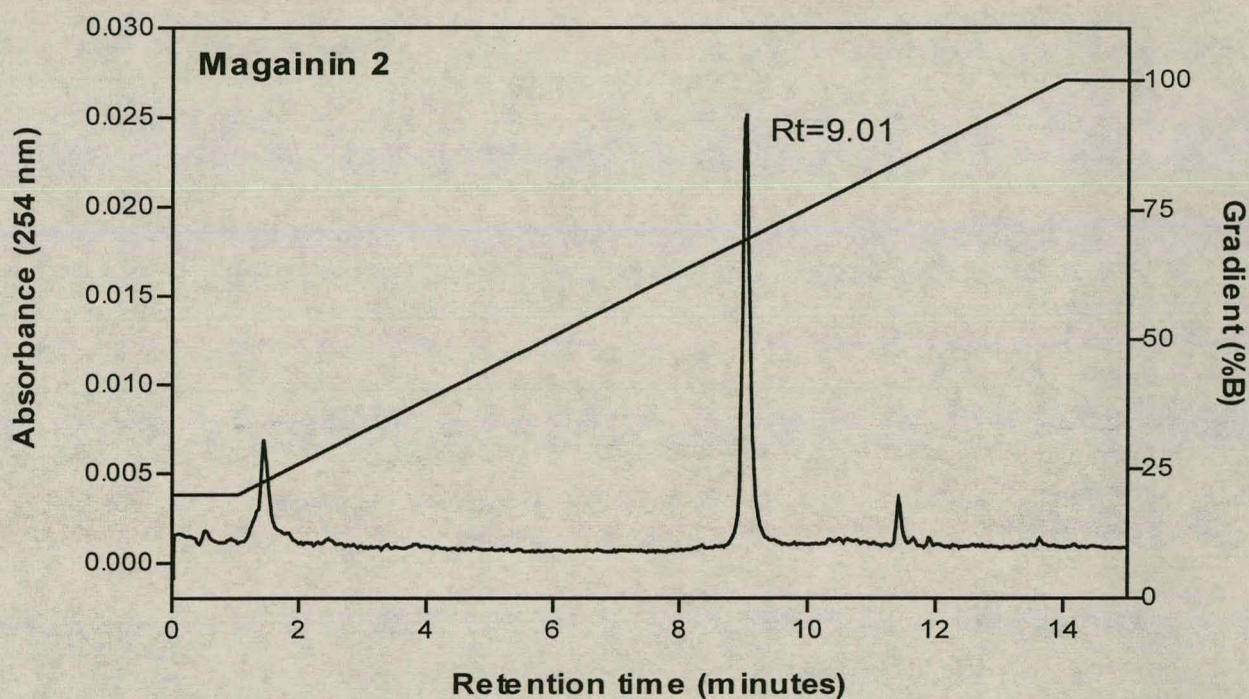


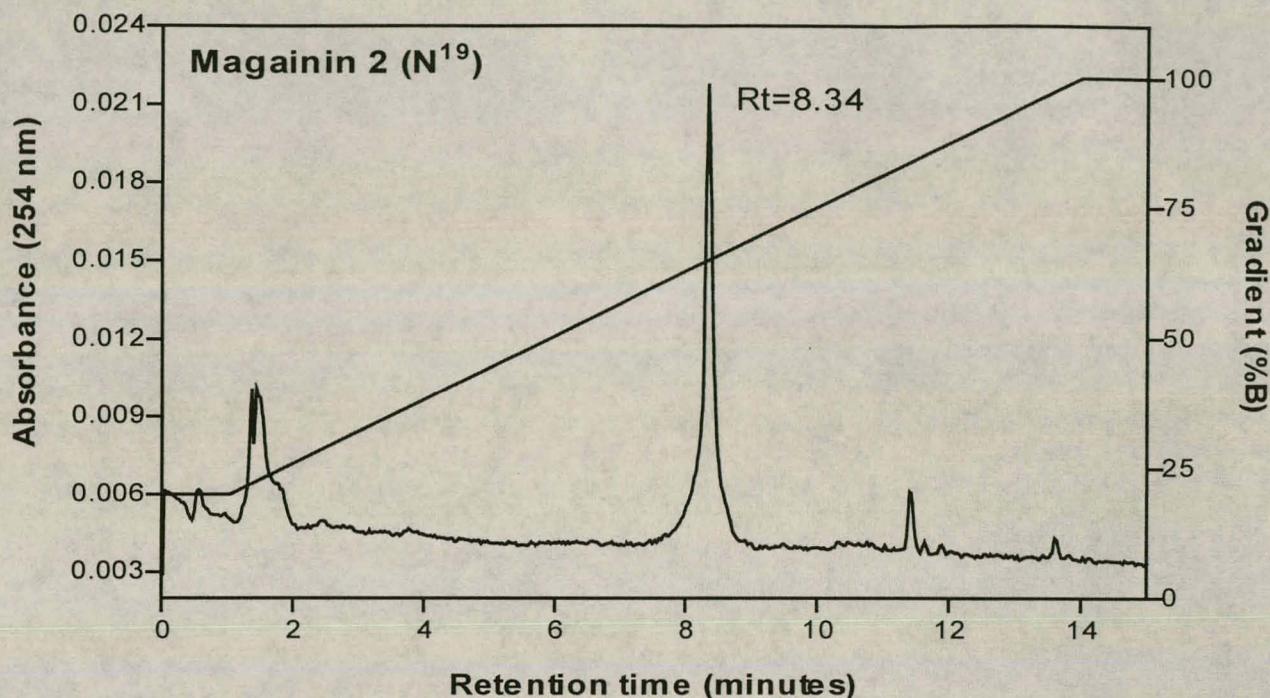
*Figure 2.5* Semi-preparative HPLC chromatograms of the peptide preparations. Peak fractions collected are indicated by the dashed lines. The linear gradient was developed over 13 minutes with 0.1% TFA in water as solvent A and acetonitrile plus 10% A as solvent B at a flow rate of 3 mL/min.

## 2.4.3 Analysis of purified peptides

### 2.4.3.1 Analytical HPLC of the purified peptides

Analytical HPLC was used to assess the semi-preparative purification of the peptides. All three peptides were of a purity  $>96\%$ <sup>4</sup> (Fig. 2.6).





*Figure 2.6* Analytical HPLC chromatograms of the purified peptide products from synthesis 2. The linear gradient was developed over 13 minutes with 0.1% TFA in water as solvent A and acetonitrile plus 10% A as solvent B at a flow rate of 1 mL/min

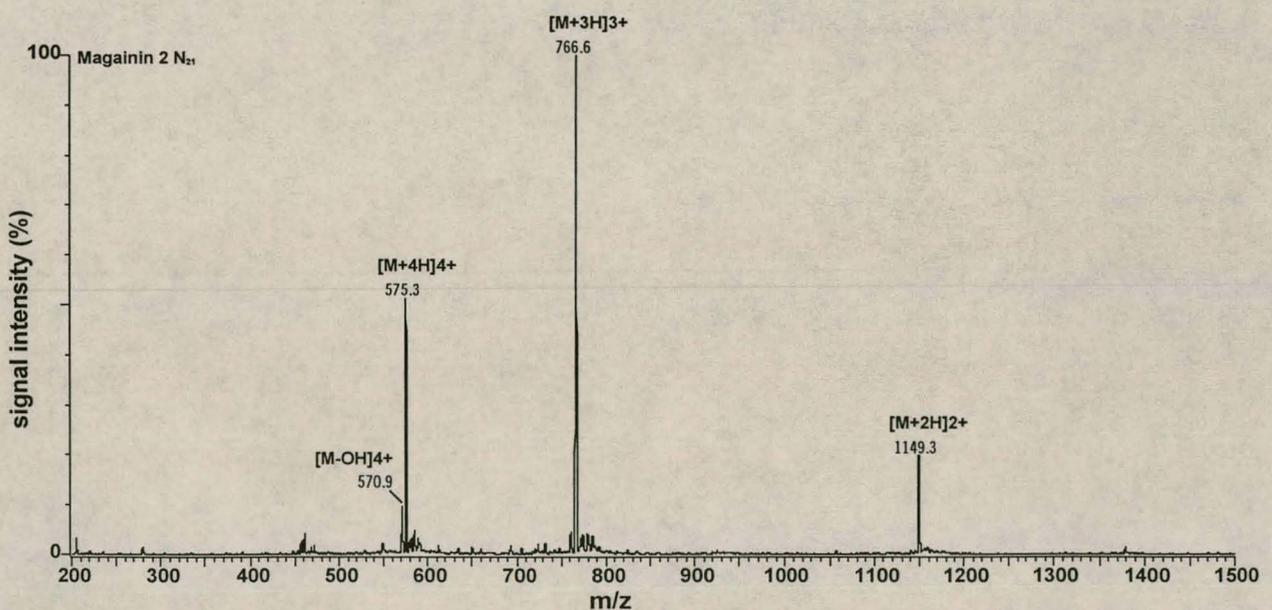
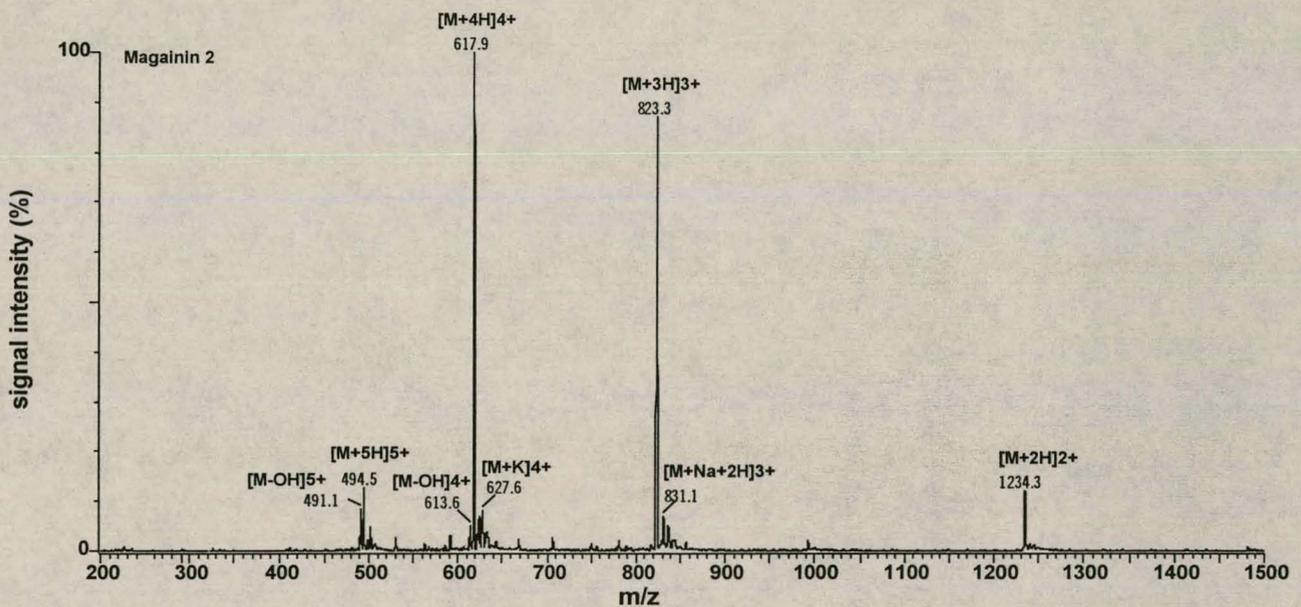
Each of the peptides exhibited slight aggregation as shown by the small peak eluting at the void volume of 1.4 mL. The two deletion analogues showed almost no difference with regard to their retention on the C<sub>18</sub> HPLC matrix. The slight increase in retention (possibly also hydrophobicity) of magainin 2 N<sup>21</sup> was attributed to its extra glycine residue. The full-length magainin 2 had a retention time  $\pm 30$  seconds greater than its deletion analogues. Its increased retention and hydrophobicity was attributed to the extra two glycine residues and isoleucine residue in its peptide sequence.

