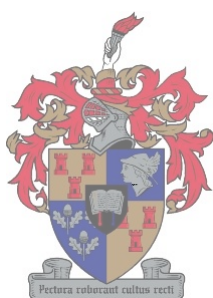# The influence of immobilisation and peptide structure on the bioactivity of model antimicrobial peptides

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.

...19/11/99...................

Gertrude D. Lourens   Date

Summary

Antimicrobial peptides play an integral role in the innate immunity of many plants and animals. An essential aspect of the activity of linear antimicrobial peptides is that they are cationic and have the ability to form an amphipathic α-helical secondary structure. Blondelle *et al.* identified cationic amphipathic peptides with pronounced antimicrobial activity in combinatorial libraries of model 18-residue peptides consisting mostly of Leu and Lys. One of these peptides (YKLLKLLLPKLKGLLFKL-NH$_2$) and three N-terminal deletion analogues (16-, 14- and 12-residue peptides) were investigated in a structure-function study. Four peptide amides were synthesised using the Fmoc-polyamide solid phase peptide synthesis protocol. These four peptides were also prepared in immobilised form by an irreversible coupling of their carboxy-terminal amino acid to a (Gly)$_3$ linker on an ethylenediamine-modified polydimethyl-acrylamide resin. The three longer peptide amides (NLK peptides) were purified using gel permeation chromatography and the shortest peptide by high performance chromatography (HPLC). The high chemical purity of the free peptide amides were confirmed by electrospray ionisation mass spectrometry (ES-MS) and analytical HPLC, while the integrity of the immobilised peptides were confirmed by amino acid analysis and protein sequencing.

In the structure-function study the influence of peptide length, charge and amphipathicity on activity against *Micrococcus luteus, Escherichia coli* and red blood cells was evaluated. The influence of immobilisation on activity was tested against *M. luteus*. The three longer free peptide amides had substantial antibacterial activity, but were more active towards the gram-positive bacterium, *M. luteus*, than towards the gram-negative bacterium, *E. coli*. This specificity could be attributed to the difference in membrane structure of these two organisms, with the *E. coli* membrane differing because of its lipopolysaccharide layer. Interestingly, the activity against *M. luteus* was in the same range than against the red blood cells. The haemolytic activity of these model peptides thus somehow correlated with their activity against gram-positive organisms. Similar activities were observed for 18-residue and the 16-residue peptides when tested against the same organism. However, the 16-residue peptide was more than five fold less active against *E. coli* than *M. luteus*, while the other two peptides were about two fold less active. The difference in activity is probably because the 16-residue

peptide having one less positive charge than the 18-residue peptide. The 14-residue peptide was less active than the two longer peptides, which could be the result of its smaller hydrophobic face it has when compared to the longer peptides. The 12-residue peptide amide tended to aggregate and was inactive. The reduced peptide length of the 12-residue peptide, its aggregation and its smaller hydrophobic face therefore influenced the peptide's activity. Some surface activity was, however, observed for the immobilised 12-residue peptide, probably because immobilisation prevented aggregation. Similar trends in activity against *M. luteus* were observed when comparing the different peptide amides with their immobilised analogues, although the immobilised peptides were about 1000 fold less active.

In conclusion, the amphipathic character, with particular emphasis on the size and density of the hydrophobic face, must be maintained to preserve antibacterial activity. Furthermore, immobilisation restricted the membrane activity of these peptides to only surface interaction, thus limiting any other interaction essential for activity.

Opsomming

Antimikrobiese peptiede speel 'n integrale rol in die aangebore immuniteit van verskeie plante en diere. 'n Baie belangrike eienskap vir aktiwiteit van lineêre antimikrobiese peptiede is dat hulle kationiese moet wees en 'n amfipatiese α-helikse sekondere struktuur moet aanneem. Blondelle *et al.* het kationiese amfipatiese peptiede met verhoogde antimikrobiese aktiwiteit, vanuit kombinatoriese biblioteke van model 18-residu peptiede wat meestal uit Leu en Lys bestaan, geïdentifiseer. Een van hierdie peptiede (YKLLKLLLPKLKGLLFKL-NH$_2$) en drie N-terminaal delesie analoë (16-, 14- en 12-residu peptiede) is in 'n struktuur-funksie verwantskap ondersoek. Die vier peptiede is as peptiedamiede gesintetiseer deur gebruik te maak van die Fmoc-poliamied soliede-fase peptiedsintese protokol. Die vier peptiede is ook voorberei in die geïmmobiliseerde vorm deur hul karboksie-terminale residu onomkeerbaar te koppel aan 'n (Gly)$_3$ verlengingsarm op 'n etileendiamien-gemodifiseerde polidimetielakrielamied hars. Die drie langer peptiedamiede (NLK-peptiede) is deur middel van gelpermeasie chromatografie gesuiwer en die kortste peptied deur middel van hoë doeltreffendheid chromatografie (HPLC). Die hoë chemiese suiwerheid van die peptiedamiede is deur elektrosproei-ionisasie massaspektrometrie (ES-MS) en analitiese HPLC bevestig, terwyl die integriteit van die geïmmobiliseerde peptiede deur aminosuur analise en proteien volgorde bepaling bevestig is.

In die struktuur-funksie studie, is die invloed van peptiedlengte, lading en amfipatisiteit op die aktiwiteit teen *Micrococcus luteus, Escherichia coli* en rooibloed selle ondersoek. Die invloed van immobilisering op antimikrobiese aktiwiteit is teen *M. luteus* ondersoek. Die drie langer peptiedamiede het beduidende aktiwiteit getoon, maar was meer aktief teen die gram-positiewe *M. luteus* as teen die gram-negatiewe *E. coli*. Die spesifisiteit is moontlik as gevolg van die verskille in membraanstruktuur tussen die twee organismes, met *E. coli* wat 'n lipopolisakkaried laag het. Wat tog interessant is, is dat die hemolitiese aktiwiteit van hierdie model peptiede ooreenkom met hul aktiwiteit teen *M. luteus*. Die 18-residu peptied en die 16-residue peptied het soortgelyke bioaktiwiteit teen dieselfde teikenselle getoon. Die 16-residu peptied is egter vyf maal minder aktief teen *M. luteus* as teen *E. coli*, terwyl die ander peptiede net twee maal minder aktief was. Die verskil in aktiwiteit kan moontlik is moontlik as gevolg van die een positiewe lading wat die 16-residu peptied minder het as die 18-residu

peptied. Die 14-residu peptied het laer aktiwiteit getoon as die langer peptiede, wat moontlik toegeskryf kan word aan die kleiner hidrofobiese kant. Die 12-residu peptiedamied was geneig om te aggregeer en het geen aktiwiteit getoon nie. Die verkorte peptiedlengte, aggregasie-tendens en die kleiner hidrofobiese kant het dus hierdie peptied se aktiwiteit beïnvloed. Oppervlakaktiwiteit is wel waargeneem vir die geïmmobiliseerde 12-residu peptied, moontlik omdat immobilisering die peptied verhoed om te aggregeer. Soortgelyke tendense in aktiwiteit teen *M. luteus* is waargeneem as die peptiedamiede se aktiwiteit met die van hul geïmmobiliseerde analoë vergelyk word. Die geïmmobiliseerde peptiede is egter omtrent 'n 1000 maal minder aktief.

Dit is dus belangrik, dat die amfipatiese karakter, spesifiek die grootte en digtheid van die hidrofobiese kant, behou moet word om antimikrobise aktiwiteit te bewaar. Verder het die immobilisering van die peptiede hulle tot oppervlakaktiwiteit beperk, wat dus enige ander interaksie wat nodig is vir aktiwiteit verklein.

Met liefde opgedra aan my ouers

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Chapter 3   The influence of structure and immobilisation on the antimicrobial activity of model α-helical peptides

Chapter 4   Conclusions

List of Abbreviations and Acronyms

Ala	alanine
Arg	arginine
CV	cone voltage
Da	dalton
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DIPEA	N, N'-diisopropylethyl amine
DMF	N, N'-dimethylformamide
EDTA	ethylenediaminetetraacetic acid
ES-MS	electrospray mass spectrometry
f	internal fragment
F	phenylalanine
FDNB	1-fluoro-2,4-dinitrobenzene
Fmoc	N^9-fluorenylmethyloxycarbonyl
G	glycine
Glu	glutamic acid
Gly	glycine
HC_{50}	peptide concentration leading to 50 % haemolysis
HOBt	1-hydroxybenzotriazol
HPLC	high performance liquid chromatography
IC_{50}	peptide concentration leading to 50 % growth inhibition
Ile	isoleucine
IR	infrared
K	lysine
L	leucine
Leu	leucine
LK-peptide	model peptide consisting of only leucine and lysine residues
Lys	lysine
[M]	molecular ion
M	molar

m/z	mass over charge ratio
NMR	nuclear magnetic resonance
P	proline
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
Pfp	pentafluorophenyl
Phe	phenylalanine
PITC	phenylisothiocyanate
Pro	proline
PTC	phenylthiocyanate
PyBOP®	benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate
RBS	red blood cells
RP-HPLC	reverse phase high performance liquid chromatography
R_t	retention time of analyte in column chromatography
SEM	standard error of the mean
t	tertiary
tBoc	N^α-t-butyloxycarbonyl
tBu	t-butyl ester
TEA	triethylamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TSB	tryptone soy broth
Tyr	tyrosine
UV	ultraviolet
Y	tyrosine

Preface

Peptide based host defence can be considered as a pervasive and evolutionarily ancient mechanism of immunity. Antimicrobial peptides have been isolated from animals and plants and so far several hundred different peptides have been characterised. A variety of peptide antibiotics are also found in bacteria. Antimicrobial peptides differ markedly in length, sequence and structure, but share two common traits in that they are generally polycationic and normally amphipathic. The recognition of the significance of the cationic amphipathic α-helix has led to *de novo* design of synthetic antimicrobial peptides that incorporate this structural motif. During the years researchers have tried to optimise the activity of antimicrobial peptides by changing net charge, peptide length and the size of the hydrophobic and hydrophilic faces. Some questions, however, still remain unanswered about the structure-function relationship of many antimicrobial peptides. Immobilisation of these peptides may provide a way to analyse the effect of surface interaction on bioactivity. Immobilisation of peptides, that retain antimicrobial activity, may also lead to the development of antimicrobial matrixes.

This thesis describes a structure-function study on model Leu-Lys containing peptides with antimicrobial activity. The influence of peptide length, charge, amphipathicity and immobilisation on the biological activity of four peptides was tested. We report on the syntheses of a model amphipathic α-helical peptide, consisting of 18-residues, and three deletion analogues, their purification, characterisation and finally their biological activity.

First, a short overview of antimicrobial peptides is given in Chapter 1. The synthesis, purification and chemical characterisation of the peptides are described in Chapter 2. The biological activity of the peptides against *Micrococcus luteus*, *Escherichia coli* and red blood cells is described in Chapter 3. In Chapter 4 an integrated overview of the work and future prospects are given.

Chapter 1

Biological activity of antimicrobial peptides

1.1 Introduction

Vertebrates offer easy access to and a favourable environment for invading parasites and potential pathogens. They can penetrate the epithelia of the respiratory and intestinal tracts as well as the excretory system. Besides the classical humoral and cellular response of the immune system against pathogenic micro-organisms, it is now recognised that vertebrates are endowed with an additional chemical defence mechanism involving antimicrobial peptides analogous to those found in invertebrates [1].

Isolation from biological sources such as mammals, amphibians, insects, bacteria and many more has served as a common means for discovery of novel antimicrobial peptides such as the defensins, magainins, cecropins, gramicidins, bacteriocins and many more [2, 3, 4, 5, 6]. Defensins, for example, are sequestered in granules of neutrophils and delivered to phagocytic vacuoles containing ingested microorganisms [7]. Frog skins contain glands that produce a large number of biologically active peptides, many of which have counterparts in neural and intestinal tissues of mammals [8]. One of these peptides, magainin, was discovered in the skin secretions of the frog X*enopus laevis* [9]. The cecropins were originally identified as highly potent antimicrobial peptides in immune haemolymph from the cecropia moth [10]. It has also been found in pig intestine, which implies that cecropins are widespread in the animal kingdom [3].

The endogenous antimicrobial peptides of plants and animals are typically cationic (i. e. contain excess Lys and Arg residues) amphipathic molecules composed of 12 to 45 amino acid residues. These peptides may be produced constitutively or only after infection or injury [11]. The antimicrobial peptides are either produced ribosomally (e.g. magainins, defensins, cecropins, and many others) or synthesised on enzymatic templates (e.g. gramicidin S, iturin A and others). The ribosomally produced peptides can be divided into four main classes [12]:

1. Linear peptides without Cys, such as magainin 2 [13] and cecropin A [14];

2. Peptides with an even number of Cys residues, always linked with disulphide bonds, such as the α– and β–defensins [15];

3. Linear peptides with a high proportion of one or two amino acids, most often Pro and Arg. These peptides do not contain any Cys-residues; examples include indolicidin [16], the histatins [17] and the cathelin-derived peptides [18].

4. Linear peptides containing unusual amino acids such as the lantibiotics in the bacteriocin group of peptides [19].

The peptides belonging to the first class often assume an amphipathic α-helical secondary structure upon interaction with the outer phospholipid bilayer of a bacterium (e.g. cecropin [10] and magainin [9]). On the other hand, the peptides belonging to the second class assume antiparallel β-sheet structures e.g. defensins [7]. The peptides in the third class all are part of the cathelicidin family [20].

Many antimicrobial peptides lyse both gram-positive and gram-negative bacteria (and certain types of liposomes), but cause little or no harm to eukaryotic cells (defensins and magainins are slightly cytotoxic) [3]. Destruction of the host cells is largely avoided by membrane specificity (which will be discussed later in this chapter). In general, their potency (almost like broad-spectrum antibiotics) and their low cost of production (in terms of time, information, and energy), justifies the use of the antimicrobial peptides as a defence mechanism. These factors enable them to form an important part of the innate defence of vertebrates against bacterial infections.

The cationic amphipathic α-helical character of the linear antimicrobial peptides in class 1 was shown to play an important role in their biological activity [2, 21, 22]. This recognition of the significance of the cationic amphipathic α-helix has led to the *de novo* design of synthetic antimicrobial peptides that incorporate this structural motif. A common approach to the design of synthetic antimicrobial peptides has involved the judicious use of hydrophobic and hydrophilic amino acids with a high α-helical propensity to generate amphipathic sequences of various lengths [23]. During the years researchers have tried to optimise the activity of antimicrobial peptides and tried to find out exactly how they work by changing net charge, peptide length and

the hydrophobic and hydrophilic faces of the peptides. Some of this work on natural and model peptides will be discussed in this chapter.

Researchers believe that the antimicrobial activity is linked to the ability of these amphipathic peptides to interact with host cell membranes. The interaction of an antimicrobial peptide with membrane lipids and other components also determines its specificity as will be discussed later. Upon interaction the formation of ion channels leads to permeability changes and consequent cell lysis. Although the exact mechanism of action of antimicrobial peptides is not known, two hypotheses will be given.

1.2 Antimicrobial specificity

Many antimicrobial peptides selectively inhibit and kill bacteria while maintaining low mammalian cell cytotoxicity. This selectivity has been attributed to the differences in the membrane structure of mammalian and bacterial cells [24]. The occurrence of some of the most common lipidic components in membranes is given in Table 1.1.

Table 1.1   Lipid composition of some biological membranes [25]

Membrane lipid	Percent of total composition in			
	Human erythrocyte plasma membrane	Human myelin	Beef heart mitochondria	*E. coli* cell membrane
Phosphatidic acid	1.5	0.5	0	0
Phosphatidylcholine	19	10	39	0
Phosphatidylethanolamine	18	20	27	65
Phosphatidylglycerol	0	0	0	18
Phosphatidylinositol	1	1	7	0
Phospatidylserine	8.0	8.0	0.5	0
Sphingomyelin	17.5	8.5	0	0
Glycolipids	10	26	0	0
Cholesterol	25	26	3	0
Others	0	0	23.5	17

The outer part of the eukaryotic cell membrane consists mainly of zwitterionic lipids such as phosphatidylcholine and sphingomyelin. Phosphatidylcholine is a derivative of glycerol-3-phosphate while sphingomyelin is a sphingolipid with a cationic choline group attached to a phosphorylated hydroxyl group [25]. The prokaryotic membranes consist mainly of phosphatidylethanolamine and are rich in negatively charged lipids such as phosphatidylglycerol and lipopolysaccharide. Antimicrobial peptides are mostly cationic and would therefore preferentially bind to the negatively charged bacterial cells rather than mammalian cells [26, 27]. Cholesterol is a weakly amphipathic substance which forms part of the mammalian cell membrane and could be another component that influences the selectivity of antimicrobial peptides. In addition to cholesterol, both spingomyelin and glycolipids are usually absent from prokaryotic membranes [28].

Selective activity against prokaryotic organisms, also exhibited by some of these peptides, is linked to the differences in cell wall structure. Cecropins and magainins are both more active against gram-negative bacteria than against gram-positive bacteria [25, 26]. Gram-positive bacteria have a thick cell wall with a polysaccharide-peptide complex, know as peptidoglycan, on the surface [25]. This peptidoglycan is a heteropolysaccharide. It is composed of amino sugars in one dimension and cross-linked through branched polypeptides in the other. The amino sugars alternate in the polymer, forming the glycan strands. This results in the bacterium's outer surface being negatively charged. Gram-negative bacterial cell walls also contain peptidoglycan, but it is single layered and covered by an outer lipid membrane layer [25]. This outer membrane, which contains negatively charged lipopolysaccharide molecules, acts as a barrier to hydrophobic molecules [24].

The interaction of magainins with the outer membrane of Gram-negative bacteria has been well studied and it is clear that the peptide has the ability to perturb the structure of the outer membrane [26, 29]. Investigations indicate that defensins also permeabilise the outer membrane of *Escherichia coli*. Upon association with the bacterial inner membrane, they form channels and thereby alter the membrane permeability property [24]. Magainins also exhibit antifungal and antiviral activity [29, 30]. Melittin, on the other hand, shows activity against both red blood cells and bacteria [31].

Many of the peptides actually lack activity against red blood cells at concentrations at which they exert antimicrobial activity [24]. Eukaryotic cells are resistant to lysis, for example, by magainins, because of the peptide-cholesterol interactions in their membranes that inhibit the formation of lytic peptide complexes. Bacterial cells on the other hand, lacking cholesterol, are susceptible to lysis [26]. Another study, with cecropins and related peptides found that sheep erythrocytes were highly resistant to lysis; whereas the introduction of D-amino acids, reversed sequence or inverted amide bonds did not make the cells significantly more susceptible to the peptides [24].

Since cecropin, magainin and melittin with D-amino acids exhibit antimicrobial activity identical to that of the L-enantiomers, it is unlikely that any chiral recognition is involved at the bacterial surface [32]. The specificity in activity could conceivably arise from differences in the architecture of the bacterial and red blood cell surfaces. It is likely that the cationic antibacterial peptides bind to the sialic acid residues on the red blood cell surface and do not even reach the lipid bilayer [24]. In contrast, it was observed that a natural haemolytic α-helical peptide, melittin (from bee venom), interacts with the negative charges on the membrane [31].

1.3 Model antimicrobial peptides

As mentioned earlier, the recognition of the significance of a cationic amphipathic α-helix has led to the *de novo* design of synthetic antimicrobial peptides that incorporate this structural motif. In these model peptides Leu and Ala are usually used as hydrophobic residues and Lys and Arg as hydrophilic residue because of their high α-helical potency [31, 28, 33, 34]. Although the length of these α-helical antimicrobial peptides varies considerably, a length of approximately 18 residues has generally been reported to be necessary to provide an α-helix capable of spanning the hydrocarbon portion of the lipid bilayer [35].

The model peptides have been used to study the influence of peptide length, charge and the size of the hydrophobic face compared to the hydrophilic face on biological activity. The importance of the α-helix was also evaluated by a number of investigators.

1.3.1 Model peptide design

Blondelle and Houghten [2] designed synthetic amphipathic peptides (consisting only of Leu and Lys residues called LK-peptides) with potent antimicrobial activity. The highest activity was found for a 18 residue peptide with the classically amphipathic α-helical sequence, which confirmed the role of an amphipathic α-helical conformation on biological activity. When an α-helix is amphipathic, i.e., one side is hydrophilic and the opposite side is hydrophobic, hydrogen bonding within the helix becomes distorted and causes a curvature for the helix. The hydrogen bonds formed by the hydrophilic carbonyls with the NH-groups within the helices have been found to be longer and less linear than those involving hydrophobic carbonyls [36]. Since Pro does not possess a free nitrogen for hydrogen bonding, it was thought earlier that Pro will terminate α-helices. It was shown later that if Pro is right in the middle of the helix, it allows the continuation of the helix, but with a kink. Pro is the least likely of the amino acids to be present in a α-helix; when it occurs, it is found mostly at hydrophilic environments. The kinking is always away from the side with the Pro residues [36]. It was found that the inclusion of Pro in the middle of the peptide sequence, inducing a kink, sometimes improved activity [37].

In a later study, Blondelle *et al.* [37] used conformational defined combinatory libraries, derived from the original 18-residue LK-peptide, to identify compounds with enhanced antimicrobial activity. They found four peptides that showed a 10-fold increase in activity relative to the original LK-peptide. These peptides contained Pro as well as a Ile or Phe and Gly in the sequence. The peptides showed activity against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, but lower activity against *Candida albicans*. All four peptides were less haemolytic than the original LK-peptide.

Taking the diversity of natural peptides into account, Tossi *et al.* [38] compared the first 20 amino acids of natural occurring peptides, identified the most frequent amino acids in these positions and subsequently designed a novel antimicrobial peptide. They found that Gly predominates at the N-terminus, acting as a good N-capping residue for the α-helix. Gly is also present within the helix for the majority of peptides analysed. In the hydrophobic face, the aliphatic, hydrophobic residues dominates, while the hydrophilic face was found to be considerably more variable. In their biologically active designed peptides the hydrophobic face consisted of contiguous hydrophobic residues, whereas the hydrophilic face consisted of

hydrophilic residues interspersed with hydrophobic residues. Lys was the favoured charged residue in hydrophilic face. Ile and Leu dominated the hydrophobic sector at the N-terminal portion of the helix, while Ala was found to be more abundant in the C-terminal portion. Thus, they have shown that it is possible to obtain potent, broad-range artificial antimicrobial agents by comparing the sequences of naturally peptides and abstracting statistically significant patterns, which can be included in relatively short sequences.

1.3.2 Influence of peptide length

For a series of amphipathic peptides, with lengths varying from 8 to 22 residues, the highest antimicrobial activities were found for the 14-mer and 15-mer sequences [2]. When compared to the much longer magainins and cecropins, the 14-residue sequence had a higher antimicrobial activity but a lower haemolytic activity. In another study, 15-residue peptides made up from segments of cecropin and melittin were recently found to have higher antimicrobial activity than the cecropins [2]. Another group of LK-peptides (consisting of Leu and Lys (2:1)) with of 15-, 20-, and 22- residues, showed higher haemolytic activity than melittin. They have a net charge and a hydrophobic sector not very different from melittin, despite the fact that there is no relationship at all between the sequences or the charge topology. These peptides were found to be mainly α-helical in organic and aqueous solvents. In the presence of lipids, α-helicity decreased with decreased peptide length [22].

Anzai *et al.* [21] found that short cationic α-helical amphipathic model peptides (8-12 residues in length), composed solely of Leu, Arg, and Ala residues, were able to form ion channels in planar lipid bilayers [21]. These results were confirmed by McLean *et al.* [39] through the investigation of short highly α-helical peptides composed of only Leu and Lys. In this study the peptides were compared for their effectiveness in clearing turbid liposomes by examining their interactions with phosphatidylcholine. Peptides of 10-12 residues were optimal for the rapid decrease in turbidity of liposomes when mixed at the phase transition temperature of the lipids; longer peptides were less effective. A peptide of eight residues also formed clear micelles, which demonstrated demonstrating that an effective lipid-binding, amphipathic α-helical peptide need only be eight residues in length. In another study [28], model peptides were designed using Lys, Leu, Ala and Gly and prepared as the C-terminus amides. These peptides were designed to determine the effect

of length, sequence, and structure on biological activity. The short peptides (heptamers) were inactive against Gram-positive and Gram-negative bacteria when tested. The Leu-containing 21-mers were about twice as potent as the 14-mers [28]. It was proposed that the mechanism of action of the shorter 14-mer peptides involve the dimerisation of the peptide, forming α-helixes long enough to span the lipidbilayer.

1.3.3 The hydrophobic face vs. the hydrophilic face

Bessalle *et al.* [40] showed that the relative proportion between the hydrophobic and basic residues of the peptide is very important. In order to achieve high bioactivity in their peptides it, was necessary that at least 30–45% of the amino acid residues were basic, the rest being hydrophobic.

The two parameters, namely, hydrophobicity and net positive charge, were investigated by Oren *et al.* [27]. The ratio of Lys and Leu residues was varied to investigate its effect on bio-activity. To eliminate the effect of the helical structure D-amino acids and L-amino acids were used. Increasing the positive charge drastically reduced the haemolytic activity while the antibacterial activity was preserved. Modulating hydrophobicity and positive charge is sufficient to confer antibacterial activity and cell selectivity.

In the work of Blondelle and Houghten [2] an unexpected increase in antimicrobial activity in the majority of the omission analogues of LK-peptides was observed. These results indicated that a hydrophobic presenting face of five, as compared to a presenting face of nine contiguous residues, is sufficient for the peptide to interact with the lipids and/or disrupt the organisation of the lipids of the bacterial cell wall [2].

Bessalle *et al.* [40] showed that decreasing the hydrophobic face of an experimental amphiphilic peptide decreases its haemolytic activity. In another study, 14- and 15-residue LK-peptides, containing a larger hydrophilic than hydrophobic face, were antibacterial whereas a larger hydrophobic face resulted in peptides that were more haemolytic [2]. A wider hydrophobic face correlated with haemolytic activity, whereas a wider hydrophilic face correlated with antimicrobial activity. The omission of any of the hydrophobic residues throughout the sequence

of melittin, and particular the omission of any of the Leu residues, dramatically decreased haemolytic activity [34].

Kiyota *et al.* [41] used five amphiphatic α-helical peptides containing Leu and Lys in different ratios to determine the effect of the size of the hydrophobic and hydrophilic faces of the peptide on its interaction with lipid- and bio-membranes. Increasing the hydrophobic face area not only increased the affinity for lipid, but also increased the trend of self-association. The structure-activity relationship, estimated by means of ability to cause leakage and haemolytic activity, demonstrated that the ability to perturb model- and biomembrane correlates entirely with the area of the hydrophobic face [41].

Most of these studies mentioned, however, simultaneously examined at least three parameters, making it difficult to distinguish between the effect of each individual parameter on the overall biological activity. Studies using model antimicrobial peptides focused on chain length and amino acid composition as well as amphipathic structure. Oh *et al.* [42], using short (<18-residues) antimicrobial peptides, showed that a high value of some structural parameters (net charge, hydrophobicity and α-helicity) is not enough for the best antimicrobial activity, but that an optimal balance in structural parameters, particularly amphipathic balance, is required.

