Amphibian Antimicrobial Peptides: Their Structures and Mechanisms of Action.


by

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Corrections in pencil (by [Illegible]) in answer to examiner's comments.
Abstract

In the last decade or so, there has been an alarming increase worldwide in the number of antibiotic-resistant strains of pathogens. Efforts to find new drugs to combat this scourge have so far been of limited success. Amphibian skin is a rich source of potent antimicrobial peptides that has the potential to be developed into a new class of antibiotics. Most are specific only to pathogens and are found to exert their effects by disrupting their cytoplasmic membranes. The mechanisms by which they exert their biological effects are still a subject of controversy. Currently, two mechanisms have been proposed: (i) the channel mechanism where the peptides aggregate on the membrane surface to form a transmembrane pore. This disrupts the cell's osmoregulatory capability and results in osmolysis, and (ii) the carpet mechanism where the peptides orient themselves parallel to the membrane surface, forming a 'carpet' layer(s) before immersing themselves into the bacterial membrane, causing cell lysis.

Recently, three antimicrobial peptides, maculatin 1.1,uperin 3.6, caerin 4.1 were isolated from the respective skin glands of the Australian amphibians *Litoria genimaculata*, *Uperoleia mjobergii* and *Litoria caerulea*. To gain a deeper insight in their mechanism of action, three-dimensional structural studies have been conducted using circular dichroism, two-dimensional nuclear magnetic resonance and computer modelling techniques.

Experimental results reveal that all three peptides adopt an overall amphipathic α-helical conformation in membrane-mimetic environments. One of the peptides (maculatin 1.1) forms a helical structure with a 'kink' in the central region due to Pro15. Orientation studies using solid-state NMR studies suggest that 15N-labelled Ala7 maculatin 1.1 interacts with bacterial membranes via the carpet mechanism.

Further investigations involving structure-activity relationship studies and the solution structures of synthetic variants were also conducted and compared. Results suggest that: (i) the bactericidal mechanism did not involve stereospecific binding sites or enzymes and (ii) the synthetic Ala15 analogue of maculatin 1.1 has markedly reduced activity, suggesting that the kink is important for biological activity.
comparison was also made between using trifluoroethanol (TFE), a membrane-mimicking solvent and dodecylphosphocholine (DPC) micelles in water to investigate the reliability of TFE as a membrane-mimicking solvent. Results indicate that the three-dimensional structures produced in both systems are very similar, suggesting that membrane-interacting peptides in trifluoroethanol/water mixtures are representative of those adopted in a membrane environment. The role of central flexibility within antibiotic peptides in their interaction with bacterial membranes is also discussed.

Like maculatin 1.1, uperin 3.6 and caerin 4.1 also adopt overall α-helical conformations in TFE/water mixtures. Substituting the cationic residues in uperin 3.6 for neutral amino acid residues result in the loss of bioactivity, suggesting that electrostatic interactions are involved in the mechanism of bactericidal action. Caerin 4.1 is shown to possess narrow-spectrum antibiotic activities. Although it does not contain a central proline kink along the length of the helix like maculatin 1.1, it was found to have a central flexible region between Gly11 to Gly16. It is believed that its relatively rigid helical backbone conformation can only undergo limited conformational changes and thereby loses its wide-spectrum antibiotic capability.

*Litora chloris*
Outline of Thesis Presentation

This thesis is presented in the following manner:

Chapter 1 gives a brief introduction to naturally-occurring bioactive peptides and their potential for development into therapeutics. Particular attention will be paid to antimicrobial peptides isolated from the skin of amphibians.

Chapter 2 reviews the mechanisms of action of a number of membrane-disrupting peptides. A summary of different experimental techniques will also be mentioned briefly.

Chapter 3 serves not only as an introduction to the experimental techniques (particularly nuclear magnetic resonance spectroscopy and molecular modelling) used to elucidate the three-dimensional structures of peptides in solution, it also explains in detail how the ‘quality’ and ‘realism’ of calculated structures are gauged using putative protocols. These protocols will be employed for all the peptides studied in this thesis (Chapters 5 to 7) and hence, will only be mentioned briefly in these chapters.

Chapter 4 explains how solid-state NMR techniques are used to probe the mechanism of action of membrane-lytic peptides by studying their interactions with lipids.

Chapters 5 to 7 contain the detailed structure-activity relationship study of selected amphibian peptides and their synthetic analogues using circular dichroism and nuclear magnetic resonance spectroscopy (discussed in Chapter 3).

Finally, Chapter 8 points out some future directions that can be undertaken: (i) to gain a deeper insight to the mechanisms of bactericidal action of these peptides and (ii) as a rationale to future drug design.

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Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no materials previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the university library, being available for loan and photocopying.

Brian Cheng San Chia
3/3/2000
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My sincere thanks to my supervisor, Professor John H. Bowie, for his guidance and advice in all aspects of my work in The University of Adelaide.

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I would like to thank The University of Adelaide for awarding me an Overseas Postgraduate Research Scholarship.

Last but not least, to my father, mother and Kent for their support and encouragement without which all this would not have been possible.
### Abbreviations used in this thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>one-dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
</tr>
<tr>
<td>DHPC</td>
<td>1,2-dihexanoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPC</td>
<td>dodecylphosphocholine</td>
</tr>
<tr>
<td>DQF</td>
<td>double-quantum filter</td>
</tr>
<tr>
<td>DSS</td>
<td>2,2-dimethyl-2-silapentane-5-sulphonate</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
</tr>
<tr>
<td>FT</td>
<td>Fourier transform</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single-quantum coherence</td>
</tr>
<tr>
<td>IR</td>
<td>infra-red</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOESY</td>
<td>nuclear Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>PES</td>
<td>potential energy surface</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>RMD</td>
<td>restrained molecular dynamics</td>
</tr>
<tr>
<td>RMSD</td>
<td>root-mean-square deviation</td>
</tr>
<tr>
<td>SA</td>
<td>simulated annealing</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TFE</td>
<td>2,2,2-trifluoroethanol</td>
</tr>
<tr>
<td>TOCSY</td>
<td>total correlation spectroscopy</td>
</tr>
<tr>
<td>TPPI</td>
<td>time proportional phase incrementation</td>
</tr>
</tbody>
</table>
**Abbreviations used to represent amino acids**

![Amino Acid Structure](image.png)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>R</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>-CH₃</td>
<td>Ala (A)</td>
</tr>
<tr>
<td>Asparagine</td>
<td>-CH₂CONH₂</td>
<td>Asn (N)</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>-CH₂COOH</td>
<td>Asp (D)</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>-C₂H₄COOH</td>
<td>Glu (E)</td>
</tr>
<tr>
<td>Glycine</td>
<td>-H</td>
<td>Gly (G)</td>
</tr>
<tr>
<td>Histidine</td>
<td></td>
<td>His (H)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>-CH(CH₃)(C₂H₅)</td>
<td>Ile (I)</td>
</tr>
<tr>
<td>Leucine</td>
<td>-CH₂CH(CH₃)₂</td>
<td>Leu (L)</td>
</tr>
<tr>
<td>Lysine</td>
<td>-(CH₂)₄NH₃⁺</td>
<td>Lys (K)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td></td>
<td>Phe (F)</td>
</tr>
<tr>
<td>Proline</td>
<td>*</td>
<td>Pro (P)</td>
</tr>
<tr>
<td>Serine</td>
<td>-CH₂OH</td>
<td>Ser (S)</td>
</tr>
<tr>
<td>Threonine</td>
<td>-CH(CH₃)OH</td>
<td>Thr (T)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td></td>
<td>Trp (W)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>-CH₂-OC₃H₃</td>
<td>Tyr (Y)</td>
</tr>
<tr>
<td>Valine</td>
<td>-CH(CH₃)₂</td>
<td>Val (V)</td>
</tr>
</tbody>
</table>

* The proline residue has the structure: ![](image.png)
**Nomenclature of amino acids in a peptide sequence**

The nomenclature used for the naming of individual amino acid residues in a peptide sequence is described as follows: taking a tripeptide as an example, the naming procedure begins at the N-terminal end of the peptide sequence. In this case, the first amino acid is glycine, so it would be named Gly 1. The adjacent alanine residue will be named Ala 2 etc. In summary, the tripeptide shown below can be represented as: Gly Ala Phe or GAF.

![Diagram of amino acids](image)

*Nomenclature of individual amino acids in a tripeptide.*
Chapter 1
Biologically Active Peptides: An Introduction
1.1 Amphibian Skin - A Chemical Arsenal

To increase their chances of survival in the wild, many organisms employ noxious chemicals (e.g., amines, steroid derivatives, alkaloids and peptides) to defend themselves against predators and pathogenic microorganisms (Bevins and Zasloff, 1990; Ganz, 1994; Boman, 1991 and 1995; Barra and Maurizio, 1995; Hancock et al., 1995; Epand and Vogel, 1999). Many are membrane-disrupting peptides and can be found in a wide variety of organisms including bacteria, fungi, plants, insects, fish, amphibians and mammals. Some examples are shown in Table 1.1:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Amino acid sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>VGAIAvVvWIWIW \textit{gramicidin A} Lowercase residues are D isomers.</td>
<td>Andersen, 1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Andersen, 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arseniev et al., 1985</td>
</tr>
<tr>
<td>Fungi</td>
<td>B*PBABABEVBGLBPVBEEQF \textit{alamethicin} * B = aminoisobutyric acid</td>
<td>Beven et al., 1999</td>
</tr>
<tr>
<td>Barley</td>
<td>KSCCKDLARNCYNTCRFAG \textit{thionin}</td>
<td>Bohlmann, 1994</td>
</tr>
<tr>
<td>Silkworm</td>
<td>AKIPIKAIKTVGKAVGKLRAINIASTANDVFNL KPKKRRKH \textit{moricin}</td>
<td>Hara and Yamakawa, 1995</td>
</tr>
<tr>
<td>Moth</td>
<td>KWKLFKKIEKVQGQNRDGIIKAGPAVAVGQAT QIAK \textit{cecropin A}</td>
<td>Boman and Hultmark, 1981</td>
</tr>
<tr>
<td>Honey Bee</td>
<td>GIGAVLKVTTLGLPALISWIKRKRQO \textit{melittin}</td>
<td>Tosteson and Tosteson, 1984</td>
</tr>
<tr>
<td>Blackfly</td>
<td>ATCDLLSGTGINHSACAAHCLLRGNRGGYCNGIK AVCVCRRN \textit{sepacin}</td>
<td>Hanzawa et al., 1990</td>
</tr>
<tr>
<td>Horse-shoe crab</td>
<td>KWCFRVCYRGICYRRCR \textit{tachyplesin}</td>
<td>Nakamura et al., 1988</td>
</tr>
</tbody>
</table>
It is also interesting to note that bacteria are also a source of cytotoxic peptides such as bacteriocins (Jack et al., 1995), gramicidin A and S (Katz and Demain, 1977; Ovchinnikov and Ivanov, 1982; Crease, 1989; Prenner et al., 1999), α-toxin (Bhakdi et al., 1981) and δ-lysin (Fitto et al., 1980; Lee et al., 1987; Tappin et al., 1988), some of which are now commercially available as pesticides (Thomas and Ellar, 1983). However, none of the above mentioned organisms can compare with amphibian skin with regards to the variety of these biologically active compounds (Erspermer et al., 1984, Simmaco et al., 1998).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androctonin</td>
<td>Ehret-Sabatier et al., 1996</td>
</tr>
<tr>
<td>Pardaxin</td>
<td>Lazarov et al., 1986; Shai, 1994;</td>
</tr>
<tr>
<td></td>
<td>Oren and Shai, 1996</td>
</tr>
<tr>
<td>Magainin 1</td>
<td>Zasloff, 1987</td>
</tr>
<tr>
<td>Magainin 2</td>
<td>Terry et al., 1988</td>
</tr>
<tr>
<td>Cryptdin</td>
<td>Selsted et al., 1992</td>
</tr>
<tr>
<td>Protegrin 1</td>
<td>Kokryakov et al., 1993</td>
</tr>
<tr>
<td>Protegrin 2</td>
<td>Sokolov et al., 1999</td>
</tr>
<tr>
<td>Bactenesin</td>
<td>Romeo et al., 1988; Gennaro et al.,</td>
</tr>
<tr>
<td>Indolicidin</td>
<td>1989; Selsted et al., 1992</td>
</tr>
<tr>
<td>Pardaxin</td>
<td></td>
</tr>
<tr>
<td>Defensin HNP 1</td>
<td>Lehrer et al., 1991; Lehrer et al.,</td>
</tr>
<tr>
<td></td>
<td>1993; Ganz and Lehrer, 1997</td>
</tr>
</tbody>
</table>
Amphibians evolved from fish that emerged from Devonian lakes and streams some 370 million years ago, and they and their chemistry have been evolving since then. Their remarkable ability to survive from predators (both large and small) and changing climatic conditions is due, in part, to their arsenal of host defence chemicals. These include toxins, analgesics, neuropeptides, antimicrobial and antifungal agents (Lazarus and Attila, 1993).

Man has used the medicinal and therapeutic properties of amphibian skin for more than two thousand years (Tyler, 1987). For example, the skin secretions of the Giant Monkey Frog *Phyllomedusa bicolor* (Fig. 1.1a) were used by Peruvian natives to increase their physical strength, awareness and resistance to hunger (Erspamer et al., 1993) while the skin secretions of the toad *Bufo gargarizans* (Fig. 1.1b) has been used as a wound-healing agent in traditional Chinese and Korean medicine (Park et al., 1995).

![Fig. 1.1](image.png) (a) *Phyllomedusa bicolor* (b) *Bufo gargarizans*.

Amphibian skin secretions are not only used as therapeutic agents; the highly toxic skin secretions of poison dart frogs (Fig. 1.2) are traditionally employed by the native Indians of Central and South America to tip their blowgun darts for hunting (Fig. 1.3) (Carraway and Cochrane, 1987). Such toxins are lethal in the microgram
concentrations and each frog is known to store over two hundred micrograms of toxin in their skin (Labler et al., 1968).

Fig. 1.2 Four species of poison dart frogs.

Fig. 1.3 (a) Coating a dart with poisonous skin secretions of Phyllobates terribilis, (b) a South American native with a blowgun.
During the last three decades, a number of biologically active peptides from amphibian skin have been isolated and identified. Some are neuropeptides and have been shown to be analogues of hormones present in neurosecretory cells of mammalian gut and brain (Erspamer and Melchiorri, 1973; Erspamer and Melchiorri, 1980a). A few other examples are listed in Table 1.2.

Table 1.2  *Physiological functions of some peptides from amphibian skin.*

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amphibian</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin</td>
<td><em>Crinia georgiana</em></td>
<td>Vasodilator</td>
<td>Erspamer <em>et al.</em>, 1979</td>
</tr>
<tr>
<td>Bombesin</td>
<td><em>Bombina bombina</em></td>
<td>Muscle stimulant</td>
<td>Erspamer <em>et al.</em>, 1972</td>
</tr>
<tr>
<td>Bombinin</td>
<td><em>Bombina variegata</em></td>
<td>Antibacterial</td>
<td>Simmaco <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Buforin 1</td>
<td><em>Bombina gargarizans</em></td>
<td>Antibacterial</td>
<td>Park <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Bradykinin</td>
<td><em>Rana temporaria</em></td>
<td>Vasodilator</td>
<td>Anastasi <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Caerulein</td>
<td><em>Hyla caerulea</em></td>
<td>Hypotensive</td>
<td>Gibson <em>et al.</em>, 1986</td>
</tr>
<tr>
<td>Dermorphin</td>
<td><em>Phyllomedusa bicolor</em></td>
<td>Analgesic</td>
<td>Negri <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Dermaseptin</td>
<td><em>Phyllomedusa sauvagii</em></td>
<td>Antibacterial</td>
<td>Mor <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Magainin</td>
<td><em>Xenopus laevis</em></td>
<td>Antibacterial</td>
<td>Mor and Nicolas, 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antitumour</td>
<td>Soravia <em>et al.</em>, 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Berkowitz <em>et al.</em>, 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cruciani <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Ranalexin</td>
<td><em>Rana catesbeiana</em></td>
<td>Antibacterial</td>
<td>Clark <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>Ranatensin</td>
<td><em>Rana pipiens</em></td>
<td>Muscle stimulant</td>
<td>Erspamer and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Melchiorri, 1973</td>
</tr>
<tr>
<td>Rugosin</td>
<td><em>Rana rugosa</em></td>
<td>Antibacterial</td>
<td>Suzuki <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Sauvagine</td>
<td><em>Phyllomedusa sauvagii</em></td>
<td>Vasodilator</td>
<td>Erspamer and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Melchiorri, 1980a</td>
</tr>
<tr>
<td>Tachykinin</td>
<td><em>Phyllomedusa bicolor</em></td>
<td>Hypotensive</td>
<td>Erspamer <em>et al.</em>, 1993</td>
</tr>
</tbody>
</table>
Chapter 1

These biologically active compounds are secreted from granular glands located on the dorsal skin surface of the animal in response to a variety of stimuli (Erspamer, 1994). For example, the South African Clawed Frog *Xenopus laevis* (Fig 1.4a) secretes chemicals that induce a yawning reflex on snakes, allowing the frog to escape unharmed (Barthalmus and Zielinski, 1988).

Not surprisingly, some of these peptides have attracted pharmaceutical interest. For example, medical researchers are currently developing muscle relaxants and anaesthetics from the neurotoxins of poison dart frogs (Negri *et al.*, 1995) while an analogue of magainin has been shown to possess anticancer activities and is currently undergoing clinical trials (Baker *et al.*, 1993).

Besides protecting the host from large predators, many of these compounds also serve as *antimicrobial agents*. Consisting mainly of peptides, they are discharged onto the skin either by adrenergic stimulation, as a response to infection or as a result of physical injury (Giovannini *et al.*, 1987). Many possess broad-spectrum antimicrobial activities, killing most strains of bacteria, fungi and protozoans. Experiments have shown that some of these peptides are specific only to microorganisms, making them good candidates for the development of antimicrobial agents (Maloy and Kari, 1995). For example, a magainin analogue (MSI-78) is now commercially available as a topical antibiotic for the treatment of impetigo and foot ulcers, common infections caused by *Staphylococcus aureus* of which many strains are now drug-resistant (Fig. 1.4) (Marshall *et al.*, 1991; Jacob and Zasloff, 1994; Lipsky *et al.*, 1997; Fuchs *et al.*, 1998). Thus, by acting as sterilising agents for the skin, these compounds are responsible for the extraordinary freedom from infection
experienced by amphibians, even though they live and breed in bacteria-infested environments (Zasloff, 1987).

Fig. 1.4 (a) The African Clawed Frog *Xenopus laevis*. (b) Magainin analogue MSI-78 antibiotic sold under the trade name Cytolex™. (c) Impetigo, a form of skin infection (d) a foot ulcer (e) *Staphylococcus aureus*.

These host defence peptides can be promptly synthesised at a low metabolic cost due to their relatively small sizes (as compared to antibodies or macrophages) and are stored as inactive, processed peptides in large granules within the granular glands of the skin (Moore *et al.*, 1991). Fig. 1.5 illustrates a magnified view of the granular glands of a frog. In some amphibian species such as the Magnificent Tree Frog *Litoria splendida*, these glands are enlarged and localised in the head and neck regions of the body while species like *Limnodynastes terraereginae* have glands on their legs (Fig. 1.6).
Fig. 1.5 (a) Magnified view of amphibian dermal granular glands showing secretory cells (Crook and Tyler, 1981) (b) scanning electron micrograph of a skin duct (Green, 1979) and (c) transverse view of granular glands with ducts opening onto the skin surface (arrowed).

Fig. 1.6 (a) Litoria splendida and (b) Limnodynastes terraereginae dorsal and (c) ventral view showing hypertrophied glands.
1.2 Peptide Biosynthesis and Production

All active peptides are encoded and synthesised by the animal as part of larger parent peptides. These generally consist of three components: a signal peptide, a spacer peptide and the bioactive peptide (Nicolas and Mor, 1995; Amiche et al., 1999). Following biosynthesis in endocrine glands, an endoprotease enzyme cleaves the signal peptide off and the remaining spacer-active peptide is transported and stored in the skin glands. At the appropriate time, another endoprotease removes the spacer peptide, releasing the active peptide onto the skin surface (Fig. 1.7) (Gibson et al., 1986; Giovannini et al., 1987; Terry et al., 1988; Ketchum et al., 1993; Ganz, 1994).

<table>
<thead>
<tr>
<th>Body Tissue</th>
<th>signal</th>
<th>spacer</th>
<th>active peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>enzymatic cleavage 1</td>
</tr>
<tr>
<td>Skin Glands</td>
<td></td>
<td>spacer</td>
<td>active peptide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>enzymatic cleavage 2</td>
</tr>
<tr>
<td>Skin Surface</td>
<td></td>
<td></td>
<td>active peptide</td>
</tr>
</tbody>
</table>

*Fig. 1.7 Schematic diagram showing the biosynthetic route of active peptide formation. The active peptide is represented by the red box.*

1.3 Bioactive Peptides from Australian Amphibians

To date, more than two hundred species of frogs have been identified in Australia since testing of their dermal secretions for pharmacological activity commenced in the 1960s (Erspamer et al., 1966a and 1966b; Tyler, 1991). This has led to the isolation of the hypotensive peptide *caerulein* from the Australian Green Tree Frog *Litoria caerulea* (Fig. 1.8) (Anastasi et al., 1968). Caerulein was also found to be an
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analgesic several thousand times more potent than morphine and has been used clinically (Lazarus and Attila, 1993).

The skin secretions from a number of native Australian Litoria species of frogs (illustrated in Fig. 1.8): L. caerulea (Stone et al., 1993), L. chloris (Steinborner et al., 1998), L. ewingi (Steinborner et al., 1997a), L. gilleni (Waugh et al., 1993), L. splendida (Stone et al., 1992a; Stone et al., 1992b) and L. xanthomera (Steinborner et al., 1997b) contain two different families of peptides which have been named caerins and caeridins (Waugh et al., 1995; Bowie et al., 1999). Certain caerins were found to have appreciable biological activity. For example, caerin 1.1 has been shown to be active against a wide range of pathogens (Table 1.3) (Wong et al., 1997). Caerin 1.1 is also active against herpes simplex I and II and the human immunodeficiency virus (HIV) [Unpublished communications with Prof. J.H. Bowie]. Caeridins, on the other hand, do not show any antibiotic activity and their function in amphibian skin is unknown at this time. New families of biologically active peptides have been recently isolated from other Litoria species, including two families of broad-spectrum bactericidal peptides from L. aurea and L. citropa (Wegener et al., 1999; Wegener et al., 2000).

It is also interesting to know that some Litoria species like L. rubella and L. electrica secrete peptides that have quite similar structures to human brain endomorphins (e.g. YPWF-NH$_2$ and YPWG-NH$_2$) (Zadina, 1997). These peptides have been named the tryptophyllins and are suspected to function as neurotransmitters or neuromodulators (Steinborner et al., 1996; Wabnitz et al.,
Table 1.3  **Antimicrobial activities of caerin 1.1 against some pathogens.**
(MIC* values in brackets).

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Pathogenicity and MIC (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em></td>
<td>Related to <em>Bacillus anthracis</em>, it is usually found in soil and livestock. Produces enterotoxins that causes food poisoning in meat and dairy products and cytotoxic enzymes such as haemolysins and phospholipase. (50)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>A common bacteria found in our gastrointestinal and urinary tracts. Virulent stains produce enterotoxins which leads to diarrhoea, blood poisoning and kidney failure. Some strains are now drug-resistant. (100)</td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td>Commonly found in the soil and can be transmitted to livestock. It produces toxins that contaminate meat, dairy and poultry products. (3)</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
<td>A food-borne pathogen commonly found in ‘ready-to-eat’ meat and dairy products. Causes food poisoning, septicaemia and listeriosis - a fatal form of meningitis. (100)</td>
</tr>
<tr>
<td><em>M. hiitols</em></td>
<td>Primarily found in mammalian skin but also found in dairy products, soil and water. Causes meningitis, pneumonia and urinary tract infections. Many strains produce blood toxins that can lead to septicaemia. (12.5)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>Found in domestic pets and household pests. It is transmitted to humans via rat and animal bites which can lead to septicaemia. (50)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>A common culprit that causes wound infections, pneumonia and blood poisoning. In 1996, 60% of the strains found in U.S. hospitals were antibiotic resistant, accounting for numerous human fatalities annually. (6)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>Present on human skin and is harmless in most circumstances. However, virulent strains can cause sore-eyes, impetigo, severe wound infections and endocarditis. (12.5)</td>
</tr>
<tr>
<td><em>S. faecalis</em></td>
<td>A common cause of wound and post-surgery infections leading to blood poisoning and endocarditis. They produce cytotoxins and haemolysins. Some strains are now drug-resistant and untreatable. (25)</td>
</tr>
<tr>
<td><em>S. uberis</em></td>
<td>Found commonly mammalian skin. Causes post-surgery infections that leads to blood poisoning and endocarditis. Some strains produce haemolysins that kill red blood cells. (3)</td>
</tr>
</tbody>
</table>

* MIC stands for minimum inhibitory concentration, the minimum concentration (in µg/L) of an antibiotic needed to inhibit the growth of a particular organism.
Fig. 1.8 Australian tree frogs of the genus *Litoria*.
(Pictures obtained from Barker *et al.*, 1995)
### Table 1.4 Sequences of some peptides isolated from Australian amphibians.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Organism</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>aurein 3.1</td>
<td><em>L. aurea</em></td>
<td>GLFDI VKKLA GHIAG ST-NH₂</td>
</tr>
<tr>
<td>caerulein</td>
<td><em>L. caerulea</em></td>
<td>pEQDYP(SO₃H) TGWMD F-NH₂</td>
</tr>
<tr>
<td>caerin 1.1</td>
<td><em>L. caerulea</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. ewingi</em></td>
<td>GLLSV LGSVA KHVLP HVVPV IAEHL-NH₂</td>
</tr>
<tr>
<td></td>
<td><em>L. gilleni</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. splendida</em></td>
<td></td>
</tr>
<tr>
<td>caeridin 1.1</td>
<td><em>L. gilleni</em></td>
<td>GLLDG LLGTG L-NH₂</td>
</tr>
<tr>
<td></td>
<td><em>L. splendida</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. xanthomera</em></td>
<td></td>
</tr>
<tr>
<td>citropin 1.1</td>
<td><em>L. citroPa</em></td>
<td>GLFDV IKKVA SVIGG L-NH₂</td>
</tr>
<tr>
<td>tryptophyllin 1.1</td>
<td><em>L. rubella</em></td>
<td>PWL-NH₂</td>
</tr>
<tr>
<td>tryptophyllin 1.2</td>
<td><em>L. electrica</em></td>
<td>FPWL-NH₂</td>
</tr>
</tbody>
</table>

### 1.4 Collection and Purification of Skin Secretions

Methods of collecting frog dermal secretions has evolved over the years. In the 1960s, dermal secretions were obtained *via* methanol extraction from sun-dried skins. For some species, this has resulted in the sacrifice of more than 700 specimens to obtain sufficient quantities of secretions for analysis (Roseghini *et al.*, 1976; Erspamer *et al.*, 1984; Erspamer *et al.*, 1986). Another method involved injecting noradrenalin into the amphibian to induce dermal secretion, followed by immersing it in ammonium acetate solution before scraping the precipitated material from the body (Nakajima, 1981; Gibson *et al.*, 1986; Giovanni *et al.*, 1987).
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The above methods involved killing the amphibian being studied and there is increasing evidence that frog populations in many countries are in serious decline, including Australia (Tyler, 1991a). This has led to the development of a benign collection technique known as surface electrical stimulation. This technique involves mild electrical stimulation of the skin whereby the dermal sympathetic nerves of the frog are stimulated to induce secretions (Fig. 1.9).