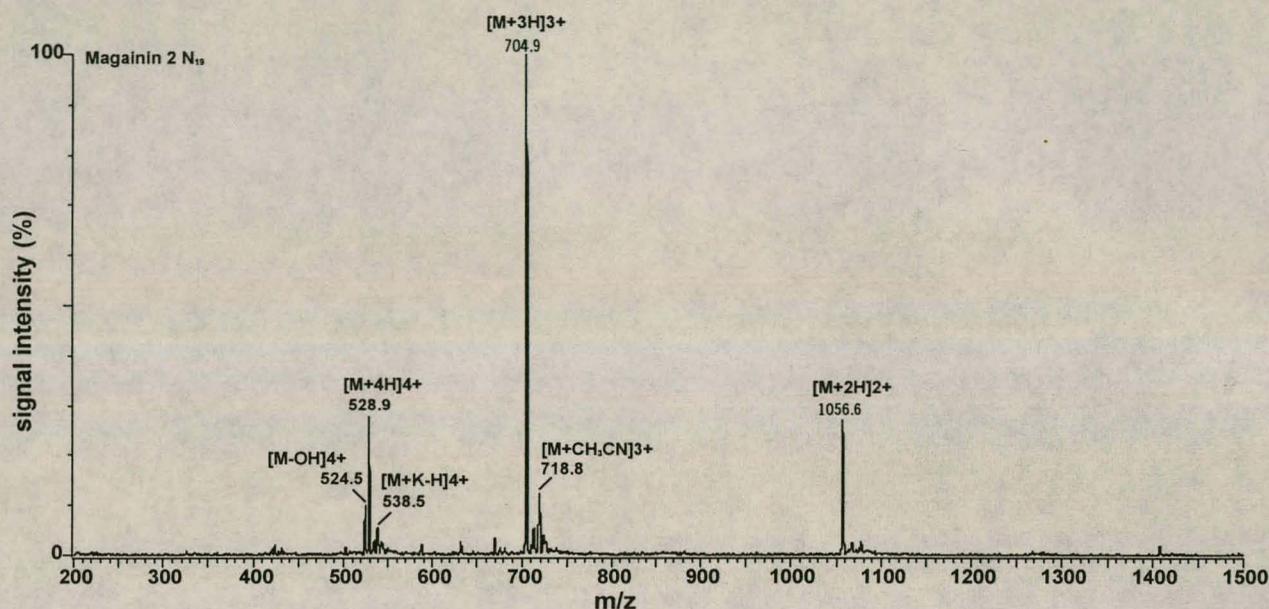
#### 2.4.3.2 Electrospray ionisation mass spectrometry of the purified peptides

The correct  $m/z$  (molecular mass/charge ratio) was found for each of the peptides, and all three peptides were shown to be pure. Spectra of magainin 2 (Fig.2.7) indicated the correct  $m/z$  in multiply charged forms, namely 494.5 ( $[M+5H]^{5+}$ ), 617.9 ( $[M+4H]^{4+}$ ), 823.3 ( $[M+3H]^{3+}$ ), and 1234.3 ( $[M+2H]^{2+}$ ). Na and K cationised species were also observed in the triply and quadruply charged forms of the peptide. The quadruply ( $m/z = 613.6$ ) and quintuply ( $m/z = 491.1$ ) charged species differing by 17 Da, indicate the loss of an OH group.

The magainin 2 N<sup>21</sup> spectrum (Fig 2.7) showed the correct  $m/z$  in multiply charged forms, namely 575.3 ( $[M+4H]^{4+}$ ), 766.6 ( $[M+3H]^{3+}$ ), and 1149.3 ( $[M+2H]^{2+}$ ). The quadruply charged specie differing by 17 Da from the molecular ion was again observed ( $m/z = 570.9$ ).

Magainin 2 N<sup>19</sup> also showed the correct  $m/z$  in multiply charged forms (Fig.2.7), namely 528.9 ( $[M+4H]^+$ ), 704.9 ( $[M+3H]^+$ ), and 1056.6 ( $[M+2H]^+$ ). A triply charged acetonitrile adduct ( $m/z = 718.8$ ), a quadruply charged specie differing by 17 Da ( $m/z = 524.5$ ), and a quadruply charged K<sup>+</sup> cationised specie ( $m/z = 538.5$ ) were also observed in the spectra of the 19-residue peptide.





*Figure 2.7* Positive mode ESI-MS spectra of the purified magainin 2 peptide and the two N-terminal deletion peptides, magainin 2 N<sup>21</sup> and magainin 2 N<sup>19</sup>. Analyses were performed as described under section 2.3.5.4.

## 2.5 Conclusions

The Fmoc-polyamide protocol proved to be successful in the synthesis of magainin 2 and the two shortened peptides. Experience gained during the first synthesis of these peptides helped to ensure the high yields and high purity peptide products obtained in the second synthesis.

The necessity for quality control checks on all derivatives, even newly acquired ones, prior to synthesis was found to be essential to ensure a successful synthesis. There are numerous reasons for the degradation of derivatives, of which the most prevalent are storage conditions (for example temperature, moisture, light, volatile organic and inorganic contamination of reagents) and it is recommended that the purity of derivatives be checked regularly [17]. Coupling of the first amino acid was also noted to be a critical step in the synthesis. In synthesis 1, the derivative Fmoc-L-Ser(tBu)-Odhbt was used for this step, and only 85% saturation of the resin could be obtained. This indicated that that the PyBOP<sup>®</sup> method was not suitable for coupling a derivative containing an Odhbt ester. Furthermore, the attempted coupling of the degraded Fmoc-L-Asn-OPfp derivative resulted in the formation of the two deletion peptides, which, owing to their similarity with the full length peptides, proved to be difficult to remove. This showed the importance of maximum coupling, and what effect sub-standard couplings have on yields and subsequent purification steps. Further

complications arose when the peptides were retained on the column during the cation exchange purification step, resulting in an undesired mixture of the peptides. This emphasised the extreme cationic nature of the peptides, and a new gradient was developed which was able to separate and partly purify the peptides.

For the second synthesis Fmoc-L-Ser(tBu)-OH was used for the initial coupling step, and maximum coupling was achieved. Pure Fmoc-L-Asn-OPfp was synthesised (section 2.3.2.4) for the second coupling step, and this too coupled successfully as shown by a negative Kaiser test. Further quality control checks were done on the other derivatives prior to coupling; and the second synthesis proceeded without complication. The use of reagent K [22] to cleave the peptide from the resin decreased the number of purification steps, as it was unnecessary to use methionine as a scavenger. Peptides were purified to purity of >96% using reverse phase HPLC, and subsequent ESI-MS analysis confirmed the purity of the peptides. ESI-MS analysis showed the pure peptides in their multiply charged forms with no evidence of any contaminants.

Finally, the peptide products were of sufficient purity for use in biological activity experiments. In Chapter 3, I shall report on the development and evaluation of an antimicrobial assay to test these peptides, and in Chapter 4 the capability of magainin 2 and its analogues to render tetracycline resistant *E. coli* susceptible to tetracycline shall be examined.

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## Chapter 3

### *A sensitive standardised micro-gell well diffusion assay for the determination of antimicrobial activity<sup>1</sup>*

#### 3.1 Introduction

With the discovery of antibiotics and vast numbers of antimicrobial peptides, numerous methods have been developed to test these compounds against various organisms. In the beginning, for lack of alternatives, bacteria were incubated with these compounds and the rate of decrease in viable counts monitored. This, however, was found to be too time and material consuming, and new, more efficient methods were sought.

A number of methods were since developed with the emphasis on the conservation of test material. The earliest, most successful methods simply entailed the formation of inhibition zones by an antibiotic in a lawn of bacteria growing in agar [1]. The inhibition zones were physically measured to determine the antimicrobial activity of the antibiotic. For example, the agar cup assay method for determining the activity of penicillin was developed as far back as 1946 [2]. Researchers favoured these methods because they required very little test material and could be carried out relatively easily. Later, however, agar was found to be unsuitable for testing certain antimicrobial molecules, as it contained specific extrinsic substances or contaminants that hampered the diffusion of these molecules. This led to agar being replaced by a high quality agarose in many of the assays [3].

To date the microtiter plate has played an integral role in the development of various micro-assays, with one of the most well-known being the enzyme-linked immunosorbent assay (ELISA) [4]. Literally hundreds of assays are now performed in microtiter plates,

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<sup>1</sup> In collaboration with M. Rautenbach to be submitted to *J. Microbiol. Methods*

because 96 wells can be used per experiment, small amounts of test material are required, and results can easily be quantitated spectrophotometrically using a microtiter plate reader.

We developed a highly sensitive micro-gel well diffusion assay by adapting an inhibition zone assay to perform it in a microtiter plate. Hereafter, our method is compared to an inhibition zone assay and a microtiter broth dilution method. Gramicidin S (an antibiotic cyclodecapeptide from *Bacillus brevis*) was used as the model antibiotic and *Micrococcus luteus* (a Gram-positive bacterium) as the indicator organism.

## 3.2 Materials

*M. luteus* (NCTC 8340) was used as indicator organism in all experiments. Dr. R. Levitt, Fine Chemicals, South Africa, donated synthetic gramicidin S. Microtiter plates (Nunc-Immuno™ Maxisorp) were from Nalge NUNC International (Roskilde, Denmark), Falcon® tubes from Becton Dickinson Labware (Lincoln Park, USA), and culture dishes from Quality Scientific Plastics, USA. Low-electroendosmosis-type agarose (D1-LE) was from Whitehead Scientific (Brackenfell, South Africa). Tween 20 and Coomassie brilliant blue R-250 were from Fluka (Buchs, Switzerland). Dulbecco's phosphate buffered saline (PBS) was either from Life Technologies (Faisley, Scotland), or prepared in the laboratory [5]. Sodium chloride and glacial acetic acid were from Saarchem (Krugersdorp, South Africa). Disodium hydrogen phosphate, methanol (99%), potassium chloride, potassium dihydrogen phosphate, tryptone soy broth (TSB), dimethylsulphoxide (DMSO), casein, and formaldehyde (99%) were from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was from Boehringer Mannheim (Germany). Analytical quality water was prepared by filtering glass distilled water through a Millipore Milli Q® water purification system.

## 3.3 Methods

### 3.3.1 Preparation of cells

#### 3.3.1.1 Radial diffusion assay (RDA) and the micro-gel well diffusion assay

Bacteria were grown overnight at 37°C in TSB, sub-cultured and grown to an optical density (OD) of 0.6 at 620 nm. The cells were centrifuged for 10 minutes at 900 g. The

supernatant was discarded and the cells were washed once with 10 mL cold Dulbecco's PBS by centrifugation. Finally, the cells were diluted to an OD of 0.6 in Dulbecco's PBS [6].

### 3.3.1.2 Microtiter broth dilution method

Bacteria were grown overnight at 37°C in TSB, sub-cultured and grown to an OD of 0.6 at 620 nm. The cells were diluted with TSB to  $5 \times 10^5$  colony forming units per mL (CFU/mL) based on the relationship  $OD_{620} 0.20 = 5 \times 10^5$  CFU/mL [6].

### 3.3.2 Radial diffusion assay

The radial diffusion assay was adapted from the method of Lehrer et al. [6]. A gel solution containing 1% (w/v) of powdered TSB medium, 1% (w/v) of low-electroendosmosis-type agarose, and 0.02% (v/v) Tween 20 made up in Dulbecco's PBS was prepared and autoclaved. Culture dishes were blocked with 0.5% casein in Dulbecco's PBS for one hour and dried under ultraviolet light for a further four hours. Ten mL of the gel, at 45°C, was aliquoted and added to 1 mL of the dilute bacterial culture and dispersed for ten seconds using a laboratory vortex. Once the bacteria were adequately dispersed, the gel was poured into a circular culture dish on a level platform. The gel was then allowed to set for one hour, before wells were made using a 5 mm punch. The test sample was dissolved in analytical quality water and then further diluted (doubling serial dilutions). After adding 15  $\mu$ L of sample material to each well, the plates were incubated for three hours at 37°C and then turned over and incubated for a further 14 hours at 37°C. The gel was stained for 24 hours in a dilute solution of Coomassie brilliant blue R-250 (2 mg dye; 27 mL methanol; 63 mL water and 15 mL 37% formaldehyde). The spent staining solution was decanted, replaced with an aqueous solution of 10% acetic acid and 2% DMSO and left for approximately ten minutes. The areas of the clear zones surrounding the wells were calculated from calliper measurements of a 190% enlarged photocopy of the stained gels. The gels were stored in water at 4°C.

### ***3.3.3 Microtiter Broth Dilution Method***

The microtiter broth dilution method, microbroth dilution assay, and versions of it have previously been described [7, 8, 9]. The test sample was dissolved in analytical quality water at twice the required maximal concentration, and then diluted 1:1 with 0.02% acetic acid, 0.4% bovine serum albumin (BSA) to the required stock concentration. Further doubling serial dilutions were made using 0.01% acetic acid, 0.2% BSA. TSB (100  $\mu$ L) was pipetted into each of the wells of the first column of the microtiter plate as a sterility control and blank. The rest of the wells each received 100  $\mu$ L of the bacterial suspension. A volume of 30  $\mu$ L of the test sample was pipetted into all wells excluding the first two rows that served as growth controls. The plate was covered and incubated at 37°C for 17 hours and the light dispersion in each well determined using a microtiter plate reader (Multiscan Titertek) at 620 nm.

