The Observation of Evolutionary Trends in Amphibians and the Analysis of Negative Ion Fragmentations in Large Peptide Systems by Mass Spectrometry.

A Thesis submitted for the Degree

of

Doctor of Philosophy

in the

Department of Chemistry

by

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July 1997

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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 material 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.

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Acknowledgements.

There are many people who I would like to thank for their help and support throughout my PhD. Initially I have to thank my supervisor Professor John Bowie for giving me the opportunity for doing a PhD. He has been a very supportive supervisor, who is willing to listen and give useful advice. Thanks also have to go to the Australian Research Council for providing the funds for my PhD.

Tom Blumenthal who did an important job by curing the ZAB of its many and varied aliments. Without him no results would have been obtained. The same must be said for Bruce May and Gino Farese for their prompt assistance when problems arose on the HPLC machines. I would like also to thank Dr. Graeme Currie at the Department of Botany at Melbourne University for running the mass spectra of the peptides from *L. chloris*.

Associate Professor Michael Tyler, Margaret Davies and Craig Williams from the Department of Zoology at the University of Adelaide must be thanked for looking after and milking frogs when a secretion was needed. They were helpful and always answered my questions with a cheerful response. Associate Professor John Wallace and Denise Turner from the Department of Biochemistry for performing the automated Edman sequencing of the peptides have to be thanked. I would like to thank the Conservation Commission of South Australia and of the Northern Territory (CCNT), Western Australian Department of Conservation and Land Management and the Queensland Department of Environment and Heritage, for advice and permits on the collection of frogs within the various states. Thanks must also go to Kevin Dawes (Farina S.A.), David Booth (University of Queensland), Jean-Marc Hero (James Cook University, Qld.), Jeff Cole (CCNT) and Jo Vandermark (Kulumburu, W.A.) for the collection of *L. rubella*. Craig

Williams is also included for helping to look locally for *L. ewingi*. Thanks also to Max Ellis for informing me when he found *L. chloris* in a shipment of ferns.

I would like to thank all the past and present members of Lab 7, newly opened Lab 6 and anyone else who helped me during my four years in the Department of Chemistry. During my PhD I have forged many friendships and have many fond memories. It is these friends that have allowed me to stay sane and keep my sense of humour.

During the writing up of my thesis I would like to thank Prof. John Bowie, Suresh Dua and Sarah Ellis for proof reading my thesis. Thanks to the other people who helped check sections during the writing up process.

Big thanks must go to some very close family and friends. To my parents, Ken and Maureen, who's understanding and financial support during my PhD and especially during my undergraduate and Honours degrees were the foundation of my tertiary education and my future. I would also like to thank my brother, Peter, for helping me whenever I asked. A special thank you to Sarah Ellis for being there every step of the way for my PhD, offering advice and any help that was needed. To Max and Dianne Ellis for their support and the ritual Sunday night tea. Finally I would like to thank my late grand mother Edna Pfitzner for just being there and for the great memories.

Legend to Figures.

Legend to Tables.

Abstract.

This thesis is divided into two main areas of research, the characterisation of peptides from frogs belonging to the genus *Litoria* and the analysis of negative ion fragmentations from large peptides.

Peptides were obtained by surface electrical stimulation from the granular glands of *Litoria rubella, L. ewingi, L. xanthomera* and *L. chloris*. The peptides were characterised by fast atom bombardment and electrospray mass spectrometry. The aims of this research were to observe the evolutionary relationships within and between frog species, as well as to discover potentially useful medicinal peptides.

Specimens of *L. rubella* were collected throughout Australia with the peptide profiles indicating several chemically distinct populations. This revealed divergent evolutionary trends within this species. Members from the tryptophyllin and rubellidin peptide families were characterised. The tryptophyllins are believed to be neurotransmitters, whereas the rubellidins show no such activity.

The secretion from *L. ewingi* contains peptides of four different families. The caerin, caeridin and tryptophyllin peptides were characterised and are observed in other frogs of the genus *Litoria*. Members of the uperin peptide family were also characterised and are only normally found in frogs belonging to the genus *Uperoleia*. Caerin 1.1 is a wide spectrum antibiotic. The uperin and tryptophyllin peptides show minor antibiotic and smooth muscle activity. The caeridin peptides show no activity.

L. xanthomera and *L. chloris* are located on the east coast of Queensland and are very closely related. Caerin and caeridin peptides were characterised, with four being identical and the others only varying by structural or amino acid variations. Comparison of the peptides characterised from these frogs represent their close relationship. The caerin peptides characterised show antibiotic activity, whereas the caeridin peptides show no such activity.

The structural information from the spectra of negative ion fragmentations of small peptides is at least complementary to that obtained from positive ion spectra. Peptides of higher molecular weights containing the amino acid residues Asp, Asn, Ser, Thr and Trp were fragmented using negative ions. The spectra were observed to be dominated by fragmentations through the amino acid side chains that undergo facile cleavage. This was at the expense of the backbone fragmentations that provide sequencing information.

CHAPTER ONE: MASS SPECTROMETRY INTRODUCTION

1.1. General Introduction.

Mass spectrometry is an analytical technique requiring minimal amounts of sample to obtain molecular weight and structural information.[1] The first mass spectrograph instruments were Thomson's parabola mass spectrograph,[2] followed by the instrument of Aston,[3] which successfully employed an electric and magnetic sector, and then Dempster's instrument,[4] which contained only a magnetic field. From these early instruments, there has been a dramatic evolution in the type and performance of mass spectrometers. Multi-sector mass spectrometers of various geometries have been developed. These include double focussing mass spectrometers (double sector instruments),[5,6] followed by three[7] and four[8] sector instruments. Differing varieties of mass spectrometers were also developed and include quadrupole[9-11] (single through to pentaquadrupoles), flowing afterglow,[12] ion-trap,[13,14] time-of-flight,[15,16] ion cyclotron resonance (ICR)[17,18] and fourier transform-ICR[19-21] mass spectrometers. Hybrid mass spectrometers, which combine quadrupole with other instruments were also developed.[14,22]

Large advances in ionisation methods and sample handling have also been observed, which have increased the range of samples suitable for mass spectrometric analysis.[23] The progression of ionisation techniques started from the discharge tube[2] with the other techniques being developed, such as electron impact (EI),[23] chemical ionisation (CI),[24,25] and field ionisation and desorption.[26] As there were larger and more polar sample molecules to be analysed, further techniques were required to introduce these compounds into the gas phase. These 'softer' ionisation techniques include plasma desorption ionisation,[27] secondary

ion mass spectrometry (SIMS),[28] fast atom bombardment (FAB)[29,30] or liquid SIMS,[31] continous flow FAB,[32] matrix assisted laser desorption ionisation[33,34] and electrospray.[35] Developments in sample handling added another dimension to mass spectrometry. These involve 'on-line' systems, where the sample(s) are mass analysed directly after separation by various chromatographic techniques. These on-line techniques include gas chromatography,[36] liquid chromatography[37] and capillary electrophoresis mass spectrometry.[38]

The research described in this thesis involves the analysis of peptide samples from natural and synthetic sources. FAB was used to ionise the peptides, which were then analysed by tandem mass spectrometry. The instrument used to record spectra is a Vacuum Generators ZAB 2HF mass spectrometer (ZAB). The ZAB was used in both positive and negative ion modes, with both MH^+ and $[M-H]^-$ (M = molecule) ions observed using low resolution magnetic field scans. Structural information was obtained by, (1) chemical derivatisation of peptides that were analysed by low resolution mass spectrometry, (2) collisional activation mass analysed ion-kinetic energy spectroscopy (CA MIKES) and (3) B/E and B^2/E linked scans where appropriate. The Finnigan LCQ mass spectrometer (LCQ), which is an electrospray quadrupole ion trap mass spectrometer, was also used for the characterisation of peptides. The LCQ was used in the normal scanning mode to detect the MH^+ ion. Structural information was obtained by performing MS^2 and MS^3 experiments for the parent and daughter ions respectively.

1.2. Vacuum Generators ZAB 2HF Mass Spectrometer.

The ZAB[39,40] is a double focussing tandem mass spectrometer designed primarily for organic applications (see Figure 1.1). The arrangement of analysers is reversed compared to that of conventional two sector instruments. The electric sector (E) follows the magnetic sector (B) and this is known as 'reverse' Nier-Johnson

geometry.[41] The instrument is capable of high resolution, a maximum of 100 000 (at 10% valley definition), and high sensitivity. The instrument also has a mass range of *m/z* 0-3 650 at 7 kV. The source housing has the capability to accept a combined EI/CI source as well as a FAB source.

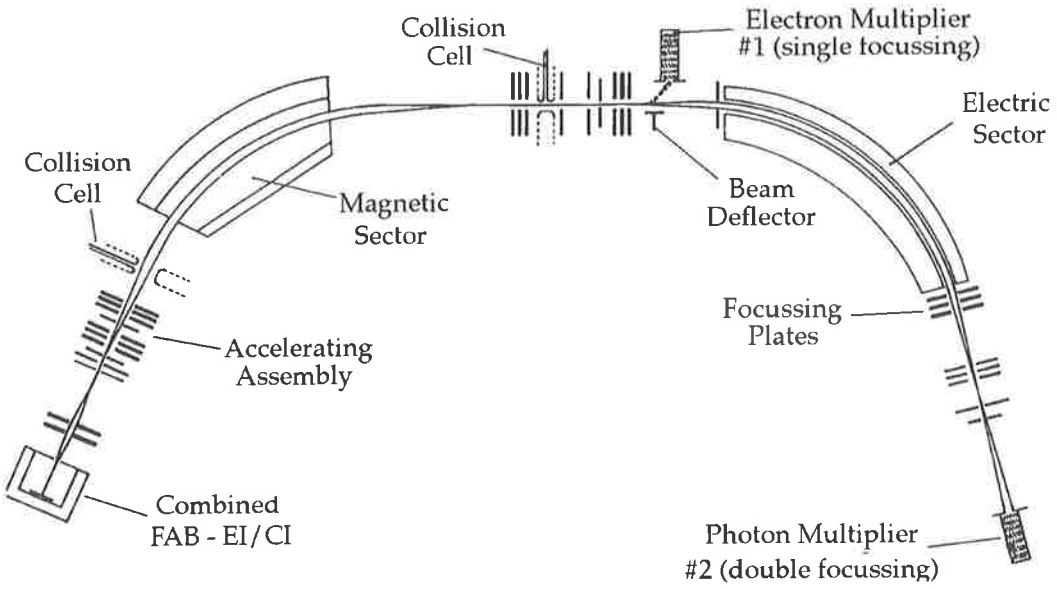

Figure 1.1. Schematic diagram of the ZAB mass spectrometer (redrawn from V.G. Instruments Handbook).[39]

Two collision cells are present in the mass spectrometer. One is located in the first field free region (FFR) between the source and the magnetic sector, and the other is located in the second FFR between the magnetic and electric sectors (see Figure 1.1).

The ions that are produced in the source are focussed through both the magnetic and electric sectors.[39,41] After the ions are produced in the source, they are accelerated by a strong electrostatic field potential, V (volts), and focussed through the exit slits into the analyser region. The kinetic energy ($1/2mv^2$) of the ion is

given by equation (1.1), where z is the charge of the ion (coulombs), m is the mass of the ion (kilograms) and v is the final velocity of the ion (meters/second).

$$1/2mv^2 = zV \tag{1.1}$$

When the ion enters the magnetic sector, the flight path is perpendicular to the lines of the magnetic field, B (tesla). The ion is then subjected to a centripetal force (BzV) that is perpendicular to both the magnetic field and path of the ion. This is balanced by a centrifugal force (mv^2/r) which allows the ions to transverse the magnetic sector, where r is the radius of the curved path. The motion of this path is described by the equation (1.2).

$$Bzv = mv^2/r \tag{1.2}$$

If this equation is rearranged, it can be seen that the magnet sector disperses ions according to their momentum (mv)-to-charge ratios as shown in Equation (1.3).[41]

$$mv/z = Br \tag{1.3}$$

Therefore, when a set magnetic field strength is focussed on a particular mass, the ions entering the magnetic field that are of a different mass, but contain the same velocity, are defocussed by their mass-to-charge ratio (m/z). This only allows the ion with the correct m/z to follow the trajectory through the central radius (r_c), of the magnetic sector. The m/z value of the ions that follow this trajectory is shown in equation (1.4),* where $r_c = r$.

$$m/z = B^2r^2/2V \tag{1.4}$$

* As the velocity of the ion remains constant, equation (1.4) is obtained by substituting v, from equation (1.1) into (1.3).