1.4 Mechanism of action

Magainins, cecropins, defensins and related peptides as well as tachyplesins exert their antibacterial activity in a manner entirely different [43, 44] from the well known antibiotics such as the penicillin's and cephalosporins, which interfere with either cell wall or macromolecular synthesis in bacteria [45]. The extensive clinical use of these antibiotics resulted in bacterial strains developing resistance, causing a serious problem in the treatment of bacterial infections. Peptides like magainins exhibit activity against a variety of Gram-positive and Gram-negative bacteria *in vitro*. It also appears to have the ability to augment antibiotic therapy [24].

It is now becoming increasingly clear that insects, invertebrates and mammals use peptides to counter bacterial infections. Considering the mechanism of action of these peptides it is likely that resistance may not develop against peptide antibacterial agents. The observation that the biological activity of *all* D-cecropins and magainins are identical to those of the *all* L-isomers

may help in overcoming the problem of proteolysis that is likely to reduce the half life of the *all* L- peptides *in vivo* [24].

The biological activity of amphipathic, α-helical peptides have been thought to result from their ability to form ion channels in membrane bilayers [44, 43]. Such channels could possibly form by self-aggregation of peptide monomers in which the hydrophobic residues interact with the phospholipid groups on the cell wall and the hydrophilic residues face inward [31]. The observation that cecropins and magainins have amphiphilic helical structures led to investigating both their interaction with lipid vesicles and their channel-forming ability by means of electrophysical techniques [44]. It was thought that magainins form voltage dependent channels in lipid bilayers, but recent research suggests that magainins form pores in membranes [46, 47]. Defensins have the ability to form anion-selective voltage-dependent channels in planar bilayer membranes [15]. Cecropins and their synthetic peptide analogues form large time-variant and voltage-dependent ion channels in planar lipid membranes at concentrations in the physiological range [44]. The ability to form channels in spite of the peptides adopting helical or β-structure indicates that channel formation is likely to have an important role in antimicrobial activity [24].

The synthetic peptides identified in the study by Blondelle *et al* [37] have activities similar to peptides isolated from natural sources. It was concluded, using conformational studies, that induction into α-helical conformations during interaction with bacterial cell membrane lipids and/or sialic acids, promotes the lysis of the cells. The structural constraint induced by this peptide-lipid association results in the perturbation of the lipid membrane. This in turn leads either to leakage of cell contents or to an initial penetration of the peptides past the membrane through hydrophobic interactions between the Leu face of the helix and the phospholipid chains. Both of these potential mechanisms result in the death of the cells [37].

Dathe *et al.* [48] used an amphipathic model peptide, KLALKLALKALKAAKLA-NH$_2$ (KLAL-peptide), to analyse the process of peptide binding to lipid vesicles with different surface charges and to determine the structure of the lipid-bound peptides using circular dichroism (CD) spectroscopy (Fig 1.1). It was found that charge interactions were the predominant cause of the accumulation of cationic KLAL-peptides on the membrane and the disruptive effect of high negative surface charge on the bilayers. Independent of any structural propensity, the cationic side chains bind to the anionic phosphatidylglycerol moieties. The peptides are held on the

bilayer surface by charge interactions, where they may disturb lipid headgroup organisation by formation of peptide-lipid clusters [48].

In contrast, KLAL-peptide-lipid interactions with bilayers that have a low negative surface charge are highly dependent on peptide helicity. Less helical peptides exhibit reduced bilayer-disturbing activity, showing that the hydrophobic helix domain is determines binding to and permeabilising membranes with low negative surface charge. It was suggested that hydrophobic interactions drive the penetration of the amphiphatic peptide structure into the inner membrane region, thus disrupting the arrangement of the lipid acyl chains and causing local disruption [48].
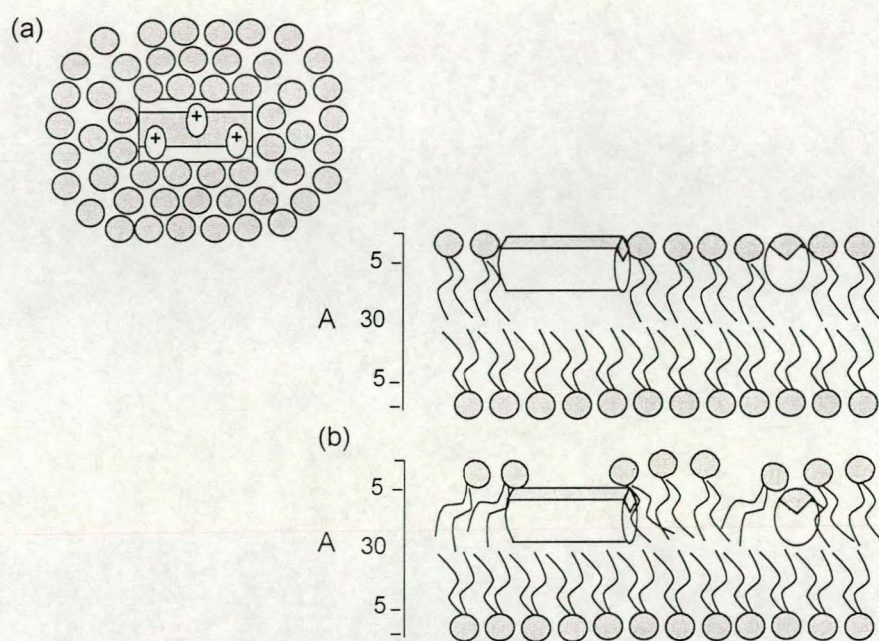
Figure 1.1   Model of interaction of the amphipathic KLAL-helix with lipid bilayers of different negative surface charge. (a) Strong electrostatic interactions between the cationic residues in the polar face of the helix and the negatively charged lipid head groups anchor the peptide in the membrane surface region, preferentially causing changes in the organisation of the lipid head groups. (b) At a low negative surface charge hydrophobic interactions between the hydrophobic face and the lipid acyl chains drives the insertion of the peptide into the inner nonpolar membrane region, causing a disturbance in the arrangement of the acyl chains [48].

Despite being studied intensely, the precise mechanism of action of the antimicrobial peptides has not yet been elucidated. Two hypotheses on the mechanism of action of the two different peptides, cecropin and magainin, are discussed here.

In Fig. 1.2 the interaction between peptide and membrane is proposed to account for the results Christensen *et al.* [44] observed in a study using cecropin and related synthetic peptides, is depicted. In the Christensen model, also called the barrel-stave model, the first step of interaction (step I in Fig 1.2) between cecropin oligomers and the bilayer is electrostatic adsorption to the bilayer-water interface. All the positively charged peptides investigated were able to do so. The next step (step II in Fig 1.2) could be the insertion of the largely hydrophobic C-terminal region into the hydrophobic membrane core, leaving the amphipathic helix in its interfacial position. This only happens with those peptides featuring a flexible link (hinge; helix-breaking Gly-Pro sequence) between the N-terminal amphipathic helix and the hydrophobic C-terminal segment. On the other hand, the molecules with a more rigid chain would not favour this insertion mechanism. This may be the reason for their lack of a voltage-dependent conductance. They probably insert directly into the membrane to form transmembane channels. The voltage sensitivity of cecropins is not due to the amphipathic helix itself but to the special structural arrangement between the helix and the more hydrophobic C-terminus. The final step (step III in Fig 1.2) of channel formation for peptides having a hinge is favoured by a positive voltage (referred to the side of peptide addition) leading to a slow increase of current. This indicates a large energy barrier between the closed and the open states of the channel. This step could be the insertion of the positively charged amphipathic α-helix in a membrane-spanning configuration with the charged residues forming the inner, water-filled pore. The positive charges on the pore walls lead to the observed anion preference. The voltage sensing could be because of the interaction between the dipole formed from the amino acid backbone of the pre-inserted hydrophobic segment with the electric field across the membrane. The voltage sensing is also affected by the surface charge and by cholesterol, possibly by means of depth to which the segment inserts into the bilayer or by means of the membrane fluidity. Cholesterol changes both the fluidity and the dipole potential of bilayers. These observations show that cecropins are potent channel-forming peptides under physiological conditions without requiring a specific target receptor on the cell membrane. The different sensitivities of bacterial and mammalian cells can be explained by the presence of cholesterol in the cell membrane of the latter [44].
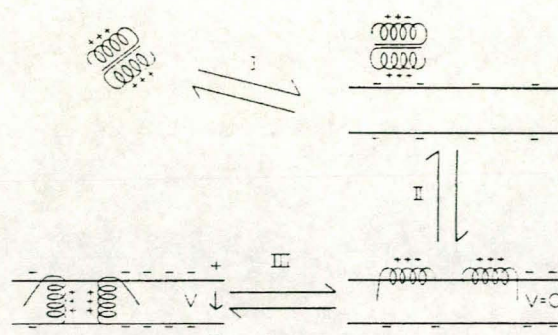
Figure 1.2   Tentative model for the interaction of ceropins with a lipid bilayer membrane. Only a dimer is sketched for the simplicity, but larger aggregates are likely to occur. Refer to text for detail [44].

On the other hand, it was shown that magainins form membrane pores rather than channels [46, 47]. Ludtke *et al.* [46] proposed a toroidal (or wormhole) model for the magainin pores (shown schematically in Fig. 1.3). In this model the lipid bilayer bends back on itself like the inside of a torus. This causes the top and bottom monolayers to be continuous. There is an energy cost for such a membrane deformation, mainly as a consequence of the bending in the toroidal area. The bending can be viewed as a lateral expansion of the head group region relative to the chains. The strain of expansion would be reduced by the incorporation of magainin monomers in the head group region, thereby stabilising the pore. The length of the peptide would require it to be orientated parallel to the pore axis. Since magainin monomers play the role of "filters" to relieve the membrane stress, there is probably no peptide-peptide contact, unlike the previously mentioned model.

The action of magainin, as depicted in Fig. 1.3, can be summarised as follows: At low peptide concentrations, magainin adsorbs in a primarily helical form parallel to the membrane surface, embedded in the head group region. At high concentrations the free energy of surface adsorption becomes too high, so that the peptide is driven to an inserted state. Inserted magainin remains associated with the head groups. The surface bends in a toroidal fashion to create a pore. Head groups and associated magainin monomers line the pore in the toroidal model. When the pore is closed, the participating magainin monomers will again adsorb in the head group region, but they may surface on either side of the membrane. This type of channel formation also provides a mechanism for peptide translocation across the bilayer [46].

Surface state   Toroidal (wormhole) model
Magainin
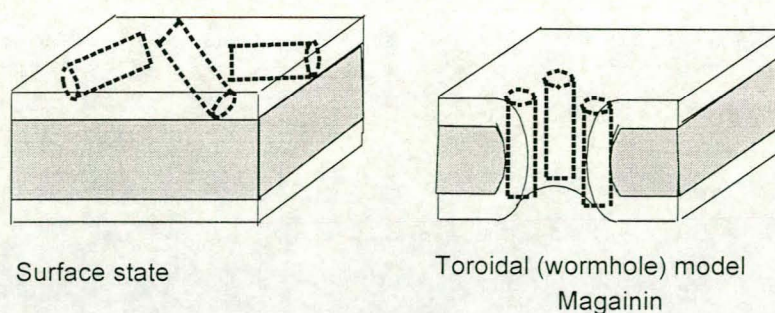
Figure 1.3   Toroidal (or wormhole) model of magainin (the cross sectional view). The small cylinders represent magainin monomers. The shaded areas represent the head group region of the lipid bilayer. Refer to text for more information [47].

The carpet mechanism proposed by Oren and Shai [43] describes a situation in which amphipathic α-helical peptides initially bind onto the surface of the target membrane and cover part of it in a carpet-like manner. This model is very similar to the toroidial one described earlier. The model however proposes that the membrane disintegrates in the final step through micellisation.

1.5 Immobilisation of peptides

According to all three of the mechanisms discussed earlier (section 1.4) antimicrobial peptides must first display some interaction with the target membrane. This surface activity may have also lead to some disruption of the membrane function, such as disturbing the membrane fluidity. Immobilisation of these peptides may provide a way to analyse the effect of this surface interaction on bioactivity. Immobilisation of peptides, that retain antimicrobial activity, may also lead to the development of antimicrobial matrixes.

Immobilised biomolecules have been exploited in many applications. For example immobilisation made it possible to utilise the unique properties of enzymes in analytical chemistry, medicine, food and pharmaceutical technology and fine organic synthesis. Investigators of enzyme mechanisms have exploited the placement of the catalyst on an insoluble support as a means of studying the roles of sub-unit and protein-protein interactions in catalytic activity [49]. The immobilisation of drugs, hormones, and neurotransmitters on insoluble supporting matrices is a technique that has found application in diverse endeavours ranging from elucidation of the sites and mechanisms of hormone action to drug receptor isolation and clinical

therapeutics [50]. In a study, Kamal *et al.* [51] found that antibiotic bonding is an efficient, safe, and cost-effective method of reducing intravascular catheter infection.

Haynie *et al.* [52] searched for an experimental design to evaluate the mechanism of action of particular antimicrobial peptides. Immobilisation restricts the range of penetration of the antimicrobial agent to loci on the surface of the microbial wall. This can be used to test whether the antimicrobial peptides are surface active or possibly use another mechanism of action. They immobilised a number of peptides on a polydimethylacrylamide resin, generally used for solid phase peptide synthesis. The model LK-peptides used in their study were originally identified by Blondelle *et al.* [2]. Magainin, a retro-magainin (reverse sequence) and non-amphiphilic, helical peptides were also immobilised in the same manner. Immobilised magainin and some of the Lys- and Leu-containing model peptides were active, which demonstrated that the outer membrane interaction with these peptides is sufficient for lethal activity [52]. As for the non-amphiphilic, helical peptides, the immobilised retro-magainin was devoid of activity, implying that the amide bond direction and the original N-terminal of magainin and, amphipathicity in general are necessary for activity. In another study it was found that the lantibiotic, nisin, only displayed antimicrobial activity when it was desorbed from the matrix onto which it had been immobilised [53].

Antimicrobial peptides such as the magainins, defensins and cecropins may therefore be attractive candidates for a support-bound antimicrobial agent because of their broad spectrum activities, their relatively low cytotoxicities, and a body of basic evidence that suggest that the membrane surface is their lethal site of action.

1.6 Conclusions

The discovery of antimicrobial peptides opened a whole new field for antibiotic research. They have been isolated from mammals, amphibia and insects, and play an important role in the host defence system. All of the antimicrobial peptides are amphipathic, some are α-helical, and some are β-sheets, while most are cationic in nature. It has been shown that for some peptide sequences a peptide length of only eight residues is necessary to give antimicrobial activity [39]. The size of the hydrophilic and hydrophobic faces of the amphipathic helix is of great importance. In general

peptides with a larger hydrophobic face are more haemolytic than peptides with a larger hydrophilic face that have similar antibacterial activity.

All antimicrobial peptides are membrane-active and form channels or pores that cause the cell to lyse. Most channels, probably formed by peptide aggregates disrupting the membrane, are ion selective or voltage dependent. Many mechanisms of action have been proposed (refer to section 1.4), but it still a major topic under investigation and of contention.

The mechanism of action is of particular importance in this study in which model antimicrobial peptides were immobilised on solid supports. Many different peptides have been immobilised on solid supports and on other proteins and successfully used as antigens in immunological studies [49]. A problem of the immobilisation of antimicrobial peptides, however, is that it could change the antimicrobial activity of the peptides. To overcome this problem, different matrices could be utilised to promote activity. Peptide synthesis is now carried out on chitin [54] and cellulose paper discs [55]. Furthermore, the solid supports can be functionalised by using different linkage agents. Antimicrobial peptides can easily be synthesised and immobilised in the laboratory and from structure-activity relationships the best peptide sequence for immobilised activity can be identified.

1.7 Summary of project and aims

In this study we decided to use a model peptide with high antimicrobial activity as described by Blondelle *et al.* [37] as lead compound. The longest model peptide amide consisted of 18 amino acid residues that form an amphipathic α-helix [37]. A helical wheel representation of the 18-residue peptide can be seen in Fig 1.4. The hydrophobic and hydrophilic faces of the α-helix are clearly visible, showing that it is an amphipathic α-helix.
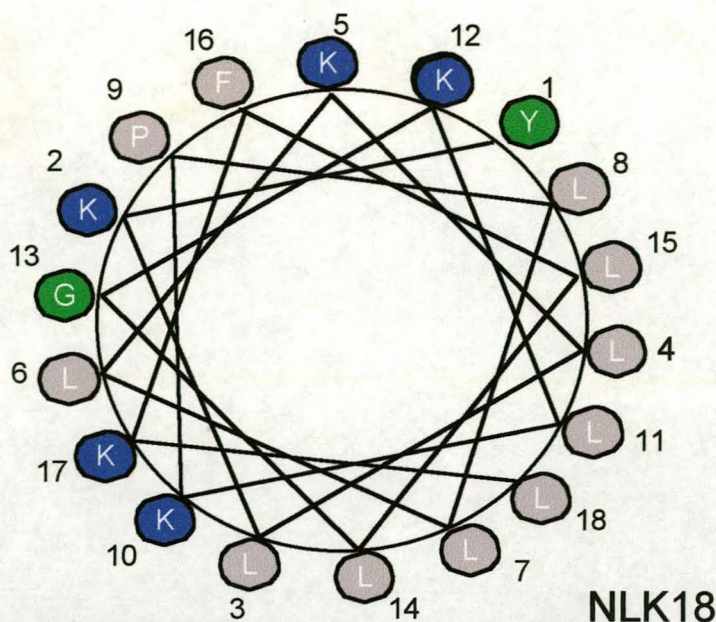
Figure 1.4   Helical wheel representation of the longest model peptide used in this study. Blue circles show the charged, polar residues, the grey circles the non-polar residues, and the green circles show the uncharged polar residues.

The aims of this study were to:

- synthesise, purify and analyse the free peptide amides of the 18-residue peptide and three of its N-terminal omission analogues;

- synthesise and analyse the four immobilised peptides, and

- compare the biological activity of these peptides towards different target cells.

The immobilised peptides (ILK peptides) were synthesised on modified Pepsyn K resin as Haynie *et al* [44] did their study. Three Gly residues functioned as an arm onto which the rest of the peptide was synthesised. This linkage arm was included to facilitate the interaction of the immobilised peptide with the target cell membrane.

In Chapter 2 the synthesis, purification and analysis of the peptides will be discussed. The structure-function relationship of the peptides will be reported in Chapter 3. Here in particular the influence of peptide length, charge, size of the hydrophobic face *vs.* the hydrophilic face and immobilisation on the antimicrobial activity of the four peptides will be addressed. A short summary of results and conclusions will then be given in Chapter 4.

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Chapter 2

Synthesis and purification of model cationic α-helical peptides

2.1 Introduction

Studies using synthetic model peptides with antimicrobial activity have been very successful in identifying highly active non-toxic peptides [1, 2, 3, 4]. In a single study some of these synthetic model peptides were used to investigate the influence of immobilisation on antimicrobial activity [5]. These peptides were full-length and N-terminal deletion analogues of a 18-residue model peptide containing only Lys and Leu, designed by Blondelle *et al.* [1]. They found that the longer immobilised peptides retained some of their activity against bacterial targets [5]. In a later study Blondelle *et al.* [4] also identified particularly active 18-residue peptides using combinatorial defined combinatorial libraries. These peptides showed a 10-fold increase in activity when compared to the original peptides and still consisted mostly of Leu and Lys. They incorporated Tyr at its N-terminus to allow accurate determination of the concentration of these peptides. The peptide, which was chosen for this study, had the highest activity against *S. aureus* and *P. aeruginosa* when compared to the other peptides. The peptide is known to be induced into an amphipathic α-helical conformation in a lipidic environment. An opportunity therefore existed for the investigation of the structure-function relationship of this model peptide using synthetic analogues, both immobilised and in a soluble form. Solid phase synthesis played an integral role in these investigations as it did in our study.

R. B. Merrifield developed solid phase peptide synthesis, and he received the Nobel Prize for this work in 1984 [6]. The solid phase approach involves covalent attachment (anchoring) of the growing peptide chain to an insoluble polymeric support (resin carrier), so that the unreacted, soluble reagents can be removed.

Peptide chain elongation is from the C-terminal to the N-terminal. Synthesis in the C→N direction leads itself to better chemical control than the N→C direction, where the activation of the acyl-amino acids results in optically impure peptides because of racemisation. Peptide bond formation in the C→N direction requires the activation of the α-carboxyl group (to facilitate bond formation) and reversible protection of the α-amino group of the incoming amino acid (to limit polymerisation).

Many polymers have been tested as an insoluble polymeric support during solid phase peptide synthesis, e.g. cellulose, polyvinyl alcohol, polymethacrylate and sulfonated polystyrene [6]. Merrifield used is a polystyrene cross-linked with 1% of m-divinylbenzene, which swells to five or six times its volume when treated with the solvents commonly used in peptide synthesis, namely dichloromethane and N, N'-dimethylformamide (DMF). The resin support is functionalised with chloromethyl groups onto which the first amino acid of the peptide is attached. A phenylacetamidomethyl (Pam) handle, which serves to link the first amino acid to the resin, was developed to reduce losses during acidolysis. The term handle, is defined as a bifunctional spacer with a smoothly cleavable protecting group on the one end, the other end coupling to a previously functionalised support [7]. This method of synthesis is based on the use of the acid labile tertiary-butyloxycarbonyl (t-Boc) α-amino protecting group and benzyl-derived side chain protection [8].

A new resin, beaded polydimethylacrylamide (polyamide), was developed by E. Atherton and R. B. Sheppard. The polar character of this support makes it useful in cases were polystyrene is unsuitable [9]. The tBoc amino acid protecting group has been replaced by a 9-fluorenylmethoxycarbonyl (Fmoc) amino protecting group. This base labile protecting group can be removed by secondary amines such as piperidine, eliminating the repetitive acid treatments necessary in the tBoc chemistry, and resulting in substantially milder reaction conditions [8].

The polyamide resin was found to be too easily deformed for convenient use in pumped-flow equipment. This problem was solved by polymerising the gel resin within a rigid macroporous framework, so as to maintain channels for solvent flow, while at the same time allowing rapid diffusion of reactants into and out of the physically supported gel. Kieselguhr is a highly absorbent material with a pore size many thousands of Å in diameter and an accessible pore volume about one-third of the total dry volume. Its rigidity, mechanical strength, and large

pore size make it a near-ideal carrier for gel resins. The poly-dimethylacrylamide-Kieselguhr resin is manufactured commercially as Pepsyn K and Novasyn K [9, 10, 11].

The polyamide gel is formed by copolymerisation of dimethylacrylamide with cross-linking and functionalising monomers that provide methyl ester groups as sites for attachment of the growing peptide chain to the resin. This support can not be used directly for peptide synthesis: it first must undergo derivatisation. Ethylene diamine is used to aminolyse the methyl ester and generate amino groups on the resin. Norleucine is coupled to act as an internal reference standard for amino acid analysis, where after a linkage agent is coupled [9]. The internal reference does not form part of the peptide being synthesised and is usually chosen not to be amongst its constituent amino acids. That it why the non-protein amino acid, commonly norleucine, is convenient [10]. A new concept of individual reversible linkage agents was introduced to import additional flexibility to the poly-dimethylacrylamide-Kieselguhr resin. Small linker molecules, usually containing a free hydroxyl group, were constructed such that the derivatised esters have stability and liability [10]. A series of benzylalcohol linkage agents were made, in which the lability of the derived benzyl esters towards acids and nucleophilic bases was adjusted by substitution in the aromatic ring.

4-hydroxymethylbenzoic acid

4-hydroxymethylphenoxyacetic acid
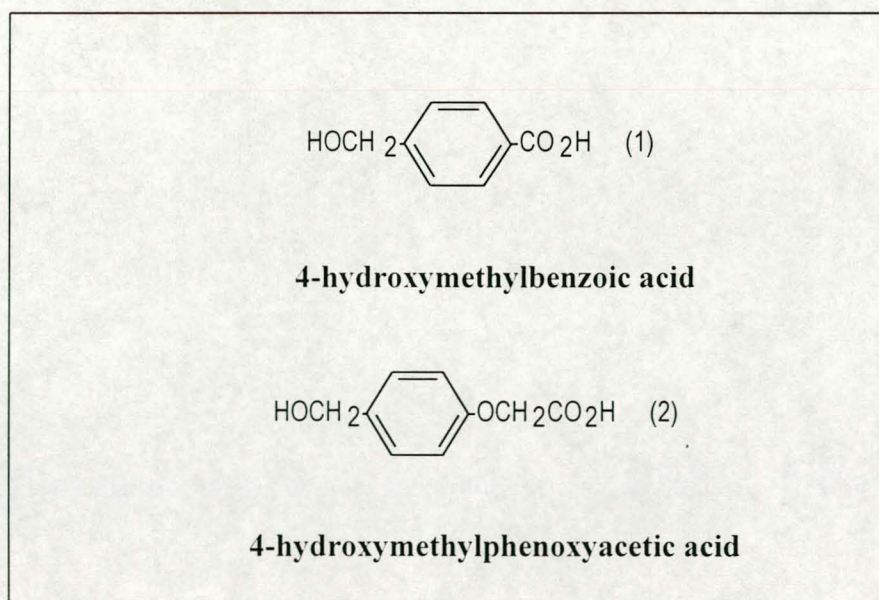
Figure 2.1   Linkage agents or handles in the Fmoc polyamide peptide synthesis protocol [10].

The linkage agent 4-hydroxymethylbenzoic acid is essentially stable even when treated with strong acids such as hydrogen fluoride (Fig. 2.1). In contrast, these esters are exceptionally easily cleaved by nucleophilic reagents, including hydroxide and ethoxide ions, ammonia, and hydrazine. This combination of properties makes this linkage agent highly suitable for the preparation of peptide amines. The acid stability allows prior cleavage of all *t*-butyl derivatives (particularly side-chain esters of aspartic and glutamic acids) while the peptide remains attached to the resin. The resin with this linkage agent is commercially manufactured as Pepsyn KB or Novasyn KB.

The linkage agent 4-hydroxymethylphenoxyacetic acid is the standard peptide-resin linkage agent in the Fmoc-polyamide technique (Fig. 2.1). Peptides linked to the resins through esters of this linkage agent can be cleaved by mild acid treatment (usually with trifluoroacetic acid) at the same time and under the same conditions used to remove *t*-butyl side-chain protecting groups 10]. The resin with this linkage agent is commercially manufactured as Pepsyn KA or Novasyn KA.

The amino acid with a temporary protecting group, such as tBoc or Fmoc, is then coupled to the chemically suitable handle by an ester bond. The tBoc protecting group is removed by acidolysis, and the Fmoc by base treatment to liberate the N^α-amine of the peptide-resin. The next incoming protected amino acid with its suitably activated carboxyl group is introduced [7]. After completion of the synthesis the peptide acid or peptide amide can be released by acidolysis or base treatment.