![Diagram of surface electrical stimulation and collection of dermal secretions]

**Fig. 1.9** (a) Surface electrical stimulation on *L. caerulea* followed by (b) collection of dermal secretions.

The secretions are washed from the skin with water, enzymes removed and the peptides separated using reverse phase high-performance liquid chromatography. Next, the primary amino acid sequences are analysed using the complementary methods of electrospray mass spectrometry and Edman sequencing. This technique can be repeated at monthly intervals as required (Tyler et al., 1992). During the last decade, about 140 peptides from the skin glands of Australian frogs and toads have been isolated and characterised. No animals were sacrificed during these studies (Bowie et al., 1998).
1.5 Antimicrobial Testing

Once a suspected antimicrobial peptide is isolated, purified and its primary sequence confirmed, it is commercially synthesised by Chiron Mimotopes, Clayton, Victoria. The synthetic peptide is sent to the Institute of Medical and Veterinary Science (IMVS), South Australia, for the testing of antimicrobial activities. The procedure involves introducing varying concentrations of peptide into cultures of bacteria colonies in a petri dish (Fig. 1.10) (Shafer, 1997). The results are reported as minimum inhibitory concentrations (MICs); the minimum concentration of an antibiotic needed to inhibit the growth of a particular organism (µg/L).

Fig. 1.10 Bacterial colonies in a petri dish.

1.6 Chapter Conclusion

Amphibian skin contains some of the most diverse yet simple biologically active compounds in the animal kingdom. These include neuro- and antimicrobial peptides. Studying all of them in detail is beyond the scope of this thesis. Instead, only selected antimicrobial peptides will be studied in detail in order to learn more about their mechanism of bactericidal action.
Chapter 2
Antibacterial Peptides and their Mechanisms of Action.
Chapter 2

2.1 Host Defence Peptides - A New Class of Antibiotics

Conventional antibiotics kill bacteria in a number of ways, generally interacting chemically with their vital biochemical functions and components. For example, many antibiotics inhibit cell wall, protein or nucleic acid synthesis by targeting specific bacterial enzymes and proteins (Tortora et al., 1982). Such antibiotics are stereospecific, i.e., they have one or more chiral functional groups that are specific to a target binding-site(s) found on the bacteria.

In the last few decades, the blatant over-use of antibiotics has caused many of these 'magic-bullets' to lose their bactericidal capabilities. Antibiotic-resistant strains of *Staphylococcus aureus* ('Golden Staph') and *Enterococcus faecium* are reportedly discovered world-wide, claiming numerous human lives (Frieden et al., 1993; Hiramatsu et al., 1997; Ewald and Cochran, 1999). How do these pathogens become drug-resistant? The answer lies primarily in their capability to mutate each time they replicate, each new generation of bacteria is likely to be genetically different from the parent. Hence, essential bacterial enzymes and proteins that were once targets for antibiotics no longer have the same structural conformations, rendering conventional antibiotics useless. For example, penicillin, the first antibiotic to be used commercially on a wide scale during the second world war is now virtually ineffective against many pathogens (Neu, 1992; Sanders and Sanders, 1992). Thus it is imperative to develop a new class of antibiotics that exert their actions via a non-stereospecific mechanism.

The recent discovery of antimicrobial peptides from amphibian skin opens a new dimension for antibiotic development and a understanding how these peptides exert
their biological action will allow for the rationale design of new antibiotics (Coghlan, 1997). These peptides were shown to be non-stereospecific; studies comparing synthetic D-enantiomers to their natural L counterparts revealed both to have similar antimicrobial activities (Bessalle et al., 1990; Wade et al., 1990; Juvvadi et al., 1996; Oren et al., 1997; Vunnam et al., 1997). How then, do these peptides exert their bactericidal action?

A number of experiments have been utilised to study the mechanisms of action of these peptides [see Blondelle et al. (1999) for a review]. Conductance experiments have revealed that such peptides disrupt bacterial cytoplasmic membrane potentials at micromolar concentrations (Ducloheir et al., 1989; Westerhoff et al., 1989; Kagan et al., 1990; Cruciani et al., 1992; Cociancich et al., 1993; Juvvadi et al., 1996; Silberstein et al., 1999) while polarised attenuated reflection (Frey and Tamm, 1991; Goormaghtigh et al., 1999) and Fourier-transform infrared (FTIR) spectroscopy revealed that such peptides interact with the lipid chains of synthetic phospholipid bilayers (Rana et al., 1990; Rana and Blazyk, 1991). In addition, fluorescence labelling studies have shown that magainin perturbs artificial bilayers (Matsuzaki et al., 1996). Such evidence suggests that these peptides interact directly with the bacterial membrane. As a result, the membrane’s osmotic regulation capabilities and transport functions are disrupted which ultimately leads to cell lysis (de Waal et al., 1991).

However, such experiments do not provide us with the complete picture of the bactericidal mechanism of action. Hence, three-dimensional (3D) structural studies are used to give us a better insight to their mechanism of action.
2.2 Solution Conformations of Antimicrobial Peptides

Structural studies involving circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy have shown that antimicrobial peptides generally adopt α-helical conformations in membrane mimicking solvents (e.g., trifluoroethanol) or when dissolved in solvents containing detergent micelles (Brown, 1979; Brown et al., 1982; Lee et al., 1987; Marion et al., 1988; Hirsch et al., 1996; Matsuzaki et al., 1989a, 1997, 1998). Closer examination of these structures revealed that these helices possess a distinct segregation of hydrophobic and hydrophilic residues along the length of the helix, i.e., hydrophobic residues are along one side of the helix with hydrophilic residues on the other. A diagrammatic representation is shown in Fig. 2.1.

Such helices were termed amphipathic helices. [This is synonymous with the term ‘amphiphilic’ which is also found in some literature (Eisenberg, 1984; Segrest et al., 1990)].

![Diagram of an amphipathic α-helix](image)

**Fig. 2.1** (a) Side and (b) axial view of an amphipathic α-helix. Hydrophilic and hydrophobic residue side chains are represented by red and blue lines respectively.
2.3 The Edmundson Helical Wheel

The possibility of a peptide adopting an amphipathic helical conformation can be assessed by the use of the Edmundson helical wheel (Schiffer and Edmundson, 1967). This is a two-dimensional representation of the 3D structure of an $\alpha$-helix with the perimeter of the wheel corresponding to the backbone of the polypeptide chain. Since one complete turn of an $\alpha$-helix consists of approximately 3.6 residues, each residue can be plotted every $360/3.6 = 100^\circ$ around a circle consecutively as shown in Fig. 2.2. Such plots show the projection of the residues onto a plane perpendicular to the helical axis.

![Fig. 2.2 The Edmundson helical wheel representation. Numbers represent amino acid sequence starting from the N-terminus.](image)

If a amphipathic peptide is projected onto the wheel, amino acids with similar biochemical properties (such as hydrophobicity and charged groups) will be found clustered in an arc on the helical surface facing one direction while those possessing different properties will cluster on the opposite surface (Fig. 2.1b). This results in a clear separation of hydrophobic and hydrophilic faces. It has been postulated that the amphipathic nature of these peptides is a prerequisite for their bactericidal properties.
The formation of such a helix is thought to facilitate electrostatic attraction between cationic residues of the peptide and the anionic surface of the bacterial membrane (Westerhoff et al., 1989). A simplified diagram of a typical membrane is shown in Fig. 2.3.

![Diagram of a bacterial membrane](image)

**Fig. 2.3 General structure of a bacterial membrane.**
(Modified from Singer and Nicolson, 1972).

A bacterial membrane is basically made up of a phospholipid bilayer which provides a natural barrier to the flow of ions and other solutes between the bacterial cell and its environment; any disruption to the bilayer will cause cell lysis and death. Antibacterial peptides are thought to cause such a disruption.

### 2.4 Membrane Specificity

To develop these peptides into commercial pharmaceuticals, an important consideration has to be taken into account: are they selective only towards bacteria? Since eukaryotic cell membranes are also made up of a phospholipid bilayer, it is possible that such peptides will also lyse them.
Experimental evidence has shown that the cecropin and magainin families of antimicrobial peptides are selective only towards bacteria and protozoa (Zasloff, 1987; Chen et al., 1988). This has been attributed to profound differences between mammalian and bacterial membrane designs. Bacteria, for reasons that are unclear, possess a much higher concentration of anionic phospholipids such as phosphatidylglycerol in their outer membranes (Duckworth et al., 1974; Rothman and Kennedy, 1977; Voelker, 1985; Ratledge and Wilkinson, 1988). In addition, Gram-positive bacteria have acidic polysaccharides (teichoic acids) in their cell wall (Brock, 1984) while Gram-negative bacteria have acidic lipopolysaccharides (LPS) located exclusively on the outer leaflet of the membrane (Lugtenberg and van Alpen, 1983; Nikaido and Vaard, 1985). This increased concentration of anionic molecules is thought to enhance the electrostatic affinity between the bacterial membrane and the highly cationic antimicrobial peptides (Miteva et al., 1999). Indeed, conductance experiments have shown that cecropins (Gazit et al., 1994; Gazit et al., 1995) and magainins (Vaz Gomes et al., 1993; Matsuzaki et al., 1995) have higher affinities towards negatively charged membranes compared to uncharged ones while fluorescence, X-ray and scanning calorimetric studies have shown that antimicrobial peptides interact more strongly with anionic than zwitterionic (electrically neutral) lipid bilayers (Polozov et al., 1997; Lohner and Prenner, 1999; Prenner et al., 1999). Furthermore, *Staphylococcus aureus* mutants possessing a higher concentration of anionic phospholipids on their cytoplasmic membranes showed greater sensitivity towards cationic bactericidal peptides (Pescher et al., 1999). In contrast, the outer bilayer surface of human cell membranes are exclusively composed of zwitterionic phospholipids, mainly phosphatidylcholine, phosphatidylethanolamine and sphingomyelin (Castano et al., 1999). Anionic phospholipids, such as
phosphatidylserine, are exclusively localized in the inner bilayer surface, oriented towards the cytoplasm (Verkleij et al., 1973). Furthermore, bacterial membranes lack cholesterol, a sterol which not only reduces the affinity of antimicrobial peptides for a phospholipid bilayer, but is also known to stabilize synthetic membranes (Nakajima, et al., 1987; Christensen et al., 1988; Tytler et al., 1995). Vertebrate cell membranes, on the other hand, contain a considerable proportion of cholesterol (Fig. 2.4) which makes them less susceptible to cell lysis (Turner and Rouser, 1970; Lange and Ramos, 1983; Bloch, 1991). Indeed, conductance experiments have shown that cholesterol reduced the bioactivity of membrane-disrupting peptides while there was a significant enhancement in their potency when introduced to human erythrocytes lacking cholesterol (Nakajima et al., 1987; Christensen et al., 1988; Matsuzaki et al., 1995).

![Cholesterol stabilise phospholipid bilayers.](image-url)
Circular dichroism studies have revealed that magainin adopts an amphipathic helical structure in aqueous solution containing anionic phosphatidylserine vesicles but was unstructured when exposed to zwitterionic phosphatidylcholine vesicles (Matsuzaki et al., 1989b; Matsuzaki et al., 1997; Matsuzaki et al., 1998). The preferential binding of these basic peptides to negatively charged phospholipids may also explain their ability to selectively lyse cancerous cells, which have three to eight-fold more anionic phospholipids (typically phosphatidylserine) on their membrane surface compared to normal cells (Conner et al., 1989; Utsugi et al., 1991; Tytler et al., 1995).

The difference in transmembrane potentials between prokaryotic and eukaryotic cells has also been suggested to play a part in cell selectivity of these peptides. Respiring prokaryotes are found to have a 'negative-inside' transmembrane potential while eukaryotic cells have no or insignificant transmembrane potential (Blondelle et al., 1992; Matsuzaki et al., 1995). This profound difference in the overall charge of the cell may be another reason for the preferential interaction of cationic membrane-lytic peptides with prokaryotes over eukaryotic cells.

Over the past two decades, a number of experimental techniques have been employed to gain a better insight to the mechanisms of bactericidal action of these peptides [see reviews by Bechinger (1999) and Shai (1998 and 1999)]. The next few sections will discuss the possible modes of action in greater detail.
2.5 The Channel Mechanism

Initially, all antimicrobial peptides were thought to undergo a transmembrane channel-forming mechanism, also known as the barrel-stave mechanism (Ojcius and Young, 1991; Shai, 1995). The process starts off with the unstructured (random coil) peptide in solution. Once in close proximity to the bacterial membrane, the cationic residues of the peptide are electrostatically attracted to the anionic headgroups of the membrane. This proposal is supported by experiments showing that adding sodium chloride inhibits the bactericidal activity of such peptides (Skerlavaj et al., 1990; Sekharam et al., 1991). Upon interacting with the membrane surface, the peptides adopt amphipathic $\alpha$-helical structures (Inagaki et al., 1989; Matsuzaki et al., 1991, Matsuzaki, 1999). The next step involves the insertion of these helical peptides vertically into the membrane once a critical peptide concentration is reached. The vertical orientation of such peptides is supported by oriented circular dichroism spectroscopy (Huang and Wu, 1991; Ludke et al., 1994) and in-plane neutron scattering studies (Ludke et al., 1996). When the helical peptides are laid in parallel with respect to each other across the membrane, a transmembrane pore is formed with the hydrophobic faces of the peptides facing outwards. These interact with the hydrophobic alkyl chains of the phospholipid bilayer. The polar hydrophilic surface of the peptides are arrayed inwardly allowing the passive flux of ions and small molecules across the membrane, disrupting the osmotic gradient of the cell, ultimately lysing the membrane. A schematic representation of the mechanism is illustrated in Fig. 2.5a and 2.5b.
Recently, the nature of these channels has come under scrutiny as they involve in a peptide arrangement that result in the accumulation of many cationic residues (e.g. lysine) in a narrow pore (Bechinger, 1997). Such channels would possess a high positive charge-density in the pore region which is energetically unfavourable due to severe electrostatic repulsion. Furthermore, experiments have shown that such pores are selective towards cations, suggesting that the conventional channel model was unacceptable (Cruciani et al., 1992). Recently, the channel model was modified: membrane phospholipids in the vicinity of the peptides are suggested to line the walls along the channel which ‘neutralises’ the highly positively-charged pore (Ludke et
al., 1996; Matsuzaki, 1998). Such channels have been termed *wormholes* (Bechinger, 1999). An example is illustrated in Fig. 2.5c.

The channel mechanism requires channel-forming peptides to be of sufficient length to span the entire phospholipid bilayer. Since each amino acid residue in an α-helix is related to the next by a distance of approximately 1.5 Å (from the N to C terminus), a peptide must have a minimum of 20 residues to be able to span a bacterial membrane typically of about 30 Å in thickness (Kaiser and Kezdy, 1987). Hence, by judging the number of amino acid residues alone, it would appear that most of the peptides listed in Table 1.1 will adopt the barrel-stave mechanism. This, in theory should include: alamethicin, cecropin, defensin, magainin, melittin and pardaxin.

How then, do shorter membrane-lytic peptides exert their biological effects? The next two sections will describe their possible mechanisms of action in detail.
2.6 Dimerisation

Antimicrobial peptides consisting less than twenty residues are too short to penetrate the entire bacterial membrane and thus cannot undergo the barrel-stave mechanism in their monomeric form. However, gramicidin A, a fifteen residue peptide isolated from \textit{Bacillus brevis} was found to possess membrane-lysing properties (Andersen, 1983). In an attempt to explain such a phenomenon, detailed two-dimensional NMR studies were conducted, revealing that gramicidin A formed N-terminal to N-terminal dimers in micelle/water mixtures (Arseniev \textit{et al.}, 1985). In addition, gramicidin A was found to be oriented perpendicular to phospholipid bilayers (Fig. 2.6) (Lazo \textit{et al.}, 1995). End-to-end dimerisation would make them long enough to form transmembrane channels (Urry, 1984; Ketchem \textit{et al.}, 1993; Separovic \textit{et al.}, 1994; Koepp et al., 1995).

![Fig. 2.6 N-terminal to N-terminal dimers of gramicidin A. Monovalent cations are postulated to traverse through the helices. (Smith \textit{et al.}, 1990; Tian and Cross, 1999)]
2.7 The Carpet Mechanism

A non-channel forming mechanism known as the carpet mechanism has also been suggested which provides another plausible explanation as to why some relatively short peptides possess antimicrobial activities (Epand et al., 1995; Gazit et al., 1995). For example, an eleven residue analogue of sapecin from the flesh fly Sacrophaga peregrina (Alvarez-Bravo et al., 1995), a twelve residue defensin analogue from beetle haemolymph (Saido-Sakanaka et al., 1999), thirteen residue peptides from bovine and porcine bone marrow (Selsted et al., 1992; Lawyer et al., 1996), a fifteen residue cecropin hybrid (Andreu et al., 1985; Andreu et al., 1992), a fifteen residue melittin hybrid (Subbalakshmi et al., 1999) and the seventeen residue tachyplesin (Rao, 1999) were found to possess broad-spectrum antibiotic activities.

The initial part of the carpet mechanism is similar to the channel mechanism: electrostatic attraction of cationic residues to the anionic phospholipids on the membrane surface occurs, causing the peptides to adopt an amphipathic α-helical conformation and forming a ‘carpet’ on the membrane surface. Stoichiometric studies have shown that the threshold concentration of peptide needed to lyse phospholipid vesicles corresponds to the amount of peptide needed to form a ‘carpet’ monolayer on each vesicle surface (Gazit et al., 1995).

Next, the peptide partially ‘immerses’ itself into the phospholipid bilayer by rotating and reorienting itself in such a way that only its hydrophobic residues interact with the membrane’s hydrophobic core while its hydrophilic face continues to interact
with the polar head groups (and possibly the aqueous solution). Spectrofluorometric studies on N-terminal labelled dermaseptin (Pouny et al., 1992; Strahilevitz et al., 1994) and cecropin (Gazit et al., 1994) showed that N-terminal fluorescent probes relocated to a more hydrophobic region while $^{13}$C NMR studies using aqueous shift reagents revealed that such peptides had their hydrophobic residues imbedded in the membrane hydrophobic core while their hydrophilic side chains were immersed in the aqueous phase (Stanislawski and Rüterjans, 1987). In addition, results from attenuated reflection Fourier transform infrared spectroscopy and molecular dynamics simulations have also suggested that cecropins were oriented parallel to the lipid membrane surface and do not insert into the lipid bilayer to form transmembrane channels (Gazit et al., 1996). Finally, solid-state NMR experiments show that the backbone amides of magainin were found near the phospholipid headgroups of artificial lipid bilayers, suggesting that the peptide was near or on the bilayer surface (Hirsch et al., 1996).

This results in the thinning of the phospholipid bilayer to accommodate the peptide 'carpet', as revealed by X-ray diffraction (Ludke et al., 1995), solid-state NMR (Bechiger et al., 1991, 1992, 1993) and stoichiometric studies (Steiner et al., 1988; Oren and Shai, 1997). When a peptide threshold concentration is reached, the membrane disintegrates, resulting in cell death. It has also been suggested that the detergent-like actions of such peptides may lead to the formation of peptide-phospholipid micelles by breaking off portions of the bilayer, destroying the integrity of the membrane (Sanders II and Prestegard, 1990; Bechinger et al., 1999). The mechanisms are shown schematically in Fig. 2.7.
Presently, it is not possible to predict which mechanism a membrane-lytic peptide will adopt just by studying its primary structure. However, amphipathic peptides consisting of twenty or more residues were thought to form transmembrane pores via the channel mechanism. In the last two decades or so, a variety of experimental techniques have been used to study the orientation and topology of these peptides.
Most experiments seem to support the fact that many membrane-lytic peptides (long enough to span a membrane) generally adopt the channel mechanism. Two extensively studied examples are alamethicin and melittin (Table 1.1). However, a number of exceptions were discovered. These include the cecropin, dermaseptin and magainin families of peptides (Epand et al., 1995; Bechinger, 1997). Although they were long enough to span a bilayer, experiments suggest that they adopt the carpet mechanism instead. Table 2.1 summarises some of these peptides and the corresponding techniques used to elucidate their membrane-lytic mechanism.

**Table 2.1 Membrane-disrupting peptides and their mechanisms of action.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Experimental method</th>
<th>Mechanism</th>
<th>References</th>
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<tbody>
<tr>
<td>Alamethicin</td>
<td>molecular modelling</td>
<td>Channel</td>
<td>Tieleman et al., 1999</td>
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<td></td>
<td>neutron-scattering</td>
<td>Channel</td>
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<td></td>
<td>Raman spectroscopy</td>
<td>Channel</td>
<td>Vogel, 1987</td>
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<td></td>
<td>solid-state NMR</td>
<td>Channel</td>
<td>North et al., 1995</td>
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<td></td>
<td>X-ray diffraction</td>
<td>Channel</td>
<td>Wu et al., 1995</td>
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<td>Cecropin</td>
<td>attenuated reflection IR</td>
<td>Carpet</td>
<td>Gazit et al., 1996</td>
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<td></td>
<td>fluorescence spectroscopy and</td>
<td>Carpet</td>
<td>Gazit et al., 1995</td>
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<td></td>
<td>resonance energy transfer</td>
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<td>molecular modelling</td>
<td>Channel</td>
<td>Durell et al., 1992</td>
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<tr>
<td>Protein</td>
<td>Technique</td>
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<tr>
<td>Carpet</td>
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<td>Gazit et al., 1994</td>
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<td>nitroxide spin-labelling</td>
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<td>Melittin</td>
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<td>Yang et al., 1999</td>
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Which mechanism will the antimicrobial peptides of Australian amphibians adopt?

In the next two chapters, experiments that can be used as probes to give a better insight to their mechanism(s) of action will be discussed in greater detail.
Chapter 3
Three-Dimensional Structural Studies

Litoria peroni
3.1 Structural Studies of Bioactive Peptides

Naturally occurring peptides have evolved to perform specific functions such as binding to biological receptors. Their functional properties depend largely on their conformation, i.e., their 3D structures. In order to gain a better understanding of their biological functions and mechanisms of action, their 3D structures must first be determined in appropriate physiological conditions.

A number of techniques have been developed to probe structural elements present in peptides and proteins. Automated Edman sequencing (Hunkapiller et al., 1983) and electrospray mass spectroscopy (Marina et al., 1999) are now routine techniques used for elucidating primary amino acid sequences. The number of new peptide sequences being discovered is increasing exponentially but primary sequences, by themselves, do not provide us with the overall picture of their biological functions. Presently, only a fraction of known bioactive peptides and proteins have had their 3D structures elucidated. Techniques such as circular dichroism (CD) (see Section 3.2), Fourier-transform and Raman IR spectroscopy (Krimm, 1963; Williams 1983; Williams et al., 1990) can reveal the presence of certain structural elements (such as α-helices and β-sheets) while ultraviolet (UV) spectroscopy can provide information on the mobility and orientation of aromatic groups in a peptide (Matsuzaki et al., 1994 and references cited therein). Although such techniques provide general information concerning the structural features of the peptide, they do not provide any quantitative structural data.
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Detailed structural information can be obtained from protein crystals using techniques such as X-ray (Glusker et al., 1994) and neutron diffraction (Kossiakoff, 1985; Helliwell, 1997; Niimura et al, 1997; Bradshaw et al., 1998). In addition, the accuracy and resolution of peptide and protein structures have been greatly improved using synchrotron radiation as an X-ray source (Helliwell, 1998a, 1998b and 1992). Such techniques have a major disadvantage: the sample has to be crystallised first. If suitable crystals are available, structures with resolution ranging from 1.0-3.5 Å are generally obtainable. However with larger peptides and proteins, it is also necessary to prepare heavy-atom (e.g. cadmium and mercury) derivatives of the crystals to help with phasing of the electron density maps obtained. In addition, crystal packing forces can sometimes distort the overall conformation of a sample, giving different results when compared to solution structures (Blundell and Johnson, 1976; Schulz and Schirmer, 1979). Furthermore, crystallographic techniques only reveal the most stable conformation of the sample in the solid phase, they do not indicate the conformation in aqueous or membrane-mimetic environments. Hence, structural data obtained from crystallographic techniques must be treated with caution in particular instances.

High-resolution electron cryomicroscopy is another diffraction technique employed to determine the 3D structures of large proteins. Presently, it is limited to a resolution of 7 Å (although work is progressing rapidly to bring this down to 3 Å) and it cannot be used to resolve structures of smaller proteins and peptides with molecular masses less than 20 kDa (Chiu and Schmid, 1997; Walz and Grigorieff, 37
1998). Thus, this technique is unsuitable for use with the small peptides which are the subject of this thesis.

Techniques such as nuclear magnetic resonance spectroscopy (NMR) have emerged as alternative methods for 3D peptide structure determination. These new approaches, with NMR in particular, enables detailed structural studies of peptides in solution. This provides new insights of the molecular mechanisms by which membrane-lytic peptides fulfil their biological functions. The next few sections will describe these two methods in greater detail.