Since r is fixed, a spectrum may be run by scanning the magnetic field or the voltage. When scanning the voltage, defocussing of the ions can occur which results in a reduction in sensitivity. Therefore, by scanning the magnetic field, ions of different m/z are separated and a routine spectrum is obtained.[42]

The electric sector is able to focus the ion beam on the basis of kinetic energy: this increases the resolution by decreasing the energy spread of the ion beam,[40] with a consequent decrease in the ion current.[43] The ions are focussed at a common point after leaving the magnetic sector. They then enter the electric field, E (volts/meter), produced between two parallel cylindrical plates (see Figure 1.1) where the lines of force are perpendicular to the path of the ion.[41,44] The ion pathway becomes circular as a result of the perpendicular force [see equation (1.5)].

$$mv^2/r = zE \tag{1.5}$$

The parameters m, v, r, E and z have been previously defined: Equation (1.5) can then be rearranged to equation (1.6).

$$mv^2/z = Er \tag{1.6}$$

When ions of a particular energy or velocity enter the electric sector, it is the electric field strength that controls the passage of the ion through the sector. Since r is fixed, an ion kinetic energy (IKE) spectra of ions containing different energies may be run by scanning the electric field strength.

The ions are detected by a photon multiplier after the electric sector when the machine is operated in double focussing mode. The ZAB can also be operated in single focussing mode, with an electron multiplier after the magnetic sector used

to detect the ions. An electron deflector is used to angle the ion beam to the detector.

1.3. Modes of Scanning.

The geometry of the ZAB allows various scans to be performed. The scans that were used for acquisition of data for this thesis were routine low resolution, CA MIKE , B/E and B^2/E scans.

1.3.1. Low Resolution Scans.[23]

Low resolution scans (i.e. accuracy to at least unit resolution[1]) were used when molecular weight information was needed for underivatised peptides or degradation products resulting from either the Edman degradation procedure or enzymatic digest. Operational procedures for low resolution scans are discussed in detail in Chapter 8. In summary the magnet was linearly scanned from m/z 3 500 to 300, using a mass resolution of 1 500 (10% valley definition). The resolution between two adjacent peaks of equal height, m and $m+\Delta m$, can be obtained from the ratio of $m/\Delta m$. An example is shown in Figure 1.2.

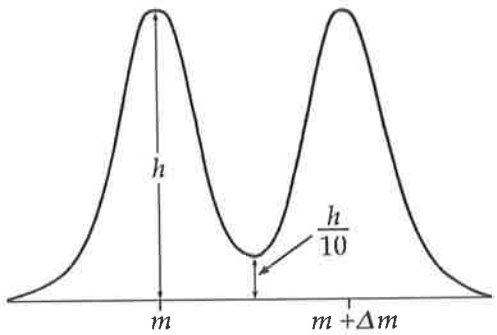

Figure 1.2. Schematic example of two adjacent peaks at a resolution of 10% valley definition (redrawn from Williams[45]).

1.3.2. Collisionally Activated Mass Analysed Ion Kinetic Energy Spectroscopy (CA MIKES).[41]

Mass analysed ion kinetic energy spectroscopy (MIKES) is defined as the change in mass and/or the charge of the source formed parent ion before detection. When a soft ionisation method such as FAB is used, the resulting parent ions (particularly anions) have little excess internal energy: this results in little fragmentation occurring. In such a circumstance, collisional activation of the ions must be used to induce fragmentation.

The technique of CA MIKES allows the analysis of induced fragmentations of the parent ion. The magnetic sector selects the parent ion of interest and focusses the ion beam into the collision cell. Fragmentation of the parent ion produces daughter ions: these are separated according to their kinetic energies by the scanning of the electric field strength. This produces a CA MIKE (or CA MS/MS) spectrum.[5]

The collisional activation process typically occurs in two sequential steps.[46] The first step is where a glancing collision occurs between the parent ion and the collision gas. A small portion of the transitional energy of the parent ion is converted into internal (rotational, vibrational and perhaps electronic) energy.[46-48] The second step involves the dissociation of the energetic ion to produce fragment ions. The number of collisions of each parent ion is dependent upon the pressure of collision gas in the cell.[46] The collision cell contains an inert gas such as argon (xenon and helium can also be used[46]), to a pressure of approximately 2×10^{-7} Torr.* The collision cell pressure produces a 10% reduction in the intensity of the parent ion beam: this equates to single collision conditions (see Stringer *et. al.*[49] and references cited therein).

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* The actual collision cell pressure is higher, since the pressure gauge is located outside the cell.

In general, consider the decomposition of the parent ion (m_p^+), to the daughter ion (m_d^+) and the neutral (m_n,) in the second collision cell [equation (1.7)].

$$m_p^+ \longrightarrow m_d^+ + m_n \qquad (1.7)$$

The energy of the parent ion is conserved during fragmentation[41] and is proportionally distributed between m_d^+ and m_n, according to their mass. Therefore by scanning the electric sector an energy spectrum, i.e. a CA MIKE spectrum will be obtained. The mass of the daughter ion is then determined by the equation (1.8) [E is the electric sector voltage required to transmit the daughter (m_d^+), and E_p is the sector voltage required to transmit the parent, m_p^+]. For the analysis of peptides by CA MIKES, m_n lost, refers to the loss of an amino acid residue.

$$m_d^+ = m_p^+ \frac{E}{E_p} \qquad (1.8)$$

1.3.3. Linked Scanning Techniques.

Linked scanning techniques (which involve three variable fields, viz. accelerating voltage, magnetic field and electric field strength) provides additional information that can be both additional and complementary to CA MIKES.[48,50] Generally, it is the magnetic and electric sectors that are scanned. Accelerating voltage scanning is seldom used since this may result in the tuning of the instrument being compromised.[50] Linked scanning techniques are used for the investigation of decompositions in the first FFR: here, the magnetic and electric sectors are scanned simultaneously at a particular ratio. B/E and B^2/E scans are discussed below and were used routinely for the investigation of fragmentation pathways.

Other linked scanning techniques can also be performed on a double focussing instrument but are not discussed here.[23,41,51]

B/E Linked Scanning.[41,51]

This technique involves setting the magnetic and consequently the electric sector to select a particular parent ion: B and E are then simultaneously scanned, with the initial ratio of B/E remaining constant. B/E daughter ion scans provide analogous information to CA MIKE scans, however with a superior resolution.* [42] The increase in resolution occurs because only ions with a small range of velocities can pass through both sectors. The velocity spread from the fragmentation process is filtered out in this scan. The daughter ion masses are determined as in equation (1.8).

B^2/E Linked Scanning.[41,51]

B^2/E linked scans indicate that the parent ions of a chosen source formed daughter ion. The instrument is set such that the daughter ion is initially transmitted through the mass spectrometer. The magnetic and electric sectors are then scanned at a constant ratio of B^2/E. The resultant peaks are broad because the ions have a range of translational kinetic energies as a result of fragmentation in the source. The parent ion masses may be determined by the equation (1.9) [E is the electric sector voltage required to transmit the parent (m_p^+) and E_d is the sector voltage required to transmit the daughter (m_d^+)].

$$m_p^+ = m_d^+ \frac{E}{E_d} \qquad (1.9)$$

* Collision gas is introduced into the first FFR to induce fragmentation (see Figure 1.1).

Artefact Peaks in CA MIKES and Linked Scans.[23,41,50]

Artefact peaks can occur in both the CA MIKE and linked scan spectra. The artefact peak is usually highly resolved (narrow) in comparison to the daughter ion peaks. It results from a higher mass ion fragmenting prior to the magnet, with a consequent metastable ion having the correct apparent mass to enable it to transverse the magnetic sector.

1.4. Fast Atom Bombardment.[29]

The FAB technique is a relatively simple process based upon that of SIMS. The sample is dissolved in a liquid matrix and is then bombarded by high velocity particles. A matrix is a liquid of low volatility in which the sample is dissolved and which aids in the production of the MH^+ or $(M-H)^-$ ions. Glycerol, thioglycerol/glycerol and eutectic dithiothreitol/dithioerythritol (5:1) mixtures are typically used as matrices for peptides.[52] However, there are many matrices that can be used for differing compounds.[53] The fast atom beam transfers momentum from the atoms to the sample/matrix mixture, where desorption of this mixture into the gas phase occurs. This is followed by analysis of the ion in the mass spectrometer (see Figure 1.3).

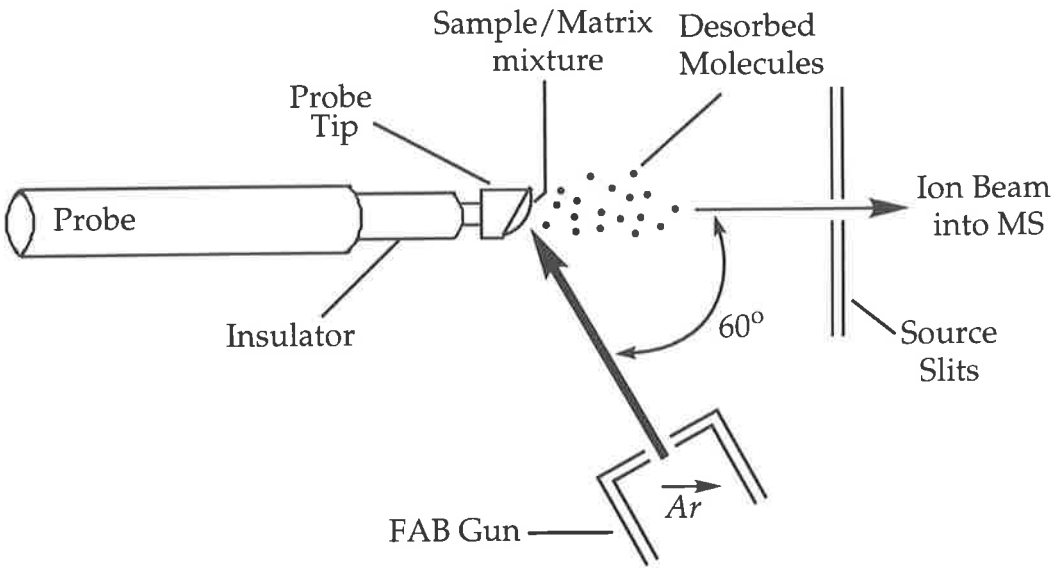

Figure 1.3. Schematic diagram of the FAB source.

The high velocity particles are a beam of atoms, usually argon or xenon, of large translational energy.[54] Argon is used for fast atom production in the ZAB. Argon atoms (*Ar*) are released in the FAB gun where they enter a field of high potential that ionises the atoms producing argon ions (Ar^+).[54,55] The Ar^+ ions are then accelerated within a range of 7-8 keV and are passed through a plasma of neutral argon atoms. The Ar^+ undergo a process of charge exchange (electron transfer) with relatively stationary *Ar* atoms, which results in a beam containing a high proportion of atoms with only minor changes in forward momentum (Equation 1.10).[29,30,47,54] Fast ions which escape the charge transfer process are removed by electrostatic deflection.

$$\underset{\text{fast ions}}{\overrightarrow{Ar^+}_\alpha} + Ar_\beta \longrightarrow \underset{\text{fast atoms}}{\overrightarrow{Ar}_\alpha} + Ar^+_\beta \qquad (1.10)$$

The sample/matrix mixture is placed upon the entire surface of the probe tip, in order to increase the surface layer available for fast atom bombardment. The probe tip is angled at 45° with reference to the probe axis: whereas the FAB gun is

angled at 60° to the probe axis.[55] This geometry has been shown to produce the maximum ion current.[56]

The type of matrix used is important. The main requirements of a matrix are that,[53] (1) it must dissolve the analyte, (2) it must have a low volatility, and (3) it must not react with the compound. The matrix must also be marginally more hydrophobic than the sample, as the sample must occupy the matrix/'vacuum' interface.[57] This helps with sensitivity since the fast atoms are only able to penetrate 10 nm into the surface.[47] The impact of the fast atoms is softened as the matrix absorbs some of the momentum and thus reduces sample damage.[30,57,58] Once desorbed, the matrix molecules evaporate from the sample and in the process reduces the internal energy of the sample molecule.[58,59] The concentration of the sample to the matrix in the clusters also affects internal energy of the sample molecule.[58] The mechanism of formation of the molecular ion occurs by two processes,[60] (1) production of the ion in the liquid phase, which is then spluttered in the gas phase. Depending on the sample and mode used, by altering the pH and/or changing the matrix, the ion currents can be optimised,[53,58-61] and (2) by ion-molecule reactions taking place in the high pressure region immediately above the matrix surface (selvedge region).[60,61] The mechanism can be considered as a 'chemical ionisation' method as charge transfer occurs from the protonated matrix molecules to the analytes.[62]

The FAB ionisation method can produce large polar molecular weight compounds in the gas phase, which have little excess internal energy and are accompanied by little or no fragmentation.[63] This allows the formation of long lasting parent ions[64] and allows CA MIKES or other techniques to deduce structural information.