Solid-phase peptide synthesis was also successfully done on chitin by Neugebauer *et al* [12]. A linker was attached to the chitin and normal Fmoc protocol was used to synthesise the peptides. Peptide-chitin complexes formed by peptide synthesis on chitin could prove useful for the induction of anti-peptide antibodies. Using chitin is advantageous because of its biocompatibility and its availability [12]. Using the Fmoc protocol, peptide synthesis was also carried out using cellulose paper discs as segmental solid supports. The rigid structure and low swelling properties of the cellulose paper allows the multiple coupling reactions to be carried out under low pressure continuous flow conditions [13].

The Fmoc-polyamide peptide synthesis methodology (Fig. 2.3) [11] was chosen in this study because of its milder chemical approach. The simplicity and elegance of the Fmoc-polyamide

chemistry allows the use of only one solvent throughout synthesis and has the additional convenience that the Fmoc-group allows UV-monitoring of coupling reactions.

Syntheses in this study were done with a shake flask method. The solid phase used for the synthesis of the immobilised peptide consisted of the polyacrylamide-Kieselguhr resin (Pepsyn K), reacted with ethylene diamine onto which the first amino acid of the peptide had been coupled. The solid phase used in the soluble peptide synthesis had a 4-hydroxymethylbenzoic acid linkage agent (Pepsyn KB) to produce the peptide amide when cleaved from the resin. The efficiency of the coupling reactions was monitored by a simple ninhydrin test, the Kaiser test, [14] or by a Fmoc test [11]. The Kaiser test was used for monitoring the disappearance of amino groups during coupling and the appearance of amino groups after deblocking. The Fmoc test was used to evaluate the coupling of the previous amino acid before the coupling of the next (refer to 2.3.3 for more detail). Amino acid side chain protection was provided by *t*-butyl ester (tBu) and t-butyloxycarbonyl (tBoc), which are both cleaved under relatively moderate acidic conditions (90% TFA).
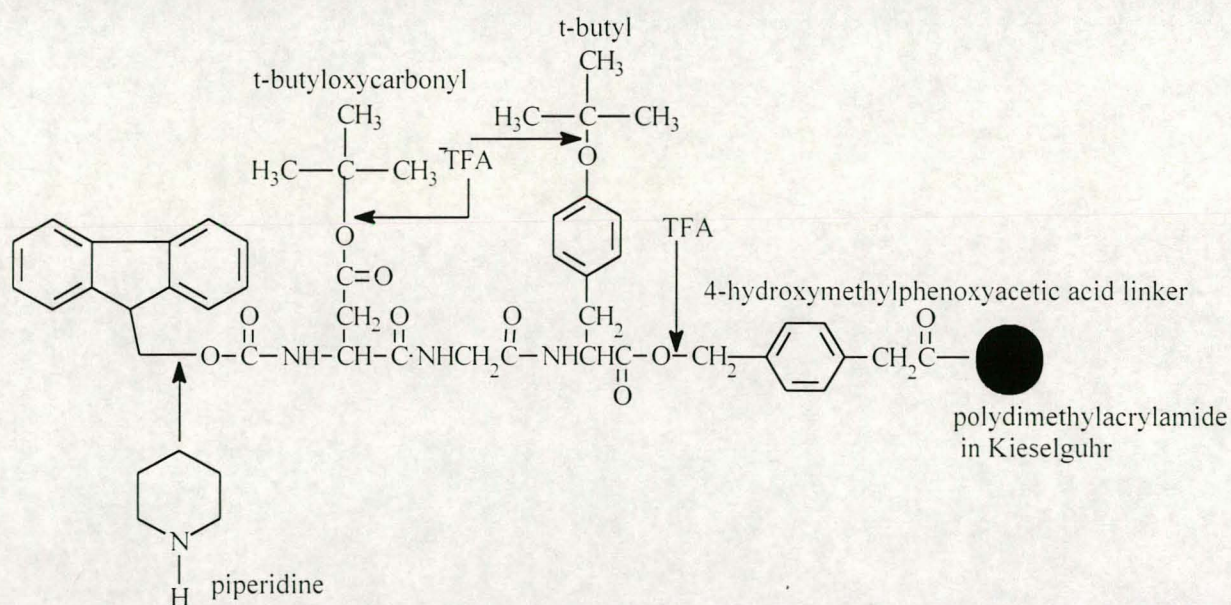
Figure 2.2   Protection scheme for solid phase synthesis in the Fmoc-polyamide methodology. The chemistry is based on mild base treatment to remove the N-terminal Fmoc-group and mild acid treatment to release the free peptide acid [7, Figure reproduced with permission from 15].

In this study the highly active model peptide from Blondelle *et al.* [4] and three N-deletion analogues were synthesised in the soluble amidated forms (NLK peptides) and the immobilised forms (ILK peptides) (Table 2.1). Two, four or six of the amino acid residues in

the conserved model sequence were omitted from the N-terminal side to form the shorter peptides. These shorter peptides were synthesised to investigate the influence of peptide length, charge and hydrophilic *vs.* hydrophobic faces on antimicrobial activity. The peptides were amidated to give the same properties to the free peptide as to the immobilised peptides. The peptides were immobilised to investigate the influence of immobilisation on the antimicrobial activity. Three glycine (Gly) residues were coupled to the resin to form an "spacer arm", onto which the rest of the peptide was synthesised. Gly was chosen because of its basic structure and ease of coupling. The C-terminal of the ILK peptides was attached to this (Gly)$_3$-linker.

Table 2.1 Primary structure of the cationic α-helical model peptide analogous synthesised in this study. (® depicts the Pepsyn K-NH(CH$_2$)$_2$NH-resin)

Peptide name	Peptide primary structure	Number of amino acids	M$_r$ of peptide moiety
ILK12	LLPKLKGLLFKLGGG-®	12+(Gly)$_3$	1536.98
ILK14	KLLLPKLKGLLFKLGGG-®	14+(Gly)$_3$	1778.31
ILK16	LLKLLLPKLKGLLFKLGGG-®	16+(Gly)$_3$	2004.63
ILK18	YKLLKLLLPKLKGLLFKLGGG-®	18+(Gly)$_3$	2295.98
NLK12	LLPKLKGLLFKL-NH$_2$	12	1381.86
NLK14	KLLLPKLKGLLFKL-NH$_2$	14	1623.19
NLK16	LLKLLLPKLKGLLFKL-NH$_2$	16	1849.51
NLK18	YKLLKLLLPKLKGLLFKL-NH$_2$	18	2140.86

The chemical purity of the immobilised peptides was evaluated using amino acid analysis and protein sequencing, while the synthesis was monitored by the picric acid test [16] (section 2.3.3.3), Kaiser test [14] (section 2.3.3.2) and Fmoc test [11] (section 2.3.3.1). The soluble peptide amides were purified using gel permeation chromatography and high performance liquid chromatography (HPLC). Chemical purity of the soluble products was evaluated by high performance liquid chromatography (HPLC) and electrospray ionisation mass spectrometry (ES-MS).

2.2 Materials

2.2.1 General reagents and solvents

N, N'-dimethylformamide (DMF; 99.5%), ethyl acetate (99.5%), glacial acetic acid (99.8%), sodium carbonate (anhydrous), phosphorus-pentoxide, molecular sieve (0.3 Å; beads approximately 3.2 mm) and self-indicating silica gel were from Saarchem (Krugersdorp, South Africa). Chloroform (> 99%), dichloromethane (DCM; 99.5%), dioxane (99.5%), diethyl ether (99.5%), ether absolute (99.5%), *n*-hexane (99.8%), tert-amylalcohol (> 99%), butan-1-ol (99.5%), ethanol (99.8%), piperidine (98%), pyridine (99.5 %), trifluoroacetic acid (TFA; > 98% and 99.5%), citric acid 1-hydrate (99.5%), magnesium sulphate, potassium cyanide (KCN), 2', 7'-dichlorofluorescein, ninhydrin, 1-fluoro-2,4-dinitrobenzene (FDNB), aluminium oxide 90 and Kieselgel 60-F_{254} thin layer plates were from Merck (Darmstadt, Germany). Piperidine (99%), pyridine (99.9%) and TFA (> 98%) were also purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Dioxane (99.5%), hydrochloric acid (31%), piperidine (98 %) and 2-methylbutan-2-ol were from BDH. High purity dry nitrogen gas was provided by Afrox, South Africa.

2.2.2 Derivatives, catalysts and resins for peptide synthesis

Pepsyn K-resin (0.1 meq/g), and Pepsyn KB-resin (0.1 meq/g), Fmoc-Gly-OPfp, Fmoc-L-Leu-OPfp, Fmoc-L-Lys-(tBoc)-OPfp, Fmoc-L-Phe-OH, Fmoc-L-Phe-OPfp, Fmoc-L-Pro-OH, Fmoc-L-Pro-OPfp, Fmoc-L-Tyr-(tBu)-OPfp were from Milligen-Millipore (Milford, USA). Glycine, Fmoc-L-Leu-OH, Fmoc-L-Lys-(tBoc)-OH, pentafluorophenol, Fmoc-pentafluorophenol, and N, N'-diisopropylethyl amine (DIPEA) were from Sigma-Aldrich Chemie (Steinheim, Germany). Benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PYBOP®) was from Calbiochem-Novabiochem Co. (La Jolla, USA). N, N'-dicyclohexylcarbodiimide (DCC) and ethylene diamine (99%) were from Merck (Darmstadt, Germany). Fluka Chemicals (Buchs, Switzerland) supplied 1-hydroxybenzotriazole (HOBt).

2.2.3 Reagents and solvents for amino acid analysis and chromatography

Constant boiling hydrochloric acid (HCl, 30%), sodium acetate (99.5%), phenol (99.5%) and TFA (99.5%), were from Merck (Darmstadt). 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 205 nm), acetonitrile (HPLC-grade, UV cut-off 190 nm) were from Romil LTD (Cambridge, UK). Pico-Tag® sample diluent, Nova-Pak analytical HPLC columns, 0.45 micron HV membrane filters were provided by Waters-Millipore (Milford, USA). Polygosil (C_{18}, 60 Å, irregular particles) packing material use to prepare semi-preparative HPLC column, was supplied by Macherey-Nagel (Düren, Germany). High quality triethylamine (TEA) was from Aldrich Chemical Co. (Gillingham, UK). Sephadex G10 and CM-Sepharose were from Pharmacia (Uppsala, Sweden). Analytical grade water was prepared by filtering glass distilled water through a Millipore Milli Q® 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 dessicators with silica gel as drying agent. The FDNB was stored at 10°C and the PYBOP® and the HOBt at -20°C, both with silica gel (with indicator) as drying agent. Before use, amino acid derivatives, resins, PYBOP® and HOBt were dried overnight under high vacuum, with phosphorous-pentoxide as drying agent. The DMF was stored over molecular sieve (0.3 Å) at room temperature. PITC and amino acid standards were stored at -20°C and TEA (high quality for amino acid analysis) at 10°C, all under N_2-atmosphere. Peptides were stored at room temperature, under vacuum in a dessicator, with silica gel as drying agent. All other reagents were stored at room temperature.

Great care was also taken to avoid detergent contamination of glassware, as such contamination of the peptide preparations could influence the antimicrobial activity studies. The glassware, after the regular washing, was rinsed three times with distilled water, three times with 60% ethanol and then washed three times with analytical grade water. It was oven-dried at temperature ranging between 110°C and 150°C. Bottles for amino acid derivatives

and purified peptides, washed in the same way, were also pyrolised at 565-570°C for 2 hours to remove all traces of contaminating chemicals.

2.3 Methods

2.3.1 Preparation of solvents

2.3.1.1 Distillation of N, N'-dimethylformamide (DMF) [adapted from 17]

N, N'-dimethylformamide (DMF) is the main solvent used in the Fmoc-polyamide peptide synthesis method. DMF could be contaminated with secondary amines and H_2O, which respectively causes the loss of the Fmoc- and active ester groups. Distilling the DMF removes these contaminants. Freshly distilled DMF is therefore used in the synthesis. DMF is kept on molecular sieve (3 Å) before and after distillation. Impurities, such as volatile secondary amines, were removed by fractional distillation under vacuum (high vacuum pump) and a dry nitrogen bleed. It was necessary to distil DMF under reduced pressure, because there is some decomposition of DMF if distilled at atmospheric pressure. The first 10-15% of the distillate was discarded and the constant boiling fraction (45°C at 10 mm Hg) was collected. The distilled DMF was stored on molecular sieve (3 Å) in dark bottles at room temperature and used if it passed the Sanger test (section 2.3.1.2).

2.3.1.2 Sanger's test for secondary amines

It is necessary to test the DMF for amines, because the Fmoc-group is very labile in the presence of secondary amines. Sanger's test [18] was carried out by adding 500 μL of each of a 1-fluoro-2, 4-dinitrobenzene (FDNB) (1.0 mg/mL in 95% ethanol) and DMF and leaving it in the dark and at room temperature for 30 minutes. The absorbance of the reaction mixture was determined at 381 nm, with 0.5 mg FDNB per mL ethanol as blank. The blank's absorbance was normally in the region of 0.2 absorbance units, while suitably pure DMF had an absorbance at 381 nm of not more than 0.08 units higher than the blank. DMF with an absorbance < 0.05 was used for the coupling steps, while the DMF with an absorbance between 0.05 and 0.08 was used for the washing of the resin after deblocking the Fmoc-group.

During the synthesis procedure, Sanger's test was used to see if all the piperine had been removed after the deblocking step (refer to sections 2.3.3.4.3). The DMF used in the final washing steps was tested and an absorbance of less than 0.02 is necessary to proceed onto the coupling step. In this case the samples were only incubated for approximately 2-5 minutes.

2.3.1.3 Distillation of piperidine and pyridine

Piperidine must be of high purity, for the same reasons as DMF. Piperidine was distilled over KOH (10 to 20 g/L) and a dry nitrogen bleed at atmospheric pressure [10]. The first 10-15% of the distillate was discarded and the constant boiling fraction (101-106°C) was collected. The distilled piperidine was stored in dark bottles. Pyridine, for the use in the Kaiser-ninhydrin test, was also prepared in the same way [10].

2.3.1.4 Distillation of diisopropylethyl amine (DIPEA)

DIPEA must be of high purity, for the same reasons as DMF. DIPEA was distilled from ninhydrin (1-2g/l) under a dry nitrogen bleed. The first 10-15% of the distillate was discarded and the constant boiling fraction (125-127°C) was collected. The distilled DIPEA was aliquotted and stored in dark bottles at 4°C [10].

2.3.2 Preparation of amino acid derivatives

2.3.2.1 Preparation of Fmoc amino acids

A method described by Carpino. *et al* [19] was used for the synthesis of Fmoc-Gly. To a solution of 0.1425 g of glycine dissolved in 5.05 mL of 10% Na_2CO_3 was added, with stirring and cooling in an ice bath, a solution of 0.49 g of Fmoc-Cl in 3.75 mL of dioxane. The mixture was stirred at room temperature for 2 hours, poured into 100 mL of analytical grade water, and extracted twice with an equal amount of diethylether. This was to remove small amounts of 9-fluorenylmethanol and the high-melting polymer of dibenzofulvene. The aqueous layer was cooled in an ice bath and acidified with concentrated HCl tot a pH of 2. The white precipitate was extracted with 50 mL ethyl acetate, the extracts were washed with analytical grade water, dried ($MgSO_4$), and evaporated. The white precipitate was dissolved in 9 mL ethylacetate, titrated with *n*-hexane and left at -20°C to crystallise. The melting point of

the product was 174°C-175°C and amounted to 0.304 g (54% yield). The melting point compared well with that of commercial Fmoc-Gly.

Thin layer chromatography (TLC) was performed on aluminium backed Kieselguhr 60-F_{254} and developed in chloroform:methanol:acetic acid (85:10:5). Plates were thoroughly air-dried and first visualised under UV-light (254 nm). The plates were then sprayed with 0.2% ninhydrin in 95% ethanol and developed at 110°C for 10 minutes to detect residual amino groups. TLC which showed an UV detectable spot (R_f = 0.55) corresponding to that of commercial Fmoc-Gly.

2.3.3 Synthesis of peptides

The peptides were synthesised at room temperature using the Fmoc-polyamide protocol in the shake flask procedure [15]. The protocol for a complete cycle of operations including washing, coupling, deblocking and sampling steps for the Fmoc-polyamide solid phase peptide synthesis is set out in Figure 2.3.

2.3.3.1 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 [11].

To determine the Fmoc-fulveen concentration in a sample, a standard curve was set up with Fmoc-leucine in 20% piperidine in DMF. 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 then removed, diluted a 100 times with DMF, and used to make up a dilution series in triplicate. The absorbance of each dilution was read at 290 nm and then used to set up a standard curve using DMF as a blank. To evaluate a coupling step, 10-15 mg of resin was removed, washed with ether, and vacuum dried for one 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 expected Fmoc groups. After deblocking, a 100 times dilution was made of the deblocking mixture, the absorbance measured at 290 nm and the percentage coupling calculated from the above equation.
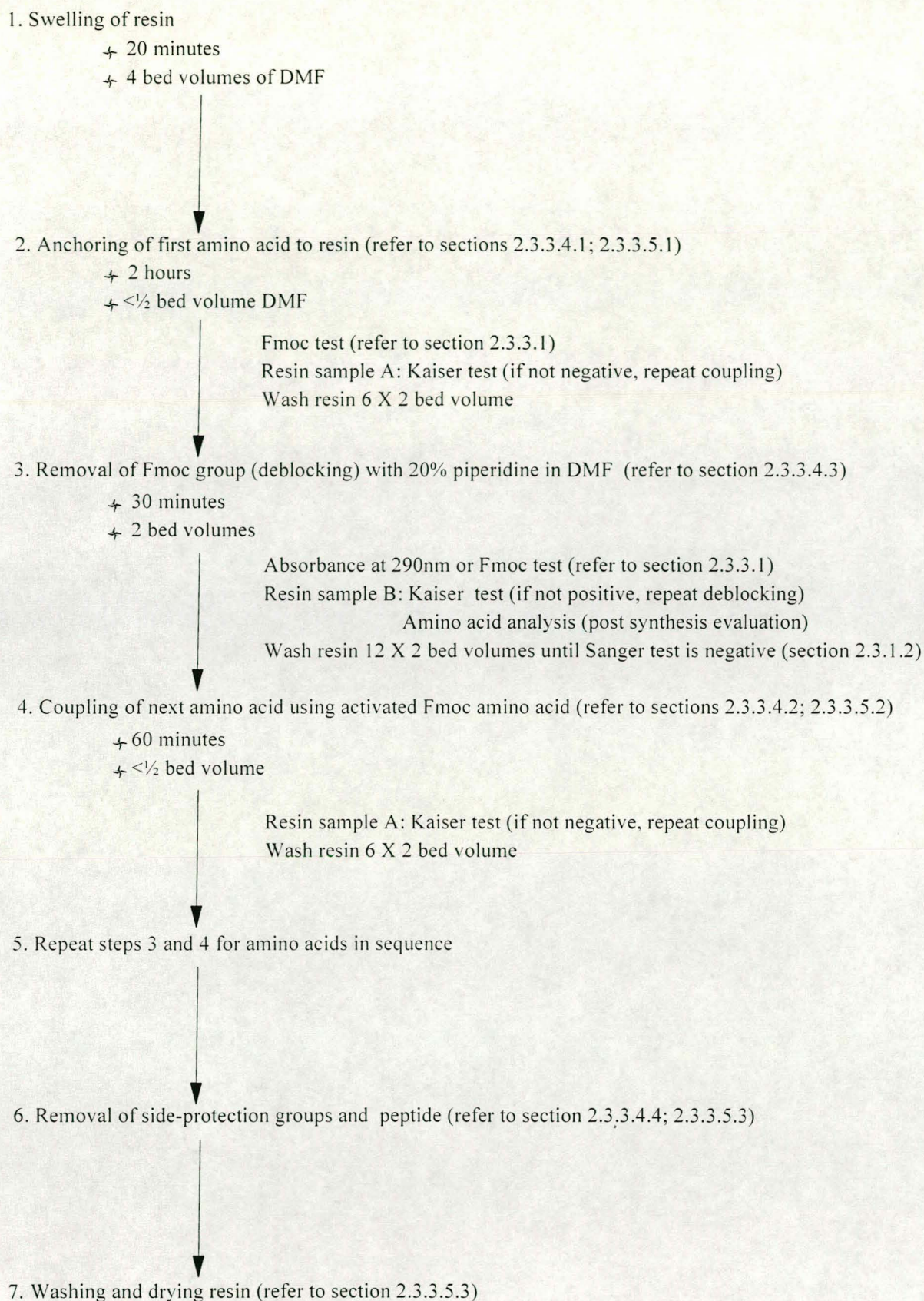
1. Swelling of resin
  + 20 minutes
  + 4 bed volumes of DMF

2. Anchoring of first amino acid to resin (refer to sections 2.3.3.4.1; 2.3.3.5.1)
  + 2 hours
  + <½ bed volume DMF

    Fmoc test (refer to section 2.3.3.1)
    Resin sample A: Kaiser test (if not negative, repeat coupling)
    Wash resin 6 X 2 bed volume

3. Removal of Fmoc group (deblocking) with 20% piperidine in DMF (refer to section 2.3.3.4.3)
  + 30 minutes
  + 2 bed volumes

    Absorbance at 290nm or Fmoc test (refer to section 2.3.3.1)
    Resin sample B: Kaiser test (if not positive, repeat deblocking)
             Amino acid analysis (post synthesis evaluation)
    Wash resin 12 X 2 bed volumes until Sanger test is negative (section 2.3.1.2)

4. Coupling of next amino acid using activated Fmoc amino acid (refer to sections 2.3.3.4.2; 2.3.3.5.2)
  + 60 minutes
  + <½ bed volume

    Resin sample A: Kaiser test (if not negative, repeat coupling)
    Wash resin 6 X 2 bed volume

5. Repeat steps 3 and 4 for amino acids in sequence

6. Removal of side-protection groups and peptide (refer to section 2.3.3.4.4; 2.3.3.5.3)

7. Washing and drying resin (refer to section 2.3.3.5.3)

Figure 2.3   Operation of one cycle of the peptide synthesis procedure (bed volume = 5 mL/g
       resin).

2.3.3.2 Kaiser test for free primary amino groups

The completeness of the acylation reaction or removal of the Fmoc-group was monitored by the Kaiser test [15], which is specific for primary amino groups. Three solutions are required, namely (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. Resin samples taken during synthesis were ether-washed and dried, four drops of each of these solutions were added to it and heated for 5 to 10 minutes in a 80°-90°C water bath. The presence of remaining free amino groups resulted in the resin beads turning characteristically deep blue (positive Kaiser test). Alternatively, the reaction solution and resin particles remained yellow (negative Kaiser test). Two (or more) samples were taken during each coupling procedure, one during the coupling step (sample A at 60 minutes), which should be negative and one after the piperidine deblocking step (sample B), which should be positive before continuation. If the test did not confirm completion of the reaction, the particular step was repeated or reaction time extended. Some of the N-terminal amino acids gave anomalous bead colours and not the intense blue of the free amino acid when reacting with the ninhydrin, e.g. proline gave a brown colour and serine, asparagine and glutamine a green-orange colour.

2.3.3.3 Picric Acid Test

The picric acid test [16] was performed on the resin to evaluate the number of amino groups available on the peptide resin (Fig. 2.4). The amino resin was reacted with an excess of 0.05 M picric acid in DMF and the excess reagent removed by thorough washing. Then the picrate anion was eluted from the resin with 5 x 5 mL 10% DIPEA in DMF. The resin was further washed with DMF between each treatment. When the yellow colour had been removed from the resin, the filtrate was made up to a known volume and measured quantitatively at 358 nm (the molar absorption coefficient of the picrate is 1.51×10^4 at 358 nm). The results show the concentration of the picrate measured as a percentage of the theoretical picrate expected. A commercial resin, with Leu attached as first amino acid, was used as positive control for 100% amino group saturation.
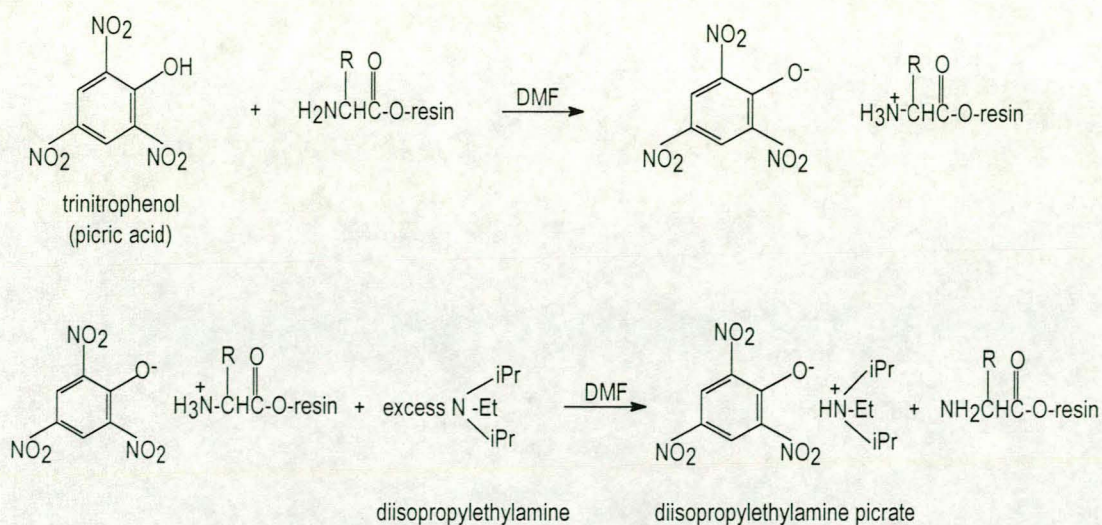
Figure 2.4   Reaction scheme of picric acid with amino groups on the peptide resin and its removal using diisopropielethylamine [16].

2.3.3.4 Synthesis of immobilised peptides (ILK peptides)

The peptides were synthesised at room temperature using the Fmoc-polyamide protocol in the shake flask procedure. Four ILK peptides synthesised simultaneously on the same batch of Pepsyn K resin. The first 15 residues were coupled, after which a quarter of the resin, containing ILK12, was removed. Two more amino acids were coupled to the remaining resin and another quarter of the resin, containing ILK14, was then removed. Again two amino acids were coupled to form ILK16, the resin split in two and the last two amino acids coupled to the remaining resin to form ILK18.