3.2 Circular Dichroism

The majority of naturally occurring peptides are constituted from L amino acids (with the exception of glycine which is not chiral). This stereo-isomeric property results in L and D peptides interacting differently with a beam of left and right-circularly polarised light. Such interactions cause both left and right-circularly polarised light to travel at different speeds as they traverse the peptide, thereby rotating the refracted beam (Brahms and Brahms, 1980; Cantor and Timasheff, 1982). The extent of rotation is measured by its molecular ellipticity (θ) and is primarily dependent on the conformational structure of the peptide. If a peptide is unstructured (i.e., random coil), a plot of θ against the wavelength of light (λ) will produce a curve with a broad, low absorbance minimum near 200 nm in the far-UV region. Peptides which adopt an α-helical conformation in solution will display a curve with characteristic minima in the vicinity of 208 nm (π-π* transition) and 220
nm (n-π* transition) (Crabbe, 1965; Crabbe, 1972; Johnson, 1990). Fig. 3.1 illustrates a schematic CD spectrum of an unstructured and an α-helical peptide.

![CD Spectrum Diagram](image)

**Fig. 3.1** A hypothetical CD spectrum of an unstructured and α-helical peptide.

Small deviations from α-helical geometry yield a CD spectrum with greatly diminished intensities at 208 and 220 nm. Used qualitatively, CD spectra can provide valuable information concerning the 3D structure of the peptide (Dyson and Wright, 1991).

### 3.3 Nuclear Magnetic Resonance Spectroscopy

In the last two decades, nuclear magnetic resonance (NMR) has emerged as an indispensable tool for the structural elucidation of biological molecules in solution, particularly proteins and peptides (Wright, 1989; Wüthrich, 1989a; Clore and Gronenborn, 1991). This is in part due to the significant developments in NMR
methodologies and hardware; in particular the introduction of multi-dimensional NMR methods, isotopic labelling techniques, superconducting high-field magnets and the use of more powerful computers for data processing. We now have a formidable second approach (alongside X-ray diffraction techniques) for accurate structural determination of peptides with molecular masses up to 40,000 Daltons (Wüthrich, 1989b; Wüthrich, 1998). NMR spectroscopy is an essential technique for structural studies of peptides because it is the only method that can provide us with high-resolution structural information of molecules in solution. In the next section, an introduction to the basic theory underlying NMR spectroscopy will be given followed by an introduction to the more complex two-dimensional (2D) NMR experiments required to obtain inter-nuclei distance restraints required for structural calculations.

3.4 The NMR Phenomenon

When a sample (dissolved in an appropriate solvent) is subjected to an external magnetic field $B_0$, all its nuclei with a non-zero nuclear spin quantum number ($I$) will orient themselves in one of the possible $(2I+1)$ directions within the field. Thus, biologically important nuclei (e.g. $^1$H, $^{13}$C, $^{15}$N) which have $I = \frac{1}{2}$ will therefore be aligned in two possible orientations: parallel or antiparallel to $B_0$. It is energetically more favourable for spins to align parallel to the magnetic field so the low energy spin state will be slightly more populated (Fig. 3.2). This results in the formation of a net macroscopic magnetisation $M$ - the sum of the magnetisation of all individual spins. It is this net magnetisation which is manipulated to generate NMR spectra.
Chapter 3

No magnetic field

In the presence of a magnetic field

Energy

Fig. 3.2 A schematic representation of the relative population of spins in the two energy states of a magnetised sample.

3.5 One-Dimensional NMR Spectroscopy

Instead of using the ‘energy states’ analogy, the behaviour of \( M \) can be more conveniently described using vector diagrams (Fig. 3.3). When a radio-frequency pulse (90°) is applied along the X axis, it exerts a torque on \( M \), represented by the movement of \( M \) into the XY plane along the Y axis.

Orientation of \( M \) under a magnetic field

Application of radio-frequency pulse

Precession of magnetisation

Fig. 3.3 Vector diagrams showing effects of a radio-frequency pulse on \( M \).

Once \( M \) is brought into the XY plane, it precesses under the influence of \( B_0 \) at its resonance frequency. This induces an electric current in a detector coil wound
around an axis perpendicular to \( \mathbf{B}_0 \). As the system relaxes back to the initial equilibrium stage, its transverse magnetisation decays with time. This free induction decay (FID) is recorded in the time domain and Fourier transformation (FT) of the transient signals into the frequency domain yields a one-dimensional (1D) NMR spectrum as illustrated in Fig. 3.4.

![Time domain and Frequency domain](image)

**Fig. 3.4 Fourier transformation of a FID.**

Since the resonance frequency exhibited by each nucleus depends primarily on its individual magnetic environment, a typical NMR spectrum contains a series of resonances from various nuclei at different frequencies relative to an internal standard expressed in parts per million (ppm) (Duncan, 1990). This makes it possible to compare the relative resonance frequencies of a particular nucleus in different compounds or when using magnets of different field strength. The internal standard of choice for biological samples (in aqueous solvent) is 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) for which the most upfield signal is referenced at 0.015 ppm (Wishart et al., 1995b; Gesell et al., 1997).
3.6 Chemical Shifts and Secondary Structures

It is known that the magnetic environment of a nucleus is sensitive not only to local electronic effects, i.e., electron withdrawing and donating elements, but also to shielding effects that result from the formation of secondary structures. For example, the $^1$H and $^{13}$C chemical shifts of amino acid residues in a peptide usually differ from random-coil values, particularly at the $\alpha$ positions. Theoretical calculations (Clayden and Williams, 1982) and statistical analysis (Szilagyi and Jardetzky, 1989) have shown that $^1$H and $^{13}$C $\alpha$-CH chemical shifts can be used to probe the presence of structural elements in proteins and peptides (Zhou et al., 1992).

The random-coil chemical shifts of the twenty common amino acids have been determined (Wishart et al., 1995b). For example, in a typical $\alpha$-helical peptide, for a window of $n = \pm 2$ residues, the smoothed plots of $^1$H chemical shifts will exhibit a distinct upfield shift and those for the $^{13}$C resonances will show a distinct downfield shift along the peptide, relative to the corresponding random-coil amino acids. This suggests that chemical shift deviations can be used to identify the presence of secondary structural elements in a peptide (Pastore and Saudek, 1990; Wishart et al., 1991; Asakura et al., 1995; Wishart et al., 1995a). Fig. 3.5 illustrates a schematic plot representing a hypothetical helical peptide.
3.7 3D Structural Elucidation: The Strategy

So far, the techniques that provide information about the general structural elements present in a peptide have been described. However, these methods do not provide details of a 3D structure at the molecular level. To achieve this, quantitative information from other NMR techniques must be used to provide stringent structural constraints (e.g. inter-nuclei angles and distances). These data can then be used as inputs for calculations to produce realistic 3D models with structural parameters that are in agreement with constraints derived from NMR experiments. The following sections will discuss the appropriate NMR techniques in greater detail.
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3.8 Coupling Constants and Dihedral Angles

The conformation of a peptide is influenced by the dihedral angles of the amide bonds (Markley et al., 1993). Fig. 3.6 defines the \( \phi \) dihedral angle (\( \phi = 0^\circ \) when the carbonyl C' is in the \textit{trans} position to the amide \( \text{^1H} \)).

![Newman projection of a peptide](image)

Fig. 3.6 The \( \phi \) dihedral angle. The Newman projection is produced by viewing along the axis of N-C\( \alpha \)-C' from the N-terminus.

The dihedral angle \( \phi \) can be obtained from the magnitude of coupling constants between vicinal NH and C\( \alpha \)H protons. The three-bond coupling constant \( (^3J) \) is related to \( \phi \) by the relationship:

\[
^3J_{\text{INCOH}} = 6.4 \cos^2 | \phi - 60^\circ | + 1.4 \cos | \phi - 60^\circ | + 1.9
\]

A \textit{Karplus plot} can be obtained from the above equation by correlating the \( \phi \) angles of bovine trypsin inhibitor (BPTI) protein and \( ^3J_{\text{INCOH}} \) values measured in solution is shown in Fig. 3.7 (Karplus, 1959; Pardi \textit{et al}., 1987; Creighton, 1993).
Fig. 3.7 Karplus plot showing relationship between coupling constant (Hz) and the $\phi$ dihedral angle.

When vicinal coupling constants are correlated with the dihedral angle $\phi$, some ambiguities may occur since as many as four different values of $\phi$ may correlate with a particular coupling constant. This problem can be solved by analysing the range of values allowed for $\phi$ in known protein structures. For example in $\alpha$-helices, $\phi$ values for all residues (with the exception of glycine and proline) are approximately between $-30^\circ$ to $-90^\circ$. As a general rule, four or more consecutive residues exhibiting a coupling constant of less than 5 Hz indicates that a helical region is present. For the purpose of structural calculations, an angular restraint of $-60 \pm 30^\circ$ is typically used in helical regions. Coupling constants between 5 and 6 Hz are used to restrain $\phi$ to $-60 \pm 40^\circ$ (Richardson, 1981; Wüthrich, 1986).

In summary, $^3J_{\text{HNCOH}}$ values can provide supporting evidence for $\alpha$-helical secondary structures. The observation of segments of three or more sequential residues with
small coupling constants (less than or equal to 5 Hz) has been considered a reliable and independent criterion for identification of helical regions in a polypeptide (Wüthrich, 1986).

3.9 Two-dimensional NMR Spectroscopy

Large molecules possess a large number of magnetically non-equivalent spins. In $^1$H NMR spectra, this results in severe overlapping of NMR resonances in the spectra due to two or more different nuclear spins resonating at similar frequencies. Hence, two-dimensional (2D) NMR techniques are required to further separate these resonances to resolve much of the overlap.

2D experiments involve a basic scheme; viz (i) a preparation period ($d_t$), (ii) an evolution period ($t_e$) during which spins are labelled according to their resonance frequencies (or chemical shifts), (iii) a mixing period ($t_m$) during which the spins are correlated to each other and finally, (iv) a detection period ($t_d$) where the FID is recorded (Wüthrich, 1986). A number of experiments are recorded with successive incremented values of $t_e$ to generate a data matrix. Fourier transformation of the data matrix in both dimensions then yields the 2D frequency spectrum. The process is summarised in Fig. 3.8.
Fig. 3.8 Generating a 2D NMR experiment.

Such spectra have both axes corresponding to the same chemical shift and are symmetrical about the diagonal. The diagonal corresponds to the one-dimensional (1D) spectrum and the off-diagonal, symmetrical cross-peaks indicate the existence of interactions between two $^1\text{H}$ nuclei, with coordinates corresponding to their respective chemical shifts.

### 3.10 2D NMR Techniques

Information on through-bond and through-space interactions between specific nuclei can be obtained by manipulating the radio-frequency pulse sequences of 2D NMR experiments. Thus, by conducting a series of 2D experiments, it is possible to obtain the complete $^1\text{H}$ and $^{13}\text{C}$ assignments of proteins consisting up to about 100 amino acid residues (Chazin and Wright, 1988). The next four sections describe the essential 2D techniques in detail.
3.11 Correlation Spectroscopy

Correlation Spectroscopy (COSY) enables the analysis of through-bond (scalar) interactions between pairs of $^1$H nuclei (Aue et al., 1975). The pulse sequence (Fig. 3.9a) involves the application of an initial 90° pulse which sends the magnetisation from the Z axis to the XY plane. Immediately after, the magnetisation evolves in the XY plane during $t_1$, leading to the labelling of nuclei that are spin-spin coupled to each other. As a consequence, each COSY cross-peak represents $^1$H-$^1$H couplings via three bonds ($^3J$ coupling).

![Diagram of COSY pulse sequence](image)

**Fig. 3.9** (a) The COSY and (b) double-quantum filtered COSY pulse sequences.

A drawback of the experiment is that the diagonal produced is a dispersion signal. This results in a ‘tailing effect’ that can obscure cross-peaks near the diagonal. To circumvent this problem, double-quantum filtering (DQF) is incorporated into the pulse sequence (Fig. 3.9b): the first 90° pulse rotates $^1$H magnetisation into the XY plane. The incremental delay ($t_1$) allows evolution of scalar-coupled chemical shifts. The second 90° pulse generates multiple quantum coherence between coupled spins. Scalar-coupled spins are rotated back to observable single-quantum coherence by the third 90° pulse and signals from uncoupled spins are eliminated via phase cycling.
DQF-COSY is the method of choice over conventional COSY, not only are the ‘tailing’ effects of the diagonal minimised, strong singlet peaks (e.g. from water) produced by uncoupled protons are also significantly reduced (Rance et al., 1993b).

3.12 Total Correlation Spectroscopy

COSY-type experiments are used to demonstrate connectivities up to three bonds in length. This is of limited value due to spectral overlap, especially for analysing $^1$H nuclei in the side chains of amino acids which tend to have chemicals shifts between 1.0 to 3.5 ppm. For this reason, total correlation spectroscopy (TOCSY) is employed to produce relayed scalar connectivities (Davies and Bax, 1985). The TOCSY experiment is similar to COSY except that the magnetisation of scalar-coupled protons is extensively transferred across all coupled spins. As a result, distant protons (up to 7 bonds apart) will show up as off-axis cross-peaks in the resultant plots unless they are isolated by a quaternary or carbonyl carbon (Braunschweiler and Ernst, 1983). This feature is essential for the identification of the different spin systems belonging to each specific residue of a peptide i.e., different residues can be identified by their distinct TOCSY pattern. Compared to the COSY pulse sequence, the TOCSY pulse sequence (Fig. 3.10) has an additional spin-lock pulse during the mixing time. This induces exchange of magnetisation between the spins of a particular spin system, causing them to temporarily become magnetically equivalent. As a consequence, the ‘relayed’ through-bond connectivities are observed in a spin system.
3.13 Heteronuclear Single-Quantum Coherence Spectroscopy

The heteronuclear single-quantum coherence (HSQC) experiment provides heteronuclear $^{13}\text{C}$-$^{1}\text{H}$ correlation information, i.e., the chemical shifts of hydrogen to carbon nuclei. It is basically similar to the COSY experiment but in this case, scalar magnetisation transfer occurs between two types of nuclei. It is an *inverse-detection* technique where the $^{1}\text{H}$ signal is observed rather than the $^{13}\text{C}$ signal (due to the greatly increased sensitivity of $^{1}\text{H}$ nucleus). The basic HSQC pulse sequence is illustrated in Fig. 3.11.

The experiment involves the use of two separate channels for the $^{1}\text{H}$ and $^{13}\text{C}$ nuclei. Once the $^{1}\text{H}$ pulse brings the magnetisation into the XY plane, the first $^{13}\text{C}$ pulse forms double (and zero) quantum coherence between scalar-coupled $^{1}\text{H}$ and $^{13}\text{C}$
spins. This coherence undergoes evolution during $t_1$. Next, a 180° $^1\text{H}$ pulse refocuses the $^1\text{H}$ chemical shift evolution, leaving the magnetisation labelled only by the $^{13}\text{C}$ chemical shifts. The second $^{13}\text{C}$ pulse converts the double-quantum coherence back to a single-quantum coherence. The $^1\text{H}$ ($^{13}\text{C}$ decoupled) spectrum detected is modulated by the $^{13}\text{C}$ chemical shift as a function of $t_1$. Finally, $^1\text{H}$ transverse magnetisation is observed after the refocusing period. Following Fourier transformation, cross-peaks are observed between the directly-bonded $^1\text{H}$ and $^{13}\text{C}$ nuclei.

3.14 Nuclear Overhauser Effect Spectroscopy

Nuclear Overhauser effect spectroscopy (NOESY) is a crucial technique that determines inter-proton through-space interactions. (Jeener et al., 1979; Wagner and Wüthrich, 1979). In this experiment, each cross-peak represents a nuclear Overhauser effect (NOE) produced by through-space dipolar interactions between $^1\text{H}$ nuclei. The pair of nuclei in question need not belong to the same residue; they simply have to be near each other in space. The NOESY pulse sequence is shown in Fig. 3.12. The second 90° pulse is applied after the $t_1$ period to bring the magnetisation to the $-Z$ axis immediately followed by a mixing period, $\tau_m$. During this period, dipolar coupling occurs between nuclei of close proximity ($\leq 5\ \text{Å}$ apart).

\begin{align*}
d_1 & \quad 90^\circ \quad t_1 \quad 90^\circ \quad \tau_m \quad 90^\circ \quad t_2 (\text{acquire})
\end{align*}

Fig. 3.12 The NOESY pulse sequence.
The relative intensities of cross-peaks reflect the magnitude of spatial separation between two nuclei: the closer apart the two nuclei are, the more intense the cross-peak. As a consequence, NOESY experiments can be utilised to deduce inter-proton distances, imposing stringent constraints on the number of possible conformations a peptide can adopt in solution.

3.15 Cross-peak Intensities and Inter-proton Distances

NOESY cross-peak intensities depend on the inter-proton distance $r$ and is a function of the rotational correlation time ($\tau_c$) of the molecule (Ernst et al., 1987):

$$\text{NOE intensity} \propto r^6 \cdot f(\tau_c) \quad \ldots \ldots (1)$$

It can be seen that the NOE intensity is inversely proportional to $r^6$ (i.e., a very short-range effect) but is also dependent on $\tau_c$ which gives a measure of the rate of molecular tumbling. Large biomolecules have a long $\tau_c$ which can result in the observation of negative NOE intensities. Fig. 3.13 illustrates the relationship between NOE intensity to $\tau_c$.

![Fig. 3.13 Plot of NOE intensity versus log $\tau_c$ ($B_0 = 11.7 \, \text{T}, 25^\circ \text{C}$). (Diagram modified from Wüthrich, 1986)]
Equation (1) assumes that all protons in the molecule possess the same $\tau_c$. In reality, however, this is not so. Intra-molecular mobility leads to non-linear averaging of distances for different $^1$H nuclei in a molecule. For example, the methyl protons of alanine and valine in a peptide may have different $\tau_c$. For this reason, it is necessary to put upper and lower limits on the distance $r$ before they can be used as distance restraints during calculations to avoid over-restraining the molecule. This will be discussed in the next section.

### 3.16 Measuring NOESY Cross-peak Intensities

In 1D NMR, the intensity of a peak is represented by its integral value and does not depend upon the line-shape or multiplet structure. In the 2D NOESY spectra however, the integral corresponds to the volume of the cross-peak. Volume integration is achieved by fitting all rows and columns of the NOESY spectrum to a set of reference line-shapes (Denk et al., 1986; Olejniczak et al., 1989). These line-shapes are defined along the F1 and F2 dimensions for each resonance by using a row (or column) containing a well-resolved crosspeak which has been unambiguously assigned to its resonance. In cases of overlapping cross-peaks, the row and column integral can be evaluated by computerised curve fitting methods. This fitting procedure is completely independent of the reference line-shapes as it uses theoretical (Lorentzian) line-shapes produced by NMR resonances (Roberts, 1995).
Chapter 3

3.17 Notations for Inter-proton Distances in Peptides

The distance between a pair of $^1$H nuclei A and B located in two different residues (at positions $i$ and $j$) of a peptide sequence is denoted by $d_{AB}(i,j)$. Distances are defined from the N to the C terminus of the peptide sequence. Some examples are given below:

$$d_{\alpha N}(i,j) = d(\alpha H_i, NH_j)$$

$$d_{\beta N}(i,j) = d(N\ H_i, NH_j) = d_{NN}(j,i)$$

Inter-proton interactions and their corresponding notations between adjacent residues are illustrated in Fig. 3.14. Arrows indicate through-space interactions between the amide proton of residue $i+1$ and the $C\alpha$H ($d_{\alpha N}$), NH ($d_{\beta N}$) and $C\beta$H ($d_{\beta N}$) of residue $i$. In peptides and proteins, these distances are short enough to give rise to strong NOEs and are used for the identification of adjacent residues during the sequential assignment procedure (see Section 3.19).

![Diagram of peptide structure with notations](image)

Fig. 3.14 Nomenclature used for defining inter-proton distances in peptides.
3.18 NOESY Patterns in an α-Helical Peptide

NOESY cross-peaks can indicate the presence of secondary structures present in a peptide. For example in amphipathic helical peptides, inter-residue hydrogen bonding takes place between the carbonyl oxygen nuclei and amide protons four residues apart (Fig. 3.15a) resulting in the formation of a α-helix (Jeffrey and Saenger, 1991). Due to this relatively stable conformation, inter-residue NOEs are usually observed between protons belonging to residues that are three to four residues apart in the sequence (Fig. 3.15b).

![Diagram of hydrogen bonding and NOESY interactions in an α-helix](image)

Fig. 3.15 Schematic representations of (a) hydrogen bonding (curved arrows) and (b) typical NOESY inter-residue interactions in an α-helix.
Some of the inter-residue NOESY interactions present in an α-helix are shown in Fig. 3.16 while the corresponding inter-proton distances are listed in Table 3.1.

Fig. 3.16 Schematic 3D view of an α-helix showing typical inter-residue NOESY interactions. (Diagram modified from Lewin, 1990).
Table 3.1 Types of inter-proton interactions observed in an α-helix.

<table>
<thead>
<tr>
<th>Type of Interaction</th>
<th>Approximate distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_{\alpha N}(i,i+1)$</td>
<td>3.5</td>
</tr>
<tr>
<td>$d_{\alpha N}(i,i+2)$</td>
<td>4.4</td>
</tr>
<tr>
<td>$d_{\alpha N}(i,i+3)$</td>
<td>3.4</td>
</tr>
<tr>
<td>$d_{\alpha N}(i,i+4)$</td>
<td>4.2</td>
</tr>
<tr>
<td>$d_{\eta N}(i,i+1)$</td>
<td>2.8</td>
</tr>
<tr>
<td>$d_{\eta N}(i,i+2)$</td>
<td>4.2</td>
</tr>
<tr>
<td>$d_{\beta N}(i,i+1)$</td>
<td>2.5-4.1</td>
</tr>
<tr>
<td>$d_{\alpha \beta}(i,i+3)$</td>
<td>2.5-4.4</td>
</tr>
</tbody>
</table>

3.19 The Sequential Assignment of Cross-peaks

Assigning $^1$H nuclei resonances to specific amino acids of a peptide is a necessary task which must be completed before detailed structural analysis can be undertaken. Central to the sequence-specific assignment of $^1$H resonances is the use of the *sequential assignment* procedure proposed by Wüthrich and co-workers (Billeter *et al.*, 1982). In essence, it employs the combined analysis of TOCSY and NOESY experiments. Sequence-specific assignments can be achieved using through-space $^1$H-$^1$H interactions (obtained from the NOESY spectrum) to identify neighbouring amino acid residues. A seventeen residue peptide, uperin 3.6 (GVIDA AKKVV NVLKN LF) is used as an illustrative example.
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The first step involves the identification and assignment of proton resonances according to their respective residue type using the TOCSY experiment. For example, the side-chains protons of Gly, Ala, Ile, Leu, Val, Pro, Lys, Arg and Thr residues give unique connectivity patterns which can usually be identified unambiguously. By using the TOCSY spectrum alone, it is possible to assign most of the peptide amide protons couplings to their respective \( \alpha \) and side-chain protons (Fig. 3.17).

![TOCSY spectrum showing NH-side chain \(^1\text{H}\) region of of uperin 3.6 in TFE/H\(_2\)O (1:1 by vol.), pH 2.4, 25°C. (\( \alpha \) and side-chain protons are indicated for each residue).](image)

Ambiguity occurs when two or more of the same type of residue is present in the peptide. For example, two asparagine (N) residues are present at positions 11 and 15 in uperin 3.6. To resolve this problem, the NOESY experiment is employed.
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The next step involves the sequential assignment of \(^1\)H resonances with respect to the peptide sequence by analysing \(d_{NN}(i,i+1)\) NOESY cross-peaks (Fig. 3.18). In \(\alpha\)-helices, the \(d_{NN}(i,i+1)\) distances are typically about 2.8 Å and thus show up as intense NOEs (Table 3.1). This allows the elucidation of the chemical shift of the adjacent amide proton resonance. Using this information, the TOCSY experiment confirms the particular residue type by analysis of the side-chain \(^1\)H pattern. This procedure is repeated sequentially along the length of the peptide.

Fig. 3.18 NOESY showing the NH-NH region of uperin 3.6 in TFE/H\(_2\)O (1:1 by vol.), pH 2.4, 25°C. NOEs between sequential NH protons of residues 8-17 are shown.
Spin systems can thus be sequentially assigned to each individual residue in the primary sequence. When the NH chemical shift of each residue has been determined, the TOCSY spectrum can be employed again to establish the chemical shifts of every proton in the peptide.

When all the $^1$H nuclei have been assigned, inter-proton distances can be calculated from NOESY cross-peak volumes as discussed in Section 3.16. This, with the angular restraints obtained from 1D NMR (Section 3.8) are used as structural constraints during molecular dynamics calculations as discussed in the following section.

### 3.20 Restrained Molecular Dynamics

Molecular dynamics (MD) is the science of simulating the motions of a system of particles over a period of time. This technique is now extensively employed for studying structure-energy relationships of large particles, in particular, biological molecules (Sternberg, 1996). In addition, MD can also be used for carrying out energy optimisation procedures in order to minimise the strain energy in calculated structures (McCammom and Harvey, 1987; Moore et al., 1988; Karplus and Petsko, 1990).

Molecular dynamics simulates the motions of a system of atoms with respect to the forces which are present based on classical Newtonian equations of motion solved as a function of time (Brünger et al., 1986; Grant and Richards, 1995):

$$ F_i = m_i a_i $$
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where $F_i$ is the force, $m_i$ is the mass and $a_i$ is the acceleration of an atom $i$. By expressing acceleration as the second derivative of displacement $r_i$,\

$$F_i = m_i \frac{d^2 r_i}{dt^2}$$

Rearranging,

$$\frac{F_i}{m_i} = \frac{d^2 r_i}{dt^2}$$

Integrating with respect to time $t$,

$$(F_i/m_i)t + c_1 = \frac{dr_i}{dt}$$

where $c_1$ is a constant. Since $a_i = F_i/m_i$, this equation can be rewritten as:

$$\frac{dr_i}{dt} = a_i t + c_1$$

When $t = 0$, the velocity $dr_i/dt$ is given by $c_1$. Further integration with respect to time gives:

$$r_i = \frac{1}{2} a_i t^2 + c_1 t + c_2$$

............... (1)

where $c_2$ is the current position of the atom.