1.5. Finnigan LCQ Mass Spectrometer.*

The Finnigan LCQ electrospray quadrupole ion trap mass spectrometer (LCQ) is primarily used for biological applications (see Figure 1.4). The LCQ has a current mass range between *m/z* 300-2 000, with a mass accuracy of 0.01% or better. The capability is due to the fact that electrospray ionisation methods produce molecules that have multiple charged states.# Other capabilities of the LCQ are that it has MS^n, as well as on-line liquid chromatography (LC)/MS and LC/MS/MS options.[68] The quadrupole ion trap is basically composed of two end caps and a ring electrode, as depicted in Figure 1.4. The LCQ allows rapid scanning to determine molecular weights of samples, as well as isolation of ions such that MS^n experiments can be performed

For collisional induced dissociation (CID), an ion of interest is isolated inside the ion trap. The internal energy of the ion is then increased such that it will undergo rapid collisions with the helium buffer gas that is inside the ion trap itself.**[65] This is an efficient ion excitation process as the CID of ions is between 40-80%, with 100% occurring in favourable cases.## To provide the MS^2 spectra, the daughter ions produced are then sequentially ejected from the ion trap and detected.[67] If the experiment is required to proceed further, the procedure for CID is repeated: the daughter ion of interest is then isolated in the ion trap, excited, inducing dissociation and the grand-daughter ions are then scanned. This process can continue until there are too fewer ions for experiments to be done.

13,14

* There are several references that overview ion trap mass spectrometry.[9-11,65-67]
# The electrospray ionisation method will be dealt with in Chapter 1.6.
** The helium gas (≈ 10^{-3} Torr) initially acts as a dampening gas to reduced energy of the ions entering the trap.
There are as much as 10,000 low energy collisions between the ions and the helium buffer gas.

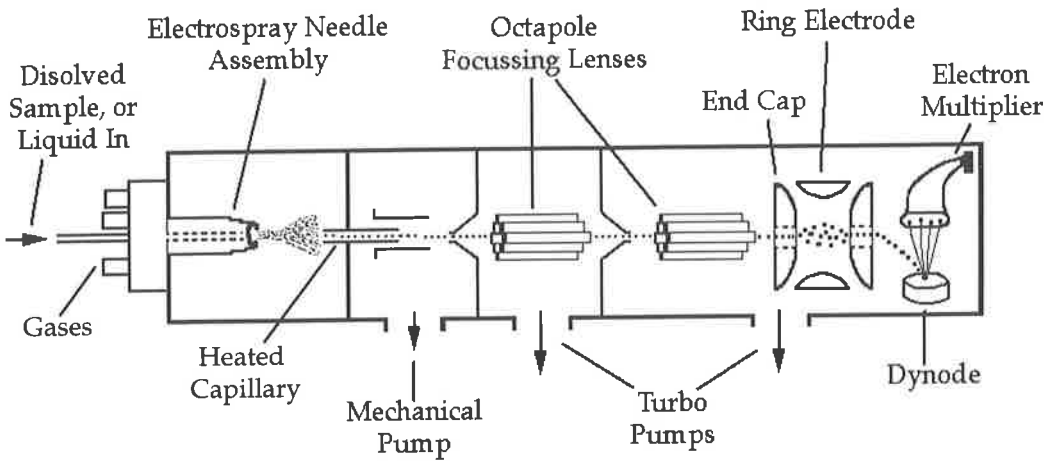

Figure 1.4. Diagram of the LCQ. The ring electrode and end caps form the ion trap (redrawn from the Finnigan MAT News Letter).[69]

1.6. Electrospray Ionisation.

Electrospray is a soft ionisation process, where gaseous ions are produced directly from a solvent system (see Figure 1.5).* [70] This enables the analysis of large biomolecules by the production of multiply charged ions.

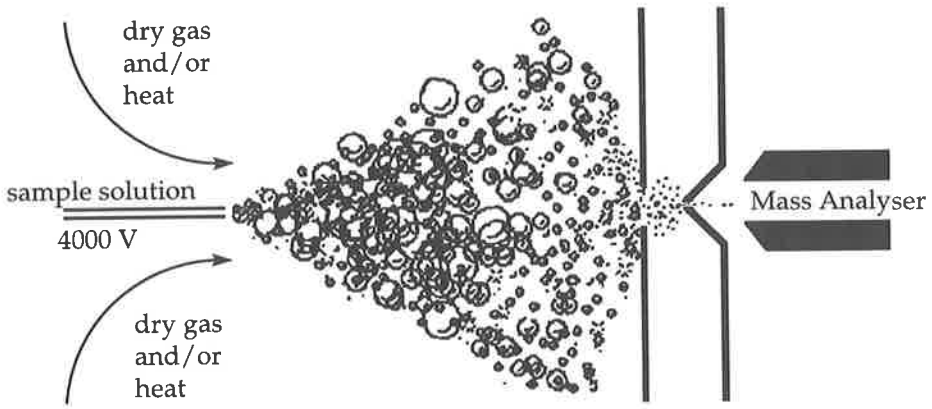

Figure 1.5. A schematic representation of an electrospray source (redrawn from Siuzdak[70]).

* The pure sample can be infused, or directly analysed from LC or electrophoretic systems.

The electrospray technique occurs through the application of a high voltage electric field to a relatively small flow of liquid from a metallic capillary tube.[71] The capillary tube is held at ≈ 4 000 V and produces a spray of charged droplets. Evaporation of the charged droplets is accelerated with the use of a dry gas and/or heat.[70] As evaporation of the droplets occurs, the surface charge density increases until the Rayleigh limit is reached: this is when forces due to electrostatic repulsion are equal to the forces produced by surface tension.[35] Beyond this limit extreme instability results and a 'coulomb explosion' occurs, producing an array of daughter droplets.[35,70] The evaporation and coulomb explosions continue to occur on each generation of daughter droplets until free ions are produced (see Figure 1.6). The free ions are then directed into the mass spectrometer through a series of lenses.

The mass determination of these multiply charged ions is relatively simple. For every multiple charge there exists an envelope of peaks, which are produced by isotopes. By resolving the isotopic envelope, the *m/z* between the isotope peaks can be determined. The difference between the isotope peaks (in Da) is inverse to the charge state of that particular envelope. For example, if the difference between the isotope peaks were 0.5 Da, the charge state of that envelope would be +2. If the difference was 0.25 Da, the charge state would be +4. From the charge state it is a simple multiplication to determine to MH^+ of the molecule.

Electrospray is a soft ionisation process, where large biomolecules are ionised and introduced into the gas phase. The amounts that are needed for molecular weight determination and structural analysis are in the low-femtomole range.[72] The advantage is the production of multiply charged ions which allows for the analysis of very high molecular weight compounds.

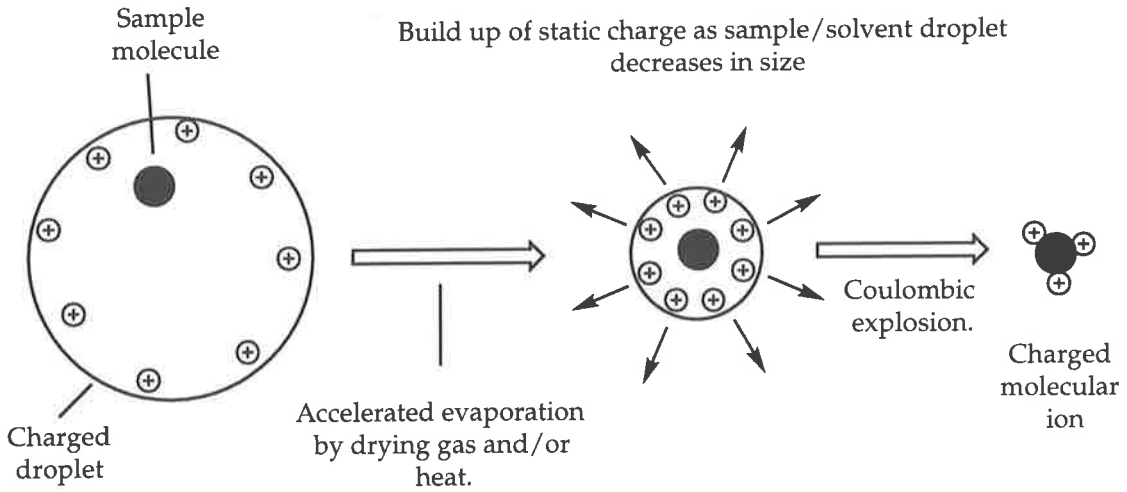

Figure 1.6. Schematic representation of the evaporation of charged droplets and coulomb explosions.

CHAPTER TWO: AMPHIBIANS AND PEPTIDES.

2.1. General Introduction.

The word amphibia is derived from the Greek words *'amphi'* meaning double, and *'bios'* meaning life, describing animals that are both terrestrial and aquatic. However, this definition is inaccurate in some instances, as a significant number of species spend their entire life upon land and do not return to the water, even to reproduce.[73]

Amphibians evolved from the Devonian sea to inhabit the land 350 million years ago. The change of environment required physical and behavioural adaptations. The major development was an epidermal layer which contained multi-cellular exocrine glands, in which a mucosal secretion was developed in adult amphibians to retard against desiccation.[74,75] This feature has evolved further to aid the general homeostasis of the frog.[76-79] It is the content of this mucus that protects the amphibian from the many and varied predators in both aquatic and terrestrial domains.[74] This protective 'shield' safeguarded amphibians during their evolution and helped them to radiate from the Devonian seas and colonise a wide variety of environments.[74,75]

2.2. Human Interest in Frog Peptides.

The contents of the secretion that assisted frogs in their spread over the bulk of the earth have been exploited by mankind, sometimes to the detriment of the frog. The frog, or more specifically the secretion, has been associated with all facets of life including healing powers, witchcraft and poisons for hunting in the folklore of

many societies such as in Europe, Asia, Africa and America.[74,80-83] The earliest recorded use is by the ancient Chinese 4 000 years ago, when frog skins and secretions were used to regulate internal bodily functions and fertility.[74,84] Today, frog secretions are still used by some indigenous people of Africa and South America.[85] One example involves the Matese Indians of northern Peru.[86] The secretion called 'sapo', obtained from *Phyllomedusa bicolor* is used to improve luck in hunting. The secretion is applied to fresh burns causing violent peripheral gastrointestinal and cardiovascular effects. This is followed by an apparent increase in physical strength, heightening of senses, resistance to hunger and thirst, and an increased capacity to cope with stressful situations. Toxins, antibiotic agents and neurotransmitters have now been isolated from the secretion.[74] From the active compounds in the secretion it is no wonder that it was believed that these frogs embodied a certain magic. In contrast, the Australian aborigines use frogs, e.g. *Cyclorana platycephala* and other burrowing species as a source of water during times of drought, but apparently not for any pharmacological or medicinal purposes.[87,88]

Erspamer and his colleagues initiated work on the isolation and activity of frog peptides in the early 1960's. They first identified phylsalaemin, a potent hypotensive agent from the South American frog *Physalaemus fuscumaculatus*.[89,90] They have reported that the frog skin is an extraordinary source of biologically active peptides waiting discovery, isolation and characterisation.[79] The main attractions of frog skin as a viable source of peptides are, (1) frogs and therefore the peptides are readily accessible, (2) the peptides occur in large quantities in amphibians (compared to analogues occurring in mammalian systems), and (3) different species of frogs store different types of pharmacologically active peptides.[79]

Erspamer has predicted that every peptide present in the frog skin should have a mammalian counterpart.[91] This prediction has been partially supported by observations such as the identification of crina-angiotensin II [structure (2.1)] from *Crina georgina*,[92] and the mammalian analogue angiotensin II, with smooth muscle and renal activity[93-97]; xenopsin from *Xenpous laevis*[98,99] [structure (2.10)], and the mammalian analogous neurotensin[100,101] with smooth muscle and gastric activity. However, very few of the ninety plus amphibian peptides characterised by the Adelaide research group correlate with reported mammalian analogues. The number of known frog species is over 4 500[102] and increasing. Only a small percentage of these species have been analysed to date, and therefore many readily accessible active peptides, i.e. hormones, neurotransmitters, antibiotics and other biologically active peptides, remain to be discovered.[84] The chemical and pharmacological investigation of these compounds found in the amphibian skin offers a promising pathway towards new medicinal discoveries.[75]

2.3. Distribution of Granular Glands.

Amphibians live in environments where there are numerous macro- and microscopic predators, and it is therefore not surprising that their dermal secretion contains a wide variety of host defence compounds. The greatest concentration of defence compounds is found on the skin, as well as in the brain,[103] circulating in the blood[104,105] and in internal organs.[106,107] In some amphibian species, toxins can also be found in the eggs and ova.[108-110] The secretion contains an array of host defence compounds of simple and complex aliphatic, aromatic and heterocyclic molecules, small and large peptides, with peptide fragments associated with prohormone processing events.[74,111,112] This variety of compounds in the skin represents a spectacular array of bio-organic chemistry.[112]

There are primarily two types of glands in the skin, the granular and the mucosal glands, with other minor glands present in other anuran species.[74,113,114] The active components are produced in the granular glands, located on the dorsal surface and legs. The glands are dispersed throughout the dermal layer of the frog, and in some species the glands are hypertrophied, as they are strategically concentrated in the areas that are most exposed to predatory attack.[75] There are eight different glands that can occur, these are named according to their anatomical position and are listed below in Table 2.1. A particular example of the rostral glands is seen in *L. splendida*, shown in Figure 2.1.