### ***3.3.4 Micro-gel well diffusion assay***

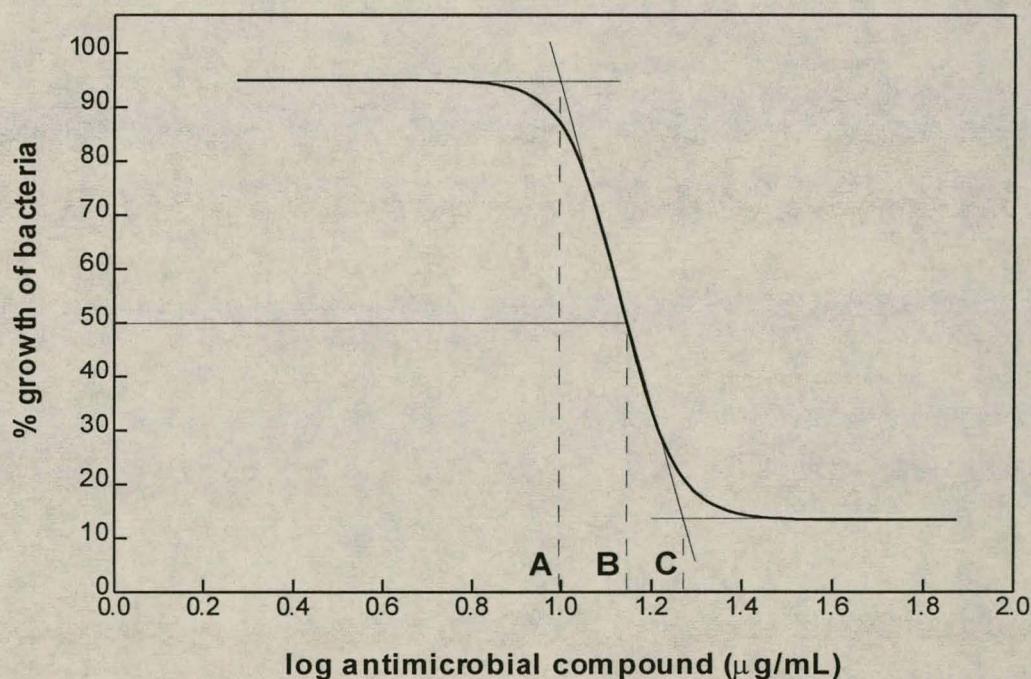
Microtiter plates were blocked with 0.5% casein in Dulbecco's PBS for one hour and sterilised under ultraviolet light for a further four hours. The gel was prepared as in the radial diffusion assay and kept in a water bath at  $46 \pm 1^\circ\text{C}$ . The following modified pipetting technique was essential for obtaining reproducible results: 70  $\mu$ L of the heated gel suspension was drawn up, using a Gilson<sup>®</sup> micropipette, by depressing the pipette plunger to its maximum, and pipetted by depressing the plunger only as far as its first stoppage point. The normal method of pipetting was therefore reversed to prevent bubble formation. The first column of the microtiter plate received only 70  $\mu$ L of gel, to serve as blank and sterility control. The other wells each received 70  $\mu$ L of a bacterial-gel suspension (70  $\mu$ L bacteria and 700  $\mu$ L gel dispersed with a laboratory vortex). The suspensions were prepared for each column immediately before application. Once the desired number of wells were prepared, the gel was allowed to set for 30 minutes. The test sample was dissolved in analytical grade water and then further diluted (doubling serial dilutions). After applying 30  $\mu$ L of sample directly onto the gel in the wells the microtiter plate was covered and incubated for 17 hours at 37°C. The light dispersion per well was determined using a Multiscan Titertek microtiter plate reader at 620 nm. The plates were preserved by adding 140  $\mu$ L of a solution containing 27 mL methanol, 63 mL water and 15

formaldehyde to each well. The gel was stained by adding 100  $\mu\text{L}$  of a 0.002% solution of Coomassie brilliant blue R-250. After 24 hours the colouring solution was discarded and replaced by water before storage at 4°C.

### 3.3.5 Data processing

All data was analysed using, Graphpad Prism version 2.01 for Windows, GraphPad Software, San Diego, California, USA ([www.graphpad.com](http://www.graphpad.com)).

Nonlinear regression was performed on the dose-response data and a sigmoidal curve with variable slope was fitted to each of the data sets. The 50% inhibition of microbial growth ( $\text{IC}_{50}$ ), the minimum inhibitory concentration (MIC), and the maximum bactericidal concentration (MBC) values were deduced as shown in the example in Fig. 3.1.



*Figure 3.1* A typical sigmoidal dose-response curve obtained with data analysis using Graphpad Prism 2.01 software. **A** represents the MIC value, **B** the  $\text{IC}_{50}$ , and **C** the MBC.

The equation used for the sigmoidal curve with variable slope was:

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$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{\log IC_{50} \times \text{Hill slope}})$$
 where :

Bottom = Y-value at the bottom plateau

Top = Y-value at top plateau

$\log IC_{50}$  = X-value of response halfway between top and bottom

Hill slope = Hill coefficient or slope factor (determines steepness of curve)

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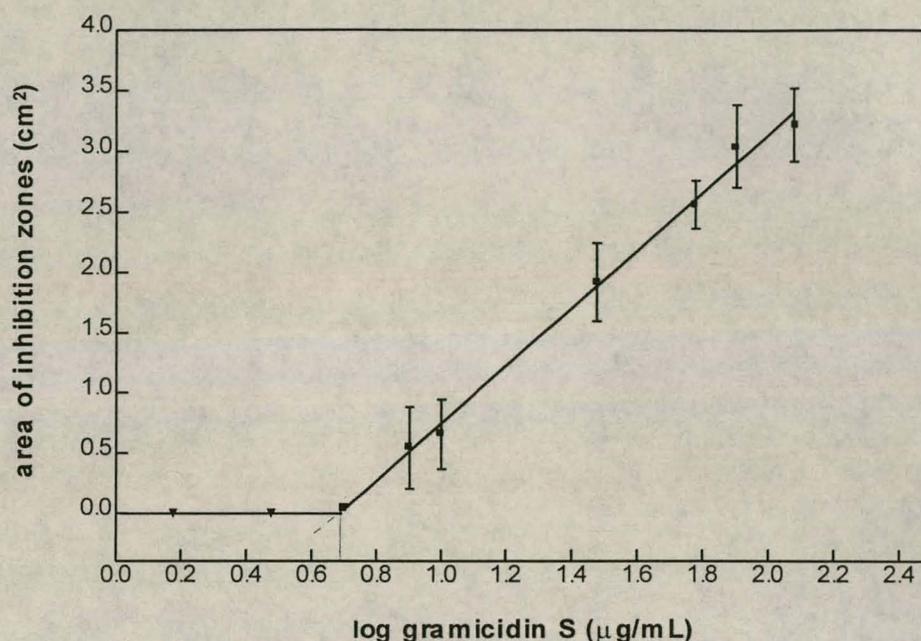
A statistical analysis of the results was done using the statistical functions of the Prism software. MIC values, obtained from all three methods, were analysed doing a one-way analysis of variance (one-way ANOVA). MBC and  $IC_{50}$  values obtained from the microtiter broth dilution method and the micro-gel well diffusion method were analysed statistically using the unpaired t-test.

### 3.4 Results and Discussion

We found that all three methods were compatible with the model antibiotic, gramicidin S, and with the indicator organism, *M. luteus*. The three methods are compared in Table 3.1. Experiments using RDA provided highly visual results. Sharply defined zones of clearing could be observed as a consequence of the growth inhibition of *M. luteus* by various concentrations of gramicidin S. The inhibition zone areas were found to be directly proportional ( $r^2=0.99$ ) to the concentration of gramicidin S over a concentration range from 5 to 120  $\mu\text{g/mL}$  (Fig. 3.2). For a concentration of 3.0  $\mu\text{g/mL}$  and less, no inhibition zones were observed (Fig. 3.2). From these results we obtained a MIC value of  $5.3 \pm 0.1 \mu\text{g/mL}$  for gramicidin S.

*Table 3.1* A comparison of the protocols for the RDA, micro-gel well diffusion assay, and microtiter broth dilution method.

	<b>Radial diffusion assay</b>	<b>Micro-gel well diffusion assay</b>	<b>Microtiter broth dilution method</b>
<b>Experimental container</b>	Petri dish	Microtiter plate	Microtiter plate
<b>Prevention of peptide-plastic interaction</b>	Coated with casein buffer	Coated with casein buffer	Sample in 0.01% acetic acid, 0.2% BSA
<b>Growth medium</b>	Low-endosmosis type agarose + TSB	Low-endosmosis type agarose + TSB	Tryptone soy broth
<b>Cell count</b>	$1.4 \times 10^7$ CFU/mL	$7.5 \times 10^5$ CFU/mL	$5 \times 10^5$ CFU/mL
<b>Incubation time</b>	17 hours	17 hours	17 hours
<b>Measurement method</b>	2× enlargement of inhibition zones	Light dispersion at 620nm	Light dispersion at 620nm



*Figure 3.2* An example of a dose-response curve from a RDA experiment. The solid line indicates a linear response ( $r^2 = 0.99$ ) over the concentration range: 5 to 120  $\mu\text{g/mL}$ . The MIC value shown on the graph is 5.4  $\mu\text{g/mL}$ . The error bars represent the SEMs (quadruplicate determinations).

Sigmoidal dose-responses curves were obtained after analysis of the results from the microtiter broth dilution method and the micro-gel well diffusion assay. From these curves the MIC, MBC, and  $\text{IC}_{50}$  values were deduced (Figs. 3.3 and 3.4, Table 3.2).

A comparison of the MIC values from the two types of microtiter-based assays showed them to be approximately twice as sensitive as the RDA (2.3 and 2.0  $\mu\text{g/mL}$  vs. 5.3  $\mu\text{g/mL}$ ). Furthermore, all three MIC values were significantly different ( $P < 0.001$ ). The MBC and  $\text{IC}_{50}$  values from the micro-gel well diffusion assay and microtiter broth dilution method were also significantly different, with  $P < 0.05$ .

Table 3.2. MIC, MBC, and IC<sub>50</sub> values of gramicidin S against *M. luteus*. Three independent experiments were performed per method and each concentration per experiment was determined in quadruplicate.

Method	MIC (µg/mL) <sup>1,2</sup>	IC <sub>50</sub> (µg/mL) <sup>1,3</sup>	MBC (µg/mL) <sup>1,3</sup>
Micro-gel well diffusion assay	2.3 ± 0.1	3.4 ± 0.1	4.8 ± 0.2
Microtiter broth dilution method	1.9 ± 0.1	2.2 ± 0.1	2.6 ± 0.1
Radial diffusion assay	5.3 ± 0.1	NA	NA

<sup>1</sup> Values are means ± standard error of the mean (SEM)

<sup>2</sup> MIC values from all three methods differed significantly from each other (P<0.001)

<sup>3</sup> MBC and IC<sub>50</sub> values from the microtiter broth dilution method and micro-gel well diffusion assay were significantly different (p<0.05).

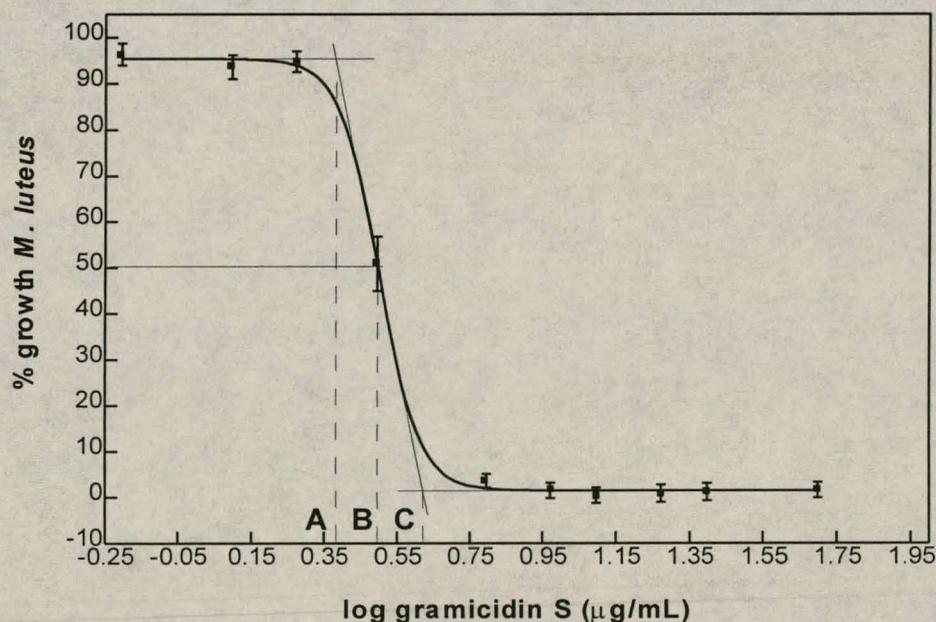
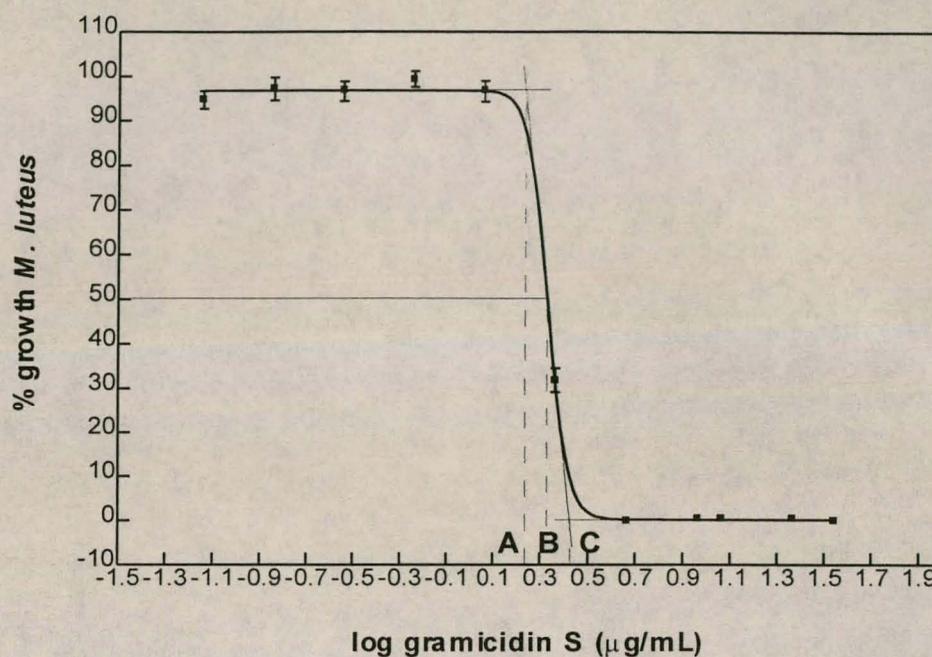