So far no account has been taken of the thermal motion of the atoms. The temperature $T$ of the system can be calculated from the mean kinetic energies of all the atoms in the system:

$$(3N/2) k_B T = \sum_{i=1}^{N} \frac{1}{2} m_i \nu_i^2$$

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where $k_B$ is the Boltzmann's constant, $m_i$ and $v_i$ are the mass and velocity of atom $i$ respectively. $N$ is the total number of atoms ($3N$ being the number of degrees of freedom of the system). The system is heated by randomly assigning initial velocities $(v_i)$ to all atoms according to a Maxwellian distribution for the given temperature (Berendsen, 1984). Once the initial velocities have been assigned, the molecular dynamics simulation is self-perpetuating; acceleration of an atom $i$ can be calculated from the forces according to the potential used:

$$a_i = F_i / m_i = (dE_i / dr_i) / m_i$$

Therefore, equation 3 allows calculation of the future position or displacement $r_i$ of an atom at time $t$ from its initial velocity $v_i$.

### 3.21 Potential Energy Terms

To perform calculations on every single atom in a large molecule will require extremely long computing times, even using modern-day supercomputers. A set of potential energy functions have been developed to simplify the calculations. Briefly, these functions consist of potential energy terms describing bonding and non-bonding interactions between nuclei (Flores and Moss, 1991). From this, the potential energy $E$ of a molecule can be described as a complex function of Cartesian coordinates of all its atoms being studied, i.e., $E = E(r_1, r_2, r_3, ..., r_N)$ from which the potential energy function, $E_{\text{total}}$ is derived. $E_{\text{total}}$ consists of a number of terms:

$$E_{\text{total}} = E_{\text{bonds}} + E_{\text{angles}} + E_{\text{improper}} + E_{\text{repel}} + E_{\text{NOE}} + E_{\text{edih}}$$

The first four empirical energy terms describe physical interactions between atoms.
Bonded interactions are defined by the $E_{\text{bond}}$, $E_{\text{angles}}$ and $E_{\text{improper}}$ terms which keep bond lengths ($b$), bond angles ($\theta$) and dihedral angles ($\phi$) at their equilibrium values respectively. In addition, $E_{\text{improper}}$ serves to maintain planarity and chirality, i.e., peptide bonds are considered trans planar while aromatic rings are considered planar. These terms are either harmonic or sinusoidal and are summarised as follows:

$$E_{\text{bond}} = \sum_{\text{bonds}} K_b (b-b_0)^2$$

$$E_{\text{angles}} = \sum_{\text{angles}} K_\theta (\theta-\theta_0)^2$$

$$E_{\text{improper}} = \sum_{\text{improper}} K_\phi [1 + \cos n\phi ]$$

$K_b$, $K_\theta$ and $K_\phi$ are force constants which are employed as weighting factors that can be varied during the course of the experiment. $b_0$ and $\theta_0$ are the equilibrium bond lengths and angles respectively and $n$ is the periodicity of the rotation.

Non-bonded interactions are represented by the van der Waals repulsion term $E_{\text{repel}}$:

$$E_{\text{repel}} = \begin{cases} 
0 & \text{if } r \geq r_{\text{min}} \\
K_{\text{repel}} (r^2_{\text{min}} - r^2)^2 & \text{if } r < r_{\text{min}}
\end{cases}$$

where $K_{\text{repel}}$ is the van der Waals repulsion force constant and $r_{\text{min}}$ is the sum of the van der Waals radii between a pair of nuclei.

The last two terms, $E_{\text{NOE}}$ and $E_{\text{coul}}$ do not correspond to any real physical force but are a means of including NMR information. Both terms allow for the introduction
of additional inter-proton distance and angular restraints during computation. $E_{\text{NOE}}$ contains NOESY restraints and has the effect of restraining proton pairs that exhibit NOE interactions to their respective coordinates $r_i$ and $r_j$. As a consequence, their measured inter-proton distance $r_{ij}$ would be restrained to a specific distance during computation. These potentials are pseudo-square-well potentials and are defined as follows:

$$
E_{\text{NOE}} = \begin{cases} 
K_{\text{NOE}} (r_{ij} - r_{ij}^u)^2 & \text{if } r_{ij} > r_{ij}^u \\
0 & \text{if } r_{ij}^l \leq r_{ij} \leq r_{ij}^u \\
K_{\text{NOE}} (r_{ij} - r_{ij}^l)^2 & \text{if } r_{ij} < r_{ij}^l 
\end{cases}
$$

where $r_{ij}^u$ and $r_{ij}^l$ are the upper and lower limits of the target distances respectively and $K_{\text{NOE}}$ is the NOE force constant. $r_{ij}^l$ is typically set to 1.79Å, the sum of the van der Waals radii of two protons.

$E_{\text{cdih}}$ contains $^3J$ coupling information restraints based on, for example, high-resolution 1D $^1$H spectra and has the effect of restraining $\phi$ dihedral angles, e.g., $-60 \pm 30^\circ$ for $^3J < 5$ Hz. Similarly, dihedral angle restraints can be incorporated into pseudo-square-well potentials of the form:

$$
E_{\text{cdih}} = \begin{cases} 
K_{\text{cdih}} (\phi_i - \phi_i^u)^2 & \text{if } \phi_i > \phi_i^u \\
0 & \text{if } \phi_i^l \leq \phi_i \leq \phi_i^u \\
K_{\text{cdih}} (\phi_i - \phi_i^l)^2 & \text{if } \phi_i < \phi_i^l 
\end{cases}
$$

where $\phi_i^u$ and $\phi_i^l$ are the upper and lower limits/bounds of the dihedral angle restraints, $\phi_i$ is its calculated value and $K_{\text{cdih}}$ is the torsion force constant.

The essence of restrained molecular dynamics (RMD) has been described in reasonable detail. The term ‘restrained’ is used because NMR angular and distance
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constraints are incorporated during MD calculations. An energy minimisation procedure that produces more 'realistic' 3D structures after MD calculations is described in the next section.

3.22 Simulated Annealing

The way the energy of a molecule varies with small changes in its structure is specified by its potential energy surface (PES). A PES is basically a mathematical relationship that links molecular structure to the corresponding potential energy. The PES can be used to illustrate the relative stabilities of different, but possible structures a molecule can adopt (Foresman and Frisch, 1993). The PES of any system consists of many dimensions but can be schematically represented in three dimensions as shown in Fig. 3.19. A typical PES contains several local potential minima but only one true global minimum.

![Diagram of a hypothetical PES](image)

**Fig. 3.19** Schematic representation of a hypothetical PES showing local minima and the global minimum.
Calculated structures based entirely on RMD may not be entirely accurate as their potential energies may represent those existing as local minima rather than the global minimum. As a consequence, these calculated structures may not correspond to the most stable conformation and may be quite different to that of the global minimum.

To circumvent this problem, simulated annealing (SA) is introduced after RMD calculations in order to locate the global minimum (Nilges et al., 1988a, 1988b and 1991). This involves simulating the introduction of kinetic energy to the molecule by raising the temperature of the system (typically to 2000 K) followed by slow cooling (to 100 K). This enables any molecule that is initially ‘trapped’ in a local minima to ‘jump out’ and reach the global minimum (shown schematically in Fig. 3.20).

---

**Fig. 3.20** Schematic diagram showing a molecule [represented by the black circle (a)] trapped in a local minimum. Introduction of kinetic energy enables the molecule to ‘jump’ out of the local minimum (b) into the global minimum (c).
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3.23 Calculation Protocol

The typical procedure of obtaining structures consistent with the NMR data constraints involves generating an ensemble of structures with random conformations using known experimental geometries (e.g. bond lengths and angles) but with random dihedral angles (Nilges et al., 1988a). Such structures are usually incompatible with most of the NMR data. The next step involves introducing inter-proton distance and \( \phi \) dihedral angle restraints based on NOE intensities and coupling constants respectively before subjecting them to RMD and SA calculations. A flow chart showing the computational strategy is shown in Fig. 3.21.

![Fig. 3.21 Calculation strategy used to solve 3D structures.](image)

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3.24 Floating Stereospecific Assignments

In the past where cases of distinguishable but unassignable resonances of methylene (-CH\textsubscript{2}-) and isopropyl groups [-CH(CH\textsubscript{3})\textsubscript{2}] were observed, distance constraints were measured relative to a pseudoatom, located centrally with respect to these protons (Wüthrich, 1983). An appropriate correction term was added to allow for the maximum possible error since experimental NOEs involve protons rather than the pseudoatom. This results in the loss of valuable NOE information. The floating stereospecific assignment method was developed to circumvent such problems (Folmer et al., 1997). Instead of using pseudoatoms, NOEs are measured for both individual resonances of a methylene or isopropyl group which are arbitrarily assigned (i.e., simply denoted H\textsubscript{β2} and H\textsubscript{β3} in the case of a β-methylene group). Force constants (e.g. K\textsubscript{β} and K\textsubscript{ρ}) used to enforce a defined chirality at the prochiral centre are initially reduced to allow stereo-related nuclei or methyl groups to ‘float’ between the pro-R and pro-S configurations to find the energetically most favourable conformation. These are slowly increased during the course of RMD/SA calculations. The final positions of the respective protons will denote the correct stereospecific assignment. The advantage of such an approach is that no correction factors have to be introduced and therefore no NOE information is lost (Weber et al., 1988; Holak et al., 1989).

3.25 Quality of Generated Structures

How is it determined whether a particular generated structure is ‘realistic’ and comparable to the final ensemble of generated structures? The conventional way to
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gauge this is by calculating the root-mean-square deviation (RMSD) of a selection of structures (typically twenty of the lowest energy structures are chosen). This value is generated by superimposing the centroids of the chosen structures and then calculating an ‘average structure’ that represents the overall ensemble:

$$\text{RMSD} = \left[ \frac{1}{N} \sum_{i=1}^{N} (r_i - r'_i)^2 \right]^{1/2}$$

where $N$ is the number of atoms being compared, $r_i$ and $r'_i$ are the atomic co-ordinates for the respective calculated and average structures respectively. Generally, a RMSD of less than 2.0 Å indicates that the calculated structures are structurally similar and close to the calculated ‘average structure’. If the RMSD is too large, then it is necessary to re-analyse the NMR spectra. Large proteins typically show RMSD of the order of 1.5 Å for all atoms in the molecule (Evans, 1995).

3.26 Angular Order Parameters

The angular order parameter ($S$) is a statistical parameter used to describe how well-defined dihedral angles ($\phi$ and $\psi$) of a peptide are over a family of structures. For example, the value of $S$ for the dihedral angles, $\alpha_j$, of residue number $i$ (where $\alpha = \phi$ or $\psi$) is defined as:

$$S = \frac{1}{N} \left| \sum_{j=1}^{N} \alpha_{i,j} \right|$$
where $N$ is the total number of structures, $j$ represents the number of the calculated structures ($j = 1, 2, \ldots, N$) and $\alpha_i \hat{j}$ is a 2D unit vector with phase equal to the dihedral angle $\alpha_i$. The relation of $S$ to the standard deviation of the dihedral angles ($\sigma$) is given by:

$$1 + 0.5 \log S = \cos (\sigma/2)$$

and is graphically represented in Fig. 3.22 (Hyberts et al., 1992). If the dihedral angles are the same for all the structures, then $S$ will have a value of 1.00. A value of $S = 0.9$ corresponds to a $\sigma$ of $\pm 24^\circ$ while a value of $S = 0.99$ corresponds to a $\sigma$ of $\pm 8^\circ$. A value of 0.01 indicates a totally disordered structure.

**Fig. 3.22** Plot of angular order parameter ($S$) vs. standard deviation ($\sigma$).

Why is the use of $S$ favoured over the more conventional standard deviation? The reason is because $S$ is easier to calculate and it gives a better 'mental picture' when
describing the precision of dihedral angles of a particular structure. It clearly defines the two limits: a totally well-defined dihedral angle (S=1) and a completely random distribution (S=0). In contrast, the standard deviation is undefined for a completely random distribution (Pallagy et al., 1993).

3.27 Ramachandran Plots

Most combinations of $\phi$ and $\psi$ angles do not occur as they lead to unfavourable steric interactions between atoms of adjacent residues. This reduces the number of possible conformations a peptide can adopt. Possible combinations of $\phi$ and $\psi$ angles that do not lead to clashes can be plotted on a conformation map known as a Ramachandran plot (Fig. 3.23) (Ramachandran et al., 1963; Ramachandran and Sassiekharan, 1968; Zubay, 1986). The plot is divided into shaded regions which indicate if a particular (non-proline) residue adopts a particular secondary structure. These regions are basically population density plots produced from known experimental $\phi$ and $\psi$ angles as determined from the analysis of 121,870 non-glycine residues obtained from 463 protein structures available from the Brookhaven databank (Morris et al., 1992). Shaded regions labelled A, B and L represent 'favoured' combinations of $\phi$ and $\psi$ angles corresponding to dihedral angles found in $\alpha$-helices, $\beta$-sheets and left-handed $\alpha$-helices respectively. Shaded regions labelled a, b and l represent 'allowed' $\phi$ and $\psi$ combinations while regions labelled $\sim$-a, $\sim$-b and $\sim$-l represent 'generous' $\phi$ and $\psi$ combinations. Unshaded regions represent disallowed combinations. Proline and glycine residues were omitted from the study because of their atypical dihedral angle distribution. Glycine residues are roughly
symmetrical around the centre of the Ramachandran plot due to the ability of glycine to adopt both right-handed and left-handed versions of any allowed conformation. Values of $\phi$ and $\psi$ angles for each residue (with the exception of glycine and proline) can be obtained from an ensemble of calculated structures and plotted to check for dihedral angle abnormalities. This method provides another way to assess the degree of 'correctness' of calculated structures.

Fig. 3.23 A Ramachandran plot. (Diagram modified from Richardson, 1981).
3.28 Membrane-Mimetic Media

To determine 3D conformations of membrane-disrupting peptides, it is necessary to conduct structural studies using suitable membrane-mimicking solvents or phospholipids. Studies have shown that 2,2,2-trifluoroethanol (TFE, Fig 3.24) is a good candidate because it promotes intra-molecular hydrogen bonding in regions of a peptide with \( \alpha \)-helical propensity but does not induce structures in regions lacking structural tendency (Goodman et al., 1971; Marion et al., 1988; Bruch and Gierasch, 1990; Blanco et al., 1994; Gesell et al., 1997). Thus, TFE is not a helix-inducing solvent, i.e., it will not induce helix formation independently of the sequence. It is rather a helix enhancing solvent which stabilises helices in peptides with helical propensity (Dyson et al., 1992; Sönnichsen et al., 1992). Previously, it has been shown that glucagon analogs adopt similar conformations in TFE/water mixtures and in phospholipid micelles, suggesting that TFE/water mixtures are suitable membrane mimicking solvents (Braun et al., 1983; Clore et al., 1986).

TFE possesses several properties responsible for its unique helix stabilising capabilities. Firstly, its dielectric constant is only about a third of that of water, enhancing electrostatic interactions between charged groups of the peptide (Llinas and Klein, 1975; Rajan and Balaram, 1996). Secondly, TFE strengthens intra-molecular hydrogen bonding in a peptide as it is a significantly weaker base (poorer proton acceptor) than water (TFE pKa \( \approx 12.4 \) vs. \( \approx 15.7 \) for water) (Nelson and Kallenbach, 1986). This reduces the amount of water molecules around the peptide. As a consequence, intra-molecular hydrogen bonds between
amide protons and carbonyl oxygen atoms in the peptide remain unperturbed, stabilising the overall secondary structure (Berliner and Reuben, 1980; Storrs et al., 1992; Brooks and Nilsson, 1993; Cammers-Goodwin et al., 1996). Based on these factors, peptides with significant helical propensity (amphipathic peptides in particular) often adopt helical structures in 30-50% (by vol.) TFE/water mixtures (Lehrman et al., 1990; Zhou et al., 1990). However, the use of TFE as a membrane-mimetic is still a subject of contention.

Detergent micelles such as sodium dodecyl sulphate (SDS), dodecylphosphocholine (DPC), 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Fig. 3.24) are now commonly utilised as membrane-mimetics to study peptide-membrane interactions (Brown and Wüthrich, 1977; Lauterwein et al., 1979; Brown and Wüthrich, 1981; Brown et al., 1982; Kaiser and Kezdy, 1983; Kaiser and Kezdy, 1984; Deber and Li, 1995; Blondelle et al., 1997).

![Fig. 3.24 Structures of TFE, SDS, DPC, DHPC and DMPC.](image-url)
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Detergent micelles are generally considered to provide a more ‘realistic’ membrane-mimetic environment for structural studies compared to TFE. The structure-inducing driving forces in isotropic solvents such as TFE and the natural, non-isotropic membrane-peptide interface are generally different. For example, TFE can weaken non-local hydrophobic interactions and favour local polar interactions such as the intra-molecular hydrogen bonding in peptides (Shiraki et al., 1995). In artificial lipid membranes, the hydrophobic interactions between amino acid sidechains and lipid alkyl chains have been suggested to be the primary forces driving secondary structure formation. (Blondelle et al., 1997). As a result of different driving forces, the solution structure of membrane-lytic peptides may be quite different. In the following chapters, TFE and the above mentioned phospholipids will be used as membrane mimetics. In particular, Chapter 5 will compare how TFE and DPC micelles influence the solution structure of the membrane-disrupting peptide maculatin 1.1.
Chapter 4
Orientation Studies
4.1 Solid-State NMR Spectroscopy

Solid-state NMR spectroscopy is fast becoming one of the methods for examining the structure and function of biological systems. In particular, it is extremely useful in studies involving membrane-associated peptides and proteins (Opella, 1997; Marassi and Opella, 1998). Both solid-state and solution NMR spectroscopy provide complementary windows on molecular information because the effects of rapid molecular reorientation present in solution are absent in the relatively immobile samples accessible to solid-state NMR methods (Opella et al., 1991). This chapter describes how solid-state NMR spectroscopy is employed to study the orientation and topology of antibiotic peptides when associated with lipid bilayers in an effort to shed light into their membrane-lytic mechanisms.

4.2 The Origin of Chemical Shifts and Powder Patterns

When a nucleus \((I \neq 0)\) is introduced to an external magnetic field \(B_0\), the moving electric charges of the surrounding electron cloud induce a local magnetic field which opposes \(B_0\). The effective field experienced by the nucleus will become:

\[
B_{\text{effective}} = B_0 - (\sigma)B_0
\]

The nucleus is said to be ‘shielded’ and the extent of shielding is given by the shielding constant, also known as the chemical shift tensor \((\sigma)\). \(\sigma\) consists of three principal components: \(\sigma_{11}\), \(\sigma_{22}\) and \(\sigma_{33}\). These principal elements are basically vectors used to characterise the 3D nature of shielding experienced by the nucleus.
and are prerequisites for the interpretation of spectral features. The chemical shift tensor of the $^{15}$N nucleus of a peptide bond has been determined (Mehring, 1983; Wu et al., 1995). Fig. 4.1 illustrates the principal elements relative to the orientation of the N-H peptide bond and $B_0$.

![Diagram](image)

**Fig. 4.1** The principal elements used to relate the orientation of the N-H peptide bond relative to $B_0$ (not drawn to scale). Diagram modified from Evans (1995).

In the solution-state, the principal elements are averaged out due to rapid molecular tumbling and only the isotropic shift tensor ($\sigma_{\text{iso}}$) is observed:

$$\sigma_{\text{iso}} = 1/3 (\sigma_{11} + \sigma_{22} + \sigma_{33})$$

Hence, only a single, averaged peak is observed for each respective nucleus in the molecule. In solids or immobile systems, however, molecular motion is severely restricted so little averaging occurs. Therefore, the observed chemical shift ($\sigma_{\text{observed}}$) for a nucleus is described as the sum of its isotropic and anisotropic parts:

$$\sigma_{\text{observed}} = \sigma_{\text{iso}} + \sigma_{\text{aniso}}$$

where $\sigma_{\text{aniso}}$, the chemical shift anisotropy, is an orientation-dependent parameter.
Thus, each nucleus with a unique orientation with respect to \( B_0 \) will give rise to a particular resonance frequency. In immobile powders, the nucleus of interest (e.g., \(^{15}\)N) will have totally random orientations with respect to \( B_0 \). This results in the formation of a broad, continuum of peaks known as a *powder pattern*. The shape of a powder pattern depends on the magnitude of the principal elements involved. When two of the principal shielding elements are identical, we obtain an axially symmetric powder pattern like that shown in Fig. 4.2.

![Diagram](image)

**Fig. 4.2** \(^{15}\)N ('H decoupled) NMR powder pattern of randomly oriented \(^{15}\)N-Ala15 labelled magainin 2. (Diagram modified from Bechinger et al., 1992).

When the N-H bond axis is parallel to \( B_0 \), the \(^{15}\)N resonance appears downfield near the principal element \( \sigma_{33} \). If the N-H bonds are perpendicular to \( B_0 \), this results in upfield resonances close to that of the principal elements \( \sigma_{11} \) and \( \sigma_{22} \). It is the observation of resonance frequencies near either extremes of the powder pattern which allows for the qualitative determination of peptide orientations in uniaxially aligned phospholipid bilayers with respect to \( B_0 \) (Teng and Cross, 1989; Bechinger et al., 1991).
4.3 Mechanical Orientation of Phospholipid Bilayers

Phospholipid bilayers can be oriented by shear mechanical forces when multilamellar vesicles are sandwiched between two plates. Geometric considerations suggest that the use of large glass microscope coverslips results in optimal bilayer alignment since edge effects are minimised (de Vries and Berendsen, 1969; Moll and Cross, 1990; Ketchum et al., 1993; Grobner et al., 1997; Marassi et al., 1997). Experiments have shown that the bilayers are large ‘onion-shaped’ structures separated by 10-30 Å thickness of water (Fig. 4.3a) (Rand and Parsegian, 1989; Cevc, 1990; Sanders et al., 1994). When a phospholipid bilayer is oriented with its axes of symmetry parallel (or perpendicular) to \( B_0 \), a single resonance peak will be observed as shown in Fig. 4.3b.

Fig. 4.3 Schematic representations of (a) oriented bilayers formed by mechanical alignment of lipids between glass plates, (b) \(^{31}\)P NMR spectrum of uniaxially oriented phospholipid bilayers between glass plates oriented with bilayer normal perpendicular to \( B_0 \).
4.4 Peptide Orientation in Phospholipid Bilayers

The orientation of a peptide incorporated into a lipid bilayer can be deduced from solid-state NMR using $^{15}$N-labelled peptides. In these experiments, the chemical shift of the $^{15}$N peptide amide resonance reflects the orientation of the labelled $^{15}$N-H peptide bond relative to the bilayer normal and $B_0$. Since the amide N-H bond of an $\alpha$-helix is aligned approximately along its length (Fig. 3.16), it is possible to deduce the general orientation of the helical peptide with respect to the lipid bilayer surface. For example, if the N-H bond is found to be oriented parallel to $B_0$, it would indicate that the peptide is oriented with the $\alpha$-helical axis parallel to $B_0$. Fig. 4.4a shows a simulation of the $^{15}$N powder pattern of $^{15}$N-Ala15 labelled magainin 2 while Fig. 4.4b shows the spectrum of an aligned sample oriented with the bilayer normal parallel to $B_0$. The former gives a single resonance near the $\sigma_{11}$ and $\sigma_{22}$ edge, indicating that the N-H amide bond of Ala15 (and those of other residues in the peptide) are oriented approximately perpendicular to the bilayer normal. This suggests that magainin 2 adopts the carpet mechanism (shown schematically in Fig. 4.4c). In contrast, the resonance of $^{15}$N-Ala15 labelled melittin (aligned in a phospholipid bilayer) appears near $\sigma_{33}$, therefore, the N-H bond of Ala15 is oriented approximately parallel to the bilayer normal (Fig. 4.4d and 4.4e). This suggests that melittin forms transmembrane pores via the channel mechanism (Fig. 4.4f) as discussed in Chapter 2. Note that for simplicity, Fig. 4.4c and 4.4f illustrate only one peptide to represent the relative orientation. In reality, the peptides are most likely to be associated as oligomers, with each peptide having a similar orientation.
Hence, by using $^{15}$N labels, the orientation of membrane-interacting peptides relative to the bilayer can be determined using solid-state NMR. Peptide-lipid interactions can further be investigated using deuterium NMR and this will be discussed in the next section.
4.5 Deuterium Quadrupolar Splittings

Deuterium ($^2$H) quadrupolar splittings reflect the dynamic and orientational order of deuterated molecules including membrane phospholipids. Since $^2$H has $I = 1$, a doublet is observed for each $^2$H resonance in the sample. For deuterated phospholipids, the magnitude of the splitting ($\Delta \nu_Q$) is dependent on the average orientation of the C-2H vector with respect to $B_0$ and can be defined by the equation:

$$<\Delta \nu_Q> = Q <S_{CD}(n)>$$

where $< >$ represent the time average of molecular conformations and orientations on the NMR timescale, $Q$ is the quadrupolar coupling constant (typically 170 kHz for C-2H bonds) and $S_{CD}$ is the orientational order parameter and is defined as:

$$S_{CD}(n) = \frac{1}{2} <3\cos^2\theta_n - 1>$$

where $\theta_n$ is the angle between the for C-2H bond for the (n)th carbon position and the axis of symmetry of the lipid acyl chain (Seelig and Niederberger, 1973; Davies, 1983).

When a peptide penetrates a phospholipid bilayer, the lipid chains become more disordered and mobile, causing a decrease in $<S_{CD}(n)>$. This decreases the magnitude of deuterium splitting and results in the narrowing of the $^2$H powder pattern. Fig. 4.5 illustrates the decrease in $^2$H order parameter of DMPC-$^2$H$_{54}$ bilayers after the addition of magainin. The broad wings with the largest splitting arise from the relatively immobile methylene doublets near the hydrocarbon-water interface.
Chapter 4

The less intense doublets with smaller splittings result from the stepwise decrease in order parameter occurring near the terminal portion of the lipid chain. The relatively mobile terminal methyl groups give rise to an intense doublet with the smallest splitting (Seelig and Niederberger, 1973; Howard and Opella, 1996).

Fig. 4.5 $^2$H NMR spectra of DMPC bilayer (a) without magainin (b) after adding magainin. (Diagram modified from Bechinger et al., 1992).