Name of Gland	**Anatomical Location.**
(1) rostral -	on the dorsal surface of the head.
(2) supralibial -	on the upper lip extending posteriorly beyond the angle of the jaw.
(3) parotoid -	on the shoulders.
(4) submandibular -	adjacent to the lower jaw.
(5) coccygeal -	on the flanks on each side of the coccyx.
(6) inguinal -	on each side of the body in the groin.
(7) femorial -	on the postural surface of each femur.
(8) tibial -	on the dorsal surface of each calf.

Table 2.1. Names and locations of the various glands present on the frog.[84]

The operation of the glands is under the control of the central nervous system,[115,116] and the release of the secretion occurs when the frog placed under stress.[84,117] A striking example comes from the African clawed frog *Xenopus laevis*. When *Nerodia sipedon*, a frog eating snake attempts to eat the frog, *Xenopus*

laevis, the secretion released by the frog induces the snake to climb, yawn and gape: all of which enable the frog to escape.[118]

Figure 2.1. The rostral glands of *Litoria splendida* (photo courtesy of M.J. Tyler, Department of Zoology, University of Adelaide).

2.4. Production of Peptides. *

The active peptides produced in the granular glands of the frog are a direct product of the genetic code. They are excised from large proteinaceous precursor molecules, prepropeptides and prohormones, by cleavage using an array of specific enzymes.[74] For example, (1) the defensin antibacterial peptides are 29-35 amino acid residues in length, and are formed from pre-pro-defensins, which are 94-100 amino acids residues long, and (2) the magainin antibacterial peptides (from *Xenopus laevis*) are 23 amino acids residues long, however the pre-pro-

* The investigation in to the biosynthesis of peptides have been done on *Xenopus leavis.*

magainin is 303 residues long, with alternating copies of anionic and cationic peptides.[81,119]

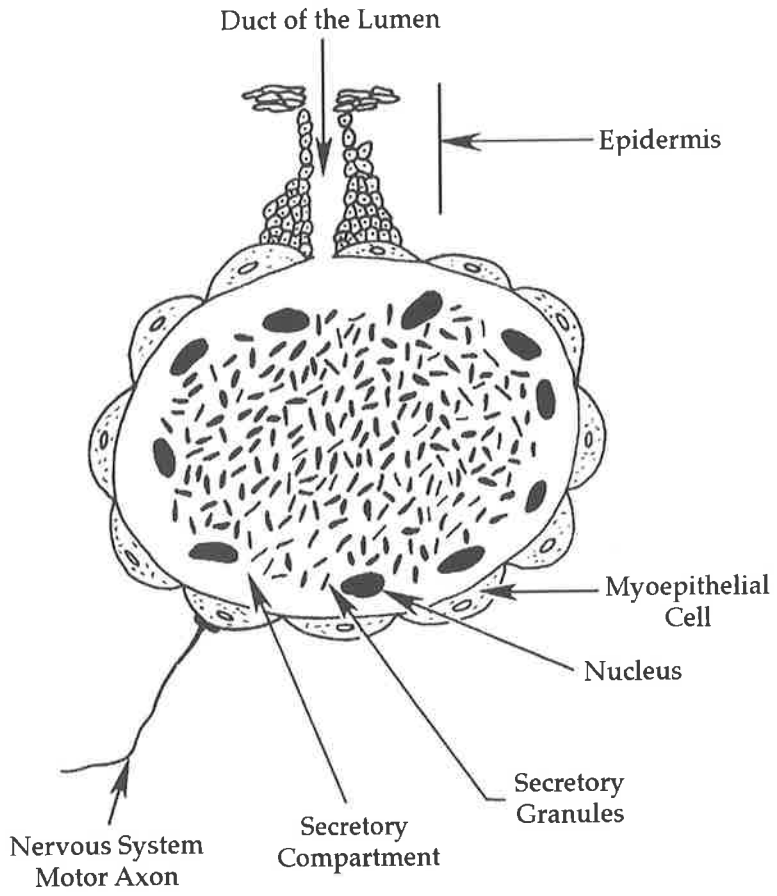

Figure 2.2. A schematic representation of the granular gland from *Xenopus laevis* (redrawn from Barthalmus[118]).

The granular gland (see Figure 2.2) is a syncytial gland# and is located just below the epidermis. The gland consists of a myoepithelial cell envelope (the outer wall), with the nuclei, endoplasmic reticulum‡ and Golgi complexes** located inside the

A syncytial gland is composed of numerous types of cells fused together to produce a giant multinucleated cell or gland.[120]

‡ The endoplasmic reticulum is composed of a sheet of membranes, extending from the outer layer of the nuclear envelope into the cytoplasm. It is involved in the biosynthesis of lipids, proteins and carbohydrates, and is also involved in sorting proteins for transportation through the cell.[121]

** The Golgi complexes are an individual stack of membranes near the endoplasmic reticulum, and are involved in both the modifications of peptides, and arranging them for transport to different cellular locations.[121]

cell, but on the periphery.[121,122] The lumen of the gland contains the secretory granules, in which the peptides are stored.

The production of the mature peptide occurs by the same fundamental process. Transcription from the deoxyribonucleic acid (DNA) to the messenger ribonucleic acid (mRNA) occurs in the nuclear envelope. This is followed by translation of the mRNA to produce the prepropeptide.[120,121] The signal peptide (prepiece) facilitates transportation within the cell and is subsequently cleaved.[121] In the granular gland production of the peptides occurs by two pathways, [123] one is when the gland is maturing, and the other when the gland needs replenishing. During the development of the gland, when the vacuolated stage## is reached, the propeptides are stored in secretory granules, which are themselves stored in the lumen.[123] Organisation of the secretory granules by the Golgi complexes does not occur.[123] In the rejuvenation state, the secretory granules are formed from the Golgi complexes.[123] In the laden mature granular gland peptide production is at a low level.[123]

The storage of the mature peptide is assisted by the spacer peptide (propiece), which counteracts the activity of the mature peptide (i.e. the propiece is generally negatively charged or neutral).[119,124] This counteracts the positive charge(s) on the mature antibiotic peptide (see Chapter 2.7.2) and assists folding of the mature peptide to inhibit any enzymatic degradation. The mature peptide is dormant in the propeptide form. Upon stimulation of the granular gland by the nervous system (see Figure 2.2), the propeptide is subjected to specific enzymatic cleavage to yield the active mature peptide.[119,124] The peptide is then released from the granule by a holocrine mechanism*** and excreted onto the dorsal surface.[122]

The vacuolated stage is where the cell membrane of secretory cells disintergrate producing the lumen.[123] The nucleus and related complexes are then located on the periphery of the lumen.

*** A holocrine mechanism involves disintergration of the cell resulting in the release of the cell contents.[125]

2.5. Collection of Frog Peptides.

When frog skins were used in early Chinese history, the custom was to orally ingest the dried frog skins.[74,84] The extraction procedures used by Erspamer also used the dried skin. The dried skins were typically soaked in aqueous methanol, peptides were then extracted and separated through alumina column chromatography.[126,127] The number of skins needed to obtain workable amounts of peptides were often in the thousands.[126-128] The evolution of analytical techniques such as HPLC, has refined the characterisation process.[81] Even so, until recently, the frogs being studied still needed to be killed to obtain the compounds under investigation.

World wide concern was generated when frog populations were universally found to be in serious decline. Thus it was crucial that a non-invasive method of extraction should be developed.[115] In addition, if the frog is killed humanly, the defence mechanism may not be activated, with the result that the peptides are still in the propeptide stage in the gland. This way may not be a viable method for obtaining the mature (active) peptide in good yield, as the release of the secretion occurs naturally following injury or stress.[79] Thus release of the secretion can be induced in the laboratory by subcutaneous injection of adrenaline or noradrenaline[129-131] or by physical handling.[132,133] Recently, a benign technique of extraction was used by Tyler and colleagues.[115] This was found to be more reliable, gave higher amounts of mature peptides and is ecologically 'friendly'.

This method is called surface electrical stimulation (SES), commonly called 'milking'. It is a variation of an earlier technique developed by Dockary and Hopkins.[122] The frog is held by the hind legs and its skin moistened with distilled water. A platinum electrode is attached to an electrical stimulator and is rubbed over the dorsal surface of the animal to contract the granular glands. The stimulus

strength varies with the size of the frog (presumably due to the thickness and conductivity of the skin). There is a delay of the discharge of the secretion, with the onset occurring between 5-15 seconds and the entire process being completed in 30-40 seconds. The gland releases between 80-90% of the secretion,[122,134] which in some species can be up to 70-100 mg of peptide material.[135] The crude secretion is then washed from the frog with distilled water and diluted with an equivalent volume of methanol. This mixture is then kept frozen for at least twelve hours, allowing for the precipitation and deactivation of any enzymes that may be able to degrade the peptides. It has been reported that the active peptide substituents from *Bombina varigeata* are enzymatically deactivated within one hour at room temperature. This is believed to be a safety mechanism in order to stop the peptides from acting upon the host.[136] The glands are replenished within several days,[115] but normally the frogs are only 'milked' at monthly intervals. Using this method the peptides may be separated and characterised from a single frog (rather than thousands sacrificed as in earlier studies).[126-128]

2.6. Families of Frog Peptides.

The frog skin is an extraordinary resource of biologically active peptides. The peptide families discovered to date can be broadly classified by their biological activity; they are either pharmacologically active (exhibit smooth muscle activity) or possess antibiotic activity (through non-specific interactions upon the bacterial cell membrane).# The structure of the peptide families are highly conserved which allows them to be categorised. This categorisation can establish a basis for structure and function relationships.[79] The pharmacologically active peptides include ten families of peptides, such as angiotensins (2.1), bombesins (2.2), bradykinins (2.3), caeruleins (2.4), dermorphins (2.5), pipinins (2.6), tachykinins

This classification by activity was obtained from Bevins and Zasloff.[79]

(2.7), tryptophyllins (2.8) uperins (2.9) and xenopsins (2.10). The mechanism of their activity is explained in more detail in Chapter 2.7.1. These peptide families are indicated below, with the name of peptide family, an example and the frog from which the peptide was first isolated.

Angiotensins; crinia angiotensin II, *Crinia georgina*.[92]

Ala Pro Gly Asp Arg Ile Tyr Val His Pro Phe (OH) (2.1)

Bombesins,# bombesin, *Bombina bombina* and *Alytes obstetricans*.[138,139]

pGlu Gln Arg Leu Gly Asn Gln Trp Ala Val Gly His Leu Met (NH_2) (2.2)

Bradykinins, bradykinin, *Rana temporaria*.[140]

Arg Pro Pro Gly Phe Ser Pro Phe Arg (OH) (2.3)

Caeruleins, caerulein, *Litoria caerulea*.[141]

pGlu Gln Asp Tyr(SO_3H) Thr Gly Trp Met Asp Phe (NH_2) (2.4)

Dermorphins, D-Ala containing peptide from *Phyllomedusa sauvagei, Phyllomedusa rohdei* and *Phyllomedusa burmeisteri*.[128,142]

Tyr D-Ala Phe Gly Tyr Pro Ser (NH_2) (2.5)

Pipinins, pipinin 1, *Rana pipinens*.[143]

Phe Leu Pro Ile Ile Ala Gly Val Ala Ala Lys Val Phe Pro Lys Ile Phe Cys Ala Ile Ser Lys Lys Cys (OH)* (2.6)

Tachykinins, physalaemin, *Physalaemin fuscumaculatus*.[89,90]

pGlu Ala Asp Pro Asn Lys Phe Tyr Gly Leu Met (NH_2) (2.7)

First bombinin isolated was a hexapeptide amide, Ala Glu His Ala Asp (NH_2) from *B.variegata*.[136,137]

* Disulphide linkage occurs between the two cystine residues.

Tryptophyllins, *Phyllomedusa rohdei*.[144]

pGlu Pro Trp Met (NH_2) (2.8)

Uperins, uperin 1.1, *Uperoleia inundata*[145]

pGlu Ala Asp Pro Asn Ala Phe Tyr Gly Leu Met (NH_2) (2.9)

Xenopsins, xenopsin, *Xenopus laevis*.[99]

pGlu Gly Lys Arg Pro Trp Ile Leu (OH) (2.10)

Antibiotic peptide families include bombinins (2.11), brevinins (related to pipinins) (2.12), caerins (2.13), frenatins (2.14), gaegurins (2.15) and the magainins (2.16). The mechanism of action will be dealt with in more detail in Chapter 2.7.2.