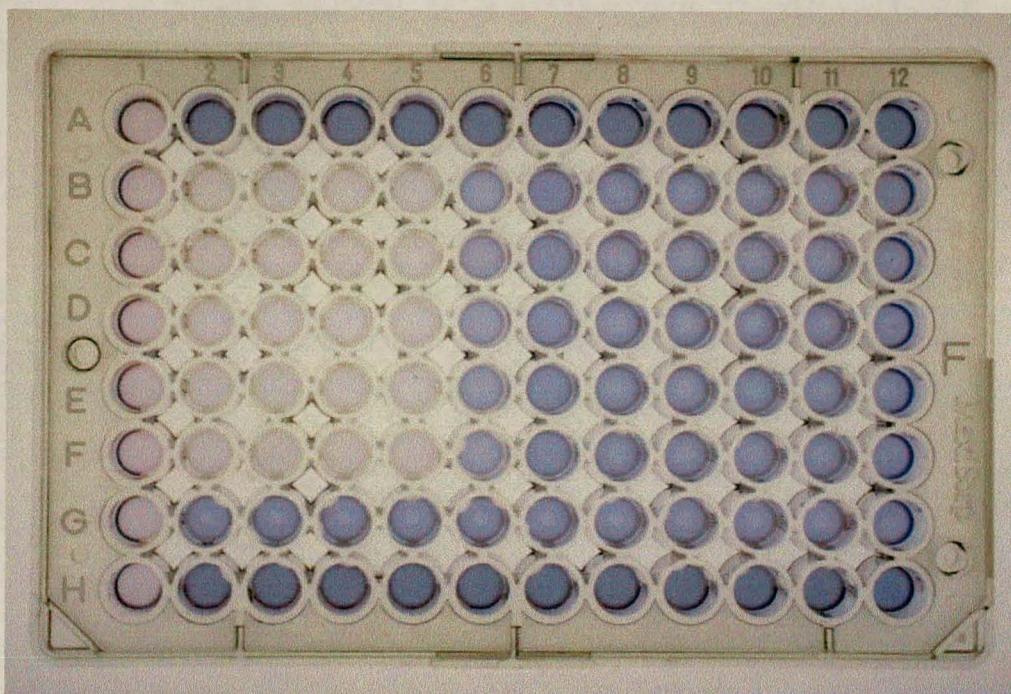
Figure 3.3 An example of a sigmoidal dose-response curve from a microtiter broth dilution method experiment. A represents the MIC value (1.6 µg/mL), B the IC<sub>50</sub> value (2.2 µg/mL), and C the MBC value (2.7 µg/mL). The error bars represent the SEMs (quadruplicate determinations).



*Figure 3.4* An example of a sigmoidal dose-response curve from a micro-gel well diffusion assay experiment. **A** represents the MIC value (2.3 µg/mL), **B** the IC<sub>50</sub> value (3.2 µg/mL), and **C** the MBC value (4.3 µg/mL). The error bars represent the SEMs (quadruplicate determinations).

Staining the gel in the micro-gel well diffusion method notably enhanced the visible inhibition in each well. This created an excellent visual record of the result (Fig. 3.5). We found, however, that staining did not enhance the sensitivity, and we therefore recommend that this be done after the plate has been scanned on the microtiter plate reader.

Comparison of the RDA and micro-gel well diffusion assay showed that blocking the plate with casein did not affect the IC<sub>50</sub> values, and that sterility was also not compromised (results not shown). Even though the IC<sub>50</sub> value remained unchanged with gramicidin S as test compound, this might not be the case for all antimicrobial compounds, therefore this blocking step is recommended.



*Figure 3.5* A photograph of a micro-gel well diffusion assay experiment after fixation and colouring. Column one contains only gel to serve as a blank and sterility control. Rows A, G, and H, starting at column two, contain the *M. luteus*-gel suspension without gramicidin S. Wells B2 to F2 contain a bacterial-gel suspension with gramicidin S (200 µg/mL), from where consecutive columns contain doubling dilutions of the peptide. The final column (wells B12 to F12) contains the lowest gramicidin S concentration (0.2 µg/mL).

### 3.5 Conclusions

In general, all three methods investigated here were easily manageable and highly repeatable results were obtained. There are, however, some advantages and disadvantages to each of them. The use of a microtiter plate allows for a much larger and more economical experiment, as opposed to a culture dish which can accommodate far fewer wells per dish. Furthermore, determining inhibition by a spectrophotometric method, using a microtiter plate reader, is more sensitive and accurate than physically measuring zones of inhibition, because of possible human bias in measuring. Comparing the microtiter broth dilution method and the micro-gel well diffusion assay, it is clear that the microtiter broth dilution method is the less labour intensive of the two, and is therefore ideal for screening large numbers of test samples. The use of a liquid broth as growth medium also allows for almost immediate antibiotic-cell interaction, as opposed to agar or agarose where it depends on

diffusion rate. Agar or agarose, on the other hand, is advantageous in that microbial heterogeneity or contamination can be detected readily by observing the nature of bacterial growth, as opposed to a broth where a contamination would not be directly discovered [1]. Results from the agarose-based assays were also easily manipulated through the use of fixing agents and staining agents. By using a fixing agent it is possible to halt the experiment at any specific time, as well as to preserve the experiment for an undetermined period of time. By using a staining agent it is possible to create a visual record of the result.

The RDA gave a good indication of antimicrobial activity through the formation of inhibition zones. This method, however, was not as sensitive as the other two. No inhibition zones were visible at gramicidin S concentrations  $< 3.0 \mu\text{g/mL}$ . This result could possibly be attributed to the fact that, at very low concentrations, the test sample might not diffuse far enough into the gel to form a visible inhibition zone. The micro-gel well diffusion assay, in contrast, only depends upon the localised downward diffusion of the test sample. Because the reaction area is not as large as that of the RDA, diffusion of molecules of varying molecular size is also no longer a limiting factor.

In other experiments we demonstrated the versatility of the micro-gel well diffusion assay by using various other antimicrobial compounds, as well as *Escherichia coli* as Gram-negative indicator organism. Antimicrobial compounds assayed included the cationic frog peptide, magainin 2 and some N-terminal deletion analogues, cationic model peptides from a combinatorial library, and the antibiotic tetracycline (see Chapter 4).

Generally, there is some difficulty in standardising antimicrobial assays because there is no single assay is compatible with all antimicrobial compounds. It seems therefore that researchers will have to decide upon, and make use of a method best suited to their work. The statistical analysis of the results from the three methods, shows that all three sets of results differed significantly from each another. This emphasises the fact that if identical assays are not used, results cannot be compared. The micro-gel well diffusion assay and our method of data analysis, using Prism software, improved the sensitivity of the current agar or agarose-based assays.

In the following chapter the micro-gel well diffusion assay shall be used to test the susceptibility of antibiotic resistant organisms to a combination of antibiotics and antimicrobial peptides.

### 3.6 References

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## Chapter 4

### *The synergistic antimicrobial effect of tetracycline and magainin 2, and N-terminal deletion analogues, on tetracycline resistant Escherichia coli*

#### 4.1 Introduction

Antibiotics are one of the most important therapeutic discoveries in medicine, but their benefits are rapidly being lost as a consequence of an ever-increasing rise in antibiotic resistance. This problem has become global, and holds significant implications for future health and patient care [1]. With the recent emergence of vancomycin insensitive *Staphylococcus aureus* it is clear that steps must be taken to curb the rise in resistant bacteria [2].

Bacteria make use of inventive and versatile mechanisms to resist antibiotics and other toxic elements. These mechanisms can either be specific or general in their mode of action. Examples of specific mechanisms of resistance include drug-inactivating enzymes [3] (e.g. beta-lactamases which destroy the beta-lactam ring of penicillin and cephalosporins), drug modifications [3] (eg. aminoglycoside and macrolide resistance), mutations in the antibiotic target site itself [3] (eg. the RNA polymerase for rifampicin), and acquisition of genes encoding antibiotic target proteins which are insensitive to the antibiotic [3]. The more general mechanisms include, for example, an organism surrounding itself with a barrier of low permeability or expelling the drug by means of an energy dependent pump [4]. Of the more general mechanisms, active efflux systems are responsible for resistance to a variety of structurally unrelated antibiotics, and are being recognised more frequently in increasing numbers of environmental and clinical isolates [4]. Antibiotics are extruded by an energy-dependent process linked to the proton-motive force [4]. The existence of multidrug efflux systems indicates that this form of resistance has become non-specific in nature, signifying the

development of bacteria resistant to numerous antibiotics [4]. (Refer to Chapter 1 for more detail)

We used an antimicrobial peptide in conjunction with a conventional antibiotic, on bacteria resistant to the antibiotic in an attempt to overcome the resistance. The mechanism of resistance targeted was the energy dependent efflux pumps. In developing a model system, tetracycline was chosen as antibiotic (Fig.4.1), *Escherichia coli* JM109 as the tetracycline resistant organism, and magainin 2, as well as two N-terminal deletion analogues, as the antimicrobial peptides.

Tetracycline curbs bacterial growth by inhibiting aminoacyl transfer RNA binding. It binds to both the 30S and 70S ribosomal subunits, and inhibits the binding of aminoacyl transfer RNAs (tRNAs) at the so-called A site [5].

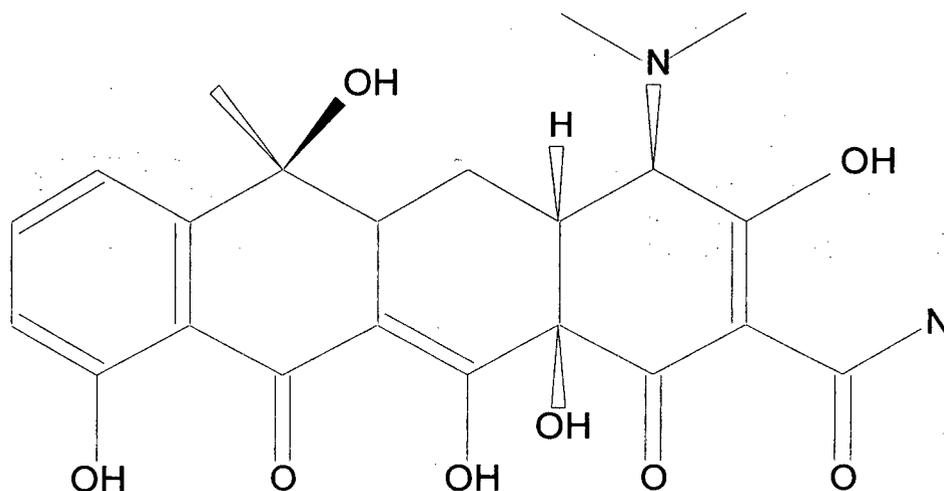
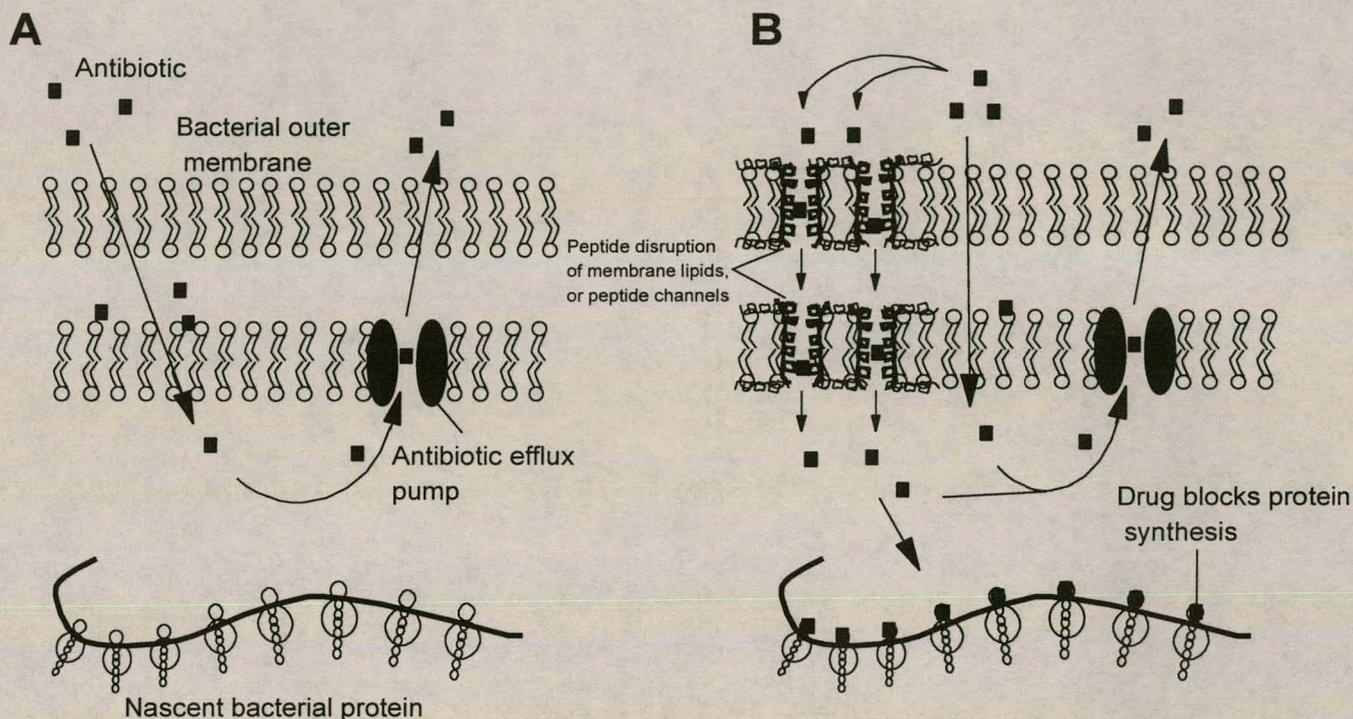


Figure.4.1 Structural formula of tetracycline.