2.3.3.4.1 Coupling of the first amino acid

Pepsyn K resin, with a capacity of 0.1 milli-equivalent per gram of dry resin, was used in the synthesis of the immobilised peptides (ILK peptides). The resin was treated with ethylene diamine (1mL/g dry resin) for 24 hours to introduce an amino group onto which the first amino acid could be irreversibly coupled. The resin was then washed three times each with *t*-amyl alcohol, acetic acid, *t*-amyl alcohol and ether. The resin was dried by placing it in a vacuum desiccator over phosphorus pentoxide. A picric acid test (section 2.3.3.3) was done to ensure that all the methyl ester groups on the resin reacted with ethylene diamine.

Before coupling the first amino acid, the resin was swollen for 50 minutes in high purity DMF (20 mL/g). The first amino acid was then coupled, using the normal peptide synthesis

procedure, to form an irreversible amide bond between the amino acid and the resin. A Kaiser test (section 2.3.3.2) and a Fmoc test (section 2.3.3.1) were performed to evaluate whether the coupling was complete.

2.3.3.4.2 Elongation of the peptide chain

For the elongation steps the required reagent quantities were calculated from the resin capacity of 0.1 milli-equivalent per gram of dry resin. A three times molar excess of the subsequent active Fmoc-amino acids and 1-hydroxybenzotriazol (HOBt) as catalyst was used in the elongation of the peptide chain. The pentafluorophenol esters of Fmoc-Gly, Fmoc-L-Leu, Fmoc-L-Lys(tBoc), Fmoc-L-Phe, Fmoc-L-Pro and Fmoc-L-Tyr(tBu) were used in the relevant coupling reactions. The total volume of DMF was limited to < 2 mL/gram of resin in the shake flask method. The reaction times of the coupling steps varied from 60 to 90 minutes, depending on the completeness of acylation, as determined with the Fmoc test (section 2.3.3.1) and the Kaiser test (section 2.3.3.2).

2.3.3.4.3 Removal of the Fmoc-group from the attached amino acid

After the coupling step was completed (as determined with a negative Kaiser test and a Fmoc test), the resin was washed thoroughly to remove any trace of active amino acid derivative and catalyst. Piperidine (20% in DMF) was applied to the resin and the deblocking reaction proceeded for 30 minutes. The absorbance of the piperidine mixture was measured at 290 nm (refer to 2.3.3.1) to confirm the liberation of the Fmoc group. The piperidine was filtered off and the resin washed with DMF until the wash tested negative with the Sanger test (refer to 2.3.1.2). For a successful synthesis it is imperative that all the piperidine is removed from the resin and the reaction glassware.

2.3.3.4.4 Removal of the side protection groups

After the final coupling reaction and DMF wash, the resin was washed on a sintered glass filter with *t*-amyl alcohol, acetic acid, *t*-amyl alcohol and finally with peroxide free diethyl ether and then dried under vacuum. The *t*-butyl groups were removed by acid treatment. Typically, peptide resin (0.25 g) was treated for 2 hours with 90% trifluoroacetic acid (5 mL) containing water (5%) and phenol (5%) as scavengers. The resin was washed again with

t-amyl alcohol, acetic acid, *t*-amyl alcohol and ether. Afterwards the peptide resin was dried by placing it in a vacuum desiccator over phosphorus pentoxide.

2.3.3.5 Synthesis of soluble peptide amides (NLK peptides)

A batch-wise procedure similar to the one used for the ILK-peptides was used to synthesise the four NLK peptides, removing the first quarter of resin with NLK12 peptide attached (refer to 2.3.3.4). The 12 amino acid peptide resulting from this synthesis will be referred to as NLK12$_1$. A second synthesis of NLK12 was done later; this batch will be referred to as NLK12$_2$.

2.3.3.5.1 Coupling of the first amino acid

For the synthesis of the amidated peptides, NLK, the commercially available resin Pepsyn KB was used. The resin was swollen for 50 minutes in high purity DMF (20 mL/g). The first amino acid was coupled to the resin (capacity 0.1 milli-equivalent per gram of dry resin) using the PyBOP® method (described hereafter). Coupling efficiency was evaluated with the Fmoc test (section 2.3.3.1) and the Kaiser test (see section 2.3.3.2) and the coupling step was repeated until the resin was saturated with the Fmoc-amino acid.

2.3.3.5.2 Elongation of the peptide chain

For the elongation steps the required reagent quantities were calculated from the resin capacity (0.1 milli-equivalent per gram of dry resin). A three times molar excess of the subsequent active Fmoc-amino acids and 1-hydroxybenzotriazol (HOBt) as catalyst was used in the elongation of the peptide chain during the first synthesis of the NLK peptides. The pentafluorophenol esters of Fmoc-Gly, Fmoc-L-Leu, Fmoc-L-Lys-(tBoc), Fmoc-L-Phe, Fmoc-L-Pro and Fmoc-L-Tyr-(tBu) were used in the relevant coupling reactions. For the synthesis of NLK12$_2$, Fmoc-Gly-OH, Fmoc-L-Leu-OH, Fmoc-L-Lys-OH, Fmoc-L-Phe-OH, Fmoc-L-Pro-OH and Fmoc-L-Tyr(tBu)-OH were used after activation by the PyBOB® reagent.

The PyBOB® reagent was used as follows: a three fold molar excess in minimum DMF (0.8-1.4 mL) of the protected amino acid and HOBt was mixed with the resin. A mixture of three fold molar excess PyBOP® and six fold molar excess of N, N'-diisopropylethylamine (DIPEA) in minimum DMF (0.8-1.4 mL) was then added and mixed thoroughly with the

resin. In the shake flask method the total volume of DMF was limited to < 2 mL/gram of resin. The coupling reaction times varied from 60 to 90 minutes, depending on the completeness of acylation, as determined with the Kaiser test and/or the Fmoc test. After the coupling step was completed, the Fmoc group was removed as described under section 2.3.3.4.3.

2.3.3.5.3 Cleavage from linkage by nucleophiles: Formation of peptide amides by aminolysis [10]

Two different cleavage reactions were used to form free peptide amides. All cleavage reactions involving attack by strong nucleophiles on the resin benzyl ester (or of other ester linkages) must usually be preceded by removal of the amino terminal Fmoc group, and by acidic cleavage of side chain *t*-butyl derivatives. Fmoc groups can be at least partly removed by nucleophilic reagents.

I.   Cleavage with methanolic ammonia

The *t*-butyl and tBoc protection groups were removed by treatment of the peptide resin (0.2 g) with 90% trifluoroacetic acid (5 mL) containing water (5%) and phenol (5%) for two hours. The treated resin was then washed three times with each *t*-amyl alcohol, acetic acid, *t*-amyl alcohol and diethyl ether and dried by placing it in a vacuum desiccator over phosphorus pentoxide. Failure to dry the resin at this stage can lead to the formation of contaminating peptide acids. The resin was swollen in DMF, the excess DMF removed by suction and transferred to a round-bottomed flask containing approximately 25 mL ice-cold methalonic ammonia. The flask was sealed and the resin stirred gently overnight at room temperature. After 24 hours the flask was cooled again, opened, and the resin filtered and washed with methanol. The combined filtrate was evaporated and the residual peptide amide dried before characterisation and purification. The NLK12₁ peptide was liberated from the resin using this method.

II.   Cleavage with liquid ammonia

Glacial acetic acid (0.4 mL) was added to the peptide resin (1 g) from which the *t*-butyl and tBoc protection groups removed as described in (I). This resin-acetic acid suspension was then cooled in a liquid nitrogen bath and dry liquid ammonia (15-20 mL) was added. The high

pressure container was sealed, allowed to warm to room temperature and kept overnight. The container was then re-cooled, opened, and the ammonia allowed to evaporate. The peptide amide was washed from the resin using 50% acetonitrile/water. The combined filtrate was evaporated and the residual peptide amide dried before characterisation and purification. The four other NLK peptides were liberated from the resins using this method.

2.3.4 Purification of the soluble peptides (NLK)

2.3.4.1 Gel permeation chromatography

A Sephadex G10 gel permeation column (500 mm x 15mm) was used for the purification of peptides. Freeze-dried crude peptides were dissolved in 1.5 to 3 mL of a 1% acetic acid solution (depending on solubility). Their absorption at 230 nm and 280 nm was determined before being applied to the Sephadex G10 column and eluted with 1% acetic acid. Flow rates were 10 to 14 mL/hour and the eluates were monitored at 230 nm or 280 nm. The major absorbing fractions were collected, freeze-dried and analysed for amino acid composition.

2.3.4.2 Cation exchange chromatography

A CM-Sepharose column (260 mm x 15mm) was used to further purify the peptide, $NLK12_1$. Before use the column was washed with two bedvolumes 0.45 M ammonium acetate and then equilibrated with three bed volumes 0.35 M ammonium acetate (pH 6.0). The freeze-dried peptide was dissolved in less than 10% of the bedvolume and loaded on the column. Elution was initiated with 0.35 M ammonium acetate (pH 6.0) and, after an hour, a linear gradient was introduced with 0.45 M ammonium acetate (pH 6.0) as the final concentration. The flow rate was 15.6 mL/hour and the eluate monitored at 230 nm or 280 nm. Major absorbing fractions were pooled, freeze-dried, weighed and analysed for purity by HPLC and ES-MS.

2.3.4.3 High performance liquid chromatography

Semi-preparative HPLC was used to purify the $NLK12_2$ and further purify $NLK12_1$ because of difficulties in purifying them sufficiently with gel permeation chromatography and cation exchange chromatography. Samples (5 mg/mL) were dissolved in 50% acetonitrile in water and centrifuged for 10 minutes at 1000 g to remove undissolved particles. Because of a high

tendency to aggregate, samples were freshly prepared just prior to injecting it onto a semi-preperative C_{18} Polygosil HPLC column (irregular particle size, 60 Å pore size, 250 mm x 10 mm). The chromatography system consisted of two Waters 510 pumps, MAXIMA software control system, Waters Model 440 detector and a WISP 712 sample processor. A linear gradient was created using eluant A (0.1% TFA in water) and eluant B (90% acetonitrile and 10% eluant A) [16] (Table 2.2). The flow rate was 3.0 mL/min and the chromatography was monitored at 254 nm. The major absorbing fractions were collected, freeze-dried and analysed.

Table 2.2   Gradient program used in the high performance liquid chromatography of NLK12$_1$ and NLK12$_2$.

Time (min)	Flow rate (mL/min)	% A	% B
0.0	3	80	20
0.5	3	80	20
13	3	0	100
14	3	0	100
20	3	80	20
25	3	80	20

2.3.5 Analysis of the immobilised peptides

2.3.5.1 Protein sequencing

Protein sequencing was done by means of Edman-degradation [20], on the Perkin Elmer Applied Biosystems Procise 491 Automated protein sequencer, using the pulsed liquid method, to confirm the sequence of the ILK peptides. This was done at the Molecular Biology Unit at the University of Natal.

2.3.5.2 Amino acid analysis

Amino acid analyses were done using a pre-column derivatisation with phenylisothiocyanate (PITC), according to the Pico-Tag® method [21]. Vacuum dried resin samples, taken after each coupling, were analysed for amino acid composition after one hour gaseous hydrolysis at 150°C. Gaseous phase hydrolysis was done with 6 N HCl containing 1% phenol under a

nitrogen atmosphere. After hydrolysis the acid was removed from the samples under high vacuum. In the next step, 20 µL ethanol:water:TEA (1:2:1) was added to each sample to ensure a sample pH of 9-10 for derivatisation. The samples were thoroughly vacuum-dried before derivatisation with 20 µL methanol:TEA:water:PITC (7:1:1:1). The derivatisation proceeded for 15 minutes at room temperature, before the samples were again vacuum-dried. Pico-Tag® diluent (200 µL) was added to the dried sample. The diluted samples were vortexed and filtered through HV 0.45 µm Millipore membrane filters.

Chromatography was done at 44°C on the Waters HPLC system, described under section 2.3.4.3, using a Nova-Pak C_{18} HPLC column (150 mm x 3.9 mm). The separation of the derivatised amino acids were accomplished with a binary non-linear gradient using eluant A (0.14 M sodium acetate, 10 mM EDTA and 0.5 mL TEA per litre water, titrated to pH 6.40 with acetic acid and then affixed with 6% acetonitrile) and eluant B (60% acetonitrile containing 10 mM EDTA) [21]. Pierce standard amino acid mixture (hydrolysis standard) was used to calibrate the analyses.

2.3.6 Analysis of the purified soluble peptides

2.3.6.1 Electrospray mass spectrometry

Electrospray mass spectrometry (ES-MS) was performed on the NKL peptides, using a Micromass Quattro triple quadropole mass spectrometer fitted with an electrospray ionisation source. The sample solution (2 ng peptide in 50% acetonitrile/water, 10µL) was introduced into the ES-MS using a Rheodyne injector valve and delivered at a flow rate of 20 µL/min. A capillary voltage of 3.5 kV was applied, with the source temperature set at 80°C. The cone voltage was set between 60 and 70 V. Data acquisition was in the positive mode, scanning the first analyser (MS$_1$), through m/z = 300-1500 at a scan rate of 100 atomic mass units/second (m/z is defined as the molecular mass to charge ratio). Representative scans were produced by combining the scans across the elution peak and subtracting the background.

2.3.6.2 Analytical high performance liquid chromatography

Reverse phase high performance liquid chromatography (RP-HPLC) was used to analyse the purity of the NLK peptide amides. The C_{18} Nova-Pak HPLC column was used with the

system described above for the semi-preparative HPLC in section 2.3.4.3. The chromatography was monitored at 254 nm. A linear gradient was created using eluant A (0,1% TFA in water) and eluant B (90% acetonitrile and 10% eluant A). The elution program was identical to the one shown in Table 2.2, except for the flow-rate, which was 1.0 mL/minute throughout chromatography.

2.4 Results and discussion

2.4.1 The Fmoc test standard curve

The standardisation of the Fmoc test was done by setting up a standard curve using Fmoc-Leu and 20% piperidine in DMF. The % Fmoc removed = % coupling of the amino acid. The linear relationship (Fig 2.5) between the mmole Fmoc fulveen and the absorbance at 290nm, with curve fit (r^2) of 0.9944, was:

$$\text{Abs.(290nm)} = 0.002848 \times \% \text{ coupling} + (-0.009975)$$
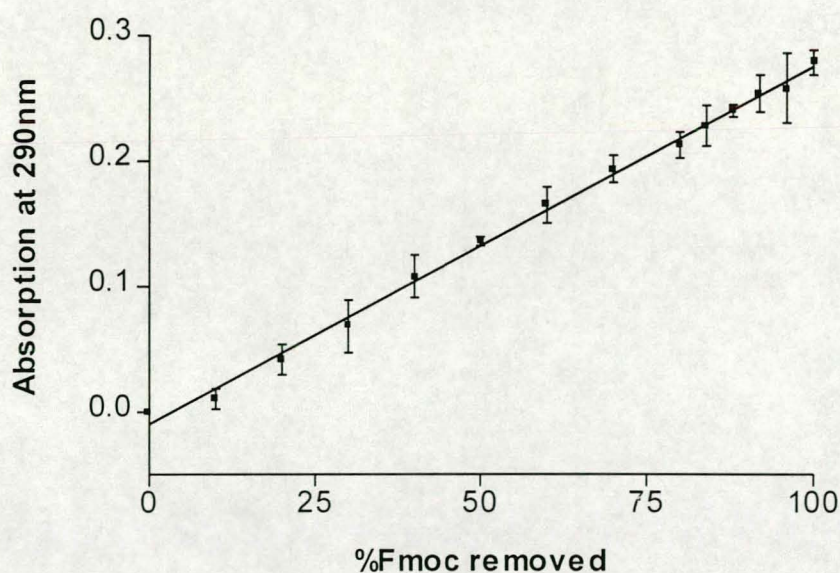
Figure 2.5   Curve showing the linear relationship between the percentage Fmoc-fulveen and the absorption at 290 nm, with curve fit (r^2) of 0.9944, n=3.

Values obtained during the Fmoc test that showed 100±10% coupling, were still sufficient because a 10% error could have been made while weighing the resin in preparation of the Fmoc test.

2.4.2 Synthesis of the immobilised peptides

The immobilised peptides were successfully synthesised using the Fmoc-polyamide methodology. Satisfactory Kaiser test results were obtained throughout the peptide synthesis (results not shown), which confirmed successful coupling and deblocking steps. The coupling time of Leu_{18} and Lys_5 was extended to 120 min because the Kaiser test during coupling was not satisfactory. The peptides being irreversibly coupled, their analyses were limited to the picric acid tests, sequencing and amino acid analysis. Other alternatives, such as solid phase NMR and IR techniques could have been useded, but they were not readily available.

2.4.2.1 Fmoc test

Because the immobilised peptides could not be purified, the Fmoc test was done after each coupling to ensure complete coupling, and therefore a pure peptide. The results in Fig 2.4 show that all Fmoc tests after each coupling step fell within the limit (100±10%) of this experiment, except Gly_3. The Kaiser test during the coupling of Gly_3, however, gave a negative result, which showed that the coupling was complete. The average coupling efficiency (Table 2.3) per step, calculated from the Fmoc tests, were between 98% and 99% for each peptide. Thus it can be said that the peptide purity is > 98%.
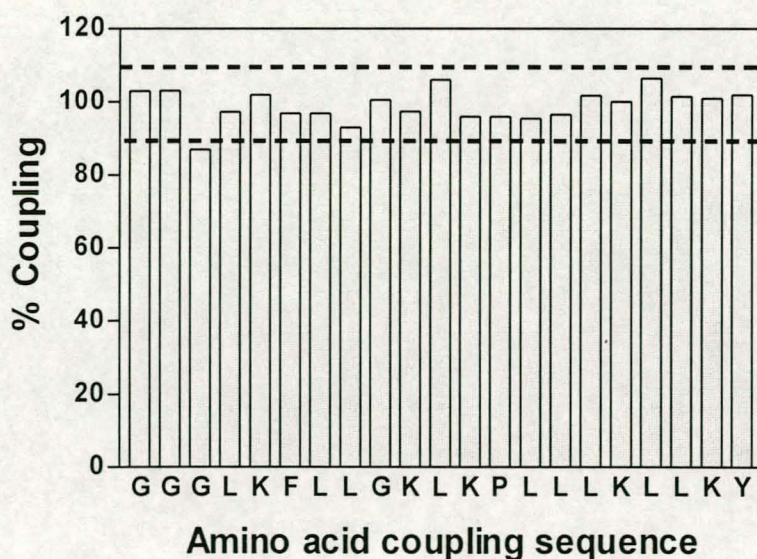
Figure 2.6   Summary of the Fmoc tests performed on the peptide resin after each amino acid coupling during the synthesis of the ILK peptides. The Fmoc test shows the % coupling of each Fmoc amino acid. The 10% error limit to still have 100% coupling is indicated by ---.

2.4.2.2 Picric acid test

The picric acid test was performed on the ILK peptides after the removal of the Fmoc and tBoc groups from the immobilised peptides. The results show the concentration of the picrate measured as a percentage of the theoretical picrate expected (Table 2.3). The control showed 76% of the expected picrate concentration. Thus, the results were adjusted to compensate for the 24% difference. The picric acid test confirmed that the side protection groups were successfully removed and showed that the peptide remained on the resin after acidic treatment.

Results confirmed that all Lys-residues were incorporated into these peptides (Table 2.3). According to this test, Lys-deletion peptides were limited, as the results of ILK16 showed a 100% incorporation of this amino acid. However, one of the next coupling reactions to form NLK18 (coupling of Lys_2 and Tyr_1), may have been incomplete, because the picric acid test revealed only 90% of the expected free amino groups. That the Fmoc test showed a 101% coupling of Lys and 102% of Tyr – may have been the result of experimental error.

Table 2.3   Summary of the coupling efficiency and the Picric acid test on the ILK peptides. The coupling efficiency was calculated from the Fmoc tests performed on the resin after each coupling. The picric acid test shows the number of free amino groups on the peptide resin after removal of all the protection groups.

Peptide	Coupling efficiency (%)	Picric acid test (%)	Number of free amino groups	Expected number of free amino groups
ILK18	99	68	5.40	6
ILK16	99	76	5.00	5
ILK14	98	73	4.80	5
ILK12	98	74	3.88	4
Control	-	76	1.00	1

2.4.3 Analysis of immobilised peptides

2.4.3.1 Protein sequencing of the longest resin bound peptide

Edman-degradation [20] was done on ILK18 by the University of Natal on the Perkin Elmer
Applied Biosystems Procise 491 Automated protein sequencer, using the pulsed liquid
method. The degradation was only done on ILK18, because of the method used to synthesise
the peptides (refer to section 2.3.3.4). The other three ILK peptides were precursors to ILK18.
Results (Table 2.4) confirm the peptide sequence as:

YKLLKLLLPKLKGLLFKLGGG

Table 2.4   Edman sequencing results of ILK18. Degradation started at the N-terminal of the
peptide.

Analysis cycle	Retention time (min)	pmol	Amino acid ID
1	11.54	40.16	Tyr (N-term)
2	19.94	35.07	Lys
3	19.15	32.58	Leu
4	19.15	31.24	Leu
5	18.97	32.68	Lys
6	19.17	30.69	Leu
7	19.17	29.28	Leu
8	19.15	28.95	Leu
9	14.00	16.43	Pro
10	18.98	14.98	Lys
11	19.15	16.53	Leu
12	18.96	19.34	Lys
13	6.42	14.06	Gly
14	19.17	18.37	Leu
15	19.17	22.22	Leu
16	18.2	15.87	Phe
17	18.98	15.24	Lys
18	19.16	16.04	Leu
19	6.43	15.28	Gly
20	6.43	19.90	Gly
21	6.40	20.64	Gly (C-term)

2.4.3.4 Amino Acid Analysis of the resin bound peptides

The amino acid analysis on the resin sample confirmed that each of the resin bound peptides had the correct amino acid composition (Table 2.5).

The values of Tyr and Pro were lower than expected due to oxidative degradation during hydrolysis. Low values for Leu were also observed, probably due to weak hydrolyses because of the hydrophobic environment. No correlation could be drawn between the values obtained from the amino acid analysis and the Fmoc test. The amino acid analysis did however show that the correct amino acids were coupled in the correct sequence.

2.4.4 Synthesis of the soluble peptides

The four soluble peptide amides were successfully synthesised using the Fmoc-polyamide protocol. Satisfactory Kaiser tests and Fmoc tests were obtained throughout the peptide synthesis. The coupling of Leu_{18} to the resin was repeated three times to give a satisfactory Fmoc test of 93%. The coupling efficiencies of the four peptides from the first synthesis varied between 97% and 99%. However, the coupling efficiency of $NLK12_2$ was much lower at 95.6% (Table 2.6).

Methanolic ammonia, one of the cleavage agents, had to be prepared prior to use. It was therefore much easier to use the liquid ammonia, which was readily available, as cleavage agent for the NLK peptides. The problem with using the liquid ammonia was that the resin had to be treated more than once to cleave all the peptide from the resin, but the peptides produced by this method was more pure than the ones cleaved with methanolic ammonia. ES-MS results showed that the peptides had the correct M_r, and amino acid sequence (section 2.4.5.1).

Analytical HPLC was done on the crude NLK peptides (Figs. 2.7, 2.8). The analytical HPLC showed more than one fraction with significant UV absorbance. The longest peptide, NLK18, had the highest retention time (R_t = 10.04 min) and the shortest peptide, NLK12, the lowest (R_t = 9.12 min). NLK16 had a retention time of 9.82 min and the retention time of NLK14 was 9.25 min.