Bombinins, bombinin, *Bombina variegata*.[146]

Gly Ile Gly Ala Leu Ser Ala Lys Gly Ala Leu Lys Gly Leu Ala Lys Gly Leu Ala Glu His Phe Ala Asn (NH_2) (2.11)

Brevinins, brevinin 1,* *Rana brevipoda*.[147]

Phe Leu Pro Val Leu Ala Gly Ile Ala Ala Lys Val Val Pro Ala Leu Phe Cys Lys Ile Thr Lys Lys Cys (OH) (2.12)

Caerins, caerin 1.1, *Litoria splendida*.[135]

Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2) (2.13)

Frenatins, frenatin 3, *Litoria infrafrenata*.[148]

Gly Leu Met Ser Val Leu Gly His Ala Val Gly Asn Val Leu Gly Gly Leu Phe Lys Pro Lys Ser (OH) (2.14)

---

* Disulphide linkage occurs between the two cystine residues.

Gaegurins, gaegurin 1, *Rana rugosa*.[149]

Ser Leu Phe Ser Leu Ile Lys Ala Gly Ala Lys Phe Leu Gly Lys Asn Leu Leu Lys Gln Gly Ala Cys Tyr Ala Ala Cys Lys Ala Ser Lys Gln Cys (OH) (2.15)

Magainins, magainin I [Gly (10), Lys (22)], *Xenopus laevis*.[150]

Gly Ile Gly Lys Phe Leu His Ser Ala Gly Lys Phe Gly Lys Ala Phe Val Gly Glu Ile Met Lys Ser (OH) (2.16)

Another group of peptides, the caeridins (2.17), dynastins (2.18) and rubellidins (2.19), have been discovered in Australian tree frogs. The peptides that belong to these families have undergone activity testing with no antibiotic nor pharmacological activity being noted.

Caeridins, caeridin 1, *Litoria splendida*.[135]

Gly Leu Leu Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2) (2.17)

Dynastins, dynastin 1, *Limnodynastes interioris*.[151]

Gly Leu Leu Ser Gly Leu Gly Leu (OH) (2.18)

Rubellidins, rubellidin 1, *Litoria rubella*.[152]

Ile Glu Phe Phe Thr (NH_2) (2.19)

2.7. Mechanism of Peptide Activity.

2.7.1. Pharmacological Activity.

There are many instances where peptides are pharmacologically active. Such examples include, (1) cholecystokinin[153] (a neuronal and intestinal peptide), (2)

substance P[154,155] (a neurotransmitter), and (3) β-endorphin[156] (exhibits opiate-like activity). Many of these mammalian peptides have amphibian analogues that are similar in both structure and pharmacological activity. The amphibian analogue of cholecystokinin is caerulein (2.4), substance P is similar to the amphibian analogues physalaemin (2.7) and eledoisin (2.20). In these cases the mammalian peptides were discovered first. There have also been cases where the discovery of amphibian peptides has initiated the search for the mammalian analogue, i.e. bombesins (2.11) and dermorphins (2.5).[112]

pGlu Pro Ser Lys Asp Ala Phe Ile Gly Met (NH_2) (2.20)

Many active compounds facilitate their activity by binding to receptors. Receptors typically have a quaternary structure, that is they are a complex of interacting proteins. The binding of a ligand to a receptor can initiate a variety of cellular effects, some of which are very rapid, for example synaptic transmissions. While others, which are produced by hormones are slow in comparison. There are various types of receptor mechanisms to effect the cellular response,* [157,158] however the principles of the ligand binding to the receptor does not change.

The binding of the active peptide (ligand) to its receptor may involve various interactions including covalent, ionic, hydrogen bonding, hydrophobic and van der Waals. Such binding typically includes several different interactions, which dictate the duration of the interaction and therefore the cellular response. The strength or intensity of a particular response is determined by the number of activated receptors that are present upon the cell.[158]

The affinity of the peptide to its binding site in the receptor is dependent upon three dimensional interactions. Minor alterations in the peptide structure can

* The types of effector receptor mechanisms are shown in references [157,158].

result in significant changes in the bioactivity.[158] The total extent of the pharmacological action is dependent upon the structure-activity relationship of the peptide and the receptor. The intensity and extent of the responses are dependent upon the location and number of receptors present on the cells throughout the body.

2.7.2. Antibiotic Activity.

Mucosal areas such as reproductive and respiratory tracts are constantly in contact with infectious micro-organisms, yet the incidence of infection from these organisms remains low. The mucosal surface has developed certain agents, including antibiotic peptides to combat these threats.[159] Examples of antibiotic peptides found include, (1) andropin (2.21), a reproductive tract peptide isolated from the giant silk moth, *Hyalophora melanogaster*,[160,161] (2) magainin 1, from the granular glands from *Xenopus laevis* (2.15),[150] and (3) the cysteine rich tracheal antibiotic peptide (TAP) (2.22) from the bovine tracheal mucosa.[162,163]

Val Phe Ile Asp Leu Asp Lys Val Glu Asn Ala Ile His Asn Ala Ala Gln Val Gly Ile Gly Phe Ala Lys Pro Phe Glu Leu Leu Ile Asn Pro Lys (OH) (2.21)

Asn Pro Val Ser Cys Val Arg Asn Lys Gly Ile Cys Val Pro Ile Arg Cys Pro Gly Ser Met Lys Gln Ile Gly Thr Cys Val Gly Arg Ala Val Lys Cys Cys Arg Lys Lys (OH) (2.22)

The primary structures of antibiotic peptides vary significantly as they can act directly with the membrane surface and are independent of any chiral receptors located on the membrane of the microbe.[164] The secondary structure of the antibiotic peptide is crucial for its activity.[159,165] All antibiotic peptides have

certain features in common, (1) the peptide has to span the width of the bacterial cell membrane (approximately 30 Å),[166] (2) have an overall positive charge due to the presence of basic amino acid residues (e.g. histidine, arginine and lysine),[167,168] and (3) have the ability to form amphipathic structures (α-helices). The interaction of the peptide and the cell membrane is not dependent upon chiral interactions. For example D- and L-magainins,[164] D- and L-caerins,[169] and D- and L-ceropins[168] have all shown identical activity.

An antibiotic peptide is often conformationally random in the extracellular fluid, with a net positive charge at neutral pH. The positively charged peptide will bind electrostatically to a membrane displaying accessible anionic phospholipid head groups.[166,170] During binding to the membrane the lipid environment induces the peptides to assume an α-helical secondary structure, which contain discrete hydrophobic and hydrophilic zones. The peptide lies parallel to the membrane allowing the hydrophobic region to be buried in the lipid environment whereas the hydrophilic region is exposed to the aqueous environment. The α-helical structure may be interpreted using the Schiffer-Edmundson wheel projection (see Figure 2.3).[171]

The formation of the α-helix has been determined by NMR studies for magainin II[172,173] and caerin 1.1.[169,174] The magainins produce a full α-helix. However, the caerins produce a structure, which contains two α-helices, Leu(2)-Lys(11) and Val(17)-His(24), separated by a short central region of a helical nature and greater flexibility (see Figure 2.3).[175] The flexible region of caerin 1.1 is produced by the presence of proline residues at positions 15 and 19 in the sequence. The central hinge 'region' enables the molecule to undergo reorientation (depending upon the pathogen involved) to retain two distinct hydrophobic and hydrophilic regions.* [169] The prolines are vital for the activity of caerin 1.1, when they are replaced with

* The Schiffer-Edmundson wheel diagram of the full caerin 1.1 sequence, does not define two distinct hydrophobic and hydrophilic regions.

glycine, the activity drops significantly.[176] Similar hinge regions occur between the helical structures of the ceropin[177] and sarcotoxin 1A antibiotic peptides.[178]

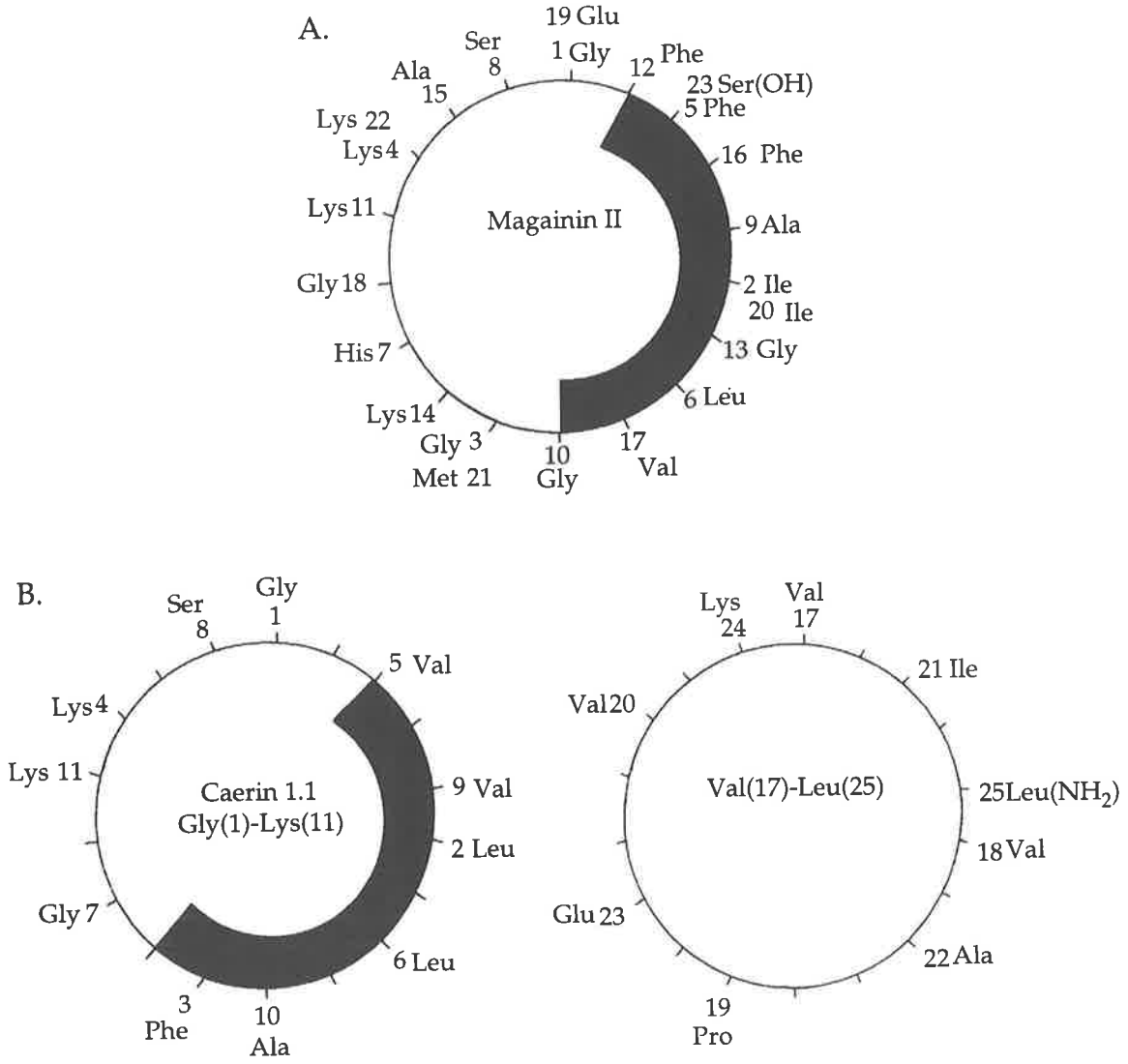

Figure 2.3. The Schiffer-Edmundson wheel projection indicating the hydrophilic and hydrophobic (shaded) regions, of (A) magainin II and (B) caerin 1.1.

The precise mechanism of the antibiotic action is not known with certainty but it may involve disruption of the microbial membrane through a 'carpet-like' mechanism.[179] When a critical concentration is reached, the peptides form a carpet or raft configuration, which produces transient holes in the membrane,

increasing the permeability of the cell membrane that leads to ion leakage and cell death.[169]

2.8. Evolutionary Trends.

Frogs are classified by traditional taxonomic methods including, somatic/osteological (bone) structures, size, markings, colours and advertisement calls.[87,113,180-183] These methods have certain limitations since some frog species are similar morphologically. Classification by colour alone can be difficult, since the colour of the frog can be changed by hormones, light intensity, temperature, humidity and stress.[87,184] Genetic methods are becoming an essential tool for taxonomy, as they provide rapid and precise measurements to allow the classification of species and genera.[87] These methods include Karyology,[185] i.e. the study of the number and size of chromosomes in each cell. Australian frogs of the *Litoria* genus typically have 13 chromosomal pairs, an exception is *Litoria infrafrenata* which has 12.[186] A more detailed approach involves the study of the specific genes in the DNA,[185] where genetic differences can be used to map evolutionary trends between different species. Biochemical genetics[185,187] is a method where the various proteins, the products of the genetic codes are compared.[79,87]

The peptides that are released on the dorsal surface of the frog are also a product of the genetic code. These peptides are highly conserved and it is this conservation that enables the comparison of peptide structures to determine evolutionary trends. Certain trends have been observed from the various studies of amphibian peptides. Different frog species may contain different types and amounts of peptide material. Different species located in the same geographic region may contain members of the same peptide family. This final trend suggests

an adaptation due to a specific feature of the ecology in a particular location.[180] Examples of such trends are observed from the common Australian green tree frog *Litoria caerulea*. *L. caerulea* is found across the top of Australia from the Northern Territory through to the east coast of Queensland. The characterisation of the peptides from specimens from different geographic locations have revealed that *L. caerulea* has evolved into two sub-species, an eastern species and a northern-central species (see Figure 2.4). Minor differences in peptide content were also observed between the populations of *Litoria caerulea* on Melville Island and from Darwin. [188] [189-191]

Figure 2.4. A map of Australia indicating two chemically different sub-species, a central and eastern species of *L. caerulea* (redrawn from Steinborner *et. al.*[192]).