Magainin 2, a linear cationic peptide originally isolated from the African clawed frog *Xenopus laevis*, forms ion permeable channels in cell membranes which ultimately leads to cell death (see section 1.6.3). We therefore set out to determine whether the permeabilisation effect of magainin 2 on the bacterial outer membrane could perhaps facilitate the entry of tetracycline into the cell to such a degree that the active efflux pumps would be neutralised, thus reducing the degree of resistance (Fig. 4.2).



*Figure 4.2* A diagram illustrating our proposed strategy to overcome the active efflux pumps that certain bacteria utilise as a mechanism of resistance. **A** shows how resistant bacteria pump the antibiotic out of the cell before it can reach its target. **B** shows how we intend to make use of an antimicrobial peptide to disrupt the bacterial membranes making them more permeable to the antibiotic [6].

## 4.2 Materials

*E. coli* strains, JM109 (containing the  $\beta$ -lactamase encoding plasmid, pBR322) and HB101 were used in all experiments. Tetracycline was from Sigma-Aldrich (St. Louis, Missouri, USA). Analytical quality water was prepared by filtering glass distilled water through a Millipore Milli Q<sup>®</sup> water purification system. HV 0.45 micron membrane filters were from Waters-Millipore (Milford, USA). Ethanol (99.8%) was from Merck (Darmstadt, Germany).

## 4.3 Methods

### 4.3.1 Peptide synthesis and purification

Peptides were synthesised at room temperature (20-25°C) according to the Fmoc-polyamide protocol using the shake flask bench procedure (see Chapter 2, section 2.3.3. Crude peptides were purified to >96% homogeneity as described in Chapter 2.

### 4.3.2 Antimicrobial assay

Peptides were filtered through HV 0.45 µm Millipore filters on the day before performing an experiment to remove any insoluble particles, and then freeze-dried overnight. Tetracycline dilutions were made from a standardised stock solution (5 mg/mL in 70% ethanol), which was used for no longer than one week, as the tetracycline tended to precipitate if kept for longer. Stock solutions were kept in amber vials at -10°C. Peptide dilutions were made from a 1 mg/mL stock solution (in analytical grade water), prepared directly before performing an assay.

For all antimicrobial determinations we used the micro-gel well dispersion assay (see Chapter 3). When performing an assay for synergism or combined inhibition studies, six dilutions of tetracycline were prepared, each inhibiting the growth of *E. coli* at a specific point over an evenly spread range of 0 to 100% bacterial growth. Tetracycline and peptide dilutions (minimum inhibitory concentrations, MIC, and the concentration resulting in 50% inhibition of microbial growth, IC<sub>50</sub>), were prepared at twice their required concentrations. Equal volumes (15 µL) of peptide and tetracycline were added to the desired wells, which resulted in a 1:1 dilution of the respective antimicrobial compounds.

For the determination of synergism, three separate controls were used to calculate bacterial growth (analytical grade water controls (30 µL), peptide at its MIC (15 µL) and water (15 µL) controls, and peptide at its IC<sub>50</sub> (15 µL) and water (15 µL) controls). Bacterial inhibition of those wells, that received tetracycline and water, was calculated in terms of growth in control wells containing water. These wells showed the effect of tetracycline alone, and IC<sub>50</sub> values obtained could be compared to previously obtained IC<sub>50</sub> values, showing the validity of the experiment. The bacterial inhibition in wells, that received peptide and tetracycline, was

calculated from growth in control wells containing the respective peptide concentration ( $IC_{50}$  or MIC), and water. These calculations facilitated the interpretation of data by ruling out the effect of the peptide and helping to identify possible synergism.

### **4.3.3 Data processing**

All data was analysed using, Graphpad Prism version 2.01 for Windows, GraphPad Software, San Diego, California, USA.

Nonlinear regression was performed on the dose-response data and a sigmoidal curve with variable slope was fitted to each of the data sets (Chapter 3, Fig. 3.1).

### **4.3.4 Structure modelling using HyperChem<sup>®</sup> 5.0**

Models of the peptide structures were generated with HyperChem<sup>®</sup> 5.0 [7].

## **4.4 Results and Discussion**

### **4.4.1 Antimicrobial activities**

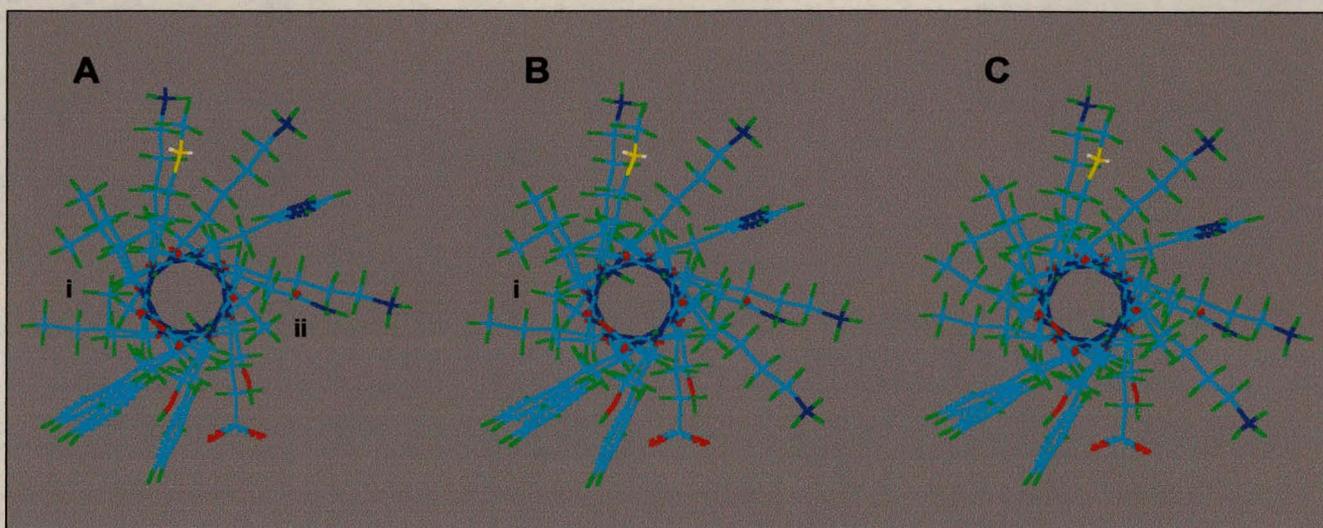
Using the micro-gel well dispersion assay, the  $IC_{50}$  and MIC values were determined for the antibiotic tetracycline against tetracycline resistant (JM109) and non-resistant (HB101) strains of *E. coli* (Table 4.1). The tetracycline resistant strain of *E. coli* showed an  $IC_{50}$  value approximately 30 times greater than that of the non-resistant strain.  $IC_{50}$  and MIC values for the peptides were determined in the same manner (Table 4.1). Magainin 2 was assayed against both strains of *E. coli*, and almost identical  $IC_{50}$  and MIC values were obtained in each case. Magainin 2 N<sup>21</sup> was assayed against the resistant strain of *E. coli* and the  $IC_{50}$  value was approximately twice that of magainin 2. Magainin 2 N<sup>19</sup> exhibited no antimicrobial activity over the concentration range 9-284 nmoles/mL. Structure modelling of the peptides using HyperChem<sup>®</sup> 5.0 showed how the absence of amino acids from the N-terminal of the peptide affected its  $\alpha$ -helical structure, and subsequently also its antimicrobial activity. Structure modelling of magainin 2 N<sup>21</sup>, which lacked a glycine and isoleucine residue, showed the definite formation of a cavity in the hydrophobic face of the amphipathic  $\alpha$ -helix of the

peptide (Fig 4.3 i). Further modelling of magainin 2 N<sup>19</sup>, which lacked additional glycine and lysine residues, showed the formation of a second cavity in the hydrophilic face of the amphipathic  $\alpha$ -helix of the peptide (Fig. 4.3 ii).

**Table 4.1.** MIC and IC<sub>50</sub> values of tetracycline, magainin 2, magainin 2 N<sup>21</sup>, and magainin 2 N<sup>19</sup> against *E. coli*. Magainin 2 was assayed against both strains of *E. coli*. Two independent experiments were performed per value and each concentration per experiment was determined in quadruplicate.

	MIC (nmol/mL) <sup>1</sup>	IC <sub>50</sub> (nmol/mL) <sup>1</sup>
<b>Tetracycline against non-resistant <i>E. coli</i> HB101 (n=2)</b>	0.2 ± 0.007	1.4 ± 0.005
<b>Tetracycline against resistant <i>E. coli</i> JM109 (n=2)</b>	14.4 ± 1.1	44.1 ± 0.5
<b>Magainin 2 against <i>E. coli</i> HB101</b>	8.7	29.7
<b>Magainin 2 against <i>E. coli</i> JM109 (n=2)</b>	8.4 ± 1.2	29.0 ± 0.9
<b>Magainin 2 N<sup>21</sup> against <i>E. coli</i> JM109 (n=2)</b>	23.6 ± 0.3	63.6 ± 0.4
<b>Magainin 2 N<sup>19</sup> against <i>E. coli</i> JM109 (n=2)</b>	> 284	> 284

<sup>1</sup> Values are means ± SEM



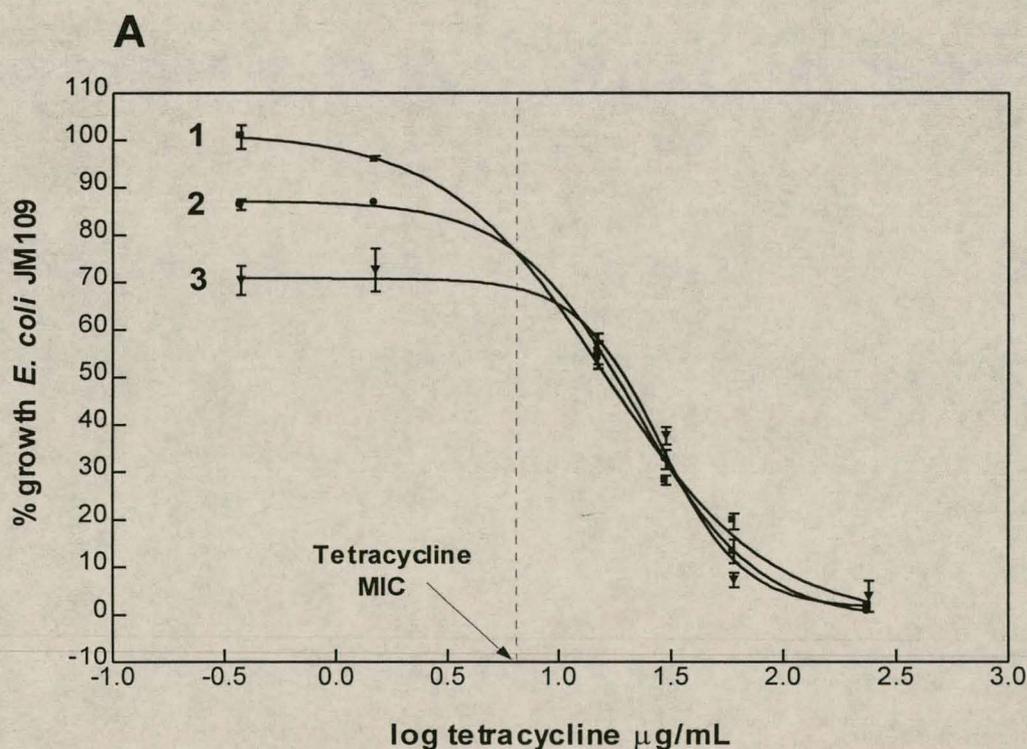
**Figure 4.3** Peptide structures modelled using HyperChem 5.0<sup>®</sup> showing the  $\alpha$ -helical conformations of the peptides as viewed from the top (N-terminal at the top). The dark blue lines depict the nitrogen, red the oxygen, light blue the carbon, yellow the sulphur and green the hydrogen atoms. **A** represents magainin 2 N<sup>19</sup>, with **i** and **ii** indicating the cavities formed in the hydrophobic and hydrophilic face of the amphipathic  $\alpha$ -helix as a result of the omission of the four residues from the N-terminal of the peptide. **B** represents magainin 2 N<sup>21</sup>, with **ii** indicating the cavity formed in the hydrophobic face of the amphipathic  $\alpha$  helix as a result of the omission of two residues from the N-terminal of the peptide. **C** represents magainin 2.

#### **4.4.2 Determination of synergism between the antimicrobial peptides and tetracycline on tetracycline resistant *E. coli***

Sigmoidal dose-response curves for tetracycline and magainin 2 (at its IC<sub>50</sub> and MIC) and tetracycline are shown in Fig. 4.4A. For tetracycline concentrations above its MIC there was no synergism, while at concentrations below its MIC there was significant synergism between the peptide and tetracycline. Magainin 2 at its MIC, and tetracycline concentrations below its MIC synergistically inhibited the growth of *E. coli* JM109 by  $\pm 12\%$ . Magainin 2 at its IC<sub>50</sub>, and tetracycline concentrations below its MIC synergistically inhibited the growth of *E. coli* JM109 by  $\pm 29\%$ . To determine whether this occurrence was as a result of the mechanism of resistance, the experiment was repeated on non-resistant *E. coli* HB101. The result, however, remained the same (Fig. 4.4B).