Table 2.5 Summary of the amino acid composition of resin samples taken during the synthesis of the ILK peptides. Values are ratios between the picomole amino acid detected and the picomole Gly detected; * theoretical value
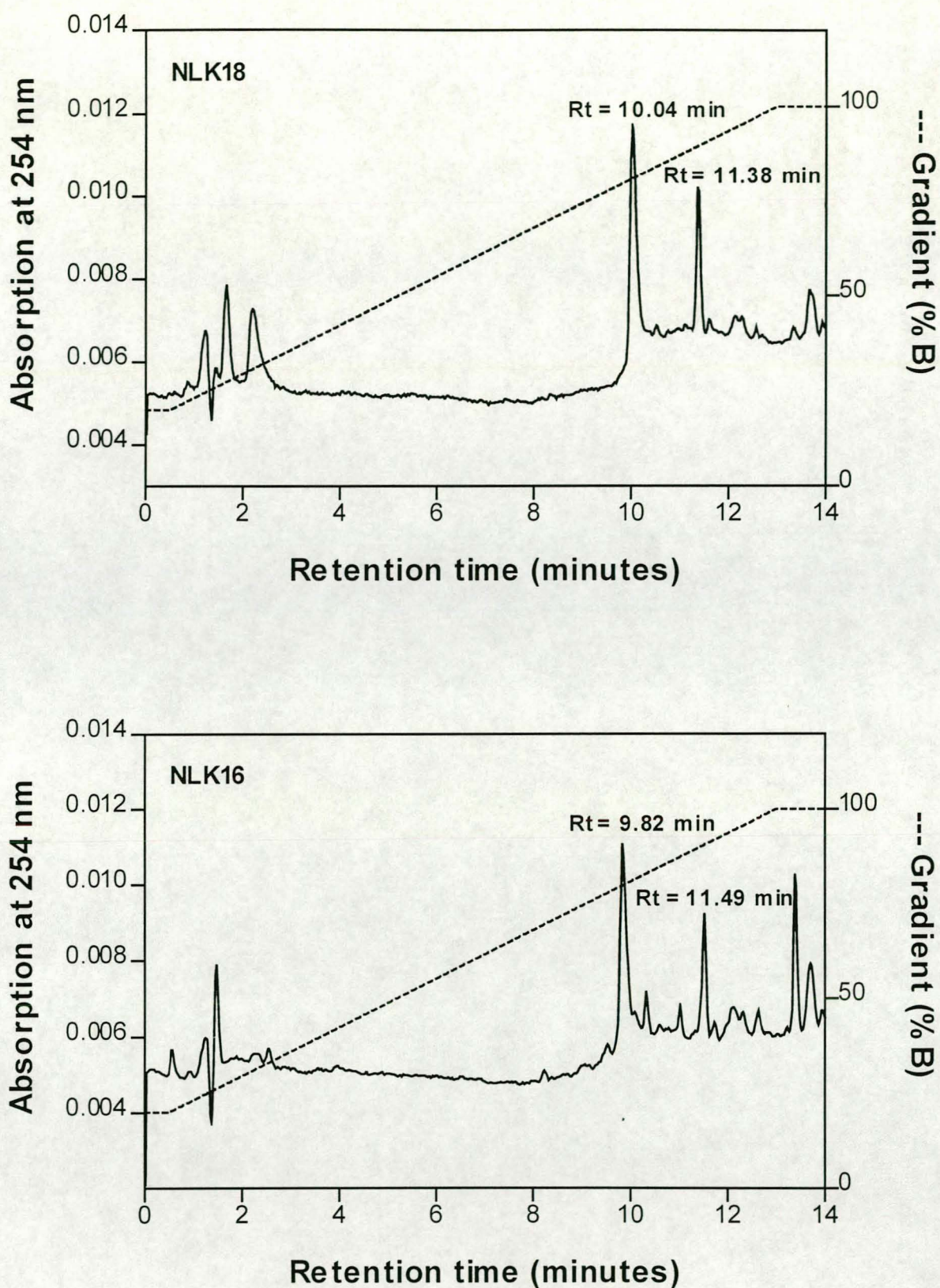
Coupling sequence →

C — Peptide sequence — N

		G	G	G	L	K	F	L	L	G	K	L	K	P	L	L	L	K	L	L	K	Y
1	G	1.00																				
2	G	-	2.00																			
3	G	-	-	3.00																		
4	L	-	-	3.00	0.63																	
5	K	-	-	3.00	-	0.99																
6	F	-	-	3.00	0.68	1.07	1.13															
7	L	-	-	3.00	-	0.89	1.04	1.30														
8	L	-	-	-	-	-	-	-	-													
9	G	-	-	-	-	1.30	0.70	-	2.04	4.00												
10	K	-	-	-	-	-	0.93	-	1.88	4.00	2.04											
11	L	-	-	-	-	-	0.97	-	-	4.00	2.08	2.74										
12	K	-	-	-	-	-	0.88	-	-	4.00	-	2.36	2.74									
13	P	-	-	-	-	-	1.07	-	-	4.00	-	2.87	3.26	1.06								
14	L	-	-	-	-	-	1.23	-	-	4.00	-	-	3.70	0.86	5.54							
15	L	-	-	-	-	-	1.25	-	-	4.00	-	-	4.92	0.81	-	8.57						
16	L	-	-	-	-	-	1.02	-	-	4.00	-	-	2.52	0.80	-	-	6.94					
17	K	-	-	-	-	-	1.24	-	-	4.00	-	-	-	0.89	-	-	7.54	4.36				
18	L	-	-	-	-	-	1.06	-	-	4.00	-	-	-	0.80	-	-	-	3.60	7.90			
19	L	-	-	-	-	-	0.92	-	-	4.00	-	-	-	0.79	-	-	-	3.32	-	7.49		
20	K	-	-	-	-	-	1.06	-	-	4.00	-	-	-	0.79	-	-	-	-	-	8.38	4.57	
21	Y	-	-	-	-	-	0.71	-	-	4.00	-	-	-	0.73	-	-	-	-	-	6.53	2.80	0.69
	Mean	1.00	2.00	3.00	0.66	1.06	1.01	1.30	1.96	4.00	2.06	2.66	3.43	0.84	5.54	8.57	7.24	3.76	7.90	7.47	3.69	0.69
	*	1	2	3	1	1	1	2	3	4	2	4	3	1	5	6	7	4	8	9	5	1

2.26

Figure 2.7   HPLC chromatograms of crude NLK18 and NLK16 using a 150 mm x 3.9 mm
C_{18} Nova-Pak column. The linear gradient was developed over 13 minutes with
0.1% TFA in water (solvent A) and acetonitrile plus 10% A (solvent B) at a flow
rate of 1 mL/min.
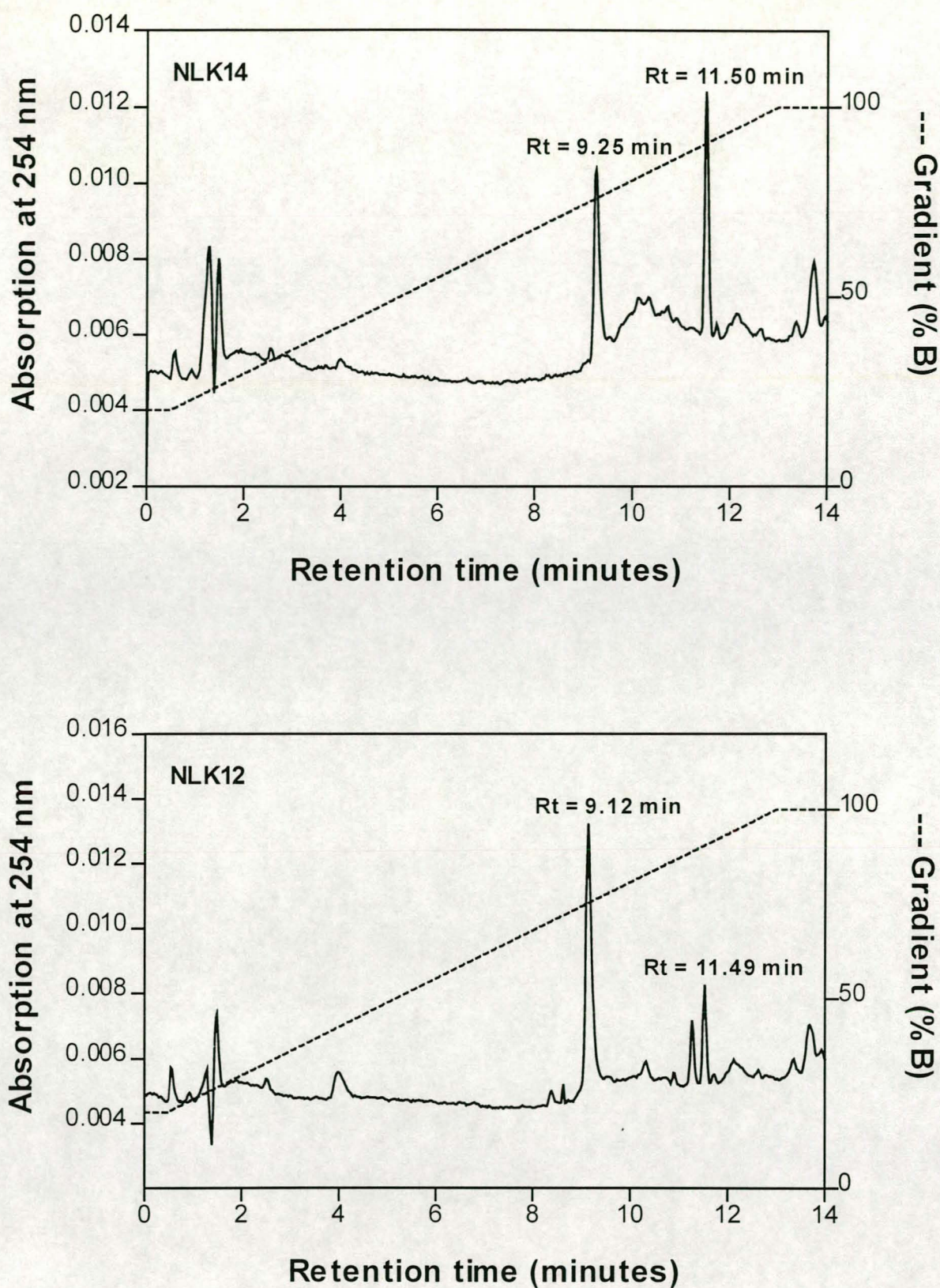
Figure 2.8 HPLC chromatograms of crude NLK14 and NLK12₁ using a 150 mm x 3.9 mm
C₁₈ Nova-Pak column. The linear gradient was developed over 13 minutes with
0.1% TFA in water (solvent A) and acetonitrile plus 10% A (solvent B) at a flow
rate of 1 mL/min.
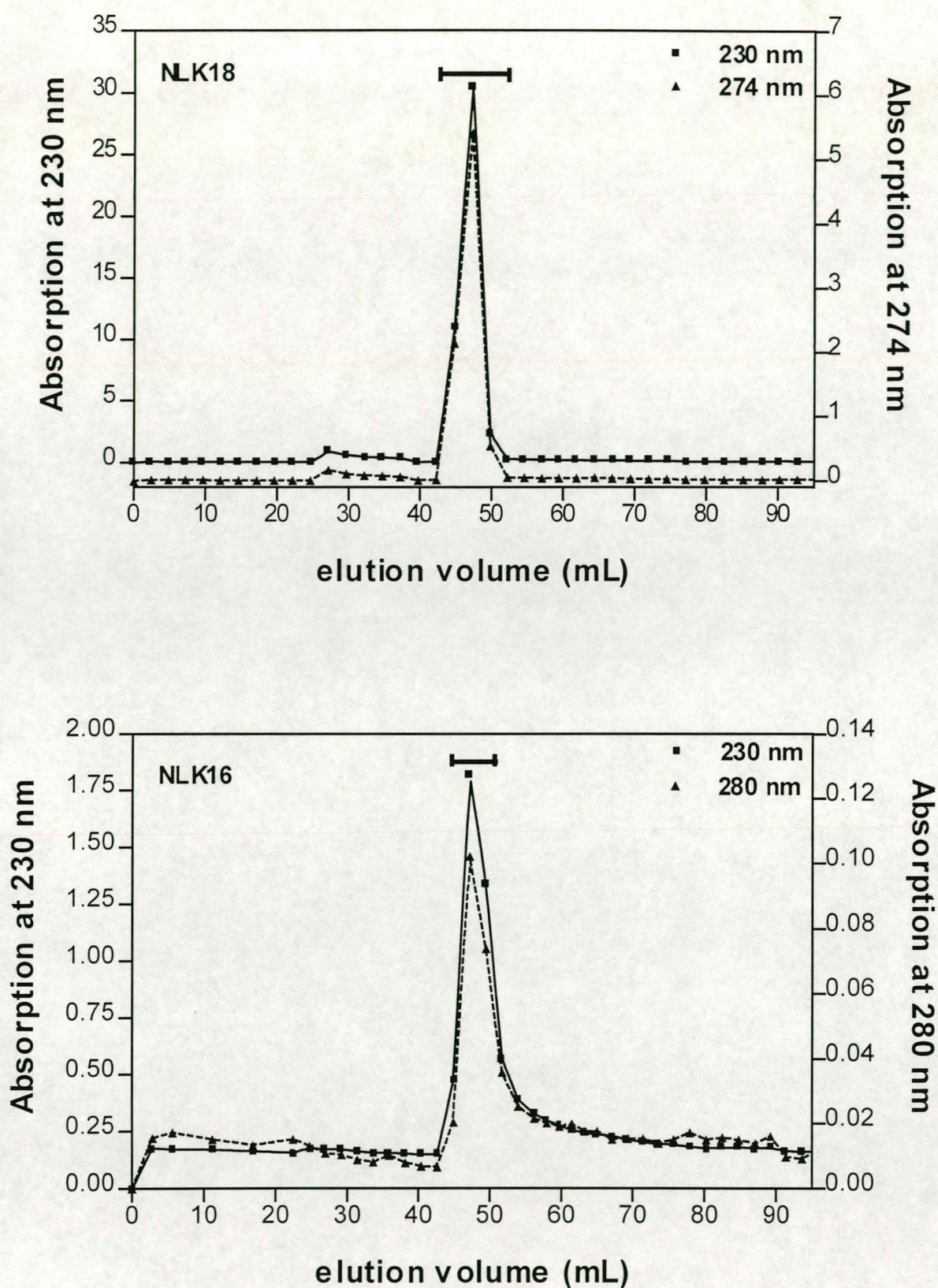
Figure 2.9   Sephadex G10 chromatography of crude NLK18 and NLK16. The eluant was 1% acetic acid in water at a flow rate of 14 mL/hour and the bedvolume of the column was 85 mL. The absorption values above 1.5 were calculated from values determined on dilutions. The bar, |—|, indicatate the peptide-containing fractions.

Figure 2.10 Sephadex G10 chromatogaphy of crude NLK14 and NLK12$_1$. The eluant was 1% acetic acid in water at a flow rate of 14 mL/hour and the bedvolume of the column was 85 mL. The absorption values above 1.5 were calculated from values determined on dilutions. The bar, |—|, indicate the peptide-containing fractions.

The purity of the crude peptide amides, NLK12$_2$, NLK14, NLK16 and NLK18, as determined by ES-MS (section 2.4.5.1), was already high with very little contaminating peptide material. The NLK12$_1$ peptide, on the other hand, had contaminating material because of the different procedure used to remove the peptide from the resin. During the use of the methanolic ammonia, methylesters formed. These peptides could not be separated from the amidated peptides and consequently it was necessary to purify NKL12$_1$ further to ensure higher purity. The other peptides were also further purified, as impurities can influence bioactivity results.

2.4.4.1 Purification of soluble peptide amides

The crude NLK12$_1$, NLK14, NLK16 and NLK18 were chromatographed on a Sephadex G10 to remove low molecular mass impurities (Figs. 2.9, 2.10). Satisfactory separations were obtained and the major absorbing fractions, containing the peptide material, were pooled and freeze-dried.

The NLK12$_1$ was further purified using chromatography with Sepharose cation exchange (results not shown). ES-MS analysis showed that contaminating peptide material still remained in the preparation (results not shown). Thus, the methylesters could not be separated from the amidated peptides by cation exchange chromatography. After cation exchange chromatography the fractions containing the peptide material were purified using semi-preparative HPLC. The second synthesis product (NLK12$_2$) was directly purified using semi-preparative HPLC.

The HPLC purification of NLK12 was successful, when eluting the fractions with the linear gradient program depicted in Table 2.2. In the case of NLK12$_1$, five peptide fractions were collected, and in the case of NLK12$_2$ four peptide fractions were collected (Fig. 2.11) and evaluated by ES-MS to determine which fraction contained the pure NLK12 peptide (results not shown).

Peptide yields were satisfactory (Table 2.6), although higher yields were expected. After the removal of the peptides from the resins, amino acid analysis was done on the resins (results not shown). The amino acid analysis showed that there remained peptide on the resin after cleavage and therefore picric acid tests were done to determine the amount of peptide on the resin (Table 2.6). Results from the picric acid tests confirmed amino acid analysis data and
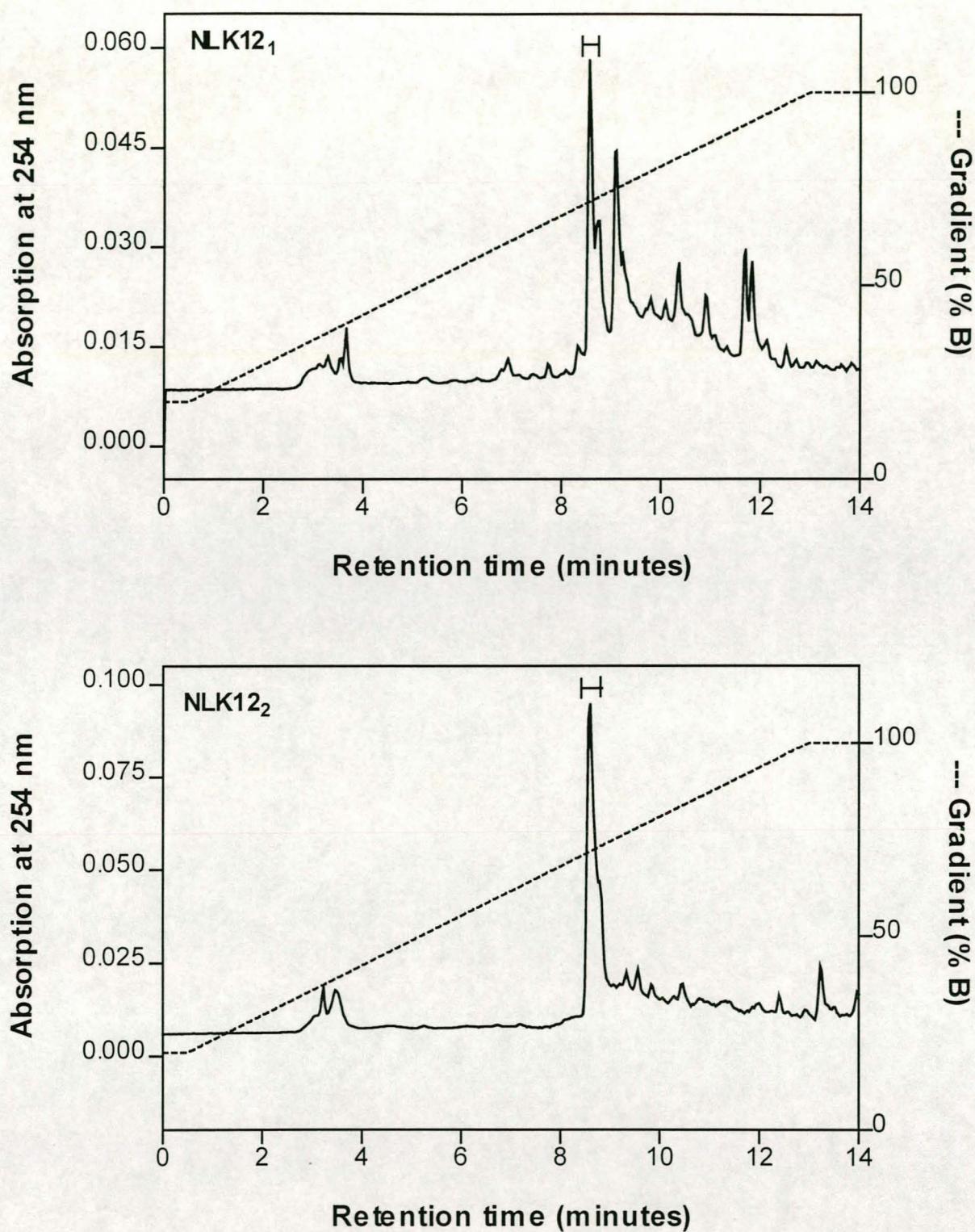
Figure 2.11 HPLC chromatograms of the crude NLK12 peptides (NLK12₁ and NLK12₂) using a 250mm x 8 mm C18 Polygosil column. The linear gradient was developed over 13 minutes with 0.1% TFA in water as solvent A and acetonitrile plus 10% A as solvent B at a flow rate of 3mL/min. Fractions that contained the peptide (as evaluated by ES-MS) are indicated by (|—|) on the chromatograms.

offered an explanation for the low crude peptide yield, namely incomplete cleavage of the peptides from the resins. Another reason for the low purified peptide yield was the high tendency of the NLK peptides to form insoluble aggregates. The best cleavage was obtained with the methanolic ammonia protocol, but extensive losses occurred because of the formation of peptide methyl esters (refer to Fig. 2.16). Large amounts of NKL18 and NKL12$_2$ remained on the resins and could still be retrieved by repeating the cleavage procedure.

Table 2.6　　Summary of peptide yields and coupling efficiency obtained for the synthesis of the four peptide amides.

Peptide	% Peptide left on the resin [1]	% Crude yield [2]	% Purified yield [3]	% Coupling efficiency [4]
NLK18	27	43.8	62.5	97.0
NLK16	14	69.1	20.6*	98.5
NLK14	7	75.3	46.2	98.4
NLK12$_1$	4	71.8	49.8	98.4
NLK12$_2$	24	45.5	44.2	95.6

[1] % peptide left on the resin was calculated from the picric acid tests on the resin samples after cleavage

[2] % crude yields = 100 x analytically weighed dry crude peptide preparation/expected yield from resin

[3] % purified yield = 100 x amount purified peptide from column/amount crude peptide loaded on column
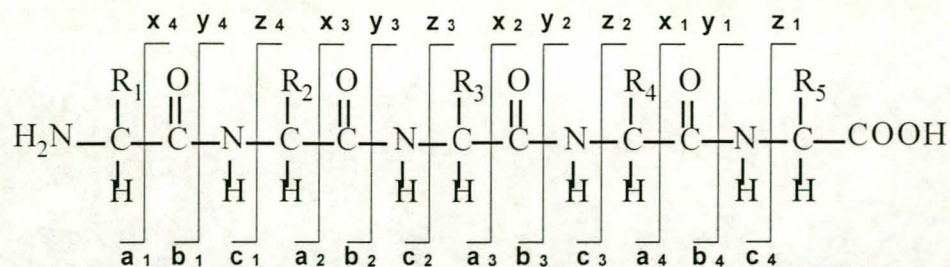
[4] % coupling efficiency = 100 x corrected crude yield$^{1/\text{number of residues}}$ (% crude yields were corrected for cleavage efficiency)

* Some of the crude NLK16 was lost during preparation for G10 chromatography

2.4.5 Analysis of the soluble peptide amides

2.4.5.1 Electrospray mass spectrometry of the peptide amides

Before discussing the ES-MS analysis of each peptide, a short background on the fragmentation of peptides during mass spectrometry is given. Fragmentation of peptides in ES-MS generally occurs at the peptide (amide) bonds (b and y product ions), but some other ion types from backbone fragmentation also occur. The nomenclature of Roepstorff and Fohlmann [22], as revised by Biemann and Martin [23], was used to identify the product ions from fragmentation of peptide molecular ions (Fig. 2.12)
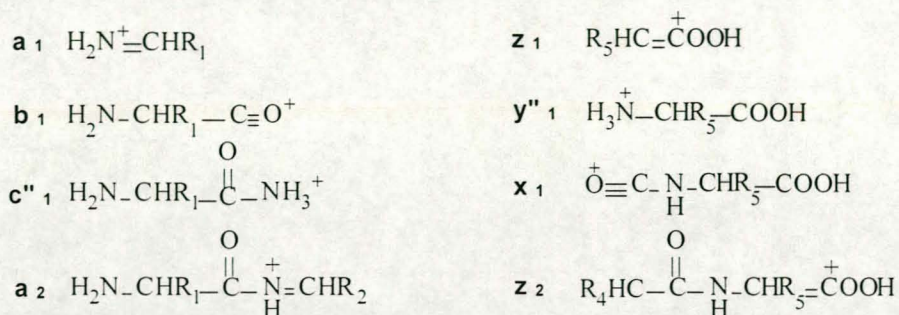
$$x_4\ y_4\ z_4\quad x_3\ y_3\ z_3\quad x_2\ y_2\ z_2\quad x_1\ y_1\quad z_1$$

$$H_2N-\overset{\overset{\displaystyle R_1}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-\overset{\overset{\displaystyle O}{\|}}{C}-N-\overset{\overset{\displaystyle R_2}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-\overset{\overset{\displaystyle O}{\|}}{C}-N-\overset{\overset{\displaystyle R_3}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-\overset{\overset{\displaystyle O}{\|}}{C}-N-\overset{\overset{\displaystyle R_4}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-\overset{\overset{\displaystyle O}{\|}}{C}-N-\overset{\overset{\displaystyle R_5}{|}}{\underset{\underset{\displaystyle H}{|}}{C}}-COOH$$

$$a_1\ b_1\ c_1\quad a_2\ b_2\ c_2\quad a_3\ b_3\ c_3\quad a_4\ b_4\ c_4$$

Peptide backbone product ions

a_1 $H_2N^+{=}CHR_1$

b_1 $H_2N-CHR_1-C{\equiv}O^+$

c''_1 $H_2N-CHR_1-\overset{\overset{\displaystyle O}{\|}}{C}-NH_3^+$

a_2 $H_2N-CHR_1-\overset{\overset{\displaystyle O}{\|}}{C}-\overset{\overset{\displaystyle +}{N}}{\underset{\underset{\displaystyle H}{}}{}}{=}CHR_2$

z_1 $R_5HC{=}\overset{+}{C}OOH$

y''_1 $H_3\overset{+}{N}-CHR_5-COOH$

x_1 $\overset{+}{O}{\equiv}C-\underset{\underset{\displaystyle H}{|}}{N}-CHR_5-COOH$

z_2 $R_4HC-\overset{\overset{\displaystyle O}{\|}}{C}-\underset{\underset{\displaystyle H}{|}}{N}-CHR_5{=}\overset{+}{C}OOH$

Figure 2.12 Fragmentation patterns of a peptide during mass spectrometry and nomenclature according to Roepstorff and Fohlmann [22], as revised by Biemann and Martin [23]. [Figure reproduced with permission from 15].

The fragment or product ions can be separated into two classes (i) a_n, b_n, and c_n product ions from the N-terminal, and (ii) x_n, y_n and z_n product ions from the C-terminal (Fig. 2.12). The subscript *n* indicates the number of amino acid residues in fragment. Because fragmentation at the peptide bond is predominant, the peptide sequence can be deduced or confirmed by the formation of certain product ions.

The correct *m/z* (molecular mass/charge ratio) was found for each of the purified NLK peptides (Figs. 2.13-2.17). For NLK18 (Fig. 2.13) the correct *m/z* of 1071 for the doubly $[M+2H]^{2+}$ charged peptide was observed with y''_5 (*m/z* 631.5) as the major product ion, confirming its sequence. The correct *m/z* of 715 was observed for the triply charged peptide ion $[M+3H]^{3+}$ and *m/z* of 536 for the quadruply charged peptide ion $[M+4H]^{4+}$. Various contaminants were removed during purification; refer to Fig 2.13 for comparison.