The peptide composition and thus chemical taxonomy can be used as a probe to study evolutionary trends of frogs,[180] it must however be used in parallel with other taxonomic methods.

2.9. Peptide Sequencing.

There are a variety of techniques that are currently used for peptide characterisation. Common techniques that are used for the determination of the primary structure are, (1) automated Edman sequencing, (2) recombinant DNA sequencing, and (3) mass spectrometry. Other techniques such as nuclear magnetic resonance spectroscopy (NMR)[193-196] and X-ray crystallography[197] are not commonly used for the primary sequencing of peptides. They are however presently used for the determination of the secondary and tertiary structures.

2.9.1. Automated Edman Sequencing.[198,199]

This method was first introduced in 1967 by Edman and Begg,[200] and has been further developed following improvements in HPLC and sample handling.[201] The technique allows the primary sequence of the peptide to be determined by the sequential removal of amino acids from the N-terminal end of the peptide. This is an effective technique and in favourable cases up to seventy amino acids may be identified using only picomole amounts of material. However this technique does have some drawbacks.[202] These problems include;

(1) the inability to sequence a peptide or protein with a blocked N-terminus[201] (i.e. if there is no primary or secondary amine on the N-terminal end). This prohibits the coupling of the peptide with phenyl isothiocyanate. An example is when the N-terminal amino acid residue is pyroglutamic acid.

(2) the inability to detect uncommon or modified amino acids.[203] This is because, (i) the chromatographic behaviour of the corresponding phenyl thiohydantoin (PTH) derivative is not known (because the modifying group is lost during the Edman procedure), or (ii) the PTH derivative may be insoluble in organic solvents and consequently not detected.

(3) as the size of the peptide decreases, it becomes more soluble and may be washed from the solid support. The remaining information of the peptide is then lost.

2.9.2. Recombinant DNA Sequencing.[204,205]

This technique was developed in the 1970's and is a versatile tool for obtaining peptide sequences from the genes or the complementary DNA (cDNA). Protein sequences up to and beyond 1 000 amino acid residues can be readily obtained.[206]

DNA contains four nucleotides, adenine (A), thymine (T), guanine (G) and cytosine (C), where a combination of three of these is a codon.[121,206] A sequence of these codons is transcribed by mRNA templates, which are then translated to an amino acid sequence.[121,206] To obtain the sequence of the peptide, the section of DNA from which it is encoded from must be known. Sequencing can commence once the section of interest is located.

This technique, although relatively non-invasive (only requiring a small amount of tissue or serum sample for analysis) has three drawbacks;

(1) the area genome which encodes for the peptide of interest has to be located before sequencing can commence.

(2) the number of nucleotides that require sequencing compared to the primary sequence of the peptide is in a ratio of 3:1 (three nucleotides/codon/amino acid).

(3) the protein sequence derived from the genetic code does not take into account any post-translational modifications or any rare amino acids that cannot be predicted.[175,203,207,208]

2.9.3. Mass Spectrometry.

Prior to 1970 the sequencing of peptides and proteins by mass spectrometry was a difficult undertaking. The development of SIMS and FAB mass spectrometry in the 1980's allowed for effective and efficient analysis of large molecular weight compounds. Further developments in mass spectrometry with electrospray and matrix assisted laser desorption ionisation have made the analysis of biologically important samples more efficient and available.

It is the ability of mass spectrometry to determine the molecular weights of peptides with an accuracy of better than 1 Da, which makes it able to by-pass problems associated with the other characterising techniques.[202] Another advantage is the ability to determine the structure of high molecular weight compounds routinely using picomole amounts of material. The ions produced are often of low internal energy, such ions have to be energised in order to effect fragmentation. Fragmentation data may be obtained from the underivatised peptide, irrespective of the amino acid composition and of any post-translational modifications.

Edman degradation/MS (Edman/MS),[209,210] provides primary sequence information from the N-terminal end of the peptide. After every cycle the peptide is checked by FAB MS and the difference between the parent and the truncated peptide indicates the amino acid residue lost. Modifications to this method allow a continuous stepwise degradation of the peptide, with a single mass spectrometric analysis producing a peptide sequencing ladder.[211,212] If the N-terminal group is a pyro-glumatate, the Edman/MS procedure is not applicable (Chapter 2.8.1). This problem can be overcome by methylation to open up the pyro-glutamate, producing a primary amine that may participate in the Edman

degradation. Methods are also available that allow C-terminal sequence determination.[213-215]

The method of CA MIKES has been discussed previously in Chapter 1.3.2. When the FAB CA MIKES technique is applied to peptides, the spectra generally provide sequence information. The positive ion fragmentations produced are predominately backbone fragmentations through and around the amide bonds. A set of fragment ions is produced and the mass differences between related fragment ions identifies the sequence. FAB CA MIKES differentiates all amino acid residues except for the isomeric leucine and *iso*leucine, and the isobaric lysine and glutamine residues.

The following nomenclature, first proposed by Roepstorff and Fohlman[216] and later modified by Biemann[52,217,218] have been used in this thesis. Fragmentations are shown in Figure 2.5.

There are basically six types of sequence determining fragmentations, 'a_n', 'b_n' and 'c_n' occur when the charge is located on the N-terminal end, and the 'x_n', 'y_n' and 'z_n' fragmentations occur when the charge is located upon the C-terminal end. The subscript 'n' indicates the fragmentation through a particular amide bond. The fragmentation is often used in pairs for sequence determination and any of the pairs above can be used for sequence determination as shown in Figure 2.5. The fragmentations used for sequence determination for this thesis are the 'b_n' and 'y_n' ions, however the 'b_n' ions are observed to be more predominate in the spectra obtained.[219,220] Side chain fragmentations also occur producing 'd_n' and 'w_n' from the 'a_n+1'[221] and 'z_n+1'[222] ions respectively. However these ions are normally seen in high energy spectra* and have been used to distinguish between the amino acid residues leucine and *iso*leucine.[222]

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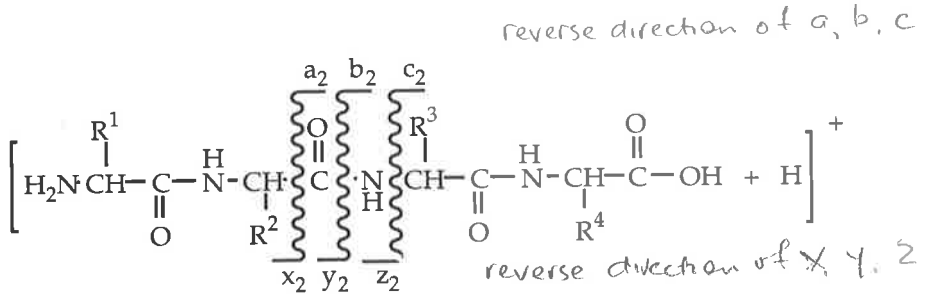
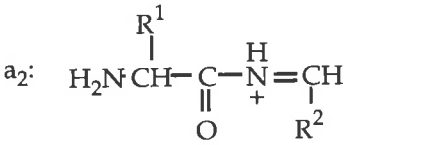
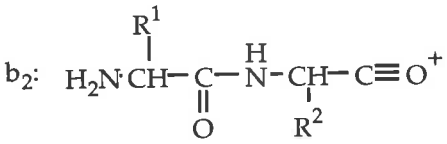
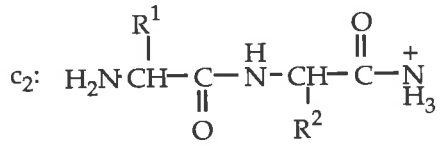
* These high energy ions are generally not observed in the CID spectra aquired with the VG ZAB 2HF mass spectrometer. Leu and Ile can also be distinguished by the fragmentations of the PTH-Leu and PTH-Ile derivatives. [223]

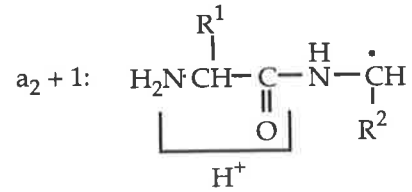


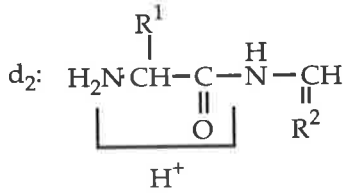
N-Terminal Fragment Ions



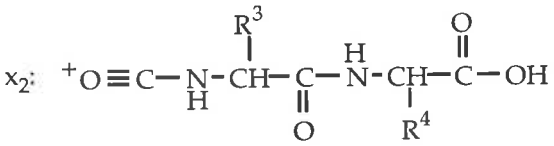


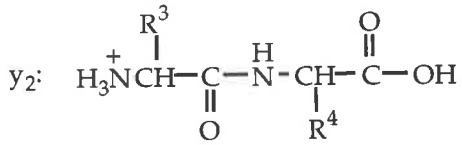


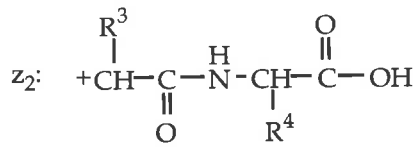


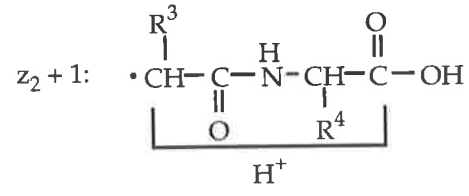


C-Terminal Fragment Ions









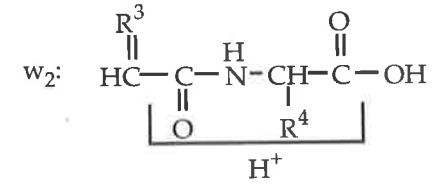


Figure 2.5. A diagram of the fragment ions produced in positive ion CA MIKE spectra using a tetrapeptide. Each fragment ion typically has two additional protons on the charged fragment unless identified, i.e. +1, for positive spectra to the right of the fragmentation identification.

Peptide mapping/MS (or FAB/mapping)[71,224,225] is a technique involving a sample being degraded either enzymatically or chemically, following analysis by mass spectrometry. Digestion with a particular enzyme produces a specific set of fragments, i.e. Endoprotease Lys-C cleaves at the C-terminal end of Lys. These fragments are then analysed by mass spectrometry. If there is post-translational modification, this will be detected directly from the mass spectrum. Peptide mapping/MS can be used in the following situations;[71] (1) for confirmation of a sequence, (2) detection and identification of post-translational modifications, (3) identification of peptide degradation products, (4) identification of peptide metabolites, (5) disulphide bond assignments, (6) ligand binding, and (7) characterisation of enzyme active sites.

2.10. Peptides from Australian Frogs.

The isolation and characterisation of peptides from Australian tree frogs was initiated by the Erspamer group. Caerulin was first isolated and characterised from *Litoria caerulea*.[141] Preliminary screenings indicated the presence of peptides in many Australian frogs and in some from Papua New Guinea.[127] Approximately one hundred species of frog had their skin dried and peptides extracted, with the activity of the peptides deduced through bioassays. The peptide families that they were screening for were caerulin, bombesin-like, tachykinin, bradykinin and angiotensin families[127] (see Chapter 2.6 for examples of these peptide families).

The Adelaide group began their survey of bioactive peptides from Australian frogs in the mid 1980's. Since that time over ninety different skin peptides have been isolated and characterised from three genera of frog, *Litoria*, *Limnodynastes* and *Uperoleia*. Peptides belonging to the caerin,[135,189,190,226,227] caeridin,[135,191,226,227]

dynastin,[151,228] fletcherin,[228] infrafrenatin,[229] rubellidin,[152] tachykinin,[145] tryptophyllin[152] and uperin[145,230] families have been characterised. Some of the peptides isolated have potent antibiotic and pharmacological activity. Uperin 1.1 (2.9),[145] isolated from the Australian flood plain toadlet, *Uperoleia inundata*, is a member of the tachykinin neuropeptide family and caerin 1.1 (2.13) isolated from *Litoria splendida*, has significant wide spectrum antibiotic activity.[135] On the other hand, several peptide families that have been discovered have been found to have neither antibiotic nor smooth muscle activity. These include the caeridin and rubellidin families of peptides, e.g. caeridin 1 (2.16) and rubellidin 1 (2.18). The role of the peptides in the dorsal secretion remains unknown at this time. With over 208 species of frogs known in Australian the possibility of obtaining more pharmacologically active or antibiotic peptides to potentially aid in medicine is high.