Identical experiments were performed with magainin 2 N<sup>21</sup> and tetracycline on *E. coli* JM109, again yielding three sigmoidal dose-response curves (Fig. 4.5). Magainin 2 N<sup>21</sup> at its MIC and tetracycline showed no noticeable synergism. Magainin 2 N<sup>21</sup>, at its IC<sub>50</sub>, together with tetracycline at concentrations below its MIC synergistically inhibited the growth of *E. coli* JM109 by ±19%. The compounds again showed a decrease in synergism at concentrations of tetracycline above its MIC.

Similar experiments were performed with magainin 2 N<sup>19</sup> and tetracycline on *E. coli* JM109. Magainin 2 N<sup>19</sup> was used at a concentration double that of the IC<sub>50</sub> of magainin 2 N<sup>21</sup>. Although magainin 2 N<sup>19</sup> had no antimicrobial activity alone, in conjunction with tetracycline, at concentrations below its MIC, the two compounds synergistically inhibited the growth of *E. coli* JM109 by ± 9% (Fig. 4.6). Fig. 4.7 compares the synergistic antimicrobial activities of all three peptides and tetracycline at concentrations below its MIC.



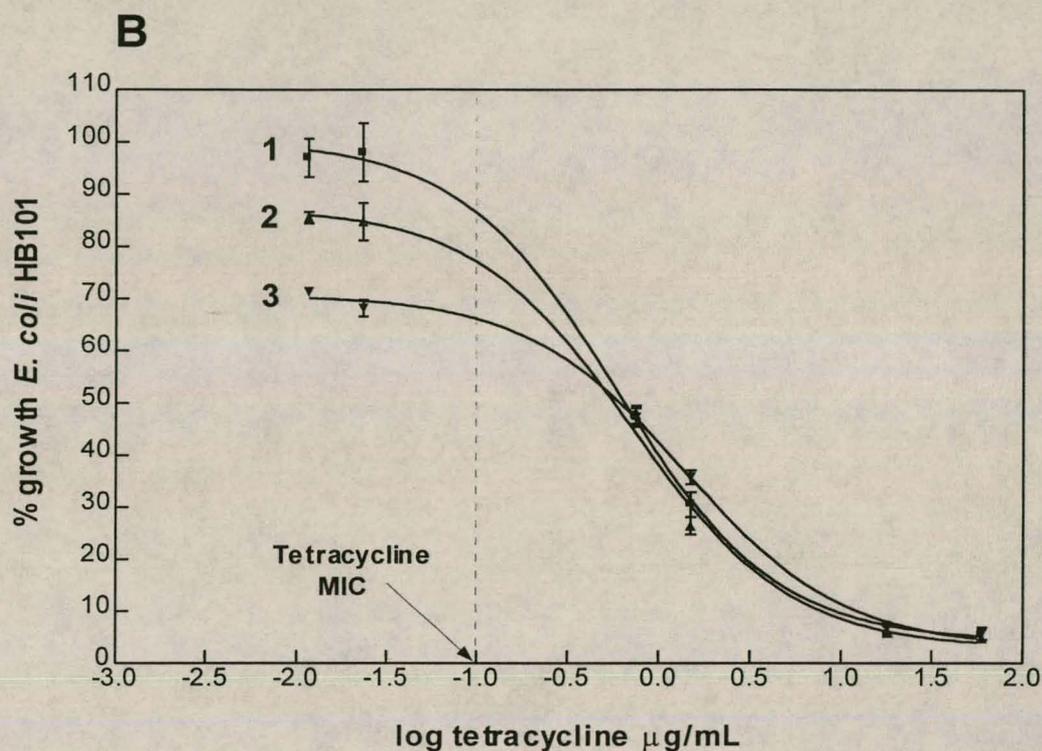


Figure 4.4 Detection of synergism using resistant (A) and non-resistant (B) *E. coli*. Lines: 1, tetracycline; 2, tetracycline and magainin 2 at its MIC; and, 3, tetracycline and magainin 2 at its  $\text{IC}_{50}$ .

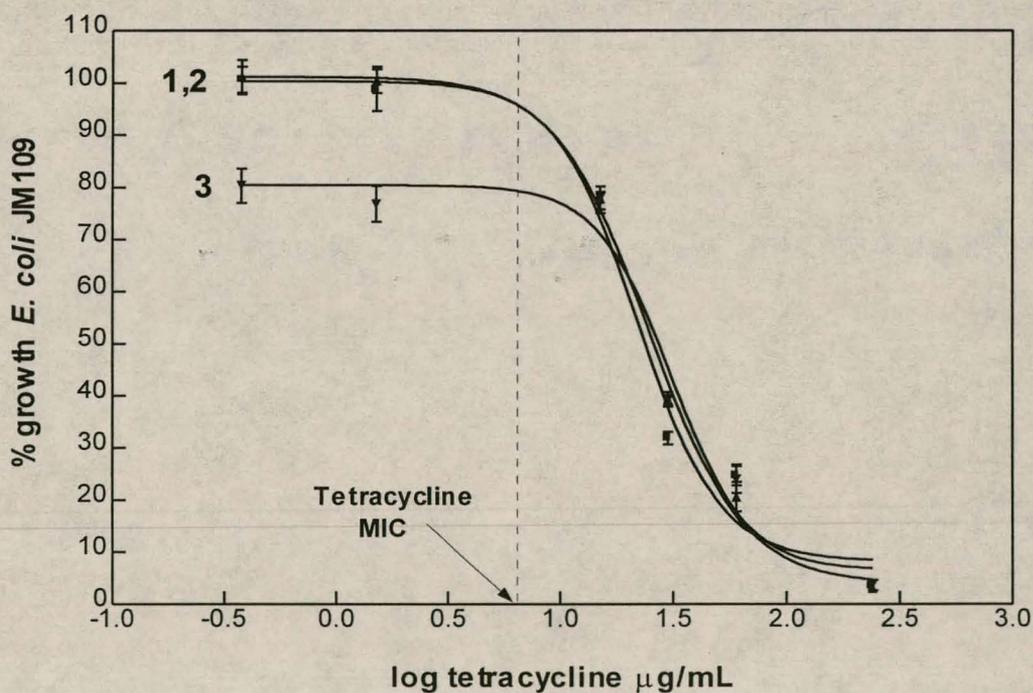


Figure 4.5 Detection of synergism using resistant *E. coli*. Line: 1, tetracycline; 2, tetracycline and magainin 2  $\text{N}^{21}$  at its MIC; and, 3, tetracycline and magainin 2  $\text{N}^{21}$  at its  $\text{IC}_{50}$ .

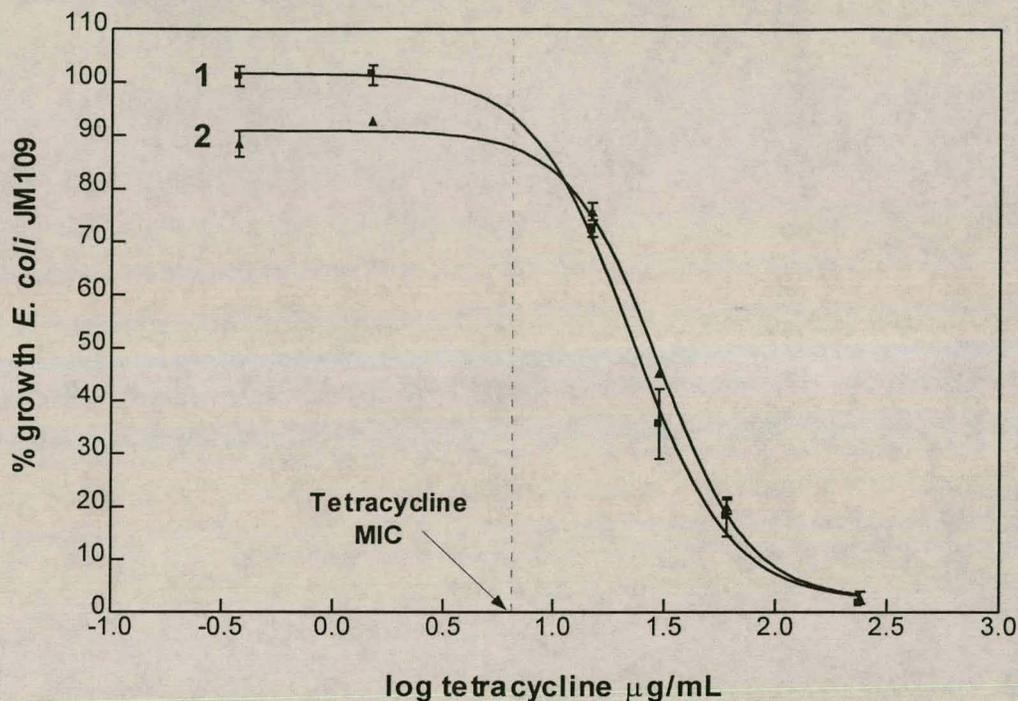


Figure 4.6 Detection of synergism using resistant *E. coli*. Line: 1, tetracycline; 2, tetracycline and magainin 2 N<sup>19</sup> at a concentration of 292 µg/mL.

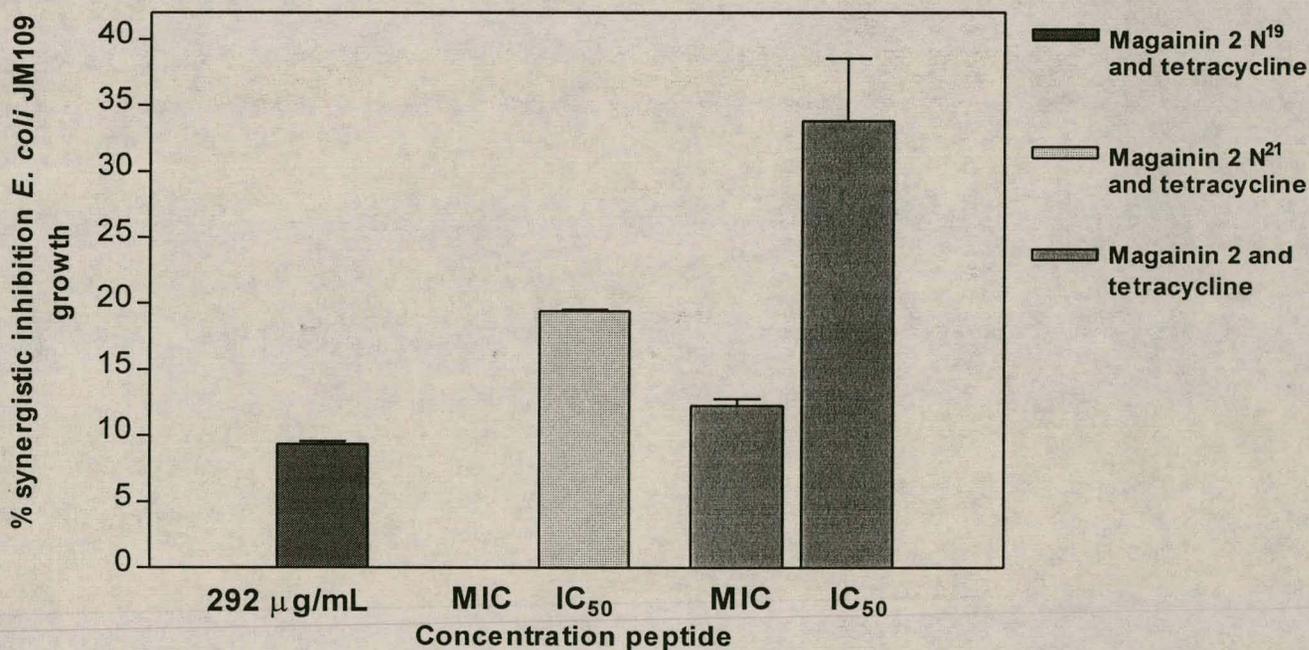


Figure 4.7 The synergistic antimicrobial activity between the peptides and tetracycline at concentrations below its MIC. Duplicate experiments were performed.

#### 4.4.3 Determination of the combined antimicrobial activity of the antimicrobial peptides and tetracycline on tetracycline resistant *E. coli* JM109

To determine the combined antimicrobial effect of the antimicrobial peptides and tetracycline, the bacterial inhibition for all data was determined from controls which contained only water. In this way the cumulative effect of the peptide and tetracycline could be seen.

Sigmoidal dose-response curves were again obtained for tetracycline alone, and tetracycline with the respective antimicrobial peptides at either their  $IC_{50}$  or MIC. In the case of magainin 2 there was a marked increase in growth inhibition. Magainin 2 at its MIC, together with tetracycline at concentrations below its MIC cumulatively inhibited the growth of *E. coli* by  $\pm 29\%$ . At its  $IC_{50}$ , together with tetracycline at concentrations below its MIC, cumulative inhibition was  $\pm 76\%$  (Fig. 4.8).