4.6 Chapter Summary

This chapter describes how solid-state NMR spectroscopy can be employed to determine the orientation of membrane-disrupting peptides when associated with lipid bilayers, providing valuable insights into their mechanism of action. The next chapter will deal with one such peptide, maculatin 1.1, in greater detail.
Chapter 5
Maculatin 1.1 and Analogues

Litoria genimaculata
Chapter 5

5.1 Maculatin 1.1 and Analogues

The dermal secretions of Australian tree frogs (*Litoria caerulea, L. chloris, L. ewingi, L. gilleni, L. splendida* and *L. xanthomera*) (see Fig. 1.8) all contain wide-spectrum antimicrobial peptides of the caerin 1 family as discussed in the first chapter. Nine members of the caerin 1 family have been identified to date; the most widespread of these peptides is caerin 1.1 which has the sequence:

\[
\text{GLLSV LGSVA KHVL}_{12} \text{HVVPV IAEHL-NH}_2
\]

2D NMR spectroscopic studies in TFE/water (1:1 vol.:vol.) together with molecular dynamics calculations indicate that caerin 1.1 adopts two well-defined \( \alpha \)-helices (Leu2 to Lys11 and Val17 to His23) separated by a hinge region of greater flexibility. Pro15 is an integral part of this region due to the flexibility that this residue imparts because of the absence of a hydrogen bonding NH group (Wong *et al.*, 1997). A computed low-energy conformation of caerin 1.1 is shown in Figure 5.1; this representation shows hydrophilic and hydrophobic zones on opposite sides of the peptide backbone. The amphipathic nature of caerin 1.1 together with the flexibility imparted by the central hinge region allows the basic peptide to interact with a variety of membrane surfaces, resulting in its wide-spectrum antibiotic activity.
Fig. 5.1 **A low-energy structure of caerin 1.1.** The marked amphipathic nature of the helical peptide is apparent from this representation with the hydrophobic side above and hydrophilic side below the polypeptide backbone, which is represented by the ribbon. Hydrophilic groups are shown in blue. The proline pyrrolidine ring is coloured purple.

Recently, another antimicrobial peptide, maculatin 1.1, was isolated from the Australian tree frog *Litoria genimaculata* (Rozek *et al.*, 1998). These frogs are found in the coastal rainforests of northern Queensland and New Guinea and typically grow up to 5cm in length (Barker *et al.*, 1995) (Fig. 5.2). Maculatin 1.1 has the primary structure:

```
GLFGV LAKVA AHVVPAIAEH F-NH₂
```

It is apparent from this sequence that maculatin 1.1 shares significant sequence similarity to caerin 1.1 except that in the former, four of the central residues (His12 to Pro15 of caerin 1.1) are missing, including the central proline residue within the hinge region in caerin 1.1. It is therefore conceivable that the solution structure of maculatin 1.1 is different from that of caerin 1.1, possibly via adoption of a more
rigid helix along its entire length. Interestingly, both caerin 1.1 and maculatin 1.1 are subjected to proteolytic cleavage which removes the first two residues of each peptide after it has been on the skin for about five to ten minutes. As a result, the bioactivity of the peptide is destroyed (Steinborner et al., 1997a; Steinborner et al., 1997b).

![Image of frog and map of Australia]

**Fig. 5.2** (a) *Litoria genimaculata* (b) a rainforest in northern Queensland (c) geographical distribution of the frog (coloured pink).

Although shorter in length than caerin 1.1 and lacking a Pro15 of caerin 1.1, maculatin 1.1 has been shown to possess similar broad-spectrum antibiotic activities against a range of pathogens (Table 5.1). The primary goal of the work presented in this chapter is to obtain insights into the mechanism of action of maculatin 1.1 and
its structure-activity relationship (SAR) when interacting with biological membranes. Towards this goal, a number of synthetically modified analogues have been synthesised in order to evaluate their ability to inhibit the growth of various pathogens:

First, the all D-amino acid form of maculatin 1.1 [(D) M1.1] was synthesised. All ‘D’ amino acids are known to form left handed α-helices as opposed to the right hand α-helices formed by naturally-occurring ‘L’ amino acids. Thus, in terms of chiral recognition, an enzyme’s active site which has evolved to recognise a right-handed helix will not recognise the same peptide consisting of ‘D’ amino acids. If both ‘D’ and ‘L’ forms are shown to possess identical biological activities, it would indicate that the peptides interact via a gross conformational feature (e.g. an amphipathic helix) and not due to some specific feature of the peptide.

Second, many bacteria have negatively charged phospholipid head groups on their membrane surface making them specific to cationic cytolytic peptides as mentioned in chapter 2. If this is true, then increasing the number of negative charges on the peptide will reduce its bioactivity because of a reduction of electrostatic attraction between the peptide and the membrane. This is investigated by synthesising the C-terminal carboxyl (-COOH) form of maculatin 1.1, i.e. without an amidated group (M1.1 acid).

Third, we would like to ascertain the effects of replacing proline with the helix forming residue, alanine, in order to determine if proline is crucial for the
antimicrobial activity of maculatin 1.1. This was investigated by synthesising maculatin 1.1 with Pro15 substituted with an alanine (A15 M1.1). The reason why proline was chosen for replacement is due to its unique properties: proline is an imino acid and thus lacks an amide proton for hydrogen bonding (see Table 3.1). As a consequence, proline cannot participate in hydrogen bonding to proton acceptors such as backbone carbonyl oxygen atoms in the peptide chain. In an α-helix, amide protons of the $i+4$ residue will hydrogen-bond to the carbonyl oxygen of the $i$ residue. Proline will therefore disrupt the hydrogen-bonding pattern of a peptide chain. Also, due to the highly rigid nature of the pyrrolidine ring, proline sterically prevents hydrogen bonding between the amide proton of its $i+1$ neighbour to the carbonyl oxygen in the preceding turn of the helix (Vanhoof et al., 1995). Both consequences allow proline to act as a ‘helix breaker’: as a result, a flexible hinge or ‘kink’ will be created in the α-helix (Kuchel and Ralston, 1988).

Lastly, we will investigate how a peptide’s helical dipole moment affects the mechanism of bactericidal action. This is done with a synthetic retro maculatin 1.1 [(R) M1.1] where the L-amino acid peptide has its primary sequence reversed (i.e., the peptide bond directions are reversed). This has the effect of inducing an equal but opposite helical dipole moment as compared to the natural isomer. If such a dipole moment makes a critical contribution to its bioactivity, then the retro isomer will be expected to lose its activity.

These synthetic analogues and the primary sequences of other natural maculatins (1.1, 1.1.1 and 1.2) (Steinborner et al., 1998) are shown on the next page.
Chapter 5

Caerin 1.1  
Maculatin 1.1  
D-Maculatin 1.1  
Maculatin 1.1 acid  
Maculatin 1.1.1  
Ala15 maculatin 1.1  
Retro maculatin 1.1  
Maculatin 1.2

GLLSV LGSVA KHVLP HVVPV IAELH-NH₂  
GLFVG LAKVA AHVVVP AIAEH F-NH₂  
GlfvG lakva ahvvp aiaeh f-NH₂  
GLFVG LAKVA AHVVVP AIAEH F-OH  
FVG LAKVA AHVVVP AIAEH F-NH₂  
GLFVG LAKVA AHVVVA AIAEH F-NH₂  
FHEAI APVHH AAVKA LGVFL G-NH₂  
GLFVG LAKVA AHNMP AIAEH FQA-NH₂

The bioactivities of the above mentioned peptides are listed in Table 5.1.

### Table 5.1 Antimicrobial activities of caerin 1.1, maculatin 1.1 and some synthetic modifications of maculatin 1.1.

<table>
<thead>
<tr>
<th>Organism</th>
<th>C1.1</th>
<th>M1.1</th>
<th>M1.1 (D)</th>
<th>M1.1 acid</th>
<th>M1.1.1</th>
<th>A15</th>
<th>M1.1 (R)</th>
<th>M1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Leuconostoc lactis</em></td>
<td>1.5</td>
<td>3</td>
<td>3</td>
<td>50</td>
<td>&gt;100</td>
<td>12</td>
<td>50</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Listeria innocua</em></td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>50</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Pasteurella multocida</em></td>
<td>25</td>
<td>50</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>3-12</td>
<td>6-12</td>
<td>12</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>50</td>
<td>&gt;100</td>
<td>50</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>12</td>
<td>12</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td>12</td>
<td>3</td>
<td>6</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>50</td>
<td>&gt;100</td>
<td>6</td>
</tr>
</tbody>
</table>

aThe data for caerin 1.1 were taken from Wong et al. (1997).
bThe data for maculatin 1.1, 1.1.1 and 1.2 were taken from Steinborner et al. (1998).
cTested against two strains (ATCC type strains 25923 and 29213).
Specific observations which arise from these data are:

(i) the spectrum of activities of caerin 1.1 and maculatin 1.1 is very similar, suggesting that the four central amino acids in caerin 1.1 are not essential for complete activity.

(ii) the antibiotic activities of natural (L) maculatin 1.1 and synthetic (D) maculatin 1.1 are the same within experimental error (to within one dilution factor), indicating that maculatin 1.1, like caerin 1.1, is a membrane-active antimicrobial peptide, i.e., activity is not dependent on interaction with chiral receptor sites or enzymes (Wong et al., 1997).

(iii) the C-terminal carboxyl analogue of maculatin 1.1 is significantly diminished in activity, suggesting that the mechanism of bactericidal action involves electrostatic peptide-membrane interactions. These data support similar SAR studies of other analogues of natural antimicrobial peptides with their amide groups substituted for carboxyl groups at their C-terminus (Li et al., 1988; Callaway et al., 1993; Mor et al., 1994).

(iv) Substituting the Pro15 residue of maculatin 1.1 with an alanine results in a peptide with greatly reduced bioactivity. This suggests that the central ‘kink’ in the helix is necessary for antimicrobial activity.
(v) the retro form of maculatin 1.1 is essentially inactive, suggesting that both H$_2$N-Gly$_1$ and Phe$_{21}$-CONH$_2$ are necessary for the activity of maculatin 1.1, i.e., the helical dipole moment is involved in the bactericidal mechanism. In contrast, similar studies on retro melittin have shown that it retains its antimicrobial properties (Juvvadi et al. 1995). This suggests that the mechanism of bactericidal action of maculatin and melittin could be different.

(vi) maculatin 1.1.1 (which arises from the action of an endogenous endoprotease to remove the first two residues of maculatin 1.1) is essentially inactive (Steinborner et al., 1998) while maculatin 1.2, which has two more residues at its C-terminus than maculatin 1.1, has markedly reduced activity compared to that of maculatin 1.1.

**5.2 Structural Studies**

Maculatin 1.1 and caerin 1.1 do not display any amphipathic character when presented on a Edmundson helical wheel (Fig 5.3). As a consequence, neither exhibit well-defined hydrophobic and hydrophilic faces. However, the proline residue(s) in the central region of these peptides may orient the C-terminal part of the helix into an appropriate conformation to allow the formation of an amphipathic $\alpha$-helix. A rotation of approximately 60° and 140° of the polypeptide chain after Pro15 is required to give such a distribution for maculatin 1.1 and caerin 1.1 respectively (Wong et al., 1997; Chia et al., 2000). Since caerin 1.1 needs to rotate at a greater angle than maculatin 1.1, it may need four additional central residues to enhance its flexibility. This may explain the extra central residues in caerin 1.1.
5.3 Circular Dichroism Studies

CD spectra were acquired on maculatin 1.1 in increasing concentrations from 0 to 50% (by vol.) of TFE in water (see Fig. 5.4). In low amounts of TFE (0-10%), maculatin 1.1 exhibits a far UV CD spectrum characteristic of an unstructured peptide, i.e., a broad minimum at 200 nm was observed. With increasing TFE, the CD spectrum underwent a marked change to one indicative of a predominantly $\alpha$-helical structure, i.e. two minima in the vicinity of 208 nm ($\pi-\pi^*$ transition) and 220 nm ($n-\pi^*$ transition) were observed. The spectra at 40% and 50% TFE showed greatest ellipticity at these minima, indicative of maximum helical conformation.
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Fig. 5.4 CD spectra of maculatin 1.1 at varying concentrations (by vol.) of TFE.

5.4 Nuclear Magnetic Resonance Studies

On the basis of the CD results, NMR spectra of maculatin 1.1 were acquired under conditions which exhibited maximal helical structure, i.e., 50% (by vol.) TFE. Spectra of Ala15 maculatin 1.1 were also acquired under the same conditions whilst a third set of spectra were acquired on maculatin 1.1 dissolved in DPC micelles. In all cases, the 1H-NMR spectrum was assigned via the standard methods discussed in chapter 3. Figs. 5.5a and 5.5b show a region of the TOCSY spectrum and the NH region of the NOESY spectrum of maculatin 1.1 in DPC/water respectively. Lines are drawn between spectra to correlate the series of side-chain proton resonance to sequential $d_{\text{NN}}(i,i+1)$ NOEs observed along the length of the peptide. Weaker $d_{\text{NN}}(i,i+2)$ NOEs were also present in this region. Similar spectra are also illustrated for maculatin 1.1 in TFE/water (Fig. 5.5c and 5.5d) and Ala15 maculatin 1.1 in TFE/water (Fig. 5.5e and 5.5f).

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Fig 5.5  (a) A region of the TOCSY spectrum showing NH to sidechain proton couplings, (b) NH region of the NOESY spectrum of maculatin 1.1 in DPC/water. Connectivities between side-chain and backbone amide protons are indicated.
Fig 5.5  (c) A region of the TOCSY spectrum showing NH to sidechain proton couplings, (d) NH region of the NOESY spectrum of maculatin 1.1 in TFE/water. Connectivities between side-chain and backbone amide protons are indicated.
Fig 5.5 (e) A region of the TOCSY spectrum showing NH to sidechain proton coupling, (f) NH region of the NOESY spectrum of Ala15 maculatin 1.1 in TFE/water. Connectivities between side-chain and backbone amide protons are indicated.
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Cross-peaks in the presence of DPC were observed to be broader than those in TFE/water mixtures, consistent with the incorporation of maculatin 1.1 into the large DPC micelle. The higher pH value of the DPC solution could also contribute to the broader NH resonances due to increased rate of exchange of NH protons with water. Using the $d_{\alpha\alpha}(i,i+1)$ and $d_{\beta\beta}(i,i+1)$ sequential connectivities, the vast majority of $^1H$ resonances of maculatin 1.1 and its Ala15 analogue were assigned under the three solvent conditions. Assignments of these resonances and the $^{13}C$ $\alpha$-CH resonances are tabulated in Table 5.2.

Table 5.2a $^1H$ and $^{13}C$ NMR chemical shifts for maculatin 1.1 in DPC/water, pH 7.0, 37°C.

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>$^\alpha$H</th>
<th>$^\beta$H</th>
<th>others</th>
<th>$^\alpha$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly 1</td>
<td>not observed</td>
<td>not observed</td>
<td>-</td>
<td></td>
<td>not observed</td>
</tr>
<tr>
<td>Leu 2</td>
<td>8.03</td>
<td>4.04</td>
<td>1.82</td>
<td>$\gamma$-CH=1.45 $\delta$-CH$_3$=0.90, 1.01</td>
<td>54.00</td>
</tr>
<tr>
<td>Phe 3</td>
<td>not observed</td>
<td>4.20</td>
<td>3.14, 3.21</td>
<td>Aromatic H=7.27, 7.45</td>
<td>61.02</td>
</tr>
<tr>
<td>Gly 4</td>
<td>8.25</td>
<td>3.85, 3.99</td>
<td></td>
<td></td>
<td>42.99</td>
</tr>
<tr>
<td>Val 5</td>
<td>7.81</td>
<td>4.05</td>
<td>2.25</td>
<td>$\gamma$-CH$_3$=0.94, 1.09</td>
<td>57.80</td>
</tr>
<tr>
<td>Leu 6</td>
<td>8.19</td>
<td>3.94</td>
<td>1.85</td>
<td>$\gamma$-CH=1.49 $\delta$-CH$_3$=0.83</td>
<td>57.51</td>
</tr>
<tr>
<td>Ala 7</td>
<td>8.36</td>
<td>3.92</td>
<td>1.42</td>
<td></td>
<td>54.91</td>
</tr>
<tr>
<td>Lys 8</td>
<td>7.53</td>
<td>4.14</td>
<td>1.98, 2.08</td>
<td>$\gamma$-CH$_2$=1.42, 1.52 $\delta$-CH$_2$=1.67, 1.74 $\varepsilon$-CH$_2$=2.98</td>
<td>58.59</td>
</tr>
<tr>
<td>Val 9</td>
<td>8.13</td>
<td>3.77</td>
<td>2.30</td>
<td>$\gamma$-CH$_3$=0.95, 1.08</td>
<td>65.09</td>
</tr>
<tr>
<td>Ala 10</td>
<td>8.65</td>
<td>3.95</td>
<td>1.47</td>
<td></td>
<td>54.99</td>
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<tr>
<td>Ala 11</td>
<td>7.81</td>
<td>3.78</td>
<td>1.45</td>
<td></td>
<td>56.12</td>
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<tr>
<td>His 12</td>
<td>7.60</td>
<td>4.12</td>
<td>3.14, 3.22</td>
<td>$\delta$-H=7.27, $\varepsilon$-H=7.01</td>
<td>58.59</td>
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Table 5.2b $^1$H and $^{13}$C NMR chemical shifts for maculatin 1.1 in $d_2$-TFE/water (1:1 by vol.) pH 2.5, 25°C.

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>$\text{C}^\alpha$H</th>
<th>$\text{C}^\beta$H</th>
<th>others</th>
<th>$\text{C}^\alpha$</th>
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<tr>
<td>Gly 1</td>
<td>not observed</td>
<td>3.87, 3.95</td>
<td>-</td>
<td>-</td>
<td>42.26</td>
</tr>
<tr>
<td>Leu 2</td>
<td>8.45</td>
<td>4.27</td>
<td>1.58</td>
<td>$\gamma\text{-CH}=1.57$</td>
<td>56.01</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>$\delta\text{-CH}_3=0.86, 0.93$</td>
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<tr>
<td>Phe 3</td>
<td>8.11</td>
<td>4.37</td>
<td>3.15</td>
<td>Aromatic H=7.25</td>
<td>59.52</td>
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<tr>
<td>Gly 4</td>
<td>8.08</td>
<td>3.86, 3.93</td>
<td>-</td>
<td>-</td>
<td>45.57</td>
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<tr>
<td>Val 5</td>
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<td>3.81</td>
<td>2.23</td>
<td>$\gamma\text{-CH}=0.98, 1.05$</td>
<td>64.91</td>
</tr>
<tr>
<td>Leu 6</td>
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<td>4.06</td>
<td>1.78</td>
<td>$\gamma\text{-CH}=1.61$</td>
<td>56.81</td>
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<td></td>
<td>$\delta\text{-CH}_3=0.91$</td>
<td></td>
</tr>
<tr>
<td>Ala 7</td>
<td>8.03</td>
<td>4.04</td>
<td>1.38</td>
<td>-</td>
<td>54.18</td>
</tr>
<tr>
<td>Lys 8</td>
<td>7.53</td>
<td>4.16</td>
<td>2.00, 2.14</td>
<td>$\gamma\text{-CH}_2=1.38, 1.51$</td>
<td>58.13</td>
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<tr>
<td></td>
<td></td>
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<td>$\delta\text{-CH}_2=1.73$</td>
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<tr>
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<td></td>
<td></td>
<td>$\varepsilon\text{-CH}_2=3.02$</td>
<td></td>
</tr>
<tr>
<td>Val 9</td>
<td>8.23</td>
<td>3.76</td>
<td>2.30</td>
<td>$\gamma\text{-CH}_3=0.98, 1.07$</td>
<td>65.70</td>
</tr>
<tr>
<td>Ala 10</td>
<td>8.80</td>
<td>4.08</td>
<td>1.51</td>
<td>-</td>
<td>54.50</td>
</tr>
<tr>
<td>Ala 11</td>
<td>7.78</td>
<td>4.10</td>
<td>1.47</td>
<td>-</td>
<td>53.10</td>
</tr>
</tbody>
</table>
### Table 5.2c $^1$H and $^1^3$C NMR chemical shifts for Ala15 maculatin 1.1 in $d_2$-TFE/water (1:1 by vol.) pH 2.5, 25°C.

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>CαH</th>
<th>CβH</th>
<th>others</th>
<th>Cα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly 1</td>
<td>not observed</td>
<td>3.83, 3.94</td>
<td>-</td>
<td>-</td>
<td>42.18</td>
</tr>
<tr>
<td>Leu 2</td>
<td>8.43</td>
<td>4.24</td>
<td>1.55</td>
<td>$\gamma$-CH$_3$=1.55</td>
<td>56.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\delta$-CH$_3$=0.85, 0.90</td>
<td></td>
</tr>
<tr>
<td>Phe 3</td>
<td>8.09</td>
<td>4.35</td>
<td>3.13</td>
<td>Aromatic H=7.23, 7.31</td>
<td>59.43</td>
</tr>
<tr>
<td>Gly 4</td>
<td>8.06</td>
<td>3.82, 3.92</td>
<td>-</td>
<td>-</td>
<td>45.39</td>
</tr>
<tr>
<td>Val 5</td>
<td>7.60</td>
<td>3.79</td>
<td>2.22</td>
<td>$\gamma$-CH$_3$=0.95, 1.04</td>
<td>64.50</td>
</tr>
<tr>
<td>Leu 6</td>
<td>8.01</td>
<td>4.37</td>
<td>1.78</td>
<td>$\gamma$-CH$_3$=1.58</td>
<td>57.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\delta$-CH$_3$=0.88</td>
<td></td>
</tr>
<tr>
<td>Ala 7</td>
<td>8.03</td>
<td>4.04</td>
<td>1.39</td>
<td>-</td>
<td>56.83</td>
</tr>
<tr>
<td>Lys 8</td>
<td>7.53</td>
<td>4.17</td>
<td>1.98, 2.15</td>
<td>$\gamma$-CH$_2$=1.50, 1.74,</td>
<td>58.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\delta$-CH$_2$=1.73, 3.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\varepsilon$-CH$_2$=3.00</td>
<td></td>
</tr>
<tr>
<td>Val 9</td>
<td>8.30</td>
<td>3.67</td>
<td>2.28</td>
<td>$\gamma$-CH$_3$=0.95, 1.04</td>
<td>66.20</td>
</tr>
<tr>
<td>Ala 10</td>
<td>8.71</td>
<td>4.02</td>
<td>1.49</td>
<td>-</td>
<td>54.22</td>
</tr>
<tr>
<td>Ala 11</td>
<td>7.91</td>
<td>4.05</td>
<td>1.52</td>
<td>-</td>
<td>53.10</td>
</tr>
</tbody>
</table>
NOEs connecting the NH, C\text{\textsuperscript{6}}H and C\text{\textsuperscript{B}}H protons can be summarised in a bar chart (Fig. 5.6) which provides a basis for a qualitative interpretation of the secondary structure of the peptides under these solvation conditions (Wüthrich \textit{et al.}, 1984).

\[ \begin{array}{|c|c|c|c|c|c|} \hline \text{His 12} & 7.89 & 4.31 & 3.45, 3.49 & \delta-H=7.35, \varepsilon-H=8.35 & 58.44 \\
\text{Val 13} & 8.34 & 3.67 & 2.31 & \gamma-\text{CH}_3=0.97, 1.07 & 65.90 \\
\text{Val 14} & 8.46 & 3.61 & 2.10 & \gamma-\text{CH}_3=0.85, 0.96 & 65.90 \\
\text{Ala 15} & 7.89 & 4.15 & 1.59 & - & 58.12 \\
\text{Ala 16} & 7.65 & 4.08 & 1.48 & - & 56.64 \\
\text{Ile 17} & 8.48 & 3.77 & 2.10 & \gamma-\text{CH}_2=1.13, 1.91 \\
& & & & \gamma-\text{CH}_3=1.00 \\
& & & & \delta-\text{CH}_3=0.85 & 64.71 \\
\text{Ala 18} & 8.82 & 4.09 & 1.53 & - & 54.26 \\
\text{Glu 19} & 8.26 & 4.10 & 2.10, 2.18 & \gamma-\text{CH}_2=2.49, 2.63 & 56.67 \\
\text{His 20} & 7.99 & 4.35 & 3.10 & \delta-H=6.77, \varepsilon-H=8.49 & 56.89 \\
\text{Phe 21} & 8.17 & 4.57 & 2.99, 3.31 & \text{Aromatic H}=7.32, 7.41 \\
& & & & \text{CONH}_2=6.90, 7.32 & 57.47 \\
\hline \end{array} \]
Fig. 5.6 Summary of NOEs used for structure calculation of maculatin 1.1 in (a) DPC/water pH 7.0, 37°C (b) TFE/water (1:1 by vol.) pH 2.5, 25°C and (c) Ala15 maculatin 1.1 in TFE/water (1:1 by vol.) pH 2.5, 25°C respectively. The amino acid sequence of the peptide is given at the top of the diagram and the values of \( J_{\text{HNCaH}} \) were obtained from high-resolution 1D 'H spectra. Asterisks (*) indicate coupling constants could not be unambiguously assigned due to overlap. @ indicates no coupling constant was detected for the particular residue. The various types of NOEs and their relative strengths are indicated by the thickness of the bars (strong, medium and weak). For this diagram, NOEs are classified on the basis of strong (< 2.7 Å), medium (between 2.7 to 3.5 Å) and weak (> 3.5 Å). Dashed lines represent connectivities that could not be unambiguously assigned due to overlap, e.g. with an intraresidue NOE.
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From inspection of Fig. 5.6, it is apparent that a large number of connectivities indicative of α-helical structure are present for all three systems (Wüthrich, 1986). For example, the presence of strong \( d_{nn}(i,i+1) \) NOEs complemented by weaker \( d_{on}(i,i+1) \) NOEs, a series of NOEs from residues three and four amino acids apart in the peptide, i.e., \( d_{nn}(i,i+3) \), \( d_{nn}(i,i+3) \), \( d_{nn}(i,i+4) \) and some weak \( d_{nn}(i,i+2) \) NOEs were observed. Finally, \( J_{\text{HINCOSH}} \) values that could be measured mostly had values less than 5 Hz, consistent with a helical structure. The NOE data and their relative intensities suggest that maculatin 1.1 adopts a helical secondary structure along the majority of its polypeptide chain in both TFE/water and DPC/water mixtures. In examining and comparing these figures for maculatin 1.1, the NOE pattern suggests that in both cases the helix commences at Leu2 and continues to His12, is partially interrupted in the vicinity of Pro15 before regaining its helical structure between Ala16 to Phe21. By contrast, the NOE pattern shown in Fig. 5.5f suggests that the Ala15 analogue of maculatin 1.1 (which does not contain the proline residue) adopts an uninterrupted α-helix along its entire length.