For NLK16 (Fig. 2.14) the correct *m/z* of the multiply charged molecular ions $[M+2H]^{2+}$ (*m/z* 925), $[M+3H]^{3+}$ (*m/z* 617) and $[M+4H]^{4+}$ (*m/z* 463) were observed. The major product ions were the triply charged y''_{14} (*m/z* 542) and the triply charged dehydrated peptide ion (*m/z* 612), which confirmed the peptide sequence. Various contaminants were removed during purification; refer to Fig 2.14 for comparison.
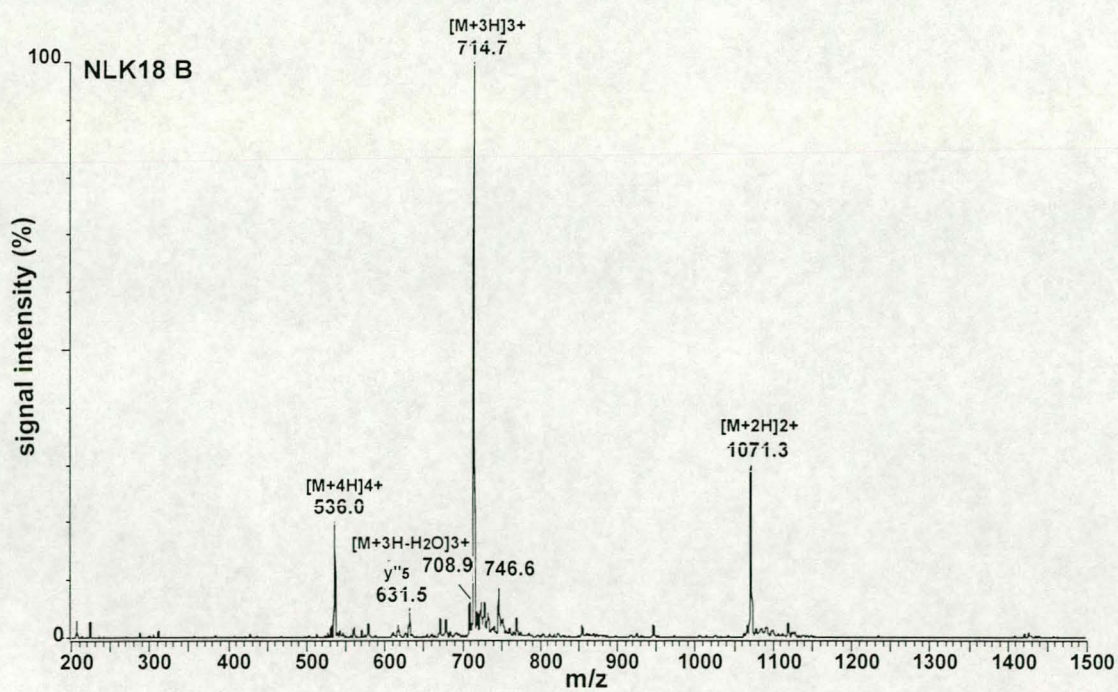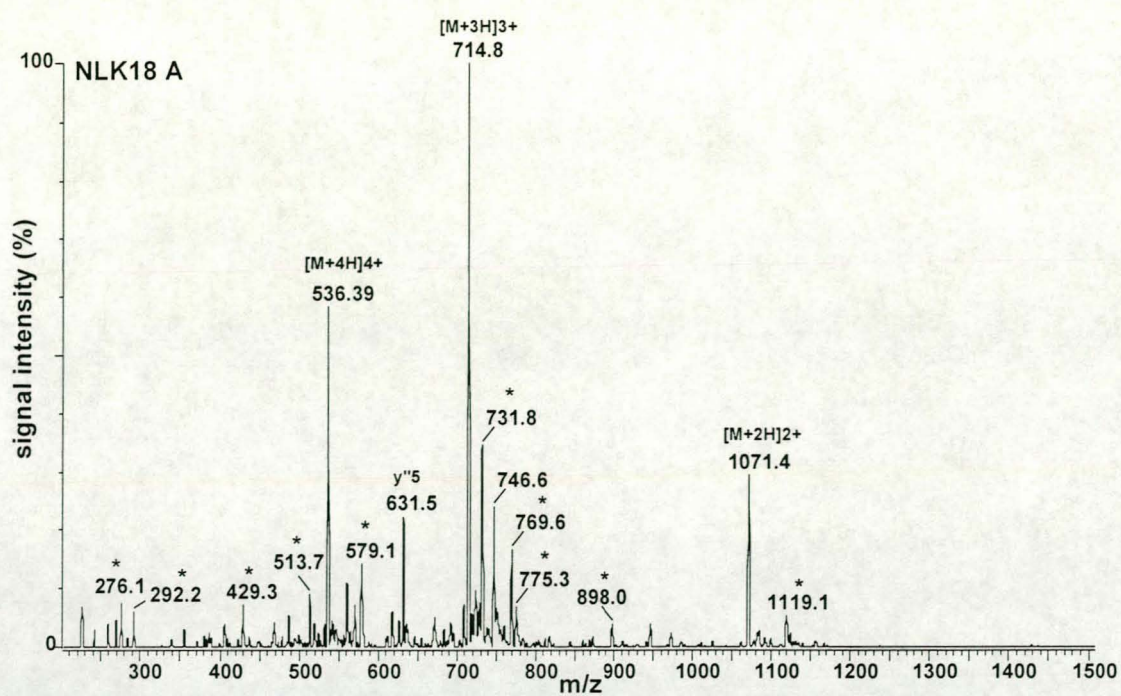
Figure 2.13 ES-MS spectra of the crude (A) and purified (B) NLK18. Analysis conditions are described under 2.3.6.1. Contaminants removed during purification are indicated by *.
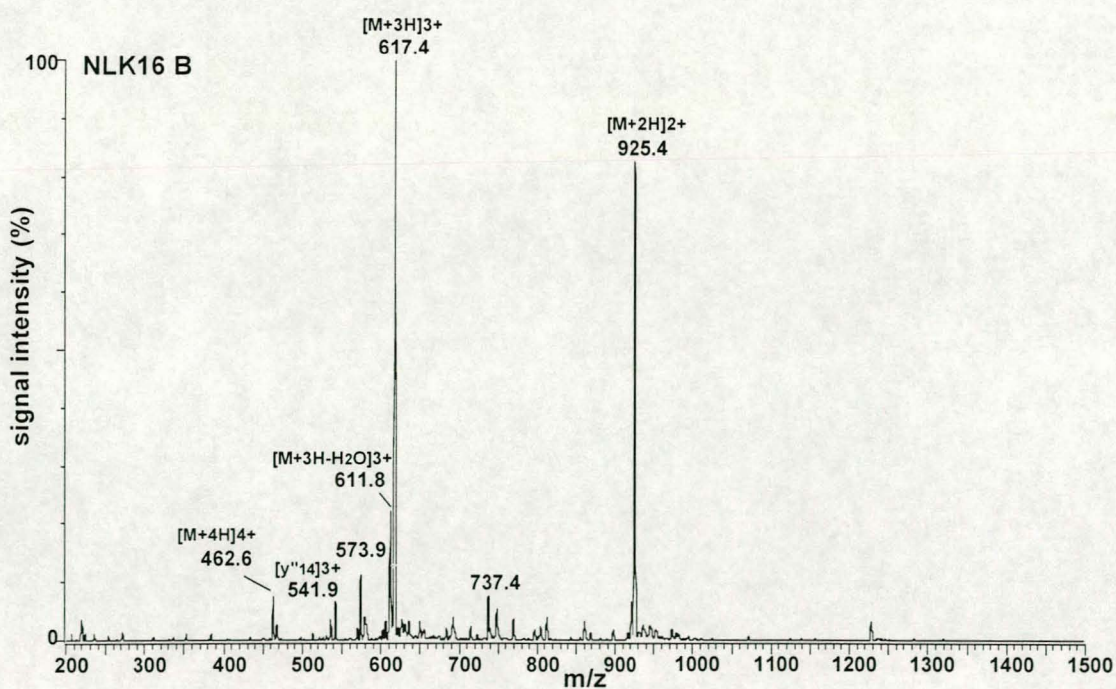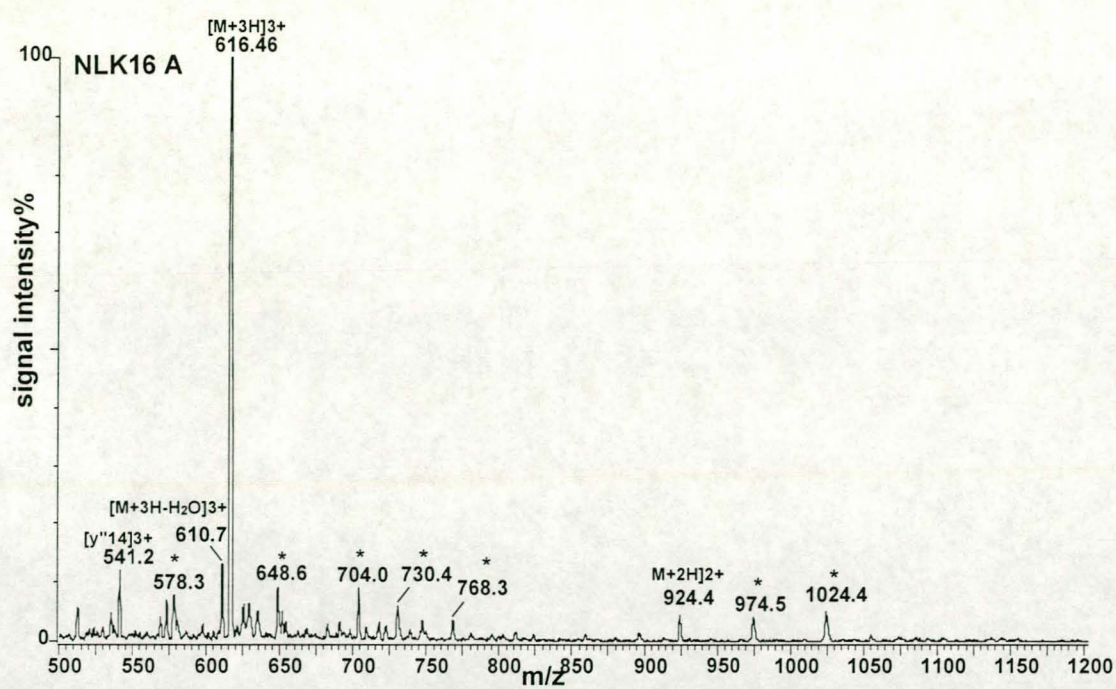
Figure 2.14 ES-MS spectra of the crude (A) and purified (B) NLK16. Analysis conditions are described under 2.3.6.1. Contaminants removed during purification are indicated by *.
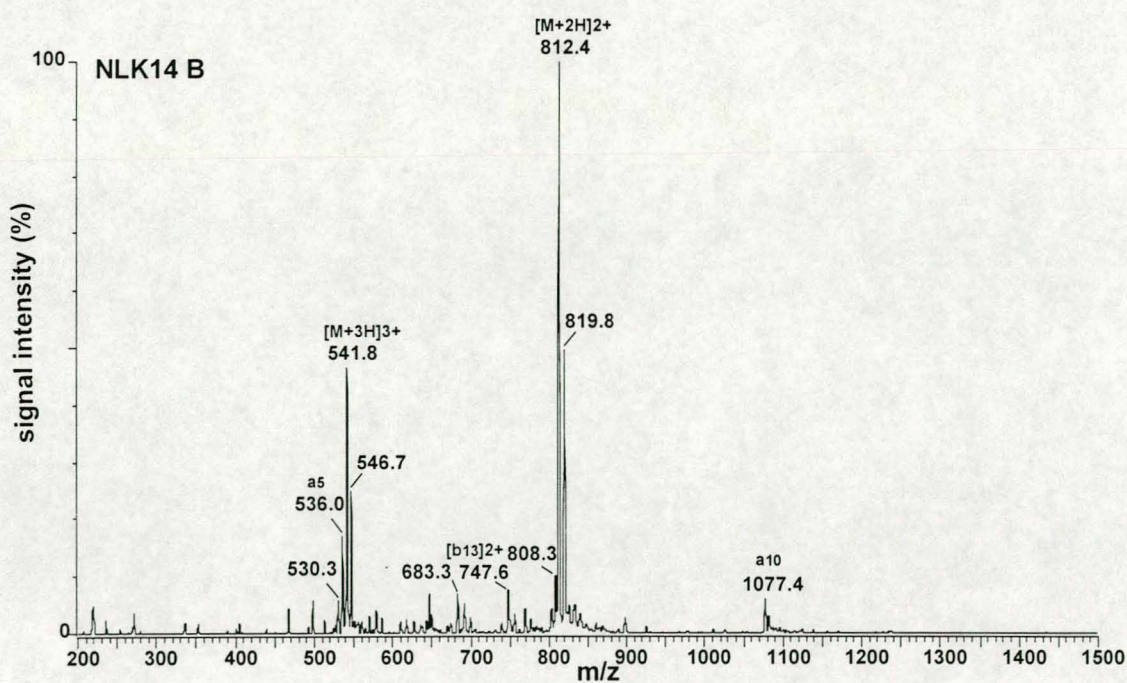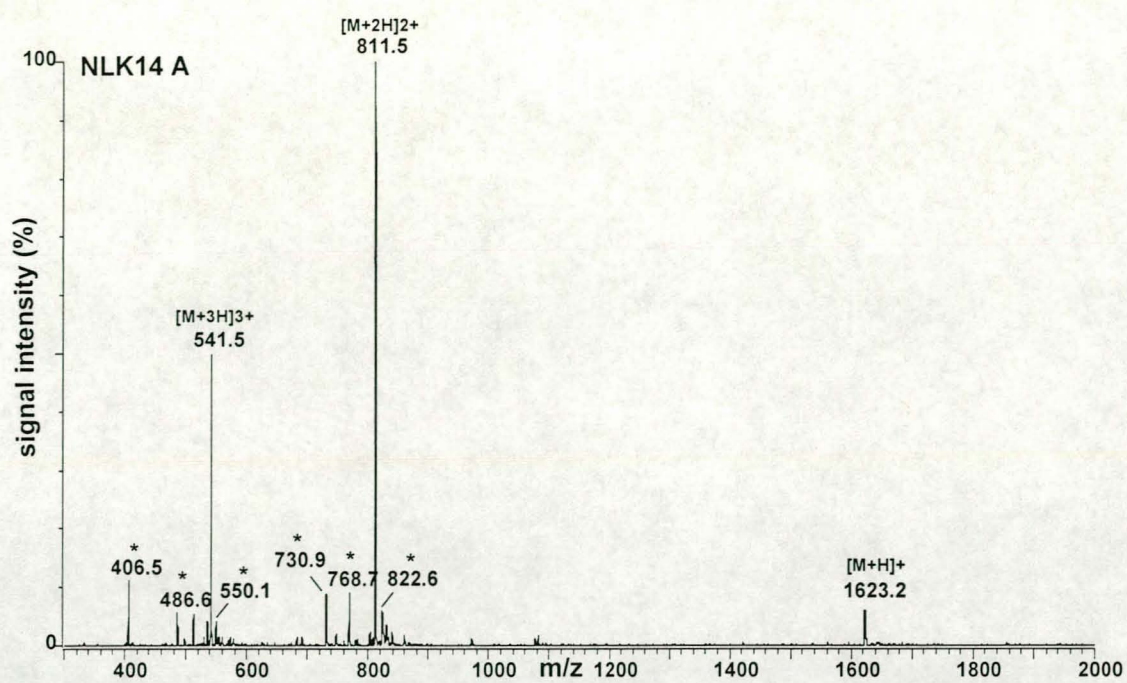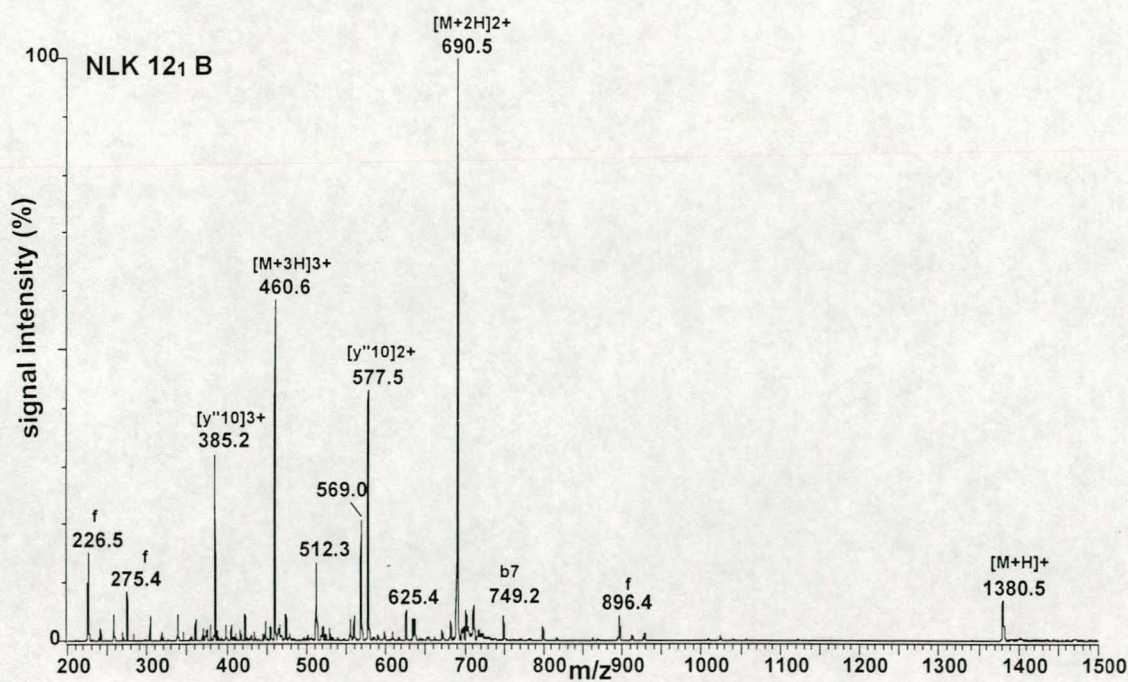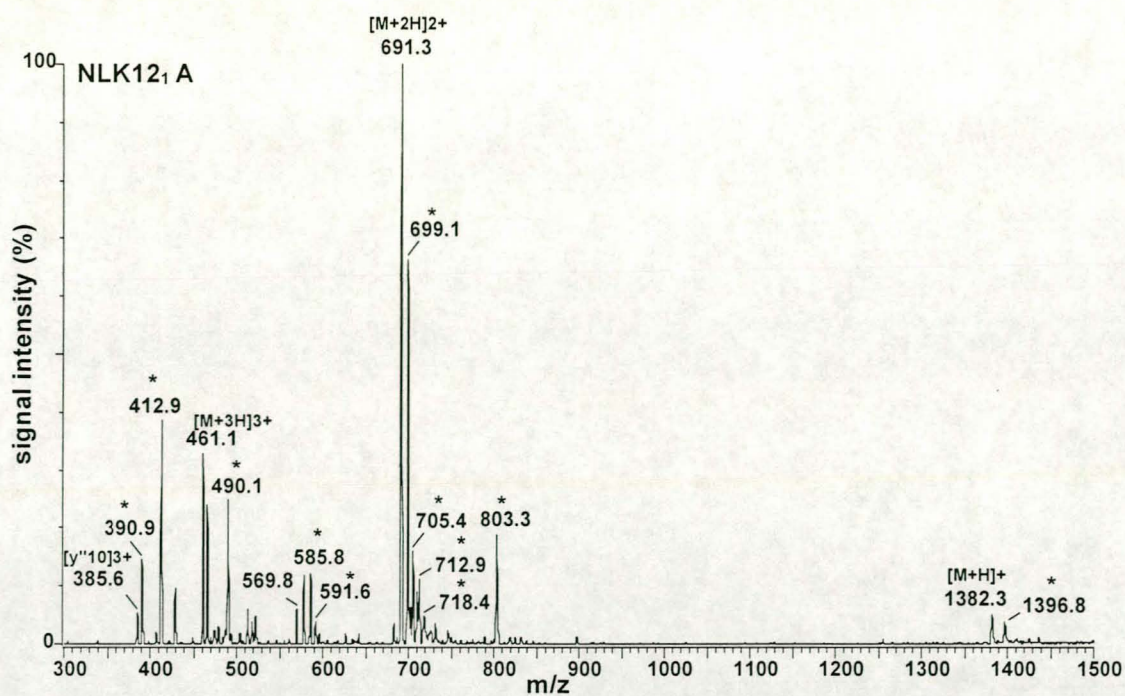
Figure 2.15 ES-MS spectra of the crude (A) and purified (B) NLK14. Analysis conditions are described under 2.3.6.1. Contaminants removed during purification are indicated by *.

2.38

Figure 2.16 ES-MS spectra of the crude (A), and HPLC purified (B) NLK12$_1$. Analysis conditions are described under 2.3.6.1. Contaminants removed during purification are indicated by *.
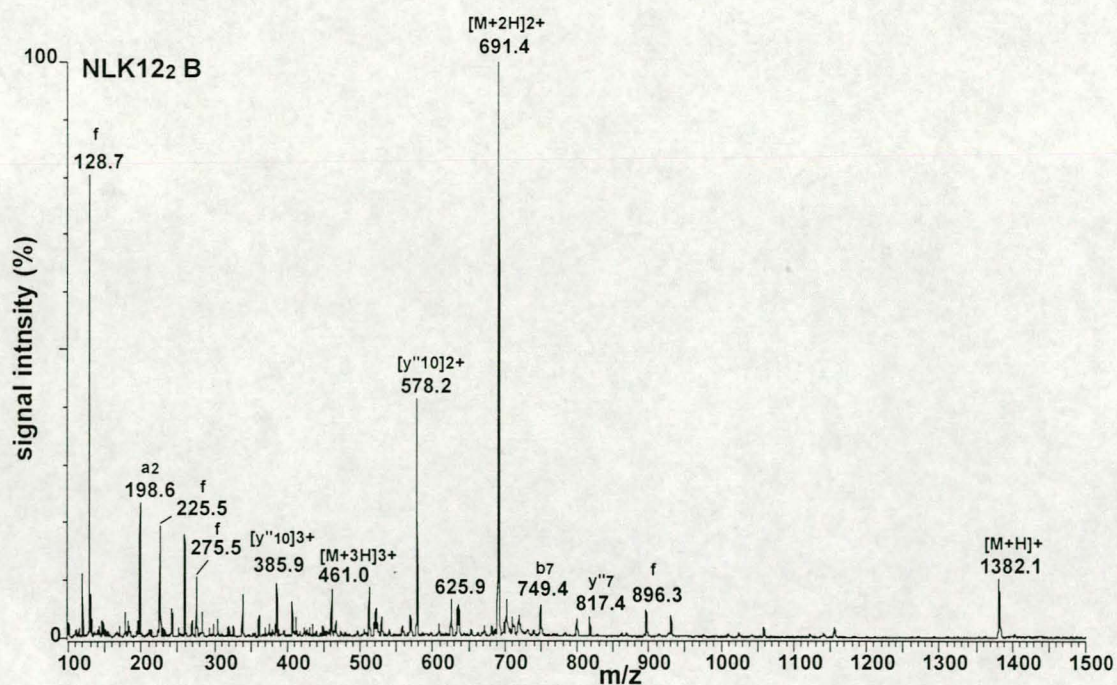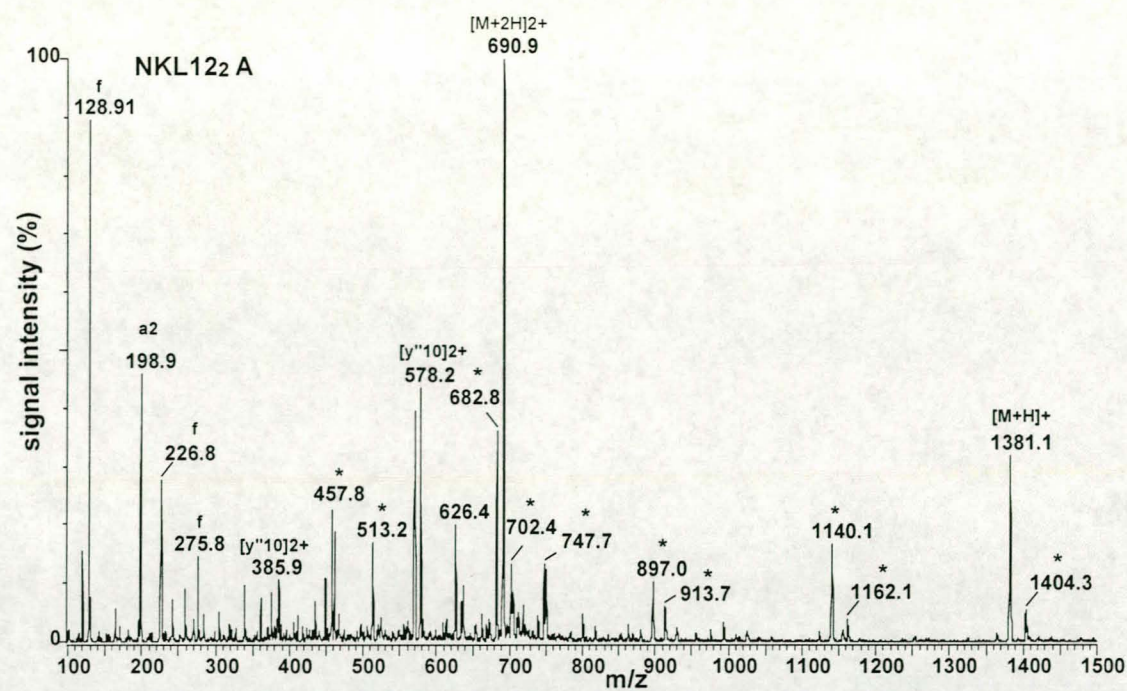
Figure 2.17 ES-MS spectra of the crude (A) and HPLC purified (B) NLK12₂. Analysis conditions are described under 2.3.6.1. Contaminants removed during purification are indicated by *.

The correct m/z of 812 for the doubly $[M+2H]^{2+}$ charged peptide ions, and 542 for the triple charged peptide ions $[M+3H]^{3+}$ of NLK14 were observed (Fig. 2.15). The major product ions, a_5 (m/z 536) and a_{10} (m/z 1077) confirmed the peptide sequence. The doubly charged product ion, b_{13} was also observed with a m/z of 748. Various contaminants were removed during purification; refer to Fig 2.15 for comparison.

Peptide NLK12 (Figs. 2.16, 2.17) fragmented extensively under the ES-MS conditions used in the analysis. The correct m/z (1382.1) of NLK12 was observed, as well as the major product ions, a_2 (m/z 198.6), b_7 (m/z 749) and $y"_7$ (m/z 817), confirming its sequence. Multiple charged peptide ions were observed ($[M+3H]^{3+}$ m/z 461; $[M+2H]^{2+}$ m/z 691) as well as the triply and doubly charged fragment, $y"_{10}$ (m/z 386 and m/z 578). Various contaminants were removed during purification; refer to Figs 2.16 and 2.17 for comparison. The major contaminant ($[M+2H]^{2+}$ m/z 699) in NLK12$_1$ was the methyl ester formed by the methanolic ammonia which was used to remove the peptide from the resin.

2.4.5.2 Analytical HPLC of the peptide amides

Analytical HPLC was done on the purified peptides, but satisfactory results could not be obtained. This was attributed to the fact that the NLK peptides have a high tendency to aggregate. This aggregation was observed in the analytical HPLC chromatograms. It seems as if the contaminating material in the crude prevents the peptide from aggregating, because the aggregation was not observed in the HPLC of the crude peptides. In Fig. 2.18 the analytical HPLC profile of the purified NKL12 changed dramatically (refer to Fig. 2.8), with most of the absorbing peptide fraction eluting in the void volume of the column. Similar results were obtained for the other peptides (results not shown).
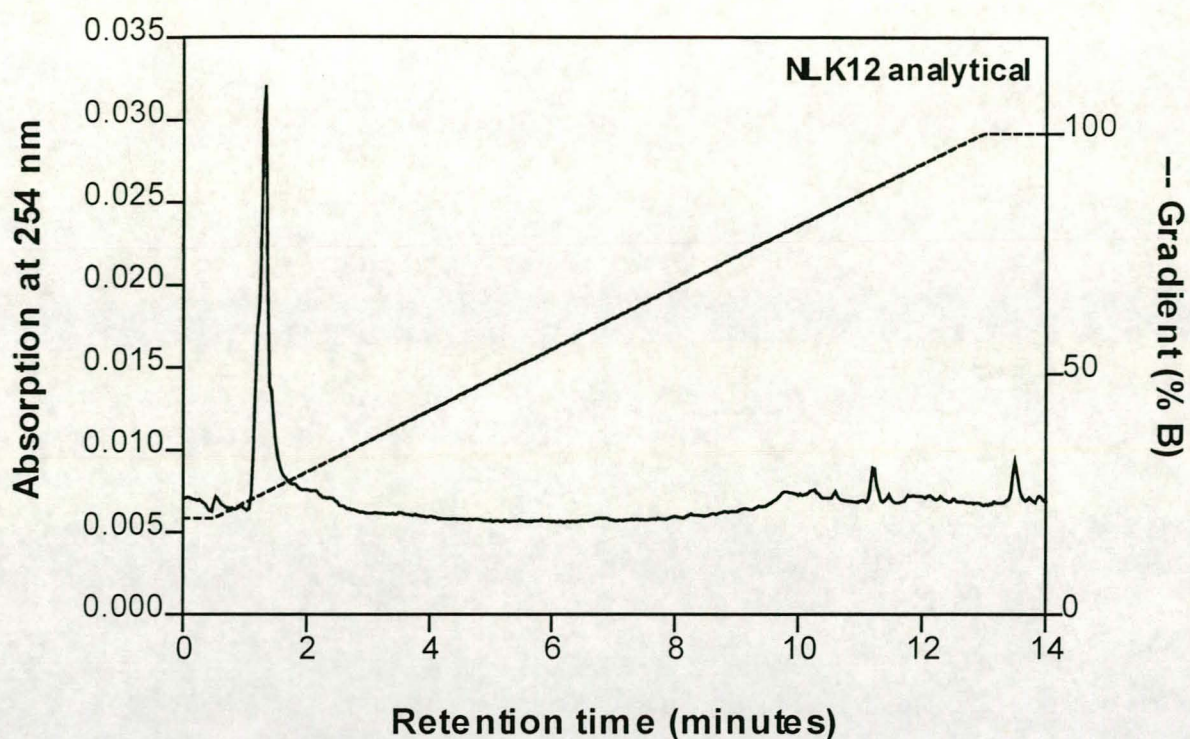
Figure 2.18 Analytical HPLC chromatogram of purified NLK12.