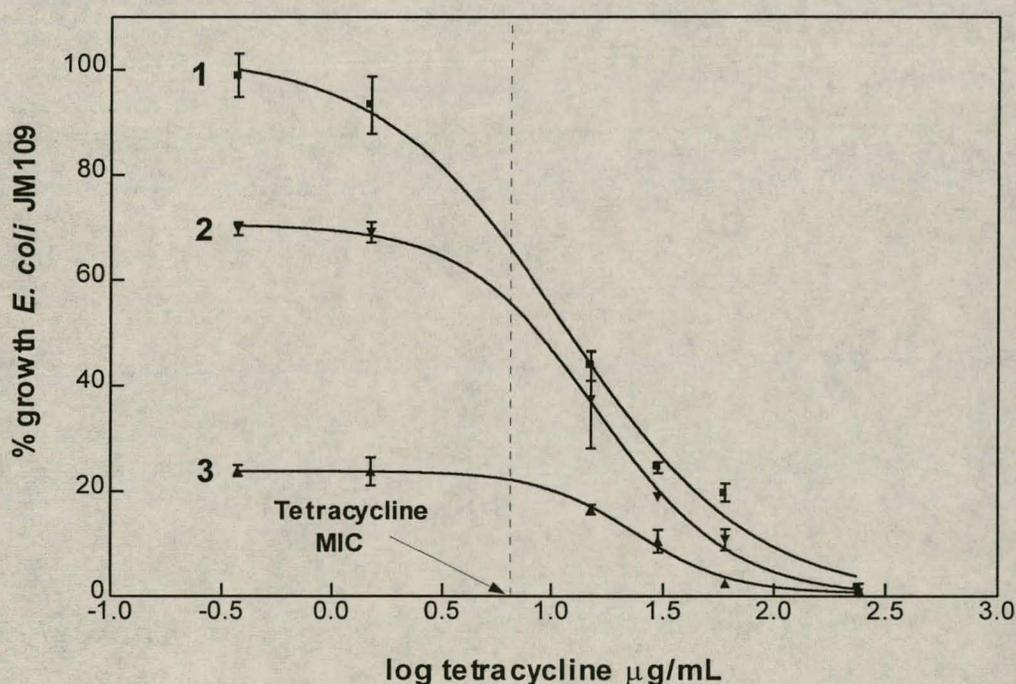
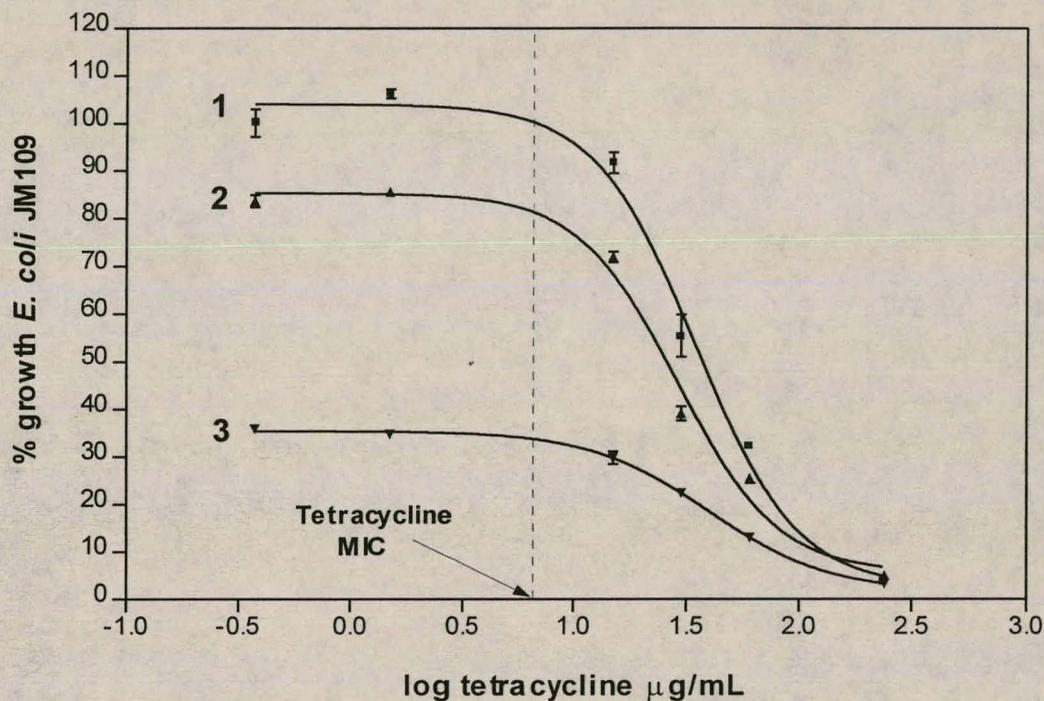


Figure 4.8 The combined antimicrobial effect of magainin 2 and tetracycline on resistant *E. coli*. Line: 1, tetracycline; 2, tetracycline and magainin 2 at its MIC; and, 3, tetracycline and magainin 2 at its  $IC_{50}$ .

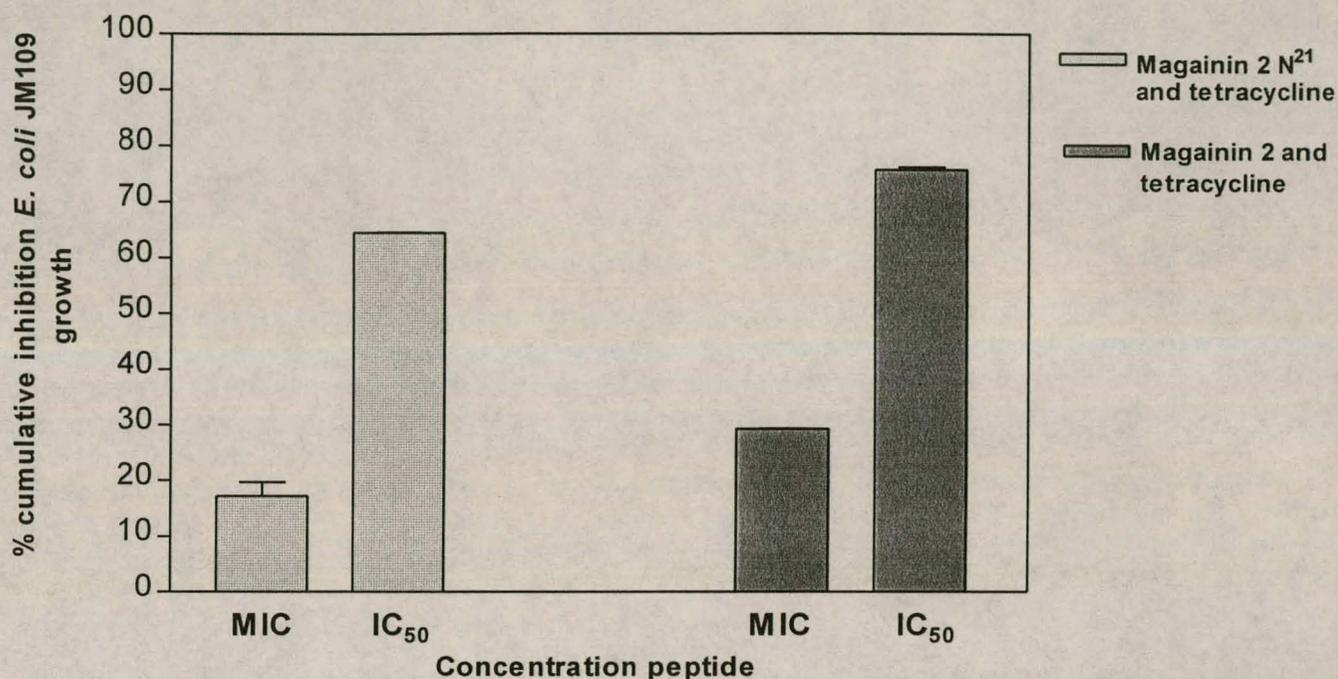
Similar results were obtained with magainin 2  $N^{21}$ . Magainin 2  $N^{21}$  at its MIC, together with tetracycline at concentrations below its MIC inhibited the growth of *E. coli* by  $\pm 15\%$ . Magainin 2  $N^{21}$  at its MIC exhibited no noticeable synergism with tetracycline (section 4.4.2), the inhibition in this case could therefore be attributed to the peptide. Magainin 2  $N^{21}$  at its

$IC_{50}$ , together with tetracycline at concentrations below its MIC cumulatively inhibited the growth of *E. coli* by  $\pm 65\%$  (Fig. 4.9).

As magainin 2  $N^{19}$  exhibited no antimicrobial activity, the synergistic and cumulative effects were assumed to be identical. Fig. 4.10 compares the combined antimicrobial activities of all three peptides and tetracycline at concentrations below its MIC.



*Figure 4.9* The combined antimicrobial effect of magainin 2  $N^{21}$  and tetracycline on resistant *E. coli*. Line: 1, tetracycline; 2, tetracycline and magainin 2  $N^{21}$  at its MIC; and, 3, tetracycline and magainin 2  $N^{21}$  at its  $IC_{50}$ .



*Figure 4.10* The combined antimicrobial activity of tetracycline, at concentrations below its MIC, and the peptides. Magainin 2 N<sup>19</sup> is not shown as its cumulative effect is identical to its synergistic effect which is shown in Fig. 4.6. Duplicate experiments were performed.

#### ***4.4.4 Determining the effect of ethanol on the antimicrobial activity of magainin 2***

Tetracycline was soluble only in 70% ethanol. Stock solutions contained 5 mg/mL tetracycline in 70% ethanol. Concentrations any higher than 5 mg/mL resulted in the tetracycline precipitating rapidly out of solution. The dilution of tetracycline with the highest concentration (1.6 mg/mL) contained 22% ethanol when diluted with analytical grade water. Further doubling dilutions were made, therefore halving the ethanol concentration each time. All concentrations of ethanol were tested with the peptide to determine what effect the ethanol had on the activity of the peptide as well as bacterial growth. Ethanol had no effect on the peptide's activity or on bacterial growth (Fig. 4.11)

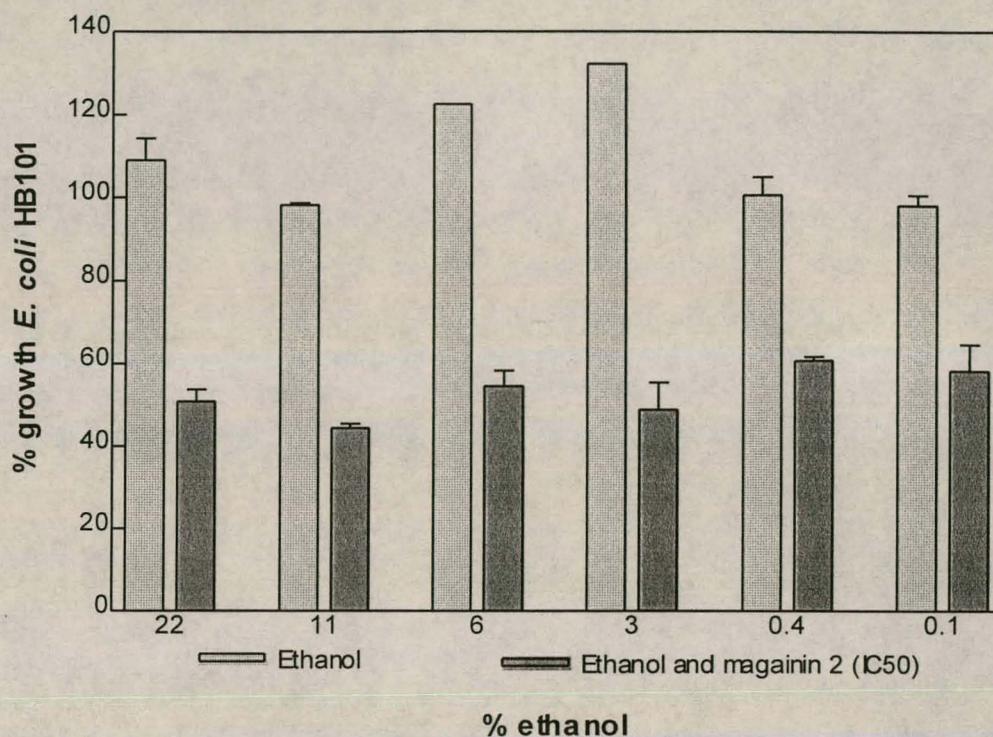


Figure 4.11 Concentrations of ethanol showing the effect of the ethanol on bacterial growth and the antimicrobial activity of magainin 2 at its  $IC_{50}$ .

## 4.5 Conclusions

The antimicrobial activity decreased rapidly with the removal of amino acids from its N-terminal. Magainin 2  $N^{21}$ , which lacked a glycine and isoleucine residue, had approximately half the antimicrobial activity of the full length peptide (comparing their  $IC_{50}$  values). Magainin 2  $N^{19}$ , which lacked two glycine residues, an isoleucine, and a lysine residue exhibited no discernable antimicrobial activity. In agreement with our results, previous studies showed that when magainin 2 is shortened to 19 residues or less from the N-terminal, its activity decreases dramatically [8]. This indicates that the amino acid residues making up the N-terminal of the peptide are vitally important for the antimicrobial activity of the peptide. Magainin 2 exhibited the same degree of activity against both strains of *E. coli*. Structure modelling of the deletion peptides indicated that the omission of the first two amino acid residues from the N-terminal of the peptide caused the formation of a cavity in the hydrophobic face of the amphipathic  $\alpha$ -helix. Omitting two more residues subsequently caused the formation of another cavity in the hydrophilic face of the amphipathic  $\alpha$ -helix. Therefore alterations to the hydrophobic face of the amphipathic  $\alpha$ -helix lead to a substantial

loss of activity, while alterations to both the hydrophobic and hydrophilic face of the amphipathic  $\alpha$ -helix lead to complete loss of activity.