2.11. Genus *Litoria*.

All frogs belong to the order Anura. In Australia, this order can be divided into five families, viz. Hylidae, Myobatrachidae (also known as Lepodactylae), Microhylidae, Ranidae and Bufonidae, which together comprise twenty nine genera. The family Hylidae contains three genera, *Litoria*, *Nyctimystes* and *Cyclorana*. The genus *Litoria* is one of the most diverse genera of frogs as it contains sixty one species of frogs which together cover the majority of the Australian continent (see Figure 2.6). Members of the genus *Litoria* live in a variety of habitats throughout Australia; above and below the ground. One species, *Litoria meriana*, even skips on the surface of the water and many are masters at camouflage.[231]

Arboreal (tree) frogs usually have the ends of their fingers and toes flattened into discs. These digits secrete a sticky mucous-like substance to aid climbing.[87,232] The characterisation of peptides from the genus *Litoria* started when Erspamer isolated the neuropeptide caerulein from *L. caerulea*.[141] The Adelaide group has studied the genus *Litoria* extensively (see Chapter 2.10), concentrating on *L. splendida*[135] because of its large hypertrophied rostral glands (see Figure 2.1).

Figure 2.6. The coverage of the genus *Litoria* on the continent of Australia. Coverage obtained by collation of geographic distributions from Tyler.[231]

Chapters 3-6 contain details of the isolation and characterisation of peptides from *L. rubella, L. ewingi, L. xanthomera* and *L. chloris*. Some of these peptides obtained have the potential to be used for medicinal purposes. It will also be shown that the peptide profile of a species may also be used to trace evolutionary trends, e.g. the development of distinct populations within a particular species.

CHAPTER THREE: *Litoria rubella.*

3.1. Introduction.

Litoria rubella (*rubell* is Latin for small red) belongs to the Anura order and is a member of the Hylidae family.[73] *L. rubella* was found and first named *Hyla rubella* by J.E. Gray at Port Essington (N.T.) in 1842.[233] It is a small frog measuring up 4.5 cm in length and is commonly known as the Red-tree frog or Desert-tree frog (see Figure 3.1).

Figure 3.1. Picture of *L. rubella* (B. Stankovich, 1975).

L. rubella is widely distributed throughout northern Australia. It reaches as far south as Wilpena Pound in South Australia (S.A.) as shown in Figure 3.2 and is also found in southern New Guinea. It is a remarkable frog, since it

flourishes in the extremes of the Australian climate, ranging from the arid conditions in central Australia through to the humid conditions of the east coast. Its adaptation is demonstrated in central Australia where the rainfall is unreliable. *L. rubella* has become so successful that it has decreased the time from egg deposition to transformation to a small frog in approximately fifteen days.[233] This time scale is remarkable when compared to *L. chloris* which has a corresponding time of forty one days, however this is in a more agreeable humid climate.[231]

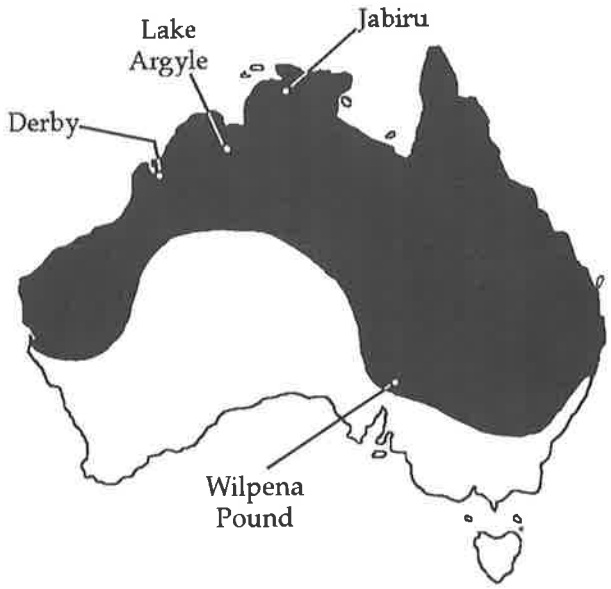


Figure 3.2. Diagram of the geographic distribution of *L. rubella*. Locations indicated are Derby, Lake Argyle and Jabiru (redrawn from Encyclopaedia of Australian Animals: Frogs[231]).

The broad geographic distribution of *L. rubella* is sufficient for biologists to question the integrity of this species, i.e. whether it is really a single species.[152,234] This hypothesis is supported by following observations, (1) the unique advertisement calls of males in central Australia, (2) differences in the

markings on the dorsal surface of the frog in different localities[234] (but not in colouration[184]), and (3) in the peptide secretions.[152]

Specimens of *L. rubella* previously studied were collected from Derby and Lake Argyle (Western Australia) and Jabiru (near Darwin in the Northern Territory)[152] (see Figure 3.2). The differences in the peptide profiles were quite spectacular between the frogs collected from these different locations. Two families of peptide were initially discovered, the tryptophyllin and rubellidin families (shown in Table 3.1).

Sequence	
Tryptophyllins	
Phe **Pro Trp** Leu (NH_2)	(3.1)
pGlu Phe **Pro Trp** Leu (NH_2)	(3.2)
Phe Leu **Pro Trp** Tyr (NH_2)	(3.3)
pGlu Ile **Pro Trp** Phe His Arg (NH_2)	(3.4)
Rubellidins	
Val Asp **Phe Phe** Ala (OH)	(3.5)
Ile Glu **Phe Phe** Ala (OH)	(3.6)
Ile Glu **Phe Phe** Thr (OH)	(3.7)

Table 3.1. Tryptophyllin peptides that have been previously characterised, from *L. rubella* from Derby, Lake Argyle and Jabiru.[152] Amino acid residues which are underlined, highlight the characteristic sequences of each peptide family.

Tryptophyllin peptides, (3.1)-(3.4), contain four to seven residues and have a characteristic Pro-Trp sequence near the C-terminal end of the peptide (Table 3.1).[82] Peptides of the tryptophyllin family were first reported by Erspamer and colleagues[235,236] from the South American tree frog *Phyllomedusa rohdei*.*

* *Phyllomedusina* have a common ancestor with the *Hylindae* of Australia, North and South America and Eurasia. Their divergence occurred approximately 75 million years ago.[237]

Tryptophyllins are found in other species like *P. sauvagei* and *P. bicolor*, as well as in frogs of the genera *Smilisa* and *Triprion*.[82,112] Only one tryptophyllin from *L. rubella*, Phe Pro Trp Leu (NH_2) (3.1), has been found previously. The tryptophyllins from *L. rubella* have no antibiotic activity,[152] but do exhibit minor smooth muscle activity.[82,144,235] The precise role of the tryptophyllins in frog skins is at present unknown. It has been suggested that tryptophyllins may play a role as neurotransmitters or neuromodulators.[236] Some produce opiate like sedation and behavioural sleep in pigeons.[236] In addition, Phe Pro Pro Trp Met (NH_2), found in *P. rohdei* was found in a set of immunoreactive cells in the rat adenhypophesis.[236,237] These cells were far more prevalent in pregnant rats than in male or non-pregnant rats,[236] suggesting that tryptophyllins may play a role in hormonal regulation. Mammalian analogues of tryptophyllins are also known and include the myleopeptides (3.8) and (3.9),[238-240] and the peptide called Tyr-W-MIF-1 (3.10).[241-244] These mammalian analogues exhibit opiate and immunoregulatory behaviour, supporting the proposal that tryptophyllins have a hormonal or neuropeptide role in the amphibian integument.

Phe Leu Gly Phe **Pro** Thr (OH) (3.8)

Leu Val Val Tyr **Pro Trp** (OH) (3.9)

Tyr **Pro Trp** Gly (NH_2) (3.10)

Three peptides in the rubellidin peptide family, (3.5)-(3.7), were also observed.[152] These are small peptides ranging from five to nine residues in length and contain a characteristic Phe-Phe sequence near the C-terminal end. The rubellidins show neither antibiotic nor smooth muscle activity.

The observed change in the peptide secretion as a function of location within a single species is not new. The example addressed in Chapter 2.8 of *L. caerulea*, indicated that there were two chemically distinct populations, an eastern and a central population.[188,226,227] The previous work on the northern populations of *L. rubella* indicated that a study of the peptide profiles of *L. rubella* throughout Australia may provide further examples of evolutionary and/or chemically distinct populations.

This chapter extends the work previously done on *L. rubella* in two ways.[152] Firstly characterising the major peptides from different locations throughout Australia and then combining and assessing the previous and current data.

3.2. Results and Discussion.

Specimens of *L. rubella* were collected live from twelve further locations throughout Australia as identified in Figure 3.7 (see page 59). The amphibians were maintained in captivity and the glandular secretions obtained by surface electrical stimulation. The 'milkings' were performed when needed, however the glands were allowed at least four weeks to replenish before the process was repeated. Each milking on average produced three milligrams of peptide material following lyophilisation of the secretion. The HPLC traces of secretions obtained from individual animals (from a specific location) have been repeated on several occasions and are reproducible. The peptide profiles of frogs obtained from various locations throughout Australia have been found to be location dependent.

3.2.1. Structural Determination of Peptides.

A detailed description of the characterisation of the peptides (isolated by HPLC) will be described later in this chapter. The sequences of all isolated peptides are shown in Table 3.2 (all the masses are nominal, i.e. those obtained by the summation of the integral masses of all of the constituent amino acids). Sixteen peptides have been characterised in total, ten of which were reported previously.[82,152,192,245] Of the six new peptides, two are the caeridin-type rubellidins 4.1 and 4.2, while four are tryptophyllins [designated L 1.1-5.1, the L (*Litoria*) identifies the tryptophyllins isolated from *L. rubella*]. The peptide profiles (as a function of location) are summarised in Table 3.3. The HPLC data (as a function of location) are shown in Figures 3.3 and 3.4.

The structures of the peptide components shown in the HPLC traces were determined primarily by positive ion FAB MS. FAB MS determined the MH^+ values of the underivatised and derivatised peptides. The peptide derivatisations include; (1) methylation, that is the difference between the methylated and underivatised peptide indicated the number of $-CO_2H$ and $-CONH_2$ units in the underivatised peptide ($CO_2H \rightarrow CO_2Me$ = 14 Da and $CONH_2 \rightarrow CO_2Me$ = 15 Da). Methylation also reveals the presence of an N-terminal pyroglutamic acid (pGlu) (evidenced by an increase of 32 Da from the underivatised peptide), (2) Edman degradation/MS.[209] The peptide is sequenced by sequential cleavage of N-terminal amino acids [peptide $n_{(residues)}$ - $[n-1]_{(residues)}$ = amino acid lost], and (3) CA MIKES of the parent MH^+ ion provides sequencing information from the underivatised peptide. The above sequencing procedures do not allow isomeric Leu and Ile to be distinguished.*[217,221,222] Where necessary this problem was overcome by the

* MS/MS data can, on occasions, differentiate between Leu and Ile by the loss of C_3H_6 from the former.[217,221,222] These fragmentations are not observed in the mass spectra of the tryptophyllins L or rubellidins.

use of automated Edman sequencing,[198] which was also used to confirm the overall structure of the peptide.

Peptide	Sequence	MH^+ (Da)
	Tryptophyllins L	
1.1	**Pro Trp** Leu (NH_2)	414
1.2*	Phe **Pro Trp** Leu (NH_2)	561
1.3*	pGlu Phe **Pro Trp** Leu (NH_2)	672
1.4#	Phe Pro Phe **Pro Trp** Leu (NH_2)	805
2.1	Ile **Pro Trp** Leu (NH_2)	527
3.1#	Phe **Pro Trp** Pro (NH_2)	545
3.2#	Phe **Pro Trp** Pro (OH)	546
3.3	pGlu Phe **Pro Trp** Phe (NH_2)	706
4.1	Leu **Pro Trp** Tyr (NH_2)	577
4.2*	Phe Leu **Pro Trp** Tyr (NH_2)	724
5.1*	pGlu Ile **Pro Trp** Phe His Arg (NH_2)	965
	Rubellidins	
1.1*	Val Asp **Phe Phe** Ala (OH)	598
2.1*	Ile Glu **Phe Phe** Ala (OH)	626
3.1*	Ile Glu **Phe Phe** Thr (OH)	655
	Caeridin-type Rubellidins	
4.1	**Gly Leu** Gly **Asp Ile Leu Gly Leu** Leu Gly Leu (NH_2)	1039
4.2	Ala **Gly Leu** Leu **Asp Ile Leu Gly Leu** (NH_2)	883

Table 3.2. Sequences of all peptides characterised from *L. rubella*. Residues which are underlined indicate the characteristic sequences in each peptide family. Peptides marked have been previously sequenced(*)[152] and (#).[192,245]

		Tryptophyllins L										Rubellidins				
	Location	414	527	545	546	561	577	672	706	724	805	965	598	626	655	883	1039
1.	Derby (W.A.)					Δ				*			*	*	*		
2.	Kalumuburu (W.A.)					Δ		*	*						*		*
3.	Lake Argyle (W.A.)					Δ		*									
4.	Jabiru (N.T.)							Δ				*		*			
5.	Adelaide River (N.T.)							Δ				*					
6.	Davenport Ranges (N.T.)					Δ			*							*	*
7.	Simpson's Gap (N.T.)	*	*	*		Δ	*							*	*	*	*
8.	Dulkaninna (S.A.)					Δ											
9.	Farina (S.A.)					Δ											
10.	Arkaroola (S.A.)					Δ											
11.	Maryborough (Qld.)			*		Δ											
12.	Gracemere (Qld.)			*		Δ											
13.	Townsville (Qld.)			Δ		*					*						
14.	Ollera Creek (Qld.)			Δ		*					*						
15.	Mt. Carbine (Qld.)			Δ	*	*					*						

Table 3.3. Summary of the peptides obtained from *L. rubella* and the locations from which they were obtained (numbers are referenced to Figure 3.9). Δ indicates the major fraction in the HPLC trace.