2.5 Conclusions

The shake flask solid phase peptide synthesis, based of the Fmoc-polyamide methodology, was successfully employed to synthesise both the immobilised and the free peptide amides. Amino acid analysis, ES-MS and protein sequencing unambiguously confirmed that the correct peptides had been synthesised. The immobilised peptides could not be removed from the resin, and therefore it could not be purified in any way. Immobilised peptides of high purity were obtained because special care was taken during the synthesis. Analysis of the free peptide amides was laborious because purified peptides tended to self-aggregate.

Two different methods were used in cleaving the peptides from the resin to form peptide amides. For the first peptide cleaved of the resin, methanolic ammonia was used, but for the others the easier method, using liquid ammonia, was used. With the use of the methanolic ammonia, methyl-esters were formed, contaminating the amidated peptide. This necessitated consequently labourious purification to obtain the dodecapeptide amide product. The use of liquid ammonia, however, gave a lower crude peptide yield because some of the peptide still

remained on the resin. More than one treatment with liquid ammonia was thus necessary, but the purification of the peptides was much easier.

The shorter peptides tended to self-aggregate more than the longer ones. Aggregation was especially observed with NLK12, which lead to diminished biological activity (discussed in Chapter 3). The differences in aggregation must have been due to the peptide structure, which changed the net charge and amphipathicity of the peptides. The next chapter describes our investigation of the influence of peptide length, charge, amphipathicity and immobilisation on the biological activity of the peptides.

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Chapter 3

The influence of structure and immobilisation on the antimicrobial activity of model α-helical peptides

3.1 Introduction

Many of the natural antimicrobial peptides that have been isolated assume a unique amphiphilic secondary structure upon interaction with the outer phospholipid bilayer of a bacterium. This characteristic may be induced by an α-helical, e.g. cecropin and magainin, or a β-sheet, e.g. defensin, molecular organisation of the peptide in hydrophobic media [1]. Blondelle *et al.* [2] designed synthetic amphipathic peptides (consisting of Leu and Lys) with potent antimicrobial activity. The highest activity was found for peptides with an amphipathic α-helical structure, which confirmed the role of an amphipathic α-helical conformation in biological activity of these peptides.

Cornut *et al.* [3] concluded that longer peptides, consisting of Leu and Lys (LK-peptides), have a more stable α-helical structure than shorter ones. The influence of peptide length has been the subject of many investigations. Anzai *et al.* [4] found that short cationic α-helical peptides (8-12 residues in length), composed solely of Leu, Arg, and Ala, were able to form ion channels. McLean *et al.* [5] demonstrated through a study with short highly α-helical peptides, composed of Glu, Leu and Lys, that an effective lipid-binding, amphipathic, α-helical peptide need only be eight residues in length. The haemolytic activity of 12 to 22-residue long LK-peptides could, on the other hand, not be correlated with peptide length [3]. It seems as if peptide length has a little influence on activity, and thus the influence of amphipathicity of the peptides was also investigated.

Not all amphipathic α-helical peptides have antimicrobial or cytotoxic activity. For example, peptides in the A_1, A_2 and Y classes of α-helices also interact with membranes without disrupting membrane function [6]. The α-helical antimicrobial peptides, however, have a markedly different sequential distribution of polar and non-polar amino acids leading to

amphipathic helices of the L-class or lytic class [7]. The importance of amphipathicity (i.e., the different presenting faces of the peptide) on biological activity of LK-peptides was evaluated by Blondelle *et al.* [2]. A large number of contiguous hydrophobic residues in an amphipathic peptide appear to be necessary for significant haemolysis to occur. An increase in antimicrobial activity was observed in the majority of the omission analogues. This suggested that a hydrophobic face of five contiguous residues as compared to a presenting face of nine contiguous residues is sufficient for the peptide to interact with the lipids and/or disrupt the organisation of the lipids of the bacterial cell wall. Bessalle *et al.* [1] confirmed that peptides that were very hydrophobic showed higher cytotoxicity. They postulated that the relative proportion between the hydrophobic and basic character of the peptide is important. Their model peptides showed that in order to achieve high bioactivity, 30-45% of the amino acid residues should be basic, whereas the rest can be hydrophobic. One of the highly basic peptides ($\approx 60\%$ basic residues) did not have antimicrobial activity.

The environment in which these peptides are presented to the target cell membrane also influences biological activity of these membrane-active peptides. Limiting the interaction of these peptides with its target membrane by immobilisation could lead to lower activity. It is, however, possible that some antimicrobial peptides may be suitable candidates for immobilisation because of their activity. Matrices with antimicrobial activity find application in industrial filter systems as well as in medicine. Microbial contamination and possible infection of the host are major concerns in the area of therapeutic medical devices and therefore new approaches are devised to avoid microbial contamination. Sequestering the antimicrobial agent close to the device surface has shown to be an effective approach in reducing the potential for catheter-related infections in patients [8].

Haynie *et al.* [9] investigated the antimicrobial activities of amphipathic α-helical peptides that were covalently bonded to a water-insoluble resin. They suggested that the surface action by magainins and structurally related antimicrobial peptides are sufficient for their lethal activities. No activity was, however, observed for either an immobilised peptide whose amino acid sequence was the exact reverse of that of magainin 2 or for several non-amphipathic, helical peptides. Amphipathic α-helical peptides with as little as seven residues still showed antimicrobial activity at high concentrations. Peptide length seems to play a role in the activity of these immobilised peptides, but it is not the major contributing factor for showing antimicrobial activity. In the activity of such peptides, immobilisation, however, restricts the range of penetration of the antimicrobial agent to loci at the surface of the microbial wall.

Immobilising the peptide is an easy way to test the extent surface activity of antimicrobial peptides and can also be used as a tool to probe mechanism of action and structure-function relationships.

The main aim of this study was to investigate the structure-function relationship of a model antimicrobial peptide, especially with regard to the influence of peptide length, charge, hydrophilic versus hydrophobic faces and immobilisation on the antimicrobial activity was investigated.

Using combinatorial libraries, Blondelle *et al.* [10] identified 18-residue peptides with a 10-fold increase in activity as compared to the original peptides. These peptides are known to be induced into an α-helical conformation in a lipidic environment. One of these 18-residue peptide amides, YKLKLLKLLLPKLKGLLFKL-NH$_2$, and three N-terminal deletion analogues were synthesised in both the free peptide amide and irreversibly immobilised form (refer to Chapter 2; Table 3.1).

Using the same water-insoluble resin, polydiacrylamide in Kieselguhr (Pepsyn K) as Haynie *et al.* [7], we tested the influence of immobilisation on the antimicrobial activity of these peptides. Three Gly-residues served as an "linker-arm" onto which the rest of the peptide was synthesised. Gly was chosen because of its basic structure and ease of coupling. The C-terminal of the ILK peptides was attached to this (Gly)$_3$-linker.

Omitting the N-terminal dipeptide unit Tyr-Lys from the 18-residue peptide, formed the 16-residue peptide. The omission of the next N-terminal dipeptide unit, Leu-Leu , formed the 14-residue peptide. The 12-residue peptide was formed by omission of Leu and Lys. The 18-residue peptide has five Lys residues, and therefore net five positive charges, whereas the 12 residue only has three Lys residues. Both the 14-residue and the 16-residue peptides have four Lys residues, but they differ in that the 14-residue peptide have two Leu residues less, and therefore a smaller hydrophobic face (see Table 3.1).

From the assumption that these peptides are all α-helical [2, 8], 3-dimensional structures were generated with HyperChem® 4.5 (Fig. 3.1). It can be seen that a "cavity" at the hydrophilic face widens and the "density" of the hydrophobic face decreases with peptide length (Fig. 3.1). In the helical wheel representations of the four peptides (Fig. 3.2) the changes in the hydrophobic face and hydrophilic face can be seen as peptide length decreases.
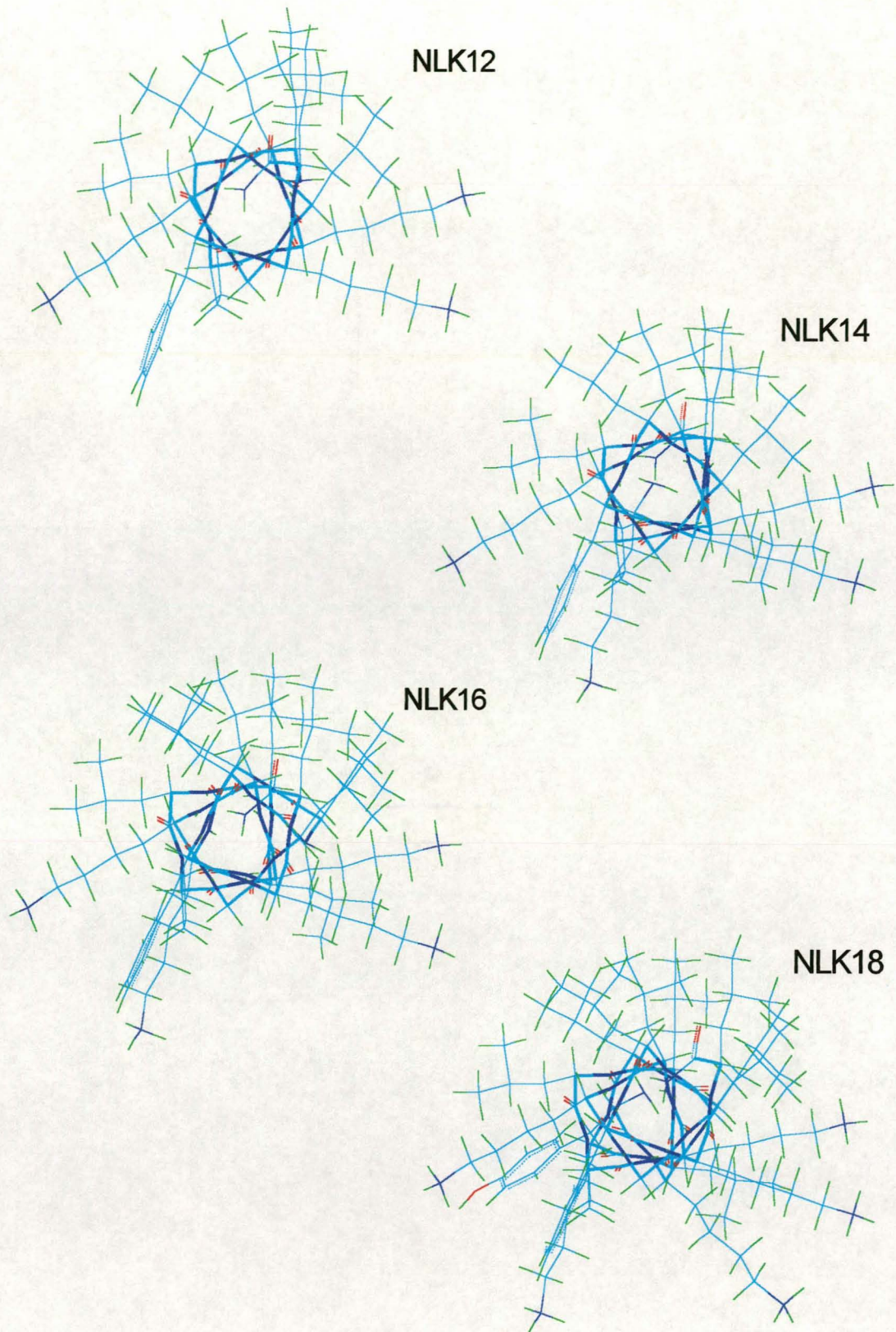
Figure 3.1 An amino- to carboxy-terminal view of the α-helical structures of the model peptides NLK12, NLK14, NLK16 and NLK18. The structures were generated with HyperChem® 4.5 [11]. (The dark blue lines depict the amino groups, red the oxygen containing groups, light blue the carbon atoms and green the hydrogen atoms.)
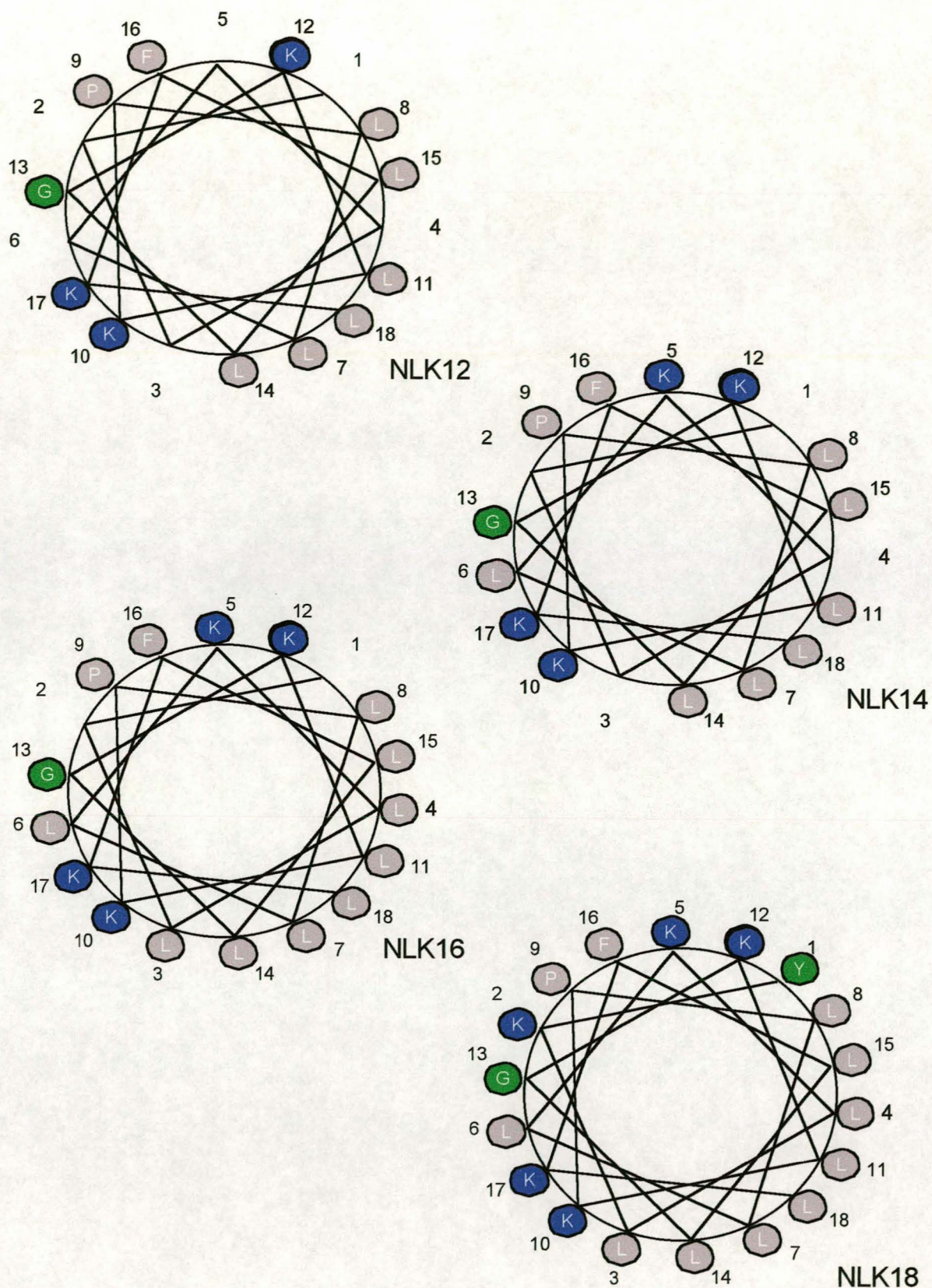
Figure 3.2   Helical wheel representation of the peptides of the α-helical structures of the model peptides NLK12, NLK14, NLK16 and NLK18. (The blue circles represent the polar charged groups, the green circles the uncharged polar groups, and the grey circles the non-polar groups.)

Table 3.1 List of the cationic α-helical model peptide analogues synthesised in this study, showing the primary structure, net charge and number of amino acids in the hydrophobic face (HØ face). (® depicts the Pepsyn K-NH(CH₂)₂NH-resin).

Peptide name	Peptide primary structure	Number of amino acids	Net charge	H Ø face
ILK12	LLPKLKGLLFKLGGG-®	$12+(Gly)_3$	3+	6
ILK14	KLLLPKLKGLLFKLGGG-®	$14+(Gly)_3$	4+	6
ILK16	LLKLLLPKLKGLLFKLGGG-®	$16+(Gly)_3$	4+	8
ILK18	YKLLKLLLPKLKGLLFKLGGG-®	$18+(Gly)_3$	5+	8
NLK12	LLPKLKGLLFKL-NH₂	12	3+	6
NLK14	KLLLPKLKGLLFKL-NH₂	14	4+	6
NLK16	LLKLLLPKLKGLLFKL-NH₂	16	4+	8
NLK18	YKLLKLLLPKLKGLLFKL-NH₂	18	5+	8

The antimicrobial tests of the free peptide amides were done using the gram-positive bacterium, *M. luteus* and the gram-negative bacterium, *E. coli*. These antimicrobial tests were done using only one strain each of *M. luteus* and *E. coli*. Peptide toxicity was tested against rabbit red blood cells. The activity of the immobilised peptides was tested against *M. luteus*.

3.2 Materials

Micrococcus luteus (NCTC 8340) and *Escherichia coli* (HB101) were used as indicator organisms in the antimicrobial activity experiments. Microtiter plates (Nunc-Immuno™ Maxisorp) were from Nalge NUNC International (Roskilde, Denmark), Falcon® tubes from Becton Dickinson Labware (Lincon Park, USA) and culture dishes from Quality Scientific Plastics, USA. Low-electroendosmosis-type agarose (D1-LE) was from Whitehead Scientific (Brackenfell, South Africa). Tween was from Fluka (Buchs, Switzerland). Dulbecco's phosphate buffered saline (PBS)[1] was either from Life Technologies (Faisley, Scotland), or prepared in the laboratory [12]. Sodium chloride was from Saarchem (Krugersdorp, South

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[1] 0.2% potassium dihydrogen phosphate, 0.115% disodium hydrogen phosphate, 0.02% potassium chloride, 0.8% sodium chloride (m/v), pH 7.2

Africa). Disodium hydrogen phosphate and citric acid were from Merck (Darmstadt, Germany). Potassium chloride, potassium dihydrogen phosphate, tryptone soy broth (TSB)[2] and casein were from Merck (Midrand, South Africa). D-glucose was from BDH Chemicals Ltd. (Poole, England). Trisodium citrate was from B&M Scientific cc. (Cape Town, South Africa). Melittin was from Sigma-Aldrich Chemie (Steinheim, Germany). Analytical quality water was prepared by filtering glass distilled water through a Millipore Milli Q® water purification system. HV 0.45 micron membrane filters were from Waters-Millipore (Milford, USA).

3.3 Methods

3.3.1 Peptide synthesis and purification

Peptides were synthesised at room temperature according to the Fmoc-polyamide protocol using the shake flask procedure (refer to Chapter 2, sections 2.3.3.4 and 2.3.3.5). The crude NLK peptides were purified using gel permeation chromatography (section 2.3.4.1). Further purification on NLK12 was by means of semi-preparative reverse phase high pressure liquid chromatography (HPLC) using a semi-preparative C_{18} Polygosil column (irregular particle size, 60Å pore size, 250 mm x 10 mm) as described in Chapter 2, section 2.3.4.3. The ILK peptides were not purified, because the peptide was irreversibly coupled to the resin after synthesis. However, special care was taken during the synthesis procedure to ensure a high purity peptide (section 2.3.3.1).

3.3.2 Preparation of cells

All experiments using bacteria, involved standard sterile techniques used in microbiology. A starter culture of the bacteria that was used as target cells was grown overnight, at 37°C, in a nutrient-rich medium (tryptone soy broth, TSB[2]). This overnight culture was inoculated into fresh TSB and grown until the mid-log phase as determined by optical density (OD) measurements (0.6 at 620 nm). For the micro-gel well diffusion assay ([13], section 3.3.3) the

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[2] 1.17% tryptone, 0.3% soy peptone, 0.5% sodium chloride, 0.25% di-sodium hydrogen phosphate, 0.25% dextrose (m/v), pH = 7.3

cells were centrifuged at 900g for 10 min. The supernatant was discarded and the cells were washed once with 10 mL cold Dulbecco's PBS[1] by centrifugation. Finally, the cells were diluted to an OD of 0.6 in Dulbecco's PBS. For the immobilised peptide assay (section 3.3.5) the cells were diluted with TSB to the desired concentration of 10^5 cells per mL (from OD_{620} 0.2 = 5 X10^7 cells, [14]).

3.3.3 Micro-gel well diffusion assaying of the free peptide amides [13]

Peptides were filtered through HV 0.45 μm Millipore filters a day prior to performing an assay and freeze-dried overnight to remove any insoluble particles. The dried filtered peptide preparations were weighed using a Sartorius analytical scale. Peptide dilutions were made from a stock solution (1 mg/mL in 50% acetonitrile/analytical grade water) just before performing an assay.

The IC_{50} (peptide concentration leading to 50% inhibition of microbial growth) of each peptide was determined by performing two dose response experiments. Eight dilutions of each peptide were prepared and microbial growth of quadruplicate determinations per peptide concentration was recorded.

The micro-gel well diffusion assay was performed according to a previously described method [13]. Microtiter plates were blocked with 0.5% casein in Dulbecco's PBS for one hour and sterilised under UV light for a further four hours. A gel solution containing 1% (m/v) of powdered TSB medium, 1% (m/v) of low-electroendosmosis-type agarose, and 0.02% (m/v) Tween 20 made up in Dulbecco's PBS, was prepared and autoclaved. The gel solution was used at 45±1°C and therefore kept in a water bath at 46±1°C. The first column of the microtiter plate received 70 μL of gel, to serve as blank and sterility control. Into each of the other wells, 70 μL of a bacterial-gel suspension, prepared by mixing 70 μL bacteria and 700 μL gel, was applied. The suspension was prepared for each column immediately before application. Once the desired number of wells was prepared, the gel was allowed to set for one hour. Hereafter the test sample was applied directly to the gel in the wells, bringing the total volume in the well to 100μL. The microtiter plate was covered and incubated for 17 hours at 37°C. The light dispersion per well was determined using a Multiscan Titertec microtiter reader at 620nm.

3.3.4 Haemolysis assays

The haemolytic activities of the free peptide amides were determined by using rabbit red blood cells (RBC). Blood was drawn from the rabbit into an equal amount of Alsever's solution (2.05% glucose, 0.80% sodium citrate, 0.42% sodium chloride and 0.055% citric acid, m/v, pH between 7.2 and 7.3). The blood was kept at 4°C at least a week to ensure plasma and erythrocyte separation and RBC stabilisation. During this week the plasma and other cells were removed by suction and Alsever's solution again added to the erythrocytes.

The Alsever's solution was removed by centrifugation (900 g for 10 minutes) just prior to the assays. The cells were then washed three times with 0.15 M phosphate buffered saline (PBS, 0.02% potassium dihydrogen phosphate, 0.115% disodium hydrogen phosphate, 0.02% potassium chloride, 0.8% sodium chloride (w/v), pH 7.2) and then finally resuspended in PBS. Peptides, dissolved in 50% acetonitrile/analytical water, were added to 100 µL of the RBC (2.5 x 10^8 cells/mL) and 800 µL PBS to reach a final volume of 1.0 mL. The peptide stock solutions were prepared as described in section 3.3.3. Following gentle mixing, the peptide-RBC suspension was incubated for 30 minutes at 37°C. Samples were then centrifuged for 5 minutes on a bench centrifuge (small type micro centrifuge). Hundred µL of the supernatants were aliquoted (in duplicate) into a microtitre plate and the absorbance measured at 405 nm with a Multiscan Titertec microtiter reader. Background haemolysis was determined by incubation of the rabbit red blood cells in PBS with 50% acetonitrile/analytical water. Complete haemolysis of the rabbit red blood cells in PBS was determined with 100 µg/mL melittin dissolved in 50% acetonitrile/analytical water.

The HC_{50} (peptide concentration leading to 50% haemolysis) of each peptide was determined by performing two dose response experiments. Six dilutions of each peptide were prepared and haemolysis of quadruplicate determinations per peptide concentration were recorded.

3.3.6 Antibacterial assaying of the immobilised peptides

The peptide-resin (ILK) was fine-ground in a pestle and mortar to increase the exposure of the insoluble peptide to the microorganisms. The peptide-containing resin was autoclaved before use. The insoluble peptide-resin conjugates were evaluated at concentrations of 1 µmole/mL and 3 µmole/mL respectively (the amount of peptide-resin ranged from 10 to 40 mg/mL).

The reaction vessels (plastic Eppendorf tubes) containing the peptide-resin were inoculated with cells in growth medium so that the cell concentration was 10^5 cells/mL. The reaction volume was 0.2 mL and the cell-resin suspensions were incubated at 37°C with gentle shaking (~250 rpm).

After 17 hours, each suspension was mixed and transferred into a sterile Eppendorf tube. The resin was removed by filtering the suspension through a 1 mL sterile plastic syringe containing the cotton wool plug (15 mg). The cotton wool plug kept the resin back and only let the cells through. TSB (200 μL) was used to wash the syringe and also transferred through the syringe into the Eppendorf tube. Two 100μL aliquots each of the filtered suspensions were pipetted into a microtiter plate and light dispersion at 620 nm was determined using a Multiscan Titertec microtiter plate reader. Cells incubated with resin containing no peptide were used to determine 100% growth.