All three peptides exhibited synergism with tetracycline at concentrations below its MIC. The degree of synergism correlated with the antimicrobial activity of the peptides. Magainin 2, with the highest antimicrobial activity, produced the most synergism in conjunction with tetracycline. Magainin 2 N<sup>19</sup> showed no antimicrobial activity alone; however, in conjunction with tetracycline at concentrations below its MIC, the two compounds displayed synergism. Magainin 2 N<sup>19</sup>, although not active enough to lyse the bacterial membrane, therefore still succeeds in permeabilising or disrupting the membrane to such an extent that the cell becomes more susceptible to tetracycline. From the curves it was clear that no synergism took place at tetracycline concentrations above its MIC. Initially it was suspected that at higher tetracycline concentrations complexes were being formed between the peptide and tetracycline; however, electrospray ionisation mass spectrometry (ESI-MS) revealed no evidence of such complexes (data not shown). The ethanol, in which the tetracycline was dissolved, had no effect on the activity of the peptide (section 4.4.4). The loss of synergism at tetracycline concentrations above its MIC seemed therefore to be caused during the initial interaction of both compounds with the bacterial outer membrane. Literature states that magnesium ions ( $Mg^{2+}$ ) function as cationic bridges between adjacent phosphates of lipopolysaccharide molecules in order to stabilise the outer membrane [6, 9]. Cationic peptides as well as antibiotics are known to compete with the  $Mg^{2+}$  cations, thus destroying the LPS cross-bridging and disturbing the outer membrane [6]. This also explains why the presence of divalent cations inhibit the antimicrobial activity of magainin [6]. We suggest that at higher tetracycline concentrations (above its MIC), tetracycline provides stronger competition for the  $Mg^{2+}$  binding sites, preventing the peptide from gaining entry into the cell. At lower tetracycline concentrations, however, there is a dual competition for the  $Mg^{2+}$  binding sites and synergism between the peptide and tetracycline is observed.

We have shown magainin 2 to overcome bacterial antibiotic resistance as a result of active efflux. On a molecular level, it is unsure how the synergism between tetracycline and magainin 2 takes place. In other experiments magainin has been shown to act synergistically with certain “standard” antibiotics in the chemotherapy of Gram-negative bacterial infections [10]. Synergy between these two antibiotic agents was hypothesised to result from the

permeabilising effect of the peptide, which disturbed the integrity of the bacterial membrane and facilitated entry of the antibiotic into the bacterial cell [11].

These results show that the unique mechanism of action of antimicrobial peptides not only allow them to lyse bacterial cells, but also synergistically enhance the antimicrobial activity of conventional antibiotics. Finally, the general implications and future prospects of this work are summarised in Chapter 5.

## 4.6 References

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## Chapter 5

### *Conclusions*

The aims of this study were first, to synthesise the antimicrobial peptide magainin 2 and two N-deletion analogues, second to develop and establish a reliable method to assess the activity of antimicrobial peptides against various organisms, and third, to investigate the influence of the membrane activity of magainin 2 and two N-deletion analogues on antibiotic resistance. The N-terminal deletion analogues were used to deduce the importance of the N-terminal with regard to the antimicrobial activity of the peptide. Most of these biologically active peptides can be isolated from a variety of sources in nature, this however is an extremely time consuming and laborious process [1]. The most effective way of acquiring large amounts of pure peptide, and peptide analogues, is through peptide synthesis, and the Fmoc-polyamide solid phase peptide synthesis protocol was used to synthesise the peptides used in this study (refer to Chapter 2).

Magainin 2 and two N-terminal deletion analogues were our peptides of choice (Chapter 1). The first deletion peptide (magainin 2 N<sup>21</sup>) was shortened by two amino acids at the N-terminal, and the second by four (magainin 2 N<sup>19</sup>). Two syntheses were done, the first providing insight and experience of the synthesis procedure and purification of these peptides, and the second being an optimisation of the procedure. Through the course of both syntheses valuable lessons were learnt about peptide synthesis, as well as the characteristics of magainin 2. During the first synthesis problems were encountered while coupling Asn<sub>22</sub> and Ser<sub>23</sub>. The asparagine derivative did not couple completely owing to degradation, and the serine derivative did not couple sufficiently because it was incompatible with the activation procedure used. This highlighted the importance of quality control checks on, and extreme purity of, all derivatives and reagents used in peptide synthesis. The unsuccessful coupling of asparagine in the first synthesis led to the formation of two deletion peptides, which proved to be extremely difficult to remove from the peptide preparations. The consequently large number of purification steps also greatly decreased the peptide yields. For the second synthesis a serine derivative compatible with the activation procedure and pure asparagine were used, both of which coupled successfully. Generally, the second synthesis was highly

successful, showing good yields and coupling efficiencies (Table 2.5). The quality of the synthesis made the subsequent purification steps relatively simple, and peptides of a high purity were obtained, as shown by ESI-MS and analytical HPLC.

The next section of this study dealt with the development and evaluation of an antimicrobial assay (Chapter 3). Assays involving antimicrobial agents are commonly performed using agar, agarose or broth media [2]. We developed an assay, termed the micro-gel well diffusion assay, by adapting an agarose-based radial diffusion method [3] to perform it in a microtiter plate. Agarose was used in place of agar to avoid electrostatic interactions between antimicrobial peptides and the polyanionic components of the standard agar [4].

Assays, such as the radial diffusion assays, are more commonly used for the detection of antimicrobial activity in the discovery stages of antimicrobial peptide research [4]. These assays involve the use of an agar or agarose as test matrix, and are exceedingly sensitive and require very little test material. In contrast, assays used for routine application in laboratories involve the use of a liquid broth medium. These assays are extremely analytical and sensitive, and are termed microtiter broth dilution methods [4, 5, 6]. The main reason they are so sensitive and analytical is because they can be analysed spectrophotometrically using a microtiter plate reader. Initially we made use of an agarose-based radial diffusion method in our laboratory [3], which was found not to be sensitive or analytical enough for our requirements. It was for these reasons that we made the above mentioned adaptations for the development of the micro-gel well diffusion assay.

In evaluating the micro-gel well diffusion assay it was compared to a radial diffusion assay [3] and a microtiter broth dilution method [4, 5, 6]. The micro-gel well diffusion assay method compared favourably, being as sensitive as the microtiter broth dilution method and approximately twice as sensitive as the radial diffusion assay. The use of light dispersion for measuring microbial growth, as opposed to measuring inhibition zones in agarose (as in the case of the radial diffusion assay) significantly enhanced sensitivity. Furthermore, agarose as a test matrix had numerous advantages over broth:

- Microbial heterogeneity or contamination can be detected easily by observing the nature of bacterial growth on the surface of the agarose;

- The medium may be supplemented with whole blood or blood products to permit testing of some of the nutritionally fastidious micro-organisms that cannot be tested satisfactorily in a clear broth medium;
- The standard procedure may be modified in a number of ways in order to permit testing of a particular type of microorganism or antimicrobial agent, which is quite acceptable as long as appropriate controls are included to demonstrate that the modification does not affect the end result;
- Using fixing agents it is possible to halt the experiment at any given time, as well as preserve the result for an undetermined period of time. Further treatment with colouring agents can also provide an excellent visual record of the result.
- Finally, with this method we also incorporated and refined a method of data analysis using Prism software for the deduction of MIC, IC<sub>50</sub>, and MBC values. A statistical analysis of the values obtained in each method showed results to be significantly different. This indicates that if identical methods are not used, it will not be feasible to compare their results. This, however, is an ongoing problem, as there is no single assay compatible with all antimicrobial compounds. Therefore for those who require agarose as test matrix, the improved sensitivity and analytical capabilities of this agarose-based assay makes the micro-gel well diffusion assay comparable to assays used for routine application in laboratories.

The final section of this study, first investigated the influence of N-terminal deletion on the antimicrobial activity of magainin 2, and second evaluated the synergistic antimicrobial activity of magainin 2 and tetracycline on tetracycline resistant *E. coli* (resistance incurred through active efflux pumps) (Chapter 4). The two N-terminal deletion analogues were also used in the synergism studies.

Initial determinations of the antimicrobial activity of magainin 2 and the N-terminal deletion analogues showed that the activity of the peptides decreased substantially with N-terminal deletions. The deletion analogue magainin 2 N<sup>21</sup>, which lacked a glycine and isoleucine residue, had approximately half the antimicrobial activity of the full-length magainin when comparing their IC<sub>50</sub> values. Magainin 2 N<sup>19</sup>, which lacked two glycine residues, an isoleucine, and a lysine residue, exhibited no discernable antimicrobial activity over the

concentration range tested. This lack of antimicrobial activity could be attributed to a number of factors: first, the shortened peptide chain could result in an inability of the peptide to completely span the bacterial membrane, or second, removing amino acids from the N-terminal of the peptide could eradicate the peptide's ability to form active peptide complexes inside the membrane. Amino acid residues making up the N-terminal of magainin 2 are therefore vitally important for antimicrobial activity. All three peptides exhibited synergism with tetracycline against tetracycline resistant *E. coli*. The degree of synergism correlated with the antimicrobial activity of the peptides. Magainin 2, at and  $IC_{50}$  concentrations, improved the activity of tetracycline by 12 % and 29% respectively. Magainin 2  $N^{21}$  improved the activity of tetracycline by 19 % at its  $IC_{50}$ , and showed no effect at its MIC. Magainin 2  $N^{19}$ , although it exhibited no antimicrobial activity, still succeeded in improving the activity of tetracycline by approximately 9% when used at an arbitrary concentration.

Synergy between these two antibiotic agents is thought to be the consequence of increased membrane permeabilisation by the peptide. By disturbing the integrity of the bacterial membrane, the entry of the antibiotic into the bacterial cell was facilitated, therefore reducing the efficacy of the efflux-pumps [7]. The decrease in antimicrobial activity of the peptides probably resulted in a decreased permeabilisation of the bacterial membrane, causing a subsequent reduction in synergistic activity. The exact molecular mechanism of this synergism is not yet known, however it is thought that increased antibiotic entry either occurs through the peptide channels, or because of the disruption of the packing of membrane lipids. Results obtained with magainin 2  $N^{19}$  indicate that synergy with this peptide relied on the latter mechanism. From these results it could be deduced that magainin 2  $N^{19}$  interacts with the membrane due to its cationic and amphipathic nature, and that this interaction sufficiently disrupts the membrane to allow tetracycline entry.

The common feature of all these observations, was the lack of synergism between the peptides and tetracycline at tetracycline concentrations above its MIC. This was found not to be a result of peptide-antibiotic complexes or of inhibition of peptide activity by experimental reagents. The lack of synergism between the peptides and tetracycline provided further information regarding the mode of action of these peptides at the outer membranes of Gram-negative organisms. Although their mechanism of action has been elucidated to a degree, the exact interaction of these peptides with bacteria is not yet fully understood [5]. These peptides have recently been shown to permeabilise both the inner and outer membranes of

Gram-negative bacteria [5]. These experiments could not, however, conclusively state whether the ultimate target of the peptide was the inner or outer bacteria membrane [5]. In explaining the lack of synergism between the peptides and tetracycline at concentrations above its MIC, we proposed a theory based on the ability of both compounds to permeabilise the outer bacterial membrane. Magnesium ions ( $Mg^{2+}$ ) function as cationic bridges between adjacent phosphates of lipopolysaccharide molecules in order to stabilise the outer membrane. Cationic peptides as well as antibiotics are known to compete with, or for the  $Mg^{2+}$  cations, thus destroying the LPS cross-bridging and disturbing the outer membrane. It is suggested that at higher tetracycline concentrations (above its MIC), tetracycline provides stronger competition for the  $Mg^{2+}$  binding sites, preventing the peptide from having any effect on the cell. At lower tetracycline concentrations, however, there is a dual competition for the  $Mg^{2+}$  binding sites and synergism between the peptide and tetracycline is observed. Therefore, an essential aspect of magainins' mode of action at the outer bacterial membrane of Gram-negative organisms is seen to be competition with  $Mg^{2+}$  binding sites to disrupt the membrane.

Therefore, the work presented here not only provided more insight into the mode of action of these  $\alpha$ -helical antimicrobial peptides, but more importantly also showed them to be an effective tool in overcoming the active efflux pumps as a mechanism of bacterial antibiotic resistance.

In conclusion there are numerous future prospects for both the micro-gel well diffusion assay, and the synergism studies. As the micro-gel well diffusion assay is specifically designed to determine the effect a compound has on microbial growth, it can be used in a diversity of fields. We have shown the assay to be compatible with cationic peptides as well as a conventional antibiotic, which also displays the versatility of the assay. As mentioned previously, supplementation of the agarose test matrix allows for the use of numerous kinds of organisms and antimicrobial compounds, which further enhances the prospects of the assay.

To further elucidate the mechanism of action of this peptide future experiments would entail the use of a chelating agent, like for instance EDTA, to remove the  $Mg^{2+}$  from binding sites and then to note the effect of the peptides and tetracycline. Future studies regarding synergistic activity between antimicrobial peptides and antibiotics would most likely entail the use of a more clinically relevant organism and antibiotic. Tetracycline and *E. coli* were used in initial studies to develop and optimise a method whereby synergism could be detected.

With *S. aureus* developing such a broad-spectrum of resistance, a clinically relevant organism would definitely be methicillin resistant *S. aureus*.

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