The presence of a helical structure is also indicated from an examination of the deviation from random coil chemical shift values of the \(^1\text{H}\) and \(^{13}\text{C}\) α-CH resonances (Wishart et al., 1995a). Using a window of \( n = \pm 2 \) residues was used (Pastore and Saudek, 1990). In all three cases, the \(^1\text{H}\) resonances showed a distinct upfield shift and the \(^{13}\text{C}\) resonances and a distinct downfield shift along the peptide (see Fig. 5.7). Statistical analysis of the \(^1\text{H}\) and \(^{13}\text{C}\) α-CH chemical shifts in structured regions of proteins has shown that α-helical regions have a marked upfield \(^1\text{H}\) and downfield
13C chemical shift respectively compared to random-coil values (Wishart et al., 1991, Wishart et al., 1992). The pattern for the 1H and 13C chemical shifts of maculatin 1.1 in TFE/water and DPC are similar, implying that the peptide adopts similar structures in both solvent systems. In particular, the 1H chemical shift data indicate a greater helical nature in the N- and C-terminal regions. The more random-coil nature of the chemical shifts in between these regions (i.e., near Pro15) suggests that the middle portion of the molecule does not have as a strong helical preference and is thus more flexible. By contrast, the same plots for Ala15 maculatin 1.1 in TFE/water show a bell-shaped trend along the peptide sequence implying that this analogue is helical along its entire length with maximal helicity in the central region. Furthermore, the 1H and 13C chemical shift deviations in the centre of Ala15 maculatin 1.1 are greater than those of maculatin 1.1 in TFE and DPC implying that a tighter helix is present in this region of the Ala15 variant.
Fig. 5.7 Deviation from random coil chemical shifts of (a) $^1$H $\alpha$-CH resonances of maculatin 1.1 in DPC/water mixture, (b) $^{13}$C $\alpha$-CH resonances of maculatin 1.1 in DPC/water mixture. A window of $\pm 2$ residues was used. Upfield and downfield chemical shifts are indicated by negative and positive values respectively.

Nobody has given a good reason for this behaviour. It maybe that when an $\alpha$-helix is formed, the $\alpha$-carbons are more shielded due to the proximity of the COO group. Thus the $^{13}$C plot shows a distinctive downfield shift.
Fig. 5.7 Deviation from random coil chemical shifts of (c) $^1$H $\alpha$-CH resonances of maculatin 1.1 in 50% (by vol.) TFE, (d) $^{13}$C $\alpha$-CH resonances of maculatin 1.1 in 50% (by vol.) TFE. A window of ± 2 residues was used. Upfield and downfield chemical shifts are indicated by negative and positive values respectively.
Fig. 5.7 Deviation from random coil chemical shifts of (e) $^1$H $\alpha$-CH resonances of Ala15 maculatin 1.1 in 50% (by vol.) TFE, (f) $^{13}$C $\alpha$-CH resonances of Ala15 maculatin 1.1 in 50% (by vol.) TFE. A window of ± 2 residues was used. Upfield and downfield chemical shifts are indicated by negative and positive values respectively.
5.5 Structural analysis

The 1D coupling constants and 2D NOESY data were used as input for calculation of the solution structures of maculatin 1.1 and Ala15 maculatin 1.1. Structural calculations for maculatin 1.1 in DPC/water were based on a total of 414 NOE distance restraints comprising of 164 intra-residue restraints, 116 sequential restraints, 132 medium and 2 long-range restraints. In the TFE/water mixture, a total of 437 NOE distance restraints comprising of 167 intra-residue restraints, 121 sequential restraints, 146 medium and 3 long-range restraints were used. For the Ala15 analogue of maculatin 1.1, a total of 366 NOE distance restraints comprising of 137 intra-residue restraints, 86 sequential restraints, 139 medium and 4 long-range restraints were employed. Following RMD, structures were minimised with respect to potential energy via conjugate-gradient methods (Sutcliffe and Dobson, 1991). The final run produced 60 conformers from which the most stable (lowest potential energy) 20 conformers were chosen for examination (Table 5.3). It is apparent from the data presented in Table 5.3 that all three structures are well ordered with very similar low RMSD values for the backbone N, Cα and C' atoms along the amino acid sequence. Fig. 5.8 shows the superimposition of the 20 structures over the backbone atoms of maculatin 1.1 and its Ala15 analogue.
Table 5.3a Structural statistics of maculatin 1.1 in DPC/water pH 7.0, 37°C following RMD/SA calculations.*

<table>
<thead>
<tr>
<th>RMSD from mean geometry (Å)</th>
<th>&lt;SA&gt;</th>
<th>(SA)_r</th>
</tr>
</thead>
<tbody>
<tr>
<td>All heavy atoms</td>
<td>0.80 ± 0.021</td>
<td>0.38 ± 0.016</td>
</tr>
<tr>
<td>All heavy backbone atoms (N, Cα and C')</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X-PLOR energies (kcal mol⁻¹)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{tct}$</td>
<td>235.43 ± 3.39</td>
<td>225.93</td>
</tr>
<tr>
<td>$E_{bond}$</td>
<td>17.61 ± 0.74</td>
<td>16.83</td>
</tr>
<tr>
<td>$E_{angle}$</td>
<td>84.63 ± 2.07</td>
<td>81.84</td>
</tr>
<tr>
<td>$E_{impr}$</td>
<td>12.96 ± 0.85</td>
<td>11.37</td>
</tr>
<tr>
<td>$E_{repel}$</td>
<td>32.81 ± 1.71</td>
<td>32.17</td>
</tr>
<tr>
<td>$E_{NOE}$</td>
<td>86.33 ± 2.53</td>
<td>82.21</td>
</tr>
<tr>
<td>$E_{edih}$</td>
<td>1.09 ± 0.14</td>
<td>0.933</td>
</tr>
</tbody>
</table>

*<SA> is the ensemble of the 20 final structures, (SA) is the mean structure obtained by best-fitting and averaging the coordinates of the backbone N, Cα and C' atoms of the 20 final structures. (SA)_r is the representative structure obtained after restrained energy minimization of the mean structure.

\[ \text{Number of NOEs} \leq 0.2 \text{ Å} = 29. \text{ Maximum violation is 2.90 Å.} \]
### Table 5.3b  Structural statistics of maculatin 1.1 in $d_2$-TFE/water (1:1 by vol.) pH 2.5, 25°C.

<table>
<thead>
<tr>
<th>RMSD from mean geometry (Å)</th>
<th>&lt;SA&gt;</th>
<th>(SA)$_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All heavy atoms</td>
<td>0.79 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>All heavy backbone atoms (N, C$\alpha$ and C')</td>
<td>0.37 ± 0.012</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X-PLOR energies (kcalmol$^{-1}$)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{tot}}$</td>
<td>200.84 ± 6.06</td>
<td>194.23</td>
</tr>
<tr>
<td>$E_{\text{bond}}$</td>
<td>19.15 ± 0.75</td>
<td>14.39</td>
</tr>
<tr>
<td>$E_{\text{angle}}$</td>
<td>66.16 ± 2.10</td>
<td>54.91</td>
</tr>
<tr>
<td>$E_{\text{impr}}$</td>
<td>11.56 ± 0.73</td>
<td>8.76</td>
</tr>
<tr>
<td>$E_{\text{repel}}$</td>
<td>16.10 ± 1.21</td>
<td>19.98</td>
</tr>
<tr>
<td>$E_{\text{NOE}}$</td>
<td>83.35 ± 4.31</td>
<td>89.39</td>
</tr>
<tr>
<td>$E_{\text{cdih}}$</td>
<td>4.55 ± 1.14</td>
<td>6.80</td>
</tr>
</tbody>
</table>

Number of NOEs violate $> 0.2\,\text{Å} = 26$. Maximum violation is 2.90 Å.

### Table 5.3c  Structural statistics of Ala15 maculatin 1.1 in $d_2$-TFE/H$_2$O (1:1 by vol.) pH 2.5, 25°C.

<table>
<thead>
<tr>
<th>RMSD from mean geometry (Å)</th>
<th>&lt;SA&gt;*</th>
<th>(SA)$_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All heavy atoms</td>
<td>0.80 ± 0.017</td>
<td></td>
</tr>
<tr>
<td>All heavy backbone atoms (N, C$\alpha$ and C')</td>
<td>0.38 ± 0.020</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X-PLOR energies (kcalmol$^{-1}$)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{tot}}$</td>
<td>196.16 ± 4.83</td>
<td>175.10</td>
</tr>
<tr>
<td>$E_{\text{bond}}$</td>
<td>15.02 ± 0.77</td>
<td>13.47</td>
</tr>
<tr>
<td>$E_{\text{angle}}$</td>
<td>58.72 ± 2.08</td>
<td>52.27</td>
</tr>
<tr>
<td>$E_{\text{impr}}$</td>
<td>20.91 ± 0.73</td>
<td>18.78</td>
</tr>
<tr>
<td>$E_{\text{repel}}$</td>
<td>12.24 ± 1.09</td>
<td>12.13</td>
</tr>
<tr>
<td>$E_{\text{NOE}}$</td>
<td>88.37 ± 4.21</td>
<td>78.21</td>
</tr>
<tr>
<td>$E_{\text{cdih}}$</td>
<td>0.91 ± 0.32</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Number of NOEs violate $> 0.2\,\text{Å} = 24$. Maximum violation is 2.80 Å.
Fig. 5.8  Superposition of backbone atoms of the 20 most stable structures of (a) maculatin 1.1 in DPC/water (b) TFE/water and (c) Ala15 maculatin 1.1 in TFE/water.
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Analysis of the angular order parameters \((S, \phi \text{ and } \psi)\) of all three groups of the twenty structures indicates that all non-terminal residues are well defined (Leu2 to His20) with \(S > 0.9\) for both their \(\phi\) and \(\psi\) angles (Fig. 5.9).

![Graph of Angular order parameters](image)

**Fig. 5.9** Plots of Angular order parameters (a) \(S(\phi)\) and (b) \(S(\psi)\) against amino acid sequence for maculatin 1.1 in DPC/water.
Fig. 5.9 Plots of Angular order parameters (c) $S(\phi)$ and (d) $S(\psi)$ against amino acid sequence for maculatin 1.1 in TFE/water.
Fig. 5.9  Plots of Angular order parameters (e) $S(\phi)$ and (f) $S(\psi)$ against amino acid sequence for Ala15 maculatin 1.1 in TFE/water.

Ramachandran plots averaged over these twenty structures indicated that in all three cases, the dihedral angles of all the 18 non-glycine and non-proline amino acids were in the sterically favoured or allowed regions for $\alpha$-helical conformation (Fig. 5.10).
Fig. 5.10a Ramachandran plot of maculatin 1.1 in DPC/water.
Fig. 5.10b  Ramachandran plot of maculatin 1.1 in TFE/water.
Fig. 5.10c  Ramachandran plot of Ala15 maculatin 1.1 in TFE/water.
The structure calculations confirmed the conclusions derived from analysis of the NOE and \(\alpha\)-CH chemical shift data (Figs. 5.6 and 5.7). Fig. 5.11 shows the lowest energy structures of maculatin 1.1 in TFE/water and DPC and its Ala15 analogue in TFE/water. In the first two cases, maculatin 1.1 adopts an overall amphipathic helical conformation with well-defined hydrophobic and hydrophilic zones. The Ala15 analogue is predominantly amphipathic except from the sidechain of His20 occurring on the hydrophobic side of the peptide (Fig. 5.11). For maculatin 1.1, a kink exists in the peptide in both solvent systems. The angle between the two helical regions in TFE/water and DPC is \(20.7 \pm 6.0^\circ\) and \(9.6 \pm 5.4^\circ\) respectively (from Leu2 to Ala10 and Val13 to His20 in the TFE/water structures and Leu2 to His12 and Pro15 to Glu19 in the DPC structures). The kink in maculatin 1.1 imparts some additional flexibility in the central region as is apparent in the greater variability in this region in the calculated structures of maculatin 1.1 in TFE/water and DPC compared to Ala15 maculatin 1.1 (Fig. 5.8). The Ala15 analogue adopts a much more linear helix. However, it does exhibit slight curvature along its length with a radius of curvature of 25 Å in the lowest energy structure of Fig. 5.11.
Fig. 5.11  Lowest energy structures illustrating (a) the side-view of maculatin 1.1 in DPC/water (b) showing axial view, (c) maculatin 1.1 in TFE/water showing side view, (d) showing axial view and (e) Ala15 maculatin 1.1 in TFE/water showing side view, (f) showing axial view. Hydrophilic groups are shown in blue. The proline pyrrolidine ring is coloured pink.

5.6 Orientation Studies using Solid-State NMR

As discussed in Chapter 4, DMPC phospholipid multilayers were prepared on glass coverslips. Solid-state $^{31}$P NMR spectra of the sample showed a single peak at −16 ppm, indicating that the phospholipids were uniaxially oriented (Fig. 5.12) (Seelig, 1984; Crowell and McDonald, 1999).
Addition of maculatin 1.1 to the sample caused the spectral width of the $^2$H NMR spectrum to decrease by approximately 4000 Hz. This decrease in order parameter of the lipid alkyl chains suggests that maculatin 1.1 has been incorporated into the lipid bilayer (see Fig. 5.13 and Section 4.5). Similar behaviour has also been observed for magainin interacting with DMPC model membranes (Bechinger et al., 1991 and 1992).
Fig. 5.14 compares the $^{15}$N-NMR spectra of $^{15}$N-Ala7 labelled maculatin 1.1: (a) in its pure form revealing a powder pattern and (b) when incorporated into multilayers. [(Note that the glass coverslips in this experiment were oriented such that the bilayer normal was perpendicular to $B_0$. This is unlike the experiments conducted by Bechinger and co-workers (1991, 1992) who orient their glass slides with the bilayer normal parallel to $B_0$ (Fig. 4.4)).]

Fig. 5.14  $^{15}$N NMR spectra of $^{15}$N-Ala7 maculatin 1.1 (a) showing powder pattern, (b) when incorporated into DMPC multilayers (lipid/peptide ratio 15:1, 35°C).

Fig. 5.15a and 5.15b show the schematic representation of the $^{15}$N solid-state NMR spectra of $^{15}$N-Ala7 labelled maculatin 1.1 while 5.15c depicts the parallel orientation of maculatin 1.1 with respect to the lipid bilayer surface. Whereas only one monomer of the peptide is shown, the NMR data are consistent with a
mechanism of membrane interaction which involves maculatin 1.1 aggregating as oligomers, forming a 'carpet' layer on the membrane surface.

Fig. 5.15 Schematic \( ^{15}\text{N} \) solid-state NMR spectra of \( ^{15}\text{N}-\text{Ala7} \) labelled maculatin 1.1: (a) powder pattern of maculatin 1.1 (b) maculatin 1.1 in oriented phospholipid multilayers. (c) Schematic representation of the 'carpet' conformation of maculatin 1.1 in a lipid bilayer. (Diagrams not drawn to scale).

The \( ^{15}\text{N} \) spectrum in Fig. 5.15b exhibits amide resonances at the downfield end of the chemical shift range, suggesting that maculatin 1.1 orients with its helical axis parallel to the bilayer surface i.e., it adopts the carpet mechanism. This behaviour has also been observed by solid-state NMR of magainin in lipid bilayers (Bechinger et al., 1991 and 1992).
5.7 Discussion

The structures of maculatin 1.1 dissolved in TFE co-solvent and DPC micelles are very similar with a definite kink in the middle of both helices [although the angle between the two helical regions is greater in the former (Figs. 5.8 and 5.11)]. The angle of this kink in both solvent systems is less than the average value for kinked helices in proteins as determined in a recent statistical survey (Kumar and Bansal, 1998) and earlier work (Barlow and Thornton, 1988). The twenty-three amino acid antimicrobial peptide, magainin 2 also adopts very similar helical structures in TFE/water and DPC micelles (Gesell et al., 1997). Likewise, the structures of the helical peptide, δ-hemolysin, are similar in DPC micelles (Lee et al., 1987) and in methanol (Tappin et al., 1988). These studies suggest that organic solvents (e.g. TFE/water mixtures or methanol solutions) give rise to representative structures for amphipathic helices in a lipid environment.

Other NMR studies have shown that other amphipathic helices containing a central proline residue have a similar central bend, e.g. melittin in methanol has a kink of 20° between the two helical portions (Bazzo et al., 1988; Dempsey et al., 1991), alamethicin in methanol has a central bend (Esposito et al., 1987), cecropin A in hexafluoropropylalcohol/water has a large degree of flexibility in its middle (Holak et al., 1988), buforin II in TFE/water is similar (Yi et al., 1996) and caerin 1.1 in TFE/water has a large kink and flexibility in its middle (Wong et al., 1997). Barlow and Thornton (1988) observed similar behaviour for these types of helices in X-ray crystal structures of proteins. In proline-containing peptides, the enhanced
flexibility in the vicinity of the proline arises from the extra freedom available to the peptide in this region due to the backbone hydrogen bonding pattern being disrupted by the absence of an NH group on the proline (Bazzo et al., 1988). Using X-ray crystallographic data, Woolfson and Williams (1990) examined the structural features of proline-containing helices within proteins. They found that in all these bent helices, the relatively hydrophobic proline residue is found on the hydrophilic side of the helix. In maculatin 1.1, Pro15 is similarly placed on the hydrophilic side of the peptide (Fig. 5.11). Likewise, in caerin 1.1, Pro15 and Pro19 are found on this side of the peptide (Fig. 5.1). From the crystal structures, the kink in these types of helices enables water or hydrogen bonding acceptor amino acid sidechains to perform the hydrogen bonding role that is lost due to the presence of the proline, i.e., to the carbonyl of the amino acid four residues earlier in the amino acid sequence (Woolfson and Williams, 1990).

Magainin 2 does not have a central proline residue but adopts an \( \alpha \)-helical structure in DPC with a bend in the middle of its sequence centred about a phenylalanine-glycine moiety such that the angle between the two helical segments is 16° (Gesell et al., 1997). Other non-proline-containing amphipathic helices are often curved (Barlow and Thornton, 1988; Zhou et al., 1992; ; McLeish et al., 1994; Kumar and Bansal, 1998; Wegener et al., 1999). Zhou et al. (1992) discovered that there is a periodic distribution of NH chemical shifts in amphipathic helices which is due to the curvature of the peptide leading to shorter hydrogen bonds on the hydrophobic face of the peptide. For maculatin 1.1 in TFE/water and DPC, this is consistent with the kink in the peptide which leads to hydrophobic amino acid side-chains being
localised on the concave side of the peptide (Fig. 5.11). The majority of helices in proteins are curved with a mean radius of curvature of \(65 \pm 30\) Å (Barlow and Thornton, 1988; Blundell et al., 1983; Kumar and Bansal, 1998) which is approximately 2.5 times greater than that observed for Ala15 maculatin 1.1. Thus, Ala15 maculatin 1.1 has only a small amount of curvature and there is reduced flexibility in the central portion compared to maculatin 1.1 (Figs 5.8 and 5.11). The greater kink and flexibility imparted by the central proline residue in maculatin 1.1 (and other proline-containing amphipathic peptides) would enable optimal amphipathic distribution of its amino acids and thereby precise interaction of the peptide with the membrane surface, e.g. the positively charged amino acids with the anionic phospholipids as discussed in Section 2.4.

In agreement with the above discussion, the structure/bioactivity data on maculatin 1.1 indicate that changing Pro15 to an alanine reduces the bioactivity of the peptide by a factor of 5-10 fold (Table 5.1). Thus, Pro15 is a necessary prerequisite for the full activity of maculatin 1.1 possibly due to its ability to rotate the C-terminal helical segment to form an amphipathic helix (Fig. 5.11). In the Ala15 analogue, however, the relative rigidity of the helix causes hydrophilic His20 to be positioned on the hydrophobic side of the peptide (Fig. 5.11). As a consequence, the amphipathic nature of the helix is lost, causing a significant decrease in bactericidal activity.

The mechanism of membrane lysis by antimicrobials is still a matter of some debate (Shai, 1995; Bechinger, 1997). Solid state NMR studies of magainin and cecropin
incorporated into lipid bilayers have shown that these peptides lie parallel to the surface of synthetic model membranes, suggesting that they both adopt the carpet mechanism when permeabilising membranes (Bechinger et al., 1992 and 1993; Marassi et al., 1999). In contrast, experimental evidence has shown that cytolytic peptides such as alamethicin and melittin adopt the channel mechanism to form transmembrane pores (see table 2.1). Both maculatin 1.1 and caerin 1.1 have central proline residues and are long enough (> 20 amino acids) to span a membrane when in a helical conformation. It is conceivable, therefore, that both peptides could utilise the channel mechanism. However, solid-state NMR studies have revealed that antimicrobial amphibian peptides magainin and its analogue, PGLa adopt the carpet mechanism (Bechinger et al., 1991, 1992, 1993, 1996 and 1998). Similar conclusions have been obtained for maculatin 1.1 using the same technique (Section 5.6). These peptides are found with their helical axis oriented parallel to the plane of the bilayer, suggesting that they exert their bactericidal action via the carpet mechanism. In this model, the peptide is most likely to be arranged so that its hydrophobic side is within the hydrocarbon chains of the lipids and its hydrophilic side interacts with the lipid polar headgroups and aqueous solution. This results in membrane thinning and destabilisation to accommodate the peptide, ultimately lysing the cell (Durell et al., 1992, Bechinger, 1999).

Presently, it is unknown why both maculatin 1.1 and caerin 1.1 are not active against Gram-negative bacteria. Although these bacteria have outer membranes which act as effective barriers against various antibiotics (Hancock, 1984), they should still be susceptible to membrane-disrupting peptides. One reason could be due to the
presence of lipopolysaccharide (LPS) on the outer membrane surface. Being anionic in nature, they may bind irreversibly to cationic bactericidal peptides, preventing the latter from reaching and disrupting the bacterial membrane. Orientation studies (using solid-state NMR techniques in particular) and molecular dynamics simulations involving maculatin 1.1 and phospholipids/LPS bilayers may resolve this issue.
Chapter 6
Uperin 3.6 and Analogues

_Uperoleia mjobergii_
6.1 Uperin 3.6 and Analogues

Toadlets of the genus *Uperoleia* have host defence peptides which fulfil similar roles to those from tree frogs of the genus *Litoria*. However, the sequences of the peptides from the two genera are quite different. These include the two related wide-spectrum antibiotic peptides named uperin 3.5 and uperin 3.6 isolated from the toadlet *Uperoleia mjobergii* (Bradford *et al*., 1996). These amphibians are found in the flood plains of the Kimberley region of Western Australia (Fig 6.1) and adult specimens typically grow up to an average of 2 cm in length.

Fig. 6.1  (a) *Uperoleia mjobergii* (note warty protuberances on the dorsal surface of the skin). (b) A flood plain in the Kimberley region of western Australia. (c) Geographical distribution on the toadlet (shaded pink).
Chapter 6

The skin secretions of these toadlets are stored in granular glands which can be seen as warty protuberances on the dorsal surface of the animal (Fig 6.1). They deliver a number of peptides onto the skin surface when the animal is stressed or attacked, in particular, the antimicrobial peptides uperin 3.5 and 3.6. Both have similar bioactivities to those of caerin 1.1 and maculatin 1.1 (see Table 6.1). Both peptides contain 17 amino acids and are therefore significantly shorter in length than the twenty-five residue caerin 1.1 and twenty-one residue maculatin 1.1 (see Section 5.1). This raises the question as to why such dissimilar structures should have similar spectra of antibiotic activities. Another interesting question is whether the positively charged residues in the uperin peptides are essential for its bactericidal properties. Most natural antibacterial and haemolytic peptides are positively charged [see Dathe and Wieprecht (1999) and Sitaram and Nagaraj (1999) for reviews]. The role of positive charges is essential for the electrostatic interaction with the anionic cytoplasmic membranes of bacteria. For example, the electrically neutral twenty-seven residue ß-haemolysin is highly haemolytic but devoid of antimicrobial activity. Substituting certain residues with lysines induced potent antibacterial activities in the peptide (Dhople and Nagaraj, 1995). Increasing the number of positive charges of magainin 2 also enhanced its bactericidal potency (Bessale et al., 1992) while a reduction of the number of positive charges resulted in the loss of antimicrobial activity (Matsuzaki et al., 1997b).

In an effort to gain a deeper insight on their mechanisms of bactericidal action, a number of synthetically modified analogues were synthesised. The primary sequences of the natural uperin peptides and their analogues are shown overleaf:
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Uperin 3.5  GVGDL IRKAV SVIKN IV-NH₂
Uperin 3.6  GVIDA AKKVV NVLKN LF-NH₂
Ala4 uperin 3.6  GVIAA AKKVV NVLKN LF-NH₂
Ala7 uperin 3.6  GVIDA AAKVV NVLKN LF-NH₂
Ala14 uperin 3.6  GVIDA AKKVV NVLAN LF-NH₂

The antibiotic activities of the uperin peptides and their synthetic analogues are summarised in Table 6.1.

Table 6.1 Antimicrobial activities of the uperin 3.5, 3.6 and three synthetic analogues.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Caerin 1.1</th>
<th>Maculatin 1.1</th>
<th>Uperin 3.5</th>
<th>Uperin 3.6</th>
<th>Ala4 uperin 3.6</th>
<th>Ala7 uperin 3.6</th>
<th>Ala14 uperin 3.6</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Leuconostoc lactis</em></td>
<td>1.5</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><em>Listeria innocua</em></td>
<td>25</td>
<td>100</td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>12</td>
<td>12</td>
<td>25</td>
<td>50</td>
<td>12</td>
<td>&gt;100</td>
<td>100</td>
</tr>
<tr>
<td><em>Pasteurella multocida</em></td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>3-12</td>
<td>6-12</td>
<td>50</td>
<td>25</td>
<td>25-50</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>&gt;100</td>
<td>100</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>25</td>
<td>25</td>
<td>12</td>
<td>12</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td>12</td>
<td>3</td>
<td>12</td>
<td>12</td>
<td>25</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

*The data for caerin 1.1 were taken from Wong et al. (1997).
†The data for maculatin 1.1 were taken from Chia et al. (2000).
‡Two strains were tested (ATCC type strains 25923 and 29213).
It can be clearly seen that substituting positively charged lysines with neutral alanine results in a significant reduction of antimicrobial activity. This suggests that positive electric charges are important for the antimicrobial activity of uperin 3.6. In the subsequent sections, detailed studies will be conducted on uperin 3.6 in order to gain a better understanding to its mechanism of action.

6.2 Structural Studies

Uperin 3.6 displays a distinct amphipathic α-helical distribution of amino acids when represented on an Edmundson helical wheel (Fig 6.2).