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). Nonlinear regression was performed on the dose-response data obtained from the micro-gel well diffusion assays and the haemolysis assays. A sigmoidal curve with variable slope and constant top of 100 and bottom of 0 was fitted to each of the data sets. The 50% inhibition of microbial growth (IC_{50}) and the 50% haemolysis of RBS (HC_{50}) were deduced from the following equation:

$$Y = 100/(1+10^{(\log IC50x \, Hill \, slope)}) \text{ where:}$$

log IC_{50} (or log HC_{50}) = X-value of response halfway between top and bottom

Hill slope = Hill coefficient (describes the steepness of the curve)

For the NLK-peptides, two experiments in quadruplicate were performed with each peptide against each target cell. The IC_{50}/HC_{50} obtained from the two experiments were analysed using Tukey's Multiple Comparison Test in GraphPad Prism 2.01 to ascertain if the observed differences were significant.

For the ILK peptides, four experiments in quadruplicate were performed with each peptide against *M. luteus*. The % growth inhibition of each of the peptides was tested at two peptide

concentrations. The data obtained from the four experiments were analysed using Tukey's Multiple Comparison Test in GraphPad Prism 2.01 to ascertain if the observed differences were significant.

3.4 Results and Discussion

3.4.1 Biological activity of the NLK peptide amides

The influence of N-terminal deletion of dipeptide units from the original 18-residue peptide amide (NH_2-YKLKLLKLLLPKLKGLLFKL), identified by Blondelle *et al.* [8], on biological activity was ascertained by using *M. luteus, E. coli* and rabbit erythrocytes as target cells.

The IC_{50} values for the four NLK peptides with *M. luteus* as target cell were determined using the micro-gel well diffusion assay (Fig 3.3, Table 3.2). Although the hexadecapeptide (NLK16) showed the highest activity against *M. luteus* over a broad concentration range (Fig. 3.3), its IC_{50} value was not significantly different ($P > 0.05$) from that of original octadecapeptide (NLK18) (Table 3.3). There was, however, a significant difference between the IC_{50} values obtained for these two longer peptides and NLK14 ($P < 0.05$). NLK14 showed a higher IC_{50} (21.9 ± 4.9 nmole/mL) and thus a lower activity than NLK18 (IC_{50} = 6.0 ± 0.7 nmole/mL) and NLK16 (IC_{50} = 3.6 ± 0.3 nmole/mL). After repetitive attempts, the IC_{50} of NLK12 could not be determined. The initial experiments, with the peptide dissolved in 50% acetonitrile/water, gave ambiguous results. In later experiments, with the peptide dissolved in water, aggregation at high concentrations was observed. From these experiments IC_{50} of > 100 nmole/mL was calculated.

The micro-gel well diffusion assay was again used to determine the IC_{50} values of the four NLK peptide amides with *E. coli* as target cell (Fig 3.4, Table 3.2). The octadecapeptide, NLK18, was the most active over a broad concentration range (Fig. 3.4). There was, however, no significant difference ($P > 0.05$) between the IC_{50} of NLK18 (13.9 ± 1.9 nmole/mL) and NLK16 (21.6 ± 2.0 nmole/mL). Both these peptides had significantly (Table 3.3) lower IC_{50} values than that of NLK 14 (31.4 ± 0.2 nmole/mL). Because of the aggregation, the IC_{50} of NLK12 against *E. coli* could not be determined. NKL12 activity (IC_{50} > 100 nmole/mL) was again compromised by self-aggregation in solution.
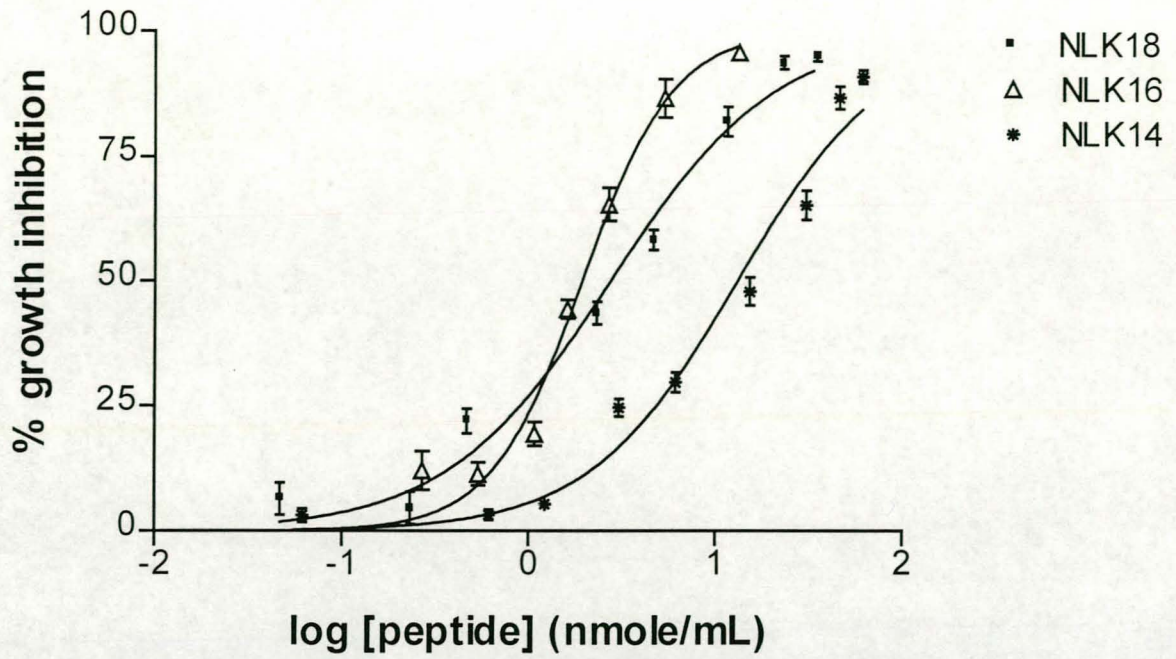
Figure 3.3   Sigmoidal dose-response curves of the NLK peptides with *M. luteus* as target cell. The combined data of two experiments are shown with standard error of the mean (SEM) for each concentration value.
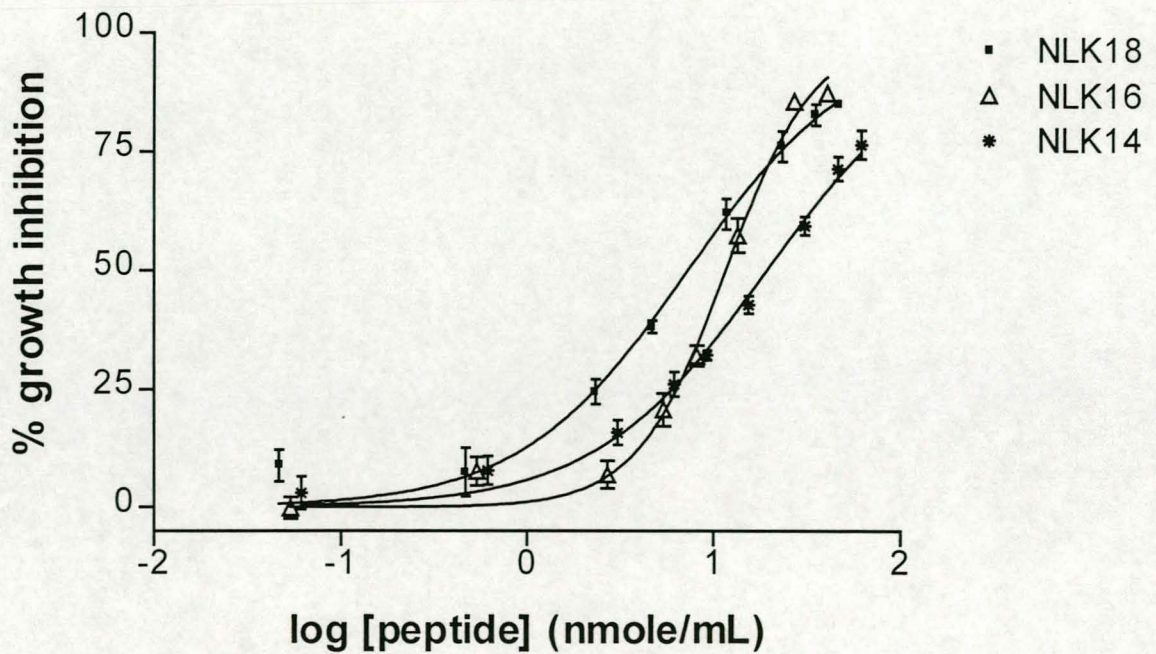

Figure 3.4   Sigmoidal dose-response curves of the NLK peptides with *E. coli* as target cell. The combined data of two experiments are shown with SEM for each concentration value.

A similar dose response effect was observed in the peptide activity against red blood cells (Fig 3.5). Overall the octadecapeptide was more haemolytic than the other peptides. There was, however, no significant difference ($P > 0.05$) between the HC_{50} values of this octadecapetide, NLK18 (3.7 ± 0.7 nmole/mL), and NLK16 (5.2 ± 0.4 nmole/mL). Their activity however, was higher than that of NLK14, whose HC_{50} (14.6 ± 2.3 nmole/mL) differed significantly from both ($P < 0.05$). Aggregation was observed for NLK12 and could again be the reason why the haemolytic activity of this peptide was negligible ($HC_{50} > 100$ nmole/mL).
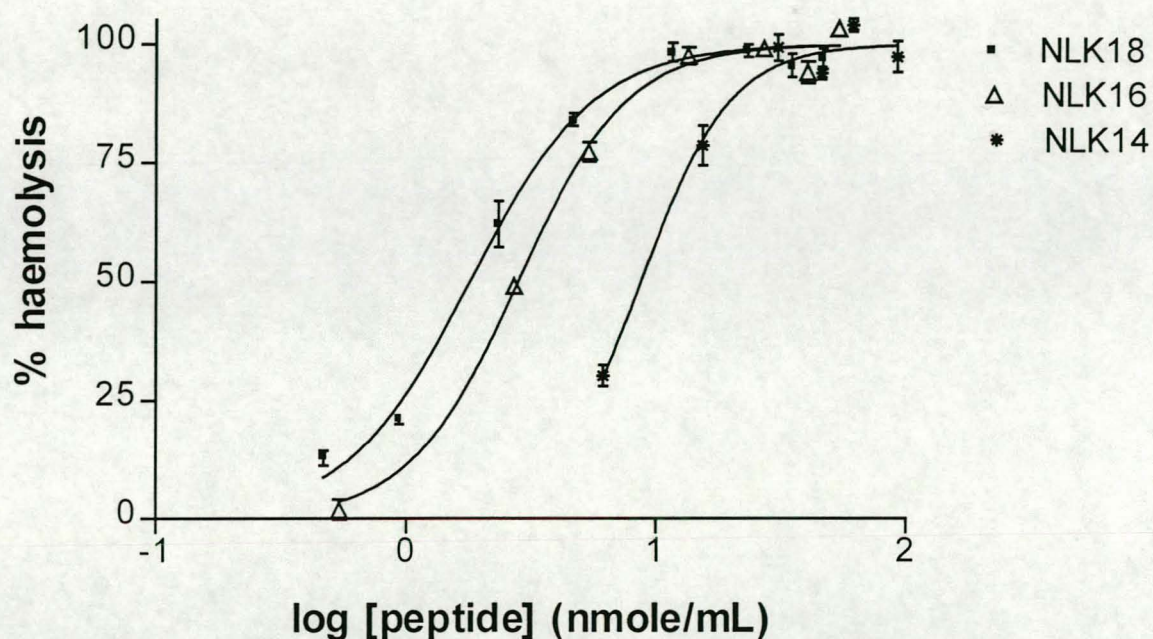

Figure 3.5   Sigmoidal dose-response curves of the NLK peptides with rabbit red blood cells as target cell. The combined data of two experiments are shown with SEM for each concentration value.

Table 3.2   Summary of the IC_{50} values obtained with *M. luteus* (NCTC 8340) and *E. coli* (HB101) as target cells and the HC_{50} values with rabbit red blood cells as target cells. Values are the average of two experiments $\pm$ SEM.

Peptide	$IC_{50} \pm$ SEM against *M. luteus* (nmole/mL)	$IC_{50} \pm$ SEM against *E. coli* (nmole/mL)	$HC_{50} \pm$ SEM against RBC (nmole/mL)
NLK18	6.0 ± 0.7	13.9 ± 1.9	3.7 ± 0.7
NLK16	3.6 ± 0.3	21.6 ± 2.0	5.2 ± 0.4
NLK14	21.9 ± 4.9	31.4 ± 0.2	14.6 ± 2.3
NKL12	>100	>100	>100

Table 3.3   Significance of differences in peptide activity (IC_{50}/HC_{50}) as determined with Tukey's Multiple Comparison Test.

Comparison	Significance of difference in peptide activity		
	M. luteus growth inhibition	*E. coli* growth inhibition	Rabbit RBC haemolysis
NLK18 vs. NLK16	$P > 0.05$ Not significant	$P > 0.05$ Not significant	$P > 0.05$ Not significant
NLK18 vs. NLK14	$P < 0.05$ Significant	$P < 0.01$ Highly significant	$P < 0.05$ Significant
NLK16 vs. NLK14	$P < 0.05$ Significant	$P < 0.05$ Significant	$P < 0.05$ Significant

3.4.2 Antimicrobial activity of the ILK peptides against M. luteus

Inhibition of the growth of *M. luteus* of each of the immobilised peptides was tested at two peptide concentrations, namely 1μmole/mL and 3μmole/mL (Table 3.3). Only two concentrations were chosen because of limited amounts of peptide. The highest activity of the free peptide amides was against *M. luteus* and thus it was decided to test the influence of immobilisation on biological activity against these target cells. The two peptide concentrations were chosen from the results given by Haynie *et al* [9]. In preliminary tests, using lower peptide concentrations, it was observed that the target cells adheres to the peptide-resin. The peptide-resin became yellow when incubated with *M. luteus*. This was not observed with the resin containing no peptide. After multiple washings, the yellow colour, and therefore the target cells, could not be removed from the peptide-resin. Therefore there was interaction between the peptide and the bacterium, but not high enough to result in the inhibition of growth. It seems as if the chosen peptide concentrations (1μmole/mL and 3μmole/mL) were too low, because growth inhibition > 55% was only observed for one of the peptides, ILK16 (Table 3.3). Therefore it could be suggested that higher peptide concentrations be used. In this case, however, the resin volume could be a problem, because all the resin (and peptide) would not be in contact with the target cells and therefore the correct peptide concentration would not be known.

As with activity on *M. luteus* of the free peptides, the highest activity was again observed for the hexadecapeptide. Tukey's Multiple Comparison Test confirmed that the activity of ILK16 was significantly different (P < 0.05) from the activity of the other ILK-peptides. This peptide in its immobilised form (ILK16) inhibited 77% of bacterial growth (shaded cell in Table 3.4) at a concentration of 3 μmole/mL. The activity was about a 1000 fold less than this free peptides' (NLK16) activity on *M. luteus* (Table 3.2). ILK12, at both concentrations, gave the lowest activity of all the ILK peptides against *M. luteus*. This result substantiates the previous observation that this free peptide (NKL12) was virtually devoid of antibacterial and haemolytic activity.

Table 3.4   Percentage growth inhibition of the ILK peptides against *M. luteus*. Values are the average of 16 measurements ± SEM.

Immobilised Peptide	% growth inhibition ± SEM	
	1 μmol/mL peptide	3 μmol/mL peptide
ILK18	37.9 ± 4.1	49.2 ± 2.2
ILK16	52.7 ± 6.6	77.3 ± 3.7
ILK14	51.9 ± 4.0	43.1 ± 4.5
ILK12	26.9 ± 4.8	22.4 ± 4.1

3.5 Conclusions

NLK12 was devoid of antibacterial and haemolytic activity over the concentration range of 10-100 nmol/mL. The three longer NLK peptides had substantial antibacterial activity, but were more active towards the gram-positive bacterium, *M. luteus*, than towards the gram-negative bacterium, *E. coli*. This specificity could be attributed to the difference in membrane structure of these two organisms, with *E. coli* having a thicker membrane because of its lipopolysaccharide layer. This result was however, the opposite of what was observed with the natural occurring peptides magainin and cecropin, which were more active against the gram-negative than the gram-positive organisms [15]. The difference in activity between the model peptides and the natural occurring peptides could be attributed to peptide sequence and peptide length. The model peptides consisted only of 18, or less, amino acids (mostly Lys and Leu) while the natural peptides consist of more aminoacids. However, both the natural and the model peptides form amphipathic α-helices and are cationic in nature. Interestingly, with

M. luteus as target cells, the HC_{50} values of the peptides were in the same range than that of the IC_{50} values. The haemolytic activity of these model peptides thus somehow correlated with their activity against gram-positive organisms.

Similar activities were observed for NLK18 and NLK16 when tested against the same organism. However, the hexadecapeptide, NLK16, was more than five fold less active against *E. coli* than *M. luteus*, while the other two peptides were about two fold less active. The difference in activity is accounted for by NLK16 having one less positive charge than NLK18. NLK14 was less active than the two longer peptides. The activity of NLK14 was therefore influenced by peptide length (it is shorter than the other 2 peptides) and the size of the hydrophobic face (it has two less Leu residues). The 12 residue peptide was devoid of antimicrobial activity in solution, but retained surface activity in the immobilised form. Having only a net positive charge of three, the peptides tend to self-aggregate in solution because there is less charge repulsion. Aggregation was restricted in the immobilised form and therefore surface activity could be observed.

Thus, peptide length is not such a crucial parameter in activity against *M. luteus* and RBC as activity probably depends on membrane thickness. The size of the hydrophobic face is very important for activity against all the organisms. The charge of the hydrophilic face is probably only important in activity against *E coli*.

The differences in activity that were observed between the different NLK peptides can therefore be related to peptide length, charge and the amphipathic helix (hydrophilic versus hydrophobic faces).

The immobilised peptides were about or more than 1000 fold less active against *M. luteus* than their free amide counterparts. The structural constraint caused by the resin therefore restricted the activity of the peptides on the target cells to surface activity. The lowest activity was also observed for the 12 residue peptide, but the fact that this immobilised peptide was slightly active imply that it still retained some surface activity.

3.6 References

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Chapter 4

Conclusions

The world of antimicrobial peptides is a complex puzzle with many missing pieces. Over the years many parts of the puzzle have been completed, but still researchers continue the quest to find out exactly how these peptides work (refer to Chapter 1).

The aim of this study was to examine the structure-function relationship of a model antimicrobial peptide. Blondelle *et al.* [1] identified, using combinatorial libraries, a model KL-peptide with 10-fold enhanced antimicrobial activity when compared to the original KL-peptide [2]. Synthesising this peptide and three N-terminal deletion analogues in both the free peptide amide and the immobilised form, allowed us to test the influence of peptide length, charge, size of the hydrophobic face *vs*. the hydrophilic face and immobilisation on their biological activity.

Peptides were synthesised at room temperature according to the Fmoc-polyamide protocol using the shake flask procedure (refer to Chapter 2, sections 2.3.3.4 and 2.3.3.5). For the immobilised peptides, one synthesis was done on the ethylene modified water-insoluble resin, polydiacrylamide in Kieselguhr (Pepsyn K), as described by Haynie *et al* [3]. During the synthesis of the ILK-peptides the average coupling efficiency was between 98 and 99%. Great care was taken to ensure maximum coupling, because these peptides remained immobilised to the resin and could thus not be purified. The free peptide amides were synthesised on a resin from which the peptide amides were liberated by either methanolic ammonia or liquid ammonia. The use of methanolic ammonia to liberate the 12-residue peptide, NLK12, lead to the formation of methyl esters as well as correct peptide amides. These two peptides were extremely difficult to separate from each other (section 2.4.4.1). The number of purification steps, necessitated by the presence of this contaminating peptide methyl esters, greatly decreased the overall peptide amide yield. Therefore, NLK12 was synthesised again to ensure a sufficient amount of peptide to perform the biological activity experiments. The longer resin-bound peptides were successfully liberated as peptide amides by liquid ammonia treatment. By using this method, however, all the

peptide was not removed from the resin and a second treatment with liquid ammonia was necessary. Some of the resins still contain peptide and complete liberation will necessitate more liquid ammonia treatments (Table 2.6). In using liquid ammonia, purer peptides were liberated, as shown by ES-MS (section 2.4.5.1). These peptides were further purified using gel permeation chromatography to remove residual contaminating material. One of the reasons for the relatively low purified peptide yield (44-62%) could be because of increased aggregation when the peptides are in the pure form. This aggregration was observed with analytical HPLC for all the peptides (section 2.4.5.2). During HPLC, the high concentrations of peptide needed for visualisation (the shorter peptides did not contain any UV-absorbing residues) aggravated the problem of aggregation.

In performing the antimicrobial assays with the free peptide amides, the biggest problem again proved to be the tendency of the peptide to aggregate. Although peptide preparations were made just prior to performing the dose response experiments, the IC_{50} and HC_{50} of the 12-residue peptide could not be successfully determined (section 3.4.1). Having only a net positive charge of three, instead of five as in the 18-residue peptide, this peptides' tendency to self-aggregate is probably because it has less charge repulsion than its longer analogues. This resulted in the peptide exhibiting IC_{50} values and a HC_{50} of greater than 100 nmole/mL.

Peptide length is not the only factor influencing antimicrobial activity in the NLK-peptides, although the 12-residue peptide showed the lowest activity. Deletion of amino acids from the N-terminal changed the net charge and the balans between the hydrophobic and hydrophilic face of the shorter peptides. It was, however, observed that the two longer peptides (NLK 16 and NLK 18) had almost similar activities, while the two shorter peptides (NLK12 and NLK 14) were less active. It was shown that a minimum peptide length of 18 residues is therefore not always necessary for antimicrobial activity. In other studies, [4, 5] it was observed that peptides with as little as eight residues still had antimicrobial activity.

The three longer NLK peptides (NLK18, NLK16 and NLK14) had substantial antibacterial activity (Table 3.2), but were more active towards the gram-positive bacterium, *M. luteus*, than towards the gram-negative bacterium, *E. coli*. This specificity could be attributed to the difference in membrane structure of these two organisms, with *E. coli* having a more complex membrane because of its lipopolysaccharide layer. This result, however, was the opposite of what

was observed for the naturally occurring peptides magainin and cecropin [6], which are more active against gram-negative than gram-positive organisms. The difference in activity between these model peptides and the naturally occurring peptides could be attributed purely to amino acid sequence. Our model peptides consisted mostly of Lys and Leu amino acids, while the natural peptides consist of a more diverse range of amino acids. However, both the natural and the model peptides form amphipathic α-helixes and are cationic in nature. Our peptides were only tested against one strain each of *M. luteus* and *E.coli*. It should have been tested against more than one strain because mutations can occur and give different results.

Taking the diversity of natural peptides into account, Tossi *et al.* [7] designed a peptide consisting of the amino acids which occur most frequently in the first 20 positions of natural occurring peptides. In the hydrophobic face the aliphatic, hydrophobic residues dominates, while the hydrophilic face is considerably more variable. The hydrophobic face in their designed peptides consists of contiguous hydrophobic residues, whereas the hydrophilic face consists of hydrophilic residues interspersed with hydrophobic residues. The importance of the number of the contiguous residues in the hydrophilic face of antimicrobial peptides to have antimicrobial activity, have been shown by Blondelle *et al* [2]. They confirmed that a hydrophobic presenting face of five contiguous residues is sufficient for the peptide to interact with the bacterial cell wall. In our 18-residue and the 16-residue peptides the hydrophobic face has eight contiguous residues. The six contiguous residues in hydrophobic faces of NLK14 and NLK12 were clearly not sufficient to maintain biological activity. They have two Leu residues less than NLK16 and NLK18, which indicates that the density of the hydrophobic face also influences the peptides antimicrobial activity.

Bessalle *et al.* [8] showed that the relative proportion between the number of hydrophobic and basic residues of the peptide is also very important for activity. In order to achieve high bioactivity, 30%-45% of the amino acid residues should be basic, whereas the rest can be hydrophobic. The peptides used in this study have between 25% and 28% basic residues. The activity of NLK18 was statistically ($P > 0.05$) similar to that NLK16 when tested against the same organism. However, the hexadecapeptide, NLK16, was more than five-fold less active against *E. coli* than *M. luteus*, while the other two peptides (NLK18 and NKL14) were about two-fold less active. NLK16 has one positive charge less when compared to NLK18, which seems to be the contributing factor in the lower activity of the 16-residue peptide against *E. coli*. It also

seems as if this single charge difference of NLK16 has a greater influence on activity against the gram-negative bacteria than against the gram-positive bacteria. Although the NKL16 and NKL14 both have a net positive charge of four, they have different activities. The reduced length of NLK14 and change in amphiphilic balance must therefore be responsible for its lower activity.

From these studies it can be seen that is not one single characteristic that influences the activity of the peptides, but a combination of the correct peptide length, positive charge and size of the hydrophilic *vs.* hydrophobic faces (refer to Chapter 3). Our results correlated with those reported by Oh *et al.* [9]. They showed, using short (< 18-residues) antimicrobial peptides, that a high value in some structural parameters (net charge, hydrophobicity and α-helicity) is not enough for the best antimicrobial activity, but that an optimal balance in structural parameters, particularly amphipathic balance, is required.

The biggest constraint in examining the antimicrobial activities of these immobilised peptides was that only a limited amount of peptide could be used. The insoluble resin support on which the peptides were immobilised did not lend itself to an simple way of assaying the peptides' purity or antimicrobial activities. If the amount of resin becomes to high, all the resin particles, and therefore the peptide, would not be in contact with the target cells and the active concentration of peptide would be unknown. Form these experiments we, however, were able to show that the immobilised peptides were about or more than 1000 fold less active against *M. luteus* than their free amide counterparts. The structural constraint caused by the resin therefore restricted the activity of the peptides on the target cells to surface activity. The highest activity was also observed for the 16-residue peptide, ILK16 (Table 3.4). The lowest activity was again observed for the 12-residue peptide, but the fact that this immobilised peptide was slightly active implies that it still retained some surface or membrane activity. In the immobilised form, self-aggregation was probably restricted and thus some activity was retained. For all the immobilised peptides it was found that they tend to very tightly to bind to *M. luteus* as we unable to reconstitute activity and remove the characteristic yellow of *M. luteus* form the resin particles after incubation.

If a higher antimicrobial activity could be obtained with the immobilised peptides, a vast array of applications could be found. In future studies we are planning to immobilise these peptides and other more active peptides in different ways (e.g. using longer linkers or labile linkers) and to

different in order to improve the eventual antimicrobial activity. An improvement in activity could possibly be obtained by immobilising the peptide onto a different more exposed support. Different types of porous membranes with large exposed surface areas would be feasible options as matrixes for immobilisation. Immobilisation onto these supports would create an antimicrobial surface that could be applied in water purification to decrease the number of bacteria. If immobilised to paper or plastic, the antimicrobial peptides could help extend the fruit ripening process and it could help in keeping fruit fresh for longer. Thus, immobilised antimicrobial peptide could form part of our daily lives, just like their natural analogues do.

4.1 References

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