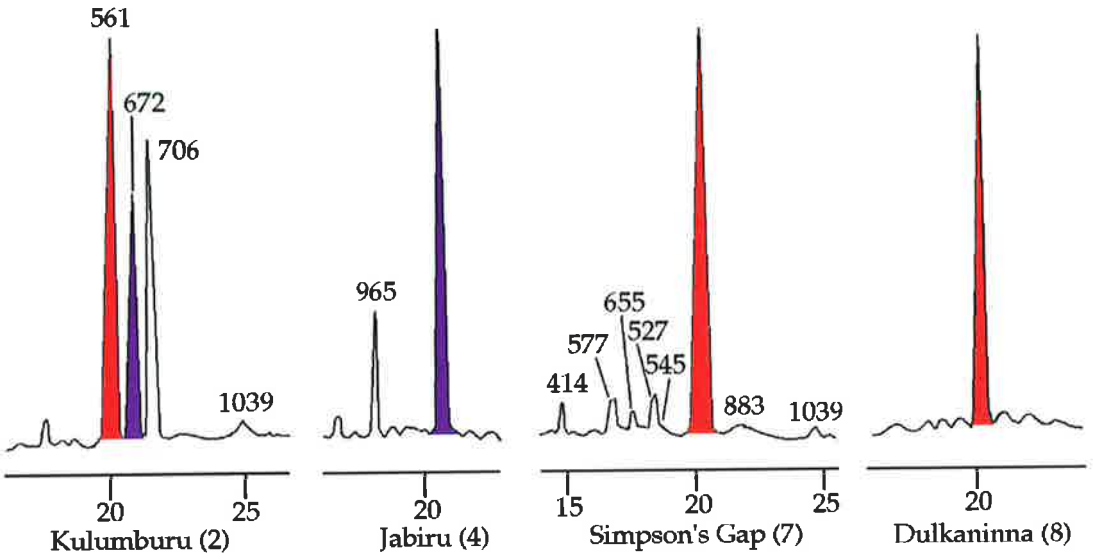


Figure 3.3. The HPLC traces from *L. rubella* specimens collected from Kalumburu, Jabiru, Simpson's Gap and Dulkaninna (numbers are referenced to Figure 3.7). The peaks are labelled using the MH^+ values of the peptides (see Table 3.2). Corresponding colours represent the presence of the same peptide.

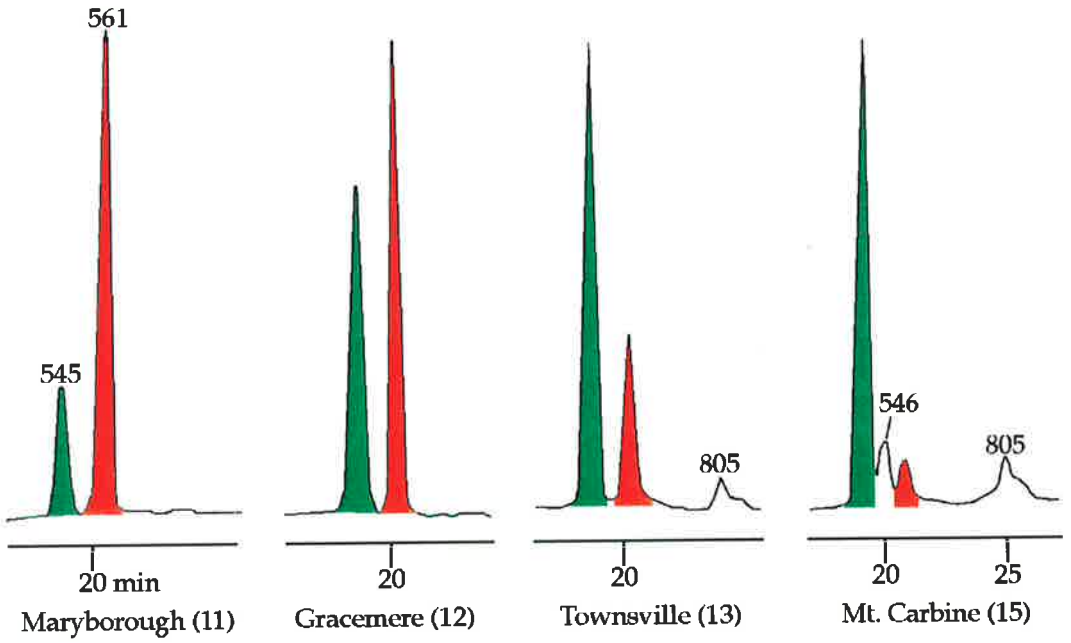


Figure 3.4. The HPLC traces from *L. rubella* specimens collected from Maryborough, Gracemere, Townsville and Mt. Carbine (numbers are referenced to Figure 3.7).* The peaks are labelled using the MH^+ values of the peptides (see Table 3.2). Corresponding colours represent the presence of the same peptide.

* Work on *L. rubella* from the Queensland locations was done in collaboration with then Honours student P. A. Wabnitz.[192,245]

Tryptophyllins L 1.1, 2.1, 3.3 and 4.1, and rubellidins 4.1 and 4.2 are all new peptides. The tryptophyllin L compounds are all post-translationally modified peptides, containing C-terminal amides and/or pGlu residues. The structure determination of individual peptides is listed in detail below. Both Ile and Leu will be designated as 'Leu', as they are unable to be distinguished by mass spectrometric methods used.

Tryptophyllin L 1.1.

(1) HPLC retention time of the peptide is 14.8 minutes* and MH^+ = 414 Da.

(2) Methylation yielded a methyl ester (MH^+ = 429 Da), identifying the C-terminal end as -$CONH_2$.

(3) CA MIKES identified the sequence as Pro Trp 'Leu' (NH_2) (see Table 3.4).

(4) Automated Edman sequencing confirms the structure to be Pro Trp Leu (NH_2).

Precursor Ion	MH^+ (m/z)	Fragment Type	Fragment Ions
Tryptophyllin L 1.1	414	'b'	397, (NH_3); 284, ('Leu').
		'y'	317, (Pro); 131, (Trp).
Structure of tryptophyllin L 1.1 obtained			Pro Trp 'Leu'(NH_2)

Table 3.4. The CA MIKES data from tryptophyllin L 1.1 (MH^+ = 414 Da). Format of fragment ions [fragment ion, m/z and (fragment lost)].

* For experimental conditions of HPLC separation, see Chapter 8.

Tryptophyllin L 2.1.

(1) HPLC retention time of the peptide is 18.2 minutes and MH^+ = 527 Da.

(2) Methylation yielded a methyl ester (MH^+ = 542 Da) identifying the presence of a C-terminal -$CONH_2$ group.

(3) (i) Edman/MS shows the first (N-terminal) residue to be 'Leu' or 'Ile' ([MH^+ - 'Leu'] = 414 Da).

(ii) The N-terminal residue was shown to be Ile using negative ion chemical ionisation CA MIKES of the PTH amino acid derivative.[223]

(4) CA MIKES identified the structure as 'Leu' Pro Trp 'Leu' (NH_2) (see Figure 3.5).

(5) Automated Edman sequencing confirmed the structure to be Ile Pro Trp Leu (NH_2).

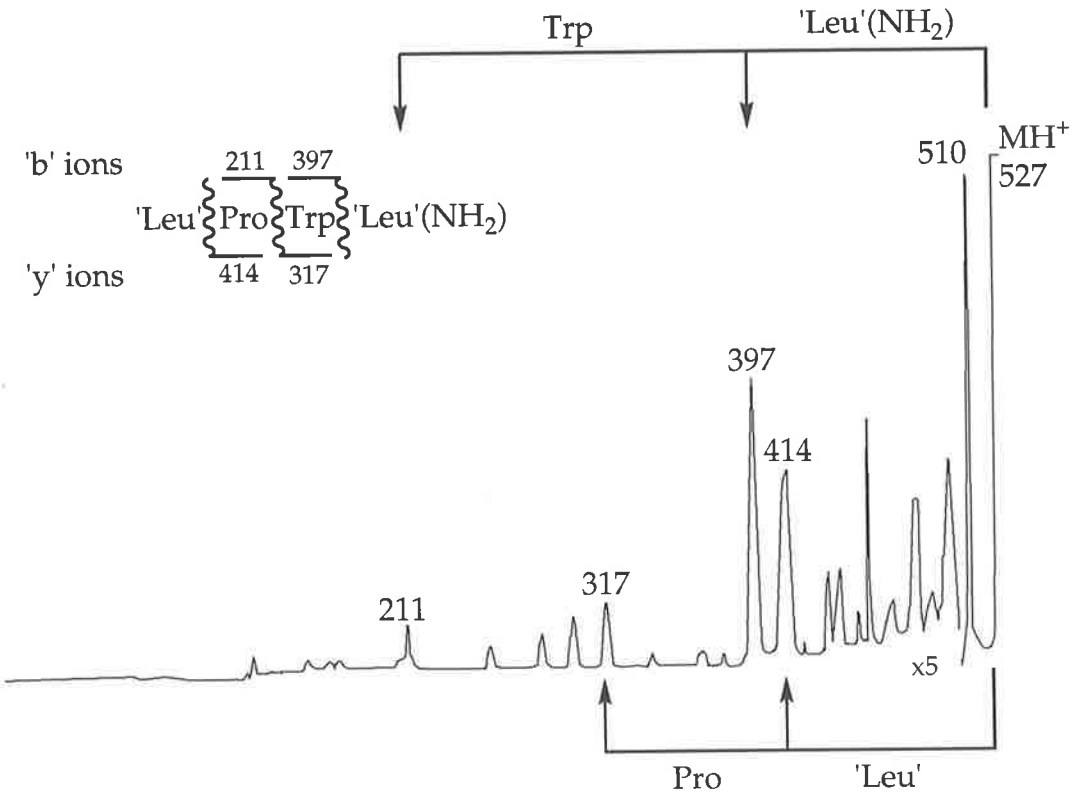


Figure 3.5. CA MIKE spectrum of tryptophyllin L 2.1 (MH^+ = 527 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

Tryptophyllin L 3.3.

(1) HPLC retention time of the peptide is 21.8 minutes and MH^+ = 706 Da.

(2) Methylation yielded two esters, (i) pGlu(OMe) ester (MH^+ = 738 Da) identifying a pGlu residue, and (ii) (bis)methyl ester (MH^+ = 753 Da) identifying a C-terminal -$CONH_2$ group.

(3) Edman/MS failed, confirming the presence of a N-terminal pGlu residue. The use of partial acid hydrolysis[246] cleaved amino acid residues from the N-terminal end, indicating the first two residues to be pGlu and Phe, ([MH^+ - pGlu] = 595 Da, [MH^+ - (pGlu + Phe)] = 448 Da).

(4) CA MIKES gave the partial sequence (pGlu + Phe) (97 Da) Trp Phe (NH_2) (see Table 3.5). The combination of all mass spectrometric data determined the structure to be pGlu Phe Pro Trp Phe (NH_2).

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Tryptophyllin L 3.3	706	'b'	689, (NH_3); 542, (Phe); 356, (Trp).
		'y'	448, (pGlu + Phe).
Partial structure of tryptophyllin L 3.3 obtained			(pGlu + Phe) (97 Da) Trp Phe(NH_2)

Table 3.5. The CA MIKES data from tryptophyllin L 3.3 (MH^+ = 706 Da). Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

Tryptophyllin L 4.1.

(1) HPLC retention time of the peptide is 16.9 minutes and MH^+ = 577 Da.

(2) Methylation yielded a methyl ester (MH^+ = 592 Da) identifying the presence of a C-terminal -$CONH_2$ group.

(3) CA MIKES indicates the structure to be 'Leu' Pro (Trp + Tyr) (NH_2) (see Table 3.6).

(4) Automated sequencing determined the structure as Leu Pro Trp Tyr (NH_2).

Precursor Ion	MH^+ (m/z)	Fragment Type	Fragment Ions
Tryptophyllin L 4.1.	577	'b'	560, (NH_3); 211 (Trp + Tyr).
		'y'	464, ('