![Edmundson helical wheel representation of uperin 3.6. Hydrophobic and hydrophilic residues are represented by black and white circles respectively.]

In this chapter, CD, NMR spectroscopy and computer based modelling techniques were used to determine the 3D structure of uperin 3.6 in TFE/water mixtures.
6.3 Circular Dichroism Studies

CD spectra of uperin 3.6 were measured under increasing concentrations from 0-50% (by vol.) of TFE in water (see Fig. 6.3). At low concentrations of TFE (0-10%), uperin 3.6 exhibits a spectrum characteristic of an unstructured peptide, i.e. a broad minimum at 200 nm was observed. On increasing the TFE concentration, the spectra underwent a marked change to one indicative of a predominantly \( \alpha \)-helical structure, i.e. two minima in the vicinity of 208 nm (\( \pi-\pi^* \) transition) and 220 nm (\( n-\pi^* \) transition) were observed. Spectra at intermediate concentration (20-30% TFE) showed reduced helical content as evidenced by both a lower ellipticity and a shift of the minima around 208 nm to lower wavelengths. The spectra at 40% and 50% TFE showed greatest ellipticity at these minima, indicative of maximum helical conformation. Based on this evidence, NMR studies were undertaken in a 50% TFE/water mixture. (It should be noted that a 50% (by vol.) TFE/water mixture corresponds to a mole fraction of 20:80 respectively, i.e. to relatively small amounts of TFE on a molar basis).

![CD spectra of uperin 3.6 at varying amounts (by vol.) of TFE.](image)

**Fig 6.3** CD spectra of uperin 3.6 at varying amounts (by vol.) of TFE.
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6.4 NMR Studies

On the basis of the CD results, NMR spectra of uperin 3.6 were acquired under conditions which exhibited maximal helical structure, i.e. 50% (by vol.) TFE. Fig. 6.4 shows a region of the TOCSY spectrum and the NH region of the NOESY spectrum of uperin 3.6 in which a series of strong sequential NH to NH NOEs \( d_{NN}(i, i+1) \) were observed along the length of the peptide. Weaker \( d_{NN}(i, i+2) \) NOEs can also be observed in this region. Using \( d_{NN}(i, i+1) \) and \( d_{ON}(i, i+1) \) sequential connectivities, all proton resonances of uperin 3.6 were assigned. Assignments for all \(^1\)H and \(^{13}\)C\(^\alpha\) resonances are tabulated in Table 6.2.

A summary of NOEs involving the NH, C\(^\alpha\)H and C\(^\beta\)H protons obtained from the NOESY spectra are presented in a connectivity diagram (Fig. 6.5). This figure shows a large number of connectivities indicative of an \( \alpha \)-helix. For example, the presence of strong \( d_{NN}(i, i+1) \) NOEs complemented by weaker \( d_{ON}(i, i+1) \) NOEs, a series of NOEs from residues three and four amino acids apart in the peptide, i.e., \( d_{ON}(i, i+3) \), \( d_{ON}(i, i+4) \) and eight \( d_{NN}(i, i+2) \) NOEs were observed. Finally, \(^3J_{HNCOH}\) values that could be measured mostly had values less than 5 Hz, consistent with a helical structure. In summary, the NOE data and intensities suggest that a helical secondary structure for uperin 3.6 commences at Val2 and continues to Phe17.
Fig 6.4  (a) A region of the TOCSY spectrum showing NH to sidechain proton couplings, (b) NH region of the NOESY spectrum of uperin 3.6 in TFE/water. Connectivities between sidechain and backbone amide protons are indicated.
Table 6.2 $^1$H and $^{13}$C NMR chemical shifts for uperin 3.6 in $d_2$-TFE/water (1:1 by vol.) pH 2.4, 25°C.

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>$\text{C}^{\alpha}\text{H}$</th>
<th>$\text{C}^{\beta}\text{H}$</th>
<th>others</th>
<th>$\text{C}^{\alpha}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly 1</td>
<td>not observed</td>
<td>3.93, 3.99</td>
<td>0.00</td>
<td>-</td>
<td>42.3</td>
</tr>
<tr>
<td>Val 2</td>
<td>8.46</td>
<td>4.04</td>
<td>2.11</td>
<td>$\gamma$-$\text{CH}_3=1.03, 1.03$</td>
<td>63.5</td>
</tr>
<tr>
<td>Ile 3</td>
<td>7.98</td>
<td>4.04</td>
<td>1.90</td>
<td>$\gamma$-$\text{CH}_3=1.28, 1.56$ $\delta$-$\text{CH}_3=0.94$</td>
<td>61.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp 4</td>
<td>7.88</td>
<td>4.52</td>
<td>2.87</td>
<td>-</td>
<td>54.2</td>
</tr>
<tr>
<td>Ala 5</td>
<td>7.82</td>
<td>4.14</td>
<td>1.49</td>
<td>-</td>
<td>54.2</td>
</tr>
<tr>
<td>Ala 6</td>
<td>8.10</td>
<td>4.08</td>
<td>1.49</td>
<td>-</td>
<td>54.1</td>
</tr>
<tr>
<td>Lys 7</td>
<td>7.87</td>
<td>3.94</td>
<td>1.91</td>
<td>$\gamma$-$\text{CH}_3=1.44, 1.49$ $\delta$-$\text{CH}_3=1.70$ $\varepsilon$-$\text{CH}_3=2.97$</td>
<td>58.5</td>
</tr>
<tr>
<td>Lys 8</td>
<td>7.62</td>
<td>4.12</td>
<td>1.90, 2.01</td>
<td>$\gamma$-$\text{CH}_3=1.47$ $\delta$-$\text{CH}_3=1.61, 1.71$ $\varepsilon$-$\text{CH}_3=2.95$</td>
<td>58.4</td>
</tr>
<tr>
<td>Val 9</td>
<td>7.87</td>
<td>3.66</td>
<td>2.24</td>
<td>$\gamma$-$\text{CH}_3=0.96, 1.07$</td>
<td>65.8</td>
</tr>
<tr>
<td>Val 10</td>
<td>8.25</td>
<td>3.65</td>
<td>2.14</td>
<td>$\gamma$-$\text{CH}_3=0.97, 1.09$</td>
<td>65.8</td>
</tr>
<tr>
<td>Asn 11</td>
<td>7.72</td>
<td>4.42</td>
<td>2.81, 2.95</td>
<td>$\delta$-$\text{CONH}_2=6.77, 7.51$</td>
<td>55.9</td>
</tr>
<tr>
<td>Val 12</td>
<td>7.96</td>
<td>3.72</td>
<td>2.38</td>
<td>$\gamma$-$\text{CH}_3=0.96, 1.10$</td>
<td>65.8</td>
</tr>
<tr>
<td>Leu 13</td>
<td>8.42</td>
<td>4.06</td>
<td>1.97</td>
<td>$\gamma$-$\text{CH}_3=1.52$ $\delta$-$\text{CH}_3=1.61, 1.71$</td>
<td>57.4</td>
</tr>
<tr>
<td>Lys 14</td>
<td>8.54</td>
<td>4.04</td>
<td>1.92, 1.98</td>
<td>$\gamma$-$\text{CH}_3=1.52$ $\delta$-$\text{CH}_3=1.62$ $\varepsilon$-$\text{CH}_3=2.95$</td>
<td>58.3</td>
</tr>
<tr>
<td>Asn 15</td>
<td>7.69</td>
<td>4.55</td>
<td>2.74, 2.93</td>
<td>$\delta$-$\text{CONH}_2=6.77, 7.42$</td>
<td>54.7</td>
</tr>
</tbody>
</table>
### Fig. 6.5  A summary of NOEs used for structure calculation of uperin 3.6 in 50% (by vol.) d₅-TFE, pH 2.4, 25°C. The amino acid sequence of the peptide is given at the top of the diagram and the values of $^3J_{\text{NH} \text{CaH}}$ were obtained from high-resolution 1D $^1$H spectra. @ indicates no coupling constants were detected for the corresponding residue. Asterisks (*) indicate that coupling constants could not be unambiguously assigned due to overlap. The various types of NOEs and their relative strengths are indicated by the thickness of the bars (strong, medium and weak). For the basis of this diagram, NOEs are classified on the basis of strong (< 2.7 Å), medium (between 2.7 to 3.5 Å) and weak (> 3.5 Å). Dashed lines represent connectivities that could not be unambiguously assign due to overlap, e.g. with an intraresidue NOE.
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The presence of a helical structure is also indicated from an examination of the deviation from random coil chemical shift values of the $\alpha$–CH $^1$H and $^{13}$C resonances. Although a complete set of random coil chemical shifts are not available for amino acids in TFE, those which have been tabulated indicate that there is little difference between random coil chemical shifts in TFE and water (Merutka et al., 1995). A comparison was made with random coil chemical shifts determined in water. For a window of $n = \pm 2$ residues, smoothed plots for the $^1$H resonances showed a distinct upfield shift (see Fig. 6.5a) and those for the $^{13}$C resonances exhibited a distinct downfield shift along the peptide (see Fig. 6.5b). A negative chemical shift difference indicates an upfield chemical shift compared to the random coil value. Statistical analysis of the $\alpha$–CH $^1$H and $^{13}$C chemical shifts in structural regions of proteins has shown that $\alpha$-helical regions give more to a marked upfield $^1$H and downfield $^{13}$C chemical shift compared to random-coil values. The directions of the deviations from random coil chemical shifts in uperin 3.6 are thus consistent with helical structure for the peptide with maximum helicity being present in the central region of the peptide.
Fig. 6.6 Deviation from random coil chemical shifts of (a) $^1$H $\alpha$-CH resonances and (b) $^{13}$C $\alpha$-CH resonances of uperin 3.6 in 50% (by vol.) $d_2$-TFE, pH 2.4, 25°C. A window of $\pm$2 residues was used. Upfield and downfield chemical shifts are indicated by negative and positive values respectively.

6.5 Structural Analysis

The conclusions derived from the examination of the NMR data presented above are confirmed when the NOESY data were used as input for calculations. Structural
calculations were based on a total of 319 NOE distance restraints comprising of 132 intra-residue restraints, 77 \((i, i+1)\) sequential restraints, 110 medium-range restraints and 13 \(\phi\) angle restraints. Following calculations, structures were minimised with respect to potential energy via conjugate-gradient methods. The final run produced 60 conformers from which the 20 of the most stable (lowest potential energy) conformers were chosen for detailed examination. The statistics for these structures are presented in Table 6.3.

**Table 6.3** Structural statistics of uperin 3.6 in 50\% (by vol.) \(d_2\)-TFE, pH 2.40, 25°C following RMD/SA calculations*.

<table>
<thead>
<tr>
<th>RMSD from mean geometry (Å)</th>
<th>(&lt;\text{SA}&gt;&gt;^*)</th>
<th>(SA)_r</th>
</tr>
</thead>
<tbody>
<tr>
<td>All heavy atoms</td>
<td>0.42 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>All heavy backbone atoms</td>
<td>0.10 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>(N, Cα and C')</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X-PLOR energies (kcalmol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E_{\text{tot}})</td>
</tr>
<tr>
<td>(E_{\text{bond}})</td>
</tr>
<tr>
<td>(E_{\text{angle}})</td>
</tr>
<tr>
<td>(E_{\text{impr}})</td>
</tr>
<tr>
<td>(E_{\text{repeel}})</td>
</tr>
<tr>
<td>(E_{\text{NOE}})</td>
</tr>
<tr>
<td>(E_{\text{cdih}})</td>
</tr>
</tbody>
</table>

* \(<\text{SA}>\) is the ensemble of the 20 final structures, (SA) is the mean structure obtained by best-fitting and averaging the coordinates of the well-defined heavy backbone N, Cα and C’ atoms of the 20 final structures. (SA)_r is the representative structure obtained after restrained energy minimisation of the mean structure.

\(\text{Number of NOE violations} > 0.2 \text{ Å} = 1\%\). \text{Maximum violation is} 2.80 \text{ Å}. 

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The superimposition of the twenty most stable structures over the entire backbone N, C\(^{\alpha}\) and C\(^{\prime}\) atoms is shown in Fig. 6.7.

Gly 1

Phe 17

Fig. 6.7 Superposition of backbone atoms of the 20 most stable structures of upperin 3.6 in 50\% (by vol.) \(d_{2}\)-TFE, pH 2.4, 25\(^{\circ}\)C

Analysis of the angular order parameters (\(S, \phi\) and \(\psi\)) of all three groups of the twenty structures indicates that all non-terminal residues are well defined (Val2 to Phe17) with \(S > 0.9\) for both their \(\phi\) and \(\psi\) angles (Fig. 6.8).
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Fig. 6.8 Plots of Angular Order Parameters (a) $S(\phi)$ and (b) $S(\psi)$ against amino acid sequence for uperin 3.6 in 50% (by vol.) $d_3$-TFE, pH 2.4, 25°C.

The Ramachandran plot of uperin 3.6 (Fig 6.9) showed all non-glycine residues had their averaged backbone dihedral angles ($\phi$ and $\psi$) within the ‘favoured’ (A) $\alpha$-helical region, the only exception being Leu16 which is in the ‘allowed’ (A) region, possibly indicating a region of flexibility near the C-terminal.
Fig 6.9 Ramachandran plot of the average backbone $\phi$ and $\psi$ angles of uperin 3.6.
The most stable structure (lowest potential energy) of uperin 3.6 is shown in Fig. 6.10. It is apparent from this representation that uperin 3.6 has an amphipathic helical structure with distinct hydrophobic and hydrophilic faces.

Fig 6.10 The most stable structure (lowest potential energy) of uperin 3.6. The projection demonstrates the well-defined hydrophobic and hydrophilic zones (blue). The polypeptide backbone is represented by the ribbon.

6.6 Discussion

The antibiotic activities of the membrane-active amphipathic uperin peptides are listed in Table 6.1. With the exception of the activity against Staphylococcus aureus, the spectra of activities of uperin 3.5 and uperin 3.6 are quite similar to that of caerin 1.1 and maculatin 1.1. Both caerin 1.1 and maculatin 1.1 are helical with a region of flexibility about Pro15 (see Chapter 5). This flexibility enables both peptides to interact with a variety of membrane surfaces by rearranging their backbone conformations to optimise their interactions with the bacterial membrane surface.
Such an ability would account, at least in part, for both their broad-spectrum antibiotic activities. In contrast, neitheruperin 3.5 and 3.6 have proline residues and the three-dimensional solution structure ofuperin 3.6 shows that it adopts a well-defined amphipathic helix along its entire length with no central region of flexibility (Fig 6.7 and 6.10). Despite these differences,uperin 3.5 and 3.6 exhibit similar broad-spectrum antibiotic activities to caerin 1.1 and maculatin 1.1 (see Table 6.1). One reason for their similar activity profile may be that the two peptides have different overall charges. Caerin 1.1 has three histidine residues and assuming that these have similar pKₐ values for an isolated histidine, they will be uncharged at neutral pH. Thus at pH 7, caerin 1.1 will have an overall charge of +1, whereasuperin 3.5 and 3.6 (which have no histidine residues) will possess an overall charge of +3. This suggests that the significantly higher overall charge ofuperin 3.5 and 3.6 compared to caerin 1.1 would enable the former to interact electrostatically more strongly with the anionic phospholipids on a typical bacterial membrane. For this reason,uperin 3.6 may be able to compensate for the lack of flexibility in its central region that caerin 1.1 possess. This proposal is supported by the bioactivity data for alanine-substituteduperin 3.6 peptides listed in Table 6.1. Substitution of the negatively charged Asp4 by an uncharged alanine residue induces only minor changes in the activity data, while replacement of either Lys7 or Lys15 with alanine reduces the activity significantly.

The studies above emphasise the importance of the overall cationic peptide charge for the interaction with bacterial membranes. However, a simple correlation between
the number and position of cationic residues on the one hand and antimicrobial specificity on the other hand does not seem to exist.

In Chapter 5, solid-state NMR studies suggest that maculatin 1.1 adopts the carpet mechanism in favour of the transmembrane channel-forming mechanism. Which mechanism will uperin 3.6 adopt? As mentioned in Chapter 2, a typical bacterial cytoplasmic membrane is about 30 Å in thickness. Since the distance between successive amino acids in an α-helix is 1.5 Å, a minimum of 20 amino acids is required for an α-helical peptide to span a membrane. Uperin 3.6, with only 17 amino acids, is not long enough to traverse a bacterial membrane. Therefore, it is unlikely that it would follow the channel mechanism, unless the peptide forms head-to-head or head-to-tail dimers in a similar manner to gramicidin A (see Section 2.6). 2D NMR results in TFE/water do not indicate uperin 3.6 undergoing dimerisation. NMR studies of the peptide incorporated into phospholipid micelles may shed light into this.

The NMR and activity data presented herein for uperin 3.6 are consistent with the initial phase of the carpet and channel mechanism, i.e., the formation of an amphipathic helix followed by electrostatic interaction to the bacterial membrane. However, the next step involving the way the peptide penetrates a phospholipid membrane is yet to be determined. Solid-state NMR studies involving the peptide interacting with phospholipid bilayers may resolve this issue.
Chapter 7
The Caerin 4 Peptides
7.1 The Caerin 4 Antimicrobial Peptides

The caerin 4 family of antimicrobial peptides (shown below) are isolated from the Australian green tree frog *Litoria caerulea* (Stone et al., 1993). It is a medium sized tree frog measuring from 6 to 11 cm in length and is found in tropical rainforests of Australia (Barker et al., 1995) (Fig 7.1).

Caerin 4.1  GLWQK IKSAA GDLAS GIVEG IKS-NH₂
Caerin 4.1  GLWQK IKSAA GDLAS GIVEG IKS-NH₂
Caerin 4.3  GLWQK IKNAA GDLAS GIVEG IKS-NH₂

Fig. 7.1  (a) *Litoria caerulea* (b) a tropical rainforest in Australia (c) geographical distribution of the frog (shaded pink).
Chapter 7

The antibiotic activities of the caerin 4 family of peptides are summarised in Table 7.1.

**Table 7.1 Antibiotic activities of the caerin 4 family of peptides as shown by MIC* tests (values given in μg/ml).**

<table>
<thead>
<tr>
<th>Organism</th>
<th>MIC (μg/mL)</th>
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<tr>
<td></td>
<td>Caerin 4.1</td>
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<tr>
<td><em>Bacillus cereus</em></td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>25</td>
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<td><em>Leuconostoc lactis</em></td>
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<td><em>Listeria innocua</em></td>
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<tr>
<td><em>Micrococcus luteus</em></td>
<td>12.5</td>
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<tr>
<td><em>Pasteurella multocida</em></td>
<td>&lt;0.4</td>
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<tr>
<td><em>Staphylococcus aureus</em></td>
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<td><em>Streptococcus faecalis</em></td>
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<tr>
<td><em>Streptococcus uberis</em></td>
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</table>

If there is no figure indicated, the MIC value is > 100 μg/ml.
All data are taken from Bradford et al. (1996)

* Caerin 4.1 has also been tested against several viruses (HIV 1, *Herpes simplex* 1 and 2) and cancers (L1210 mouse leukaemia and SKOV ovarian cancer). Unlike caerin 1.1 (see Chapter 5), caerin 4.1 showed no significant activity against these pathogens.

Caerin 4.1 is a narrow spectrum antibiotic which shows potent activity against *Pasteurella multocida* (swine fever); this activity is high enough to suggest that the
peptide should be investigated as a veterinary product. It is also interesting to note that caerin 4.1 is active against Gram-negative *Escherichia coli*, which is not a general feature of amphibian peptides studied. Indeed, the bioactivity of caerin 4.1 against *Escherichia coli* is the best yet recorded for a peptide from an Australian amphibian (MIC = 25 µg/mL). The 3D structure of caerin 4.1 was investigated using NMR spectroscopy and computer based modelling techniques in order to provide an insight into the mechanism of bactericidal action. This chapter reports the results of an NMR spectroscopic determination of the solution structure of caerin 4.1.

### 7.2 Structural Studies

Caerin 4.1 does not display a well-defined amphipathic α-helical distribution of amino acids when represented on an Edmundson helical wheel (Fig 7.2).

![Edmundson helical wheel representation showing the non-amphipathic nature of caerin 4.1. Hydrophobic and hydrophilic residues are represented by black and white circles respectively.](image)

**Fig 7.2** Edmundson helical wheel representation showing the non-amphipathic nature of caerin 4.1. Hydrophobic and hydrophilic residues are represented by black and white circles respectively.
From Fig. 7.2, it is apparent that caerin 4.1 is not amphipathic based on the helical wheel representation. More detailed structural studies on the peptide were conducted using CD, NMR spectroscopy and computer based modelling techniques.

7.3 Circular Dichroism Studies

The CD spectra were acquired on caerin 4.1 in the presence of an increasing proportion [from 0 to 50% (by vol.)] of TFE in water (see Fig 7.3). At low amounts of TFE (0-10%), caerin 4.1 exhibits a CD spectrum characteristic of an unstructured peptide, i.e., a broad minimum at 200 nm was observed. On increasing the amount of TFE, the CD spectra indicated the presence of a predominantly $\alpha$-helical structure, i.e. two minima in the vicinity of 208 nm ($\pi-\pi^*$ transition) and 220 nm ($n-\pi^*$ transition) were observed. The spectra at 40% and 50% TFE showed greatest ellipticity at these minima, indicative of maximum helical conformation. Based on this evidence, NMR studies were undertaken in a 50% (by vol.) TFE/water mixture.

![Fig 7.3 CD spectra of caerin 4.1 at varying concentrations (by vol.) of TFE.](image)
Chapter 7

7.4 NMR Spectroscopy

The $^1$H NMR spectrum of caerin 4.1 in 50% (by vol.) TFE was assigned via standard methods as discussed in Chapter 3. $^{13}$C NMR resonances were assigned from the HSQC spectrum from correlations of the $^{13}$C resonances to the assigned $^1$H resonances. Fig. 7.4 shows a region of the TOCSY spectrum and the NH region of the NOESY spectrum of caerin 4.1 in which a series of strong sequential NH to NH NOEs [$d_{NN}(i,i+1)$] were observed along the length of the peptide. Weaker $d_{NN}(i,i+2)$ NOEs can also be observed in this region. Using $d_{NN}(i,i+1)$ and $d_{NN}(i,i+1)$ sequential connectivities, all proton resonances of caerin 4.1 were assigned. Assignments for all $^1$H and $^{13}$C$^\alpha$ resonances are tabulated in Table 7.2.

A summary of NOEs involving the NH, C$^\alpha$H and C$^\beta$H protons obtained from the NOESY spectra are presented in a connectivity diagram (Fig. 7.5). This figure shows a large number of connectivities indicative of an $\alpha$-helix. For example, the presence of strong $d_{NN}(i,i+1)$ NOEs complemented by weaker $d_{NN}(i,i+1)$ NOEs, a series of NOEs from residues three and four amino acids apart in the peptide, i.e., $d_{NN}(i,i+3)$, $d_{NN}(i,i+3)$, $d_{NN}(i,i+4)$ and three $d_{NN}(i,i+2)$ NOEs were observed. Finally, $^3J_{HNCOH}$ values that could be measured mostly had values less than 5 Hz, consistent with a helical structure. In summary, the NOE data and intensities suggest that a helical secondary structure for caerin 4.1.
Fig 7.4  (a) A region of the TOCSY spectrum showing NH to sidechain proton couplings, (b) NH region of the NOESY spectrum of caerin 4.1 in TFE/ water. Connectivities between sidechain and backbone amide protons are indicated.
Table 7.2 $^1$H and $^{13}$C NMR chemical shifts for caerin 4.1 in $d_2$-TFE/water (1:1 by vol.) pH 2.8, 25°C.

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<tr>
<th>Residue</th>
<th>NH</th>
<th>C$^\alpha$H</th>
<th>C$^\beta$H</th>
<th>others</th>
<th>C$^\alpha$</th>
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<td>Leu 2</td>
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Fig. 7.5 A summary of NOEs used for structure calculation of caerin 4.1 in 50% (by vol.) d$_7$-TFE, pH 2.8, 25°C. The amino acid sequence of the peptide is given at the top of the diagram and the values of $^{3}J_{\text{NHCOH}}$ were obtained from high-resolution 1D $^1$H spectra. @ indicates no coupling constants were detected for the corresponding residue. Asterisks (*) indicate that coupling constants could not be unambiguously assigned due to overlap. The various types of NOEs and their relative strengths are indicated by the thickness of the bars (strong, medium and weak). For the basis of this diagram, NOEs are classified on the basis of strong (< 2.7 Å), medium (between 2.7 to 3.5 Å) and weak (> 3.5 Å). Dashed lines represent connectivities that could not be unambiguously assigned due to overlap, e.g. with an intrar residue NOE.
The presence of a helical structure is also indicated from an examination of the deviation from random coil chemical shift values of $^1$H and $^{13}$C $\alpha$-CH resonances determined in water. For a window of $n = \pm 2$ residues, smooth plots for the $^1$H resonances showed a distinct upfield shift and those for the $^{13}$C resonances showed a distinct downfield shift along the peptide (Fig 7.6a & b). The directions of the deviations from random coil chemical shifts are consistent with helical structure for the peptide with maximum helicity being present in its central region. However, it is apparent that these deviations do not exhibit a smooth distribution along the entire amino acid sequence. For example, the $^1$H $\alpha$-CH chemical shifts show a marked upfield shift in the region from Lys5 to Ser8 and Gly16 to Gly20 with an intervening region of $\alpha$-CH chemical shifts which approach random coil values. The implications from these data are that these two aforementioned regions are strongly helical and are separated by a region where the helicity is reduced. In the main, the $^{13}$C $\alpha$-CH chemical shift deviations were opposite in direction to the $^1$H deviations, with a significant downfield shift from Gln4 to Ser8 and shifts near random coil values from Gly1 to Trp3 and Ala9 to Gly16. However, a downfield shift was not observed for the $\alpha$-CH resonances of Ile17 to Gly20 which would have been expected on the basis of the $^1$H $\alpha$-CH shift data. In fact, these residues were shifted upfield. This suggests a degree of flexibility in the C-terminal region.
7.5 Structural Analysis

The conclusions derived from the examination of the NMR data presented above were confirmed when the NOESY data were used as input for determination of the solution structure of caerin 4.1. Structural calculations were based on a total of 415 NOE distance restraints comprising of 188 intra-residue restraints, 104 \((i, i+1)\) sequential restraints, 123 medium-range restraints and 5 \(\phi\) torsion angle restraints. Following RMD/SA calculations, structures were energy minimised with respect to potential energy via conjugate-gradient methods. The final run produced 60 conformers from which the 20 most stable (lowest potential energy) conformers
were selected for detailed examination. The structural statistics are presented in Table 7.4.

<table>
<thead>
<tr>
<th>Table 7.4 Structural statistics of caerin 4.1 following RMD/SA calculations *</th>
<th>( &lt;\text{SA}&gt; )</th>
<th>(SA)_R</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMSD from mean geometry (Å)</td>
<td>0.74 ± 0.26</td>
<td>0.50 ± 0.29</td>
</tr>
<tr>
<td>All heavy atoms</td>
<td>( &lt;\text{SA}&gt; )</td>
<td>(SA)_R</td>
</tr>
<tr>
<td>All heavy backbone atoms (N, C( \alpha ) and C')</td>
<td>260.39 ± 3.39</td>
<td>241.85</td>
</tr>
<tr>
<td>X-PLOR energies (kcal/mol(^{-1}))</td>
<td>28.12 ± 0.70</td>
<td>26.48</td>
</tr>
<tr>
<td>( E_{\text{tot}} )</td>
<td>74.82 ± 2.79</td>
<td>67.23</td>
</tr>
<tr>
<td>( E_{\text{bond}} )</td>
<td>17.56 ± 1.12</td>
<td>14.75</td>
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<tr>
<td>( E_{\text{angle}} )</td>
<td>23.14 ± 1.53</td>
<td>19.25</td>
</tr>
<tr>
<td>( E_{\text{impr}} )</td>
<td>116.73 ± 3.55</td>
<td>114.12</td>
</tr>
<tr>
<td>( E_{\text{repel}} )</td>
<td>0.07 ± 0.02</td>
<td>0.02</td>
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<tr>
<td>( E_{\text{cdih}} )</td>
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* \( <\text{SA}> \) is the ensemble of the 20 final structures, (SA) is the mean structure obtained by best-fitting and averaging the coordinates of the well-defined heavy backbone N, C\( \alpha \) and C' atoms of the 20 final structures. (SA)_R is the representative structure obtained after restrained energy minimization of the mean structure.

The superimposition of the twenty most stable structures over the entire backbone N, C\( \alpha \) and C' atoms show that caerin 4.1 forms a slightly curved helix along its entire length (Fig 7.7). The peptide is well-defined over its entire length with a RMSD of 0.50 ± 0.29 Å for the backbone atoms (Table 7.4).
Fig 7.7 Superimposition of backbone atoms (N, Cα and C') of the 20 most stable structures of caerin 4.1 in 50% (by vol.) d5-TFE, pH 2.8, 25°C.

Analysis of the angular order parameters (S, φ and ψ) of these twenty structures indicate that eighteen of the nineteen non-glycine residues are well defined with $S > 0.9$ for both their φ and ψ angles. Ser23 at the C-terminus is the exception with (S, φ and ψ) values of 0.98 and 0.80 respectively (Fig. 7.8). The N- and C-terminal residues exhibited greater conformational variability.
The Ramachandran plot of caerin 4.1 (Fig 7.9) showed seventeen of the nineteen non-glycine residues had their averaged backbone dihedral angles (\(\phi\) and \(\psi\)) within the ‘favoured’ (A) \(\alpha\)-helical region, the only exception being Val18 and Glu19 which are in the ‘allowed’ (a) region, possibly indicating a region of flexibility near the C-terminus.
Fig 7.9 Ramachandran plot of the average backbone $\phi$ and $\psi$ angles of caerin 4.1. The dots represented by Val 18 and Glu 19 are indicated.

The most stable structure of caerin 4.1 (i.e. with the lowest potential energy) is shown in Figure 7.10. Caerin 4.1 adopts a slightly curved helix along its entire length with well-defined hydrophobic and hydrophilic zones. The variation in the
middle of the peptide is due to flexibility associated with two glycine residues at positions 11 and 16, amino acids that are well known as "helix breakers" (Richardson and Richardson, 1988). Likewise, the helicity is disrupted after Gly20 which causes greater variation at the C-terminus than at the N-terminus of the molecule.

![Diagram of peptide structure]

**Fig 7.10** The most stable structure (lowest potential energy) of caerin 4.1. The projection demonstrates the well defined hydrophobic and hydrophilic zones (shown in blue).

### 7.6 Discussion

There are three members of the caerin 4 family. To date, these have only been found in the skin secretion of the granular glands of the Australian green tree frog *Litoria caerulea* (Chia *et al*., 2000b). The structure of caerin 4.1 in TFE co-solvent is that of a helical peptide along its entire length (Fig. 7.7 and 7.10). The first part of the peptide (from Gln4 to Ala10) is highly helical. However, the structures shown in Fig. 7.7 exhibit some conformational variation in the middle of the peptide (from
Chapter 7

Gly11 to Gly16) which leads to greater flexibility in this portion of the molecule.

The region of flexibility from Gly11 to Gly16 is followed by approximately one turn of well-structured helix from Ile17 to Ile21 which leads into a flexible portion at the C-terminus. The structure-disrupting ability of glycine amino acids is apparent from the structures in Fig. 7.7 where the ends of the two highly helical regions in caerin 4.1 correspond to the presence of glycine amino acids at positions 11 and 20.

Central flexibility is common in many unrelated antibiotic amphipathic helical peptides. For example, caerin 1.1 has a central region of greater flexibility than caerin 4.1 which occurs in the vicinity of Pro15 (see Chapter 5). The equivalent residue in caerin 4.1 is a serine. The larger degree of central flexibility in caerin 1.1 may explain its much broader spectrum of antibiotic activity (Table 7.1), i.e., by arranging its amino acid side chains accordingly it can interact more efficiently with membranes from a diversity of bacteria which have different membrane composition and topology. In caerin 4.1, however, its relatively rigid helical backbone conformation cannot undergo a similar conformational change which causes a loss in its wide-spectrum antibiotic capability.

Presently, it is unknown why caerin 4.1 is active only against Gram-negative bacteria and not Gram-positive ones. It has been suggested that since all Gram-negative bacteria have lipopolysaccharide (LPS) in their outer membranes (Brock et al., 1984), it could play a role in bacterial selectivity for certain antimicrobial peptides (Epand and Vogel, 1999). Indeed, cecropin was found to bind to the phosphoryl moiety of LPS (De Lucca et al., 1995) while spectroscopic studies
showed that magainin binds to and disrupt vesicles containing LPS (Rana et al., 1990, 1991a and 1991b; Matsuzaki et al., 1999b) as well as natural Gram-negative bacterial membranes (Skurmik et al., 1999). Hence, it has been suggested that cationic bactericidal peptides initially binds to LPS and disrupt the outer membrane before penetrating the second, inner membrane (Guo et al., 1998). These studies, however, do not explain why certain antimicrobial peptides are active against Gram-negative bacteria while others are not. Recently, a novel model membrane system made up of LPS and phospholipids was developed which can be employed as a membrane mimetic for Gram-negative bacteria (Matsuzaki et al., 1999b). Hence, future experiments that can be performed using caerin 4.1 include: (i) NMR structural analysis of the peptide dissolved in a solution of phospholipid bicelles containing LPS, (ii) orientation studies of the peptide when incorporated in LPS multilayers using solid-state NMR techniques and (iii) molecular dynamics simulations involving caerin 4.1 with phospholipid/LPS bilayers. Such studies can be used to probe the 3D structure, topology and dynamics of peptide-membrane systems which may shed some light as to why caerin 4.1 is selective only towards Gram-negative bacteria.
Chapter 8
Future Directions
and Conclusions
8.1 Antimicrobial Peptides: Antibiotics of the Future?

No new classes of antibiotics have been developed in the past 26 years since the introduction of quinolones in the 1970s (Hancock et al., 1996). Already, a plethora of drug-resistant pathogens are on the rise world-wide and thus there is an urgent need for the introduction of new antibiotics (Davies, 1996; Ewald and Cochran, 1999). Naturally-occurring bactericidal peptides from amphibian skin and other organisms are a potential source of such antibiotics as they possess several features that confer some advantages over existing antibiotics (Hancock, 1997; Hancock and Lehrer, 1998; Boman et al., 1999; Hancock and Chapple, 1999). For example, they are mostly broad-spectrum, highly potent and are known to kill drug-resistant pathogens such as *Staphylococcus aureus* and fungi (de Lucca and Walsh, 1995; Hancock et al., 1995). In addition, some of these peptides have shown remarkable selectivity against bacteria and are not known to induce drug-resistant mutants. These desirable properties make them promising candidates for the development of a new antibiotic class (Hancock and Lehrer, 1998; Hancock, 1999). Furthermore, these peptides are relatively small and hence, are relatively easy to produce in a large scale using solid-phase techniques (Wade et al., 1990; Maloy and Kari, 1995) and recombinant DNA procedures (Simmaco et al., 1998). One such example is an analogue of magainin (MSI-78) which is now commercially available as a topical antibiotic cream (see Fig. 1.4b) (Lipsky et al., 1997).

However, a major problem exists: these peptides are likely to be vulnerable to proteases found in humans (or any host organism in particular). Shemyakin et al.
(1969) suggested that this can be circumvented by synthesising and using the all-D analogues which would not be targeted by host proteolytic enzymes.

8.2 Peptides and Drug Design

Antimicrobial peptides have been subjected to numerous structure-activity relationship studies which have greatly enhanced our knowledge of their mechanisms of bactericidal action (Bessalle et al., 1993; Tossi et al., 1997). Understanding the structure-function relationships of these peptides has facilitated the design of more potent and/or cell-specific analogues (Bechinger et al., 1997; Hwang and Vogel, 1998). Examples include analogues that: (i) differ at one or more sequence positions, (ii) are shortened or contain deletions or (iii) are hybrids of two different peptides (Boman et al., 1989; Fink et al., 1989; Rivett et al., 1996). Of particular interest are analogues that exhibit tumouricidal activities (see Section 8.3). These studies enable the determination of important residues that modulate biological activities, resulting in the discovery of novel analogues that are more potent than the natural peptides. For example, analogues of cecropin and magainin have been synthesised in the search for potential therapeutic agents (Sabberwal and Nagaraj, 1994; Maloy and Kari, 1995) while cecropin-melittin and cecropin-magainin hybrids have been synthesised which possess enhanced activities against a wide range of pathogens without the cytotoxic effects of the natural peptides (Boman et al., 1989; Andreu et al., 1992; Wade et al., 1992; Piers and Hancock, 1994; Fernandez et al., 1996; Shin et al., 1997; Shin et al., 1999; Hedberg et al., 2000). Another approach in the design of novel peptides for use as therapeutic agents is to create peptides with maximum amphipathicity. Towards this end, two
approaches have been utilised. The first involves synthesising peptides with repeating sequences of alternating hydrophilic and hydrophobic residues (Tossi et al., 1997) while the second method involves the use of combinatorial libraries (Blondelle and Houghton, 1996; Blondelle et al., 1996; Dorner et al., 1997). These methods have not only created highly potent membrane-lytic agents, but have also provided a deeper insight to their mechanism(s) of action.

8.3 Development of Anti-Cancer Agents

The conventional treatment for cancer involves chemotherapy. Unfortunately, chemotherapeutic drugs are highly toxic because they are indiscriminate in their killing of cells, including healthy cells. These drugs work by attacking all rapidly proliferating cells, such as those of the gastrointestinal tract. Such serious side-effects restrict the dosage level of drug which can be administered. Thus, new drugs are currently being developed that will selectively target cancerous cells with minimum effect on other healthy cells in the patient. In Section 1.3, it was mentioned that caerin 1.1 is active against many types of cancer cells (see also Section 2.4 on cancer cell membranes). Its mechanism of action is suspected to be similar to the way it, and maculatin 1.1, lyse bacteria membranes (i.e., via the carpet mechanism). This makes caerin 1.1 (and possibly most membrane-lytic peptides) potential anti-cancer agents. Cell-specific targeting can be achieved by attaching appropriate immunoglobulins (antibodies) to the peptide which will then be able to seek out specific cancer cells before disrupting their membranes (Fig 8.1). Such a concept is not new, various cytolytic agents have already been chemically linked to
immunoglobulins, including radionuclides, cytolytic drugs, ribonuclease, plant and animal toxins [see Colowick and Kaplan (1985), Neville (1986), McGuire (1988), Pai and Pastan (1994) and Compans et al. (1998) for reviews]. For example, the plant toxin ricin and cobra venom have been tested as therapeutic agents for the treatment of cancer (Vallera et al., 1983; Vogel et al., 1985; Bjorn and Villemez, 1988). These studies suggest that it could be feasible to use membrane-lytic peptides as a new class of immunotoxins, thereby offering new opportunities in cancer therapy. For example, a melittin-antibody conjugate is currently under investigation (Dunn et al., 1996).

![Diagram](image)

**Fig. 8.1** A schematic representation of an immunotoxin, consisting of an immunoglobulin, a linker and the membrane-lytic peptide.
(Figure not drawn to scale).

### 8.4 Conclusions

In this thesis, a combination of structure-activity studies, CD, NMR and computer modelling techniques were employed to give a deeper insight into the mechanisms of bactericidal action of amphibian antimicrobial peptides. These studies have also enhanced our understanding of the molecular basis of peptide specificity and potency against bacteria. This serves to facilitate the design of more potent and cell-specific antibiotics as well as anti-cancer agents.
The main reason for the difficulties in establishing a correlation between peptide conformation in membrane-mimetic media and their specificity against Gram-positive and Gram-negative bacteria is the lack of experimental model systems that can accurately mimic the highly complex natural cell membranes (Blondelle et al., 1999; Dathe and Wieprecht, 1999). Future studies in this area can be undertaken using more realistic membrane-mimicking solvent systems, including phospholipid bicelles (Vold et al., 1997; Prestegard, 1998) or liposomes containing LPS to mimic Gram-negative bacterial membranes (Matsuzaki, 1999b).

The Toad, Ugly and Venomous, Wears yet a Precious Jewel in his Skin
-adapted from William Shakespeare's "As You Like It". Act II, Scene I
Chapter 9
Experimental

_Litoria caerulea_
9.1 Preparation of Synthetic Peptides

All peptides and their synthetic analogues were synthesised commercially by Chiron Mimotopes (Clayton, Victoria) using L-amino acids via the standard N-α-Fmoc method (Maeji et al., 1995).

9.2 Antimicrobial Testing

Synthetic peptides were tested by the microbiology section of the Institute of Medical and Veterinary Science (Adelaide, South Australia). The method involved the measurement of inhibition zones produced by the applied peptide on a thin agarose plate containing the micro-organisms under study. The procedures are standard and have been fully documented (Jorgensen et al., 1993). Activities were recorded as minimum inhibitory concentration (MIC) values, i.e. the mass of peptide per millilitre of water required to kill or totally inhibit the growth of the test microorganism.

9.3 CD Spectroscopy

All CD spectra were acquired on a Jobin-Yvon CD-6 spectrophotometer (located at the Department of Chemistry, University of Wollongong) at 20°C at varying concentrations of TFE in water (from 0 to 50% by volume) at peptide concentrations listed in table 10.1. Each spectrum represented an average of five scans. The instrument was routinely calibrated with an aqueous solution of recrystallised $d_{10}$-camphorsulphonic acid (1.0 g/L in a cell of pathlength 1mm). Ellipticity was
calibrated using values of 33.5 mdeg. at $\lambda=290.5$ nm and -69.5 mdeg. at $\lambda=192.5$ nm.

Table 9.1 Sample details for CD spectroscopy.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration ($\mu$M)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>caerin 4.1</td>
<td>22.9</td>
<td>4.8</td>
</tr>
<tr>
<td>maculatin 1.1</td>
<td>23.3</td>
<td>4.8</td>
</tr>
<tr>
<td>uperin 3.6</td>
<td>27.3</td>
<td>4.2</td>
</tr>
</tbody>
</table>

9.4 NMR Spectroscopy

The general procedure for the preparation of NMR samples is described below. Details can be found in Table 9.2. The appropriate peptide (A mg) was dissolved in 0.7 ml of $d_2$-TFE/water (1:1 by volume) to give a concentration of B mM. For the experiment involving dodecylphosphocholine, 9.6 mg of maculatin 1.1 and 69.8 mg of dodecylphosphocholine-$d_{38}$ (DPC) (Cambridge Isotope Laboratories) was dissolved in 0.63 ml water / D$_2$O (9:1 by volume) containing 50 mM of NaH$_2$PO$_4$ buffer giving a concentration of 6.39 mM of maculatin and 0.70 M of DPC. pH was adjusted to 7.00 by adding appropriate amounts of HCl and NaOH. All pH measurements were made using a ActiFon Model 210 pH meter and ActiFon BJ 432 Platinum Electrode (183×6 mm epoxy thin stem, Ag/AgCl reference). Model 535 NMR tubes were used for all NMR experiments.
Table 9.2  Sample details for NMR spectroscopy.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mass of Peptide (A mg)</th>
<th>Concentration (B mM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caerin 4.1</td>
<td>10.2</td>
<td>6.30</td>
<td>2.8</td>
</tr>
<tr>
<td>Maculatin 1.1</td>
<td>9.6</td>
<td>6.39</td>
<td>2.5</td>
</tr>
<tr>
<td>Ala15 maculatin 1.1</td>
<td>10.6</td>
<td>7.21</td>
<td>2.5</td>
</tr>
<tr>
<td>Uperin 3.6</td>
<td>10.7</td>
<td>8.36</td>
<td>2.4</td>
</tr>
</tbody>
</table>

All NMR spectra were acquired on a 600 MHz Varian Unity Inova spectrometer at a $^1$H frequency of 599.12 MHz and $^{13}$C frequency of 150.00 MHz. Experiments involving water / d$_6$-TFE were conducted at 25°C while that involving DPC was conducted at 37°C. Probe temperature was calibrated using ethylene glycol. The methylene resonances of residual TFE were used as $^1$H and $^{13}$C chemical shift references (at 3.918 and 60.975 ppm, respectively) for experiments acquired in TFE/water. Sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) was used as $^1$H and $^{13}$C chemical shift references (at 0.015 and 0.000 ppm, respectively) for experiments involving DPC micelles (Gesell et al., 1997).

$^1$H 2D NMR DQF-COSY (Rance et al., 1983b), TOCSY (Davies and Bax, 1985), NOESY (Jeneer et al., 1979) experiments were conducted in the phase sensitive mode using Time Proportional Phase Incrementation (TPPI) (Marion and Wüthrich, 1983) in the $t_1$ dimension. Each experiment consisted of 512 $t_1$ increments with 32 scans recorded per increment. The FID in $t_2$ consisted of 2048 data points over a spectral width of 5112.5 Hz.
Water suppression was conducted using the presaturation method (Gueron et al., 1991). The transmitter frequency was centered on the water resonance and conventional low power presaturation from the same frequency synthesizer (proton transmitter) was applied during the delay between scans. A 1.5 s presaturation pulse was used to suppress the large water signal in the TOCSY, NOESY and DQF-COSY spectra.

The TOCSY experiments used a 75 ms MLEV-17 spin-lock and employed a modified pulse sequence to minimise cross relaxation effects (i.e. "Clean-TOCSY") (Griesinger et al., 1988). All NOESY spectra were acquired with a mixing time of 200 ms.

HSQC (Kay et al., 1992) experiments using linear prediction (Gmeiner and Babcock, 1997) were acquired to assign the protonated $^{13}$C resonances via correlations to their attached protons. For this experiment, 512 t, increments were acquired over 4096 data points with a spectral width of 5521.8 Hz in the directly detected ($^1$H, $F_2$) dimension and a spectral width in the $^{13}$C ($F_1$) dimension of 24146.5 Hz.

### 9.5 NMR Data Processing

All 2D NMR spectra were processed on a Sun Microsystems Ultra Sparc 1 workstation using VNMR (version 5.3) software. All 2D NMR spectra were multiplied by a gaussian function in both dimensions before zero-filling to 2048 data
Chapter 9

points in both dimensions prior to Fourier transformation. The resulting 2D matrices consisted of 2048 × 2048 real data points.

9.6 Cross-peak Assignments and Structural Calculations

Cross-peak assignments were performed using XEASY (version 1.3.13) software (Bartels et al., 1995). Structures were generated on a Sun Microsystems Sparc 4 workstation using X-PLOR (version 3.851) software (Brünger, 1992; Brünger and Nilges, 1993). All restraints were set to a lower limit of 1.80 Å based on the sum of the van der Waals radii of two nuclei. The upper boundary limit was set to the calculated distance plus 20% to avoid excessively restraining the molecule.

NOESY cross-peak volumes was calculated by the approach proposed by Xu et al. (1995). It involves converting the cross-peak volumes into a continuum of distance restraints with values determined from the equation:

$$\text{Calculated Distance} = [A(I)/I]^{1/6}$$

where $A(I)$ is an intensity-dependent proportionality factor and $I$ is the observed crosspeak intensity. $A(I)$ can be obtained from equation:

$$A(I) = [(I - I_d)/(I_w - I_d)] [A(I_w) - A(I_d)] + A(I_d)$$

with $A(I_w)$ and $A(I_d)$ representing the average of the 10 weakest and 10 strongest NOE intensities respectively. Both can be calculated as follows:
\[ A(I_w) = (5.0 \text{ Å})^6 I_w \]
\[ A(I) = (1.8 \text{ Å})^6 I. \]

The RMD/SA protocol was based on that of Nilges et al. (1988b) which included the use of floating stereospecific assignments (Holak et al., 1989). The All Hydrogen Distance Geometry (ALLHDG) force field (version 4.03) was employed for all calculations (Engh and Huber, 1991). The protocol started with a family of 60 initial structures generated from templates with random backbone $\phi$ and $\psi$ angles. They were next subjected to 6500 steps (19.5 ps) of high temperature dynamics at 2000 K. $K_{\text{NOE}}$ and $K_{\text{repel}}$ force constants were increased from 10 to 50 kcalmol$^{-1}$Å$^{-2}$, from 5 to 200 kcalmol$^{-1}$rad$^{-2}$ and from 0.02 to 0.1 kcalmol$^{-1}$Å$^{-4}$, respectively. (Force constants for all terms were initially set to very low values to ensure that atoms can 'pass through' each other to satisfy experimental restraints if experimental constraints so dictate). This was followed by 2500 steps (7.5 ps) of cooling from 2000 K to 1000 K with $K_{\text{repel}}$ is increasing from 0.1 to 4.0 kcalmol$^{-1}$Å$^{-4}$ while the atomic radii was decreased from 0.9 to 0.75 times those in the ALLHDG parameter set in order to ensure that no close non-bonded contacts existed. The last step involved 1000 steps (3 ps) of cooling from 1000 K to 100 K. Final structures were subjected to 200 cycles of conjugate gradient Powell minimisation (Sutcliffe and Dobson, 1991). Of these, the twenty structures with the lowest potential energy were selected for analysis. INSIGHT II (version 95.0, MSI) was used to display and manipulate the structures. PROCHECK (version 3.0) was used to produce the Ramachandran plots (Laskowski et al., 1994).
9.7 Coupling Constants

$^2J_{\text{NHCOH}}$ coupling constants were measured from high-resolution 1D $^1$H spectra acquired at 25°C with 0.0105 Hz/point digital resolution (128,000 data points). Dihedral angles were restrained as follows: $^3J_{\text{NHCOH}} < 5$ Hz, $\phi = -60^\circ \pm 30^\circ$, $^3J_{\text{NHCOH}}$ between 5 to 6 Hz, $\phi = -60^\circ \pm 40^\circ$, $^3J_{\text{NHCOH}} > 8$ Hz, $\phi = -120^\circ \pm 40^\circ$. Where 6 Hz < $^3J_{\text{NHCOH}} < 8$ Hz, $\phi$ angles were not constrained. Similarly, for residues where no coupling constant could be measured, no dihedral constraints were used.

9.8 Solid-State NMR Spectroscopy

DHPC and DMPC were purchased from Avanti Polar Lipids (Alabaster, AL). DMPC-$^2$H$_{54}$ was purchased from Serdary Research Laboratories (Ontario, Canada).

The bilayer-peptide sample was prepared using $^{15}$N-Ala7 labelled maculatin 1.1 (6.0 mg) and DMPC-$d_{54}$ (30.7 mg) to give a 15:1 phospholipid/peptide ratio. The powder was dissolved in chloroform/methanol (4 ml, 3:1 by vol.) and applied evenly to 30 strips of glass coverslips. The solvent was allowed to dry, then evaporated in vacuo (4 hours) and hydrated with 50 $\mu$l of H$_2$O. Finally, the plates were stacked, inserted into a 7 mm diameter NMR tube, left to equilibrate with excess water overnight and then sealed.

$^{31}$P and $^2$H experiments were performed on a Varian Unity Plus 400 NMR spectrometer. Spectra were proton-decoupled and acquired using a 10mm broadband probe. $^{31}$P spectra were acquired between 30°-50° C at 161.8 MHz with a
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spectral width of 32 kHz and relaxation delay of 2s. Data were acquired for 512 scans with 8192 data points. H3PO4 was used as an external reference (0 ppm).

50 Hz line-broadening was applied to the spectra. 2H NMR spectra were acquired at 61.4 MHz with a spectral width of 100 kHz. The quadrupole echo pulse sequence: 90°-t1-90°-t2-acquire was employed with phase cycling and quadrature detection (Davis et al., 1976). t1 and t2 were set to 40 μs and 30 μs respectively. A relaxation delay of 0.30 s was used. Data were acquired over 36,000 scans and 16,384 data points. D2O was used as reference (0 ppm). Proton-decoupled 15N NMR spectra were acquired on a Bruker AMX-500 spectrometer operating at 50 MHz and using saturated *NH4NO3 solution as reference (0 ppm) (Levy and Lichter, 1979). 42 000 scans were used and the FID consisted of 32 000 data points over a spectral width of 21 000 Hz.
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Appendix
Top: HSQC of maculatin 1.1 in DPC/water pH 7.0, 37°C.

Bottom: HSQC of maculatin 1.1 in $d_2$-TFE/water (1:1 by vol.) pH 2.5, 25°C.
HSQC of Ala15 maculatin 1.1 analogue in $d_6$-TFE/water (1:1 by vol.) pH 2.5, 25°C.
Top: HSQC of uperin 3.6 in $d_3$-TFE/water (1:1 by vol.) pH 2.4, 25°C.

Bottom: HSQC of caerin 4.1 in $d_3$-TFE/water (1:1 by vol.) pH 2.8, 25°C.
Publications

1998 - 2000
List of Publications


*Chemistry in Australia*, v. 65(8), pp. 45-48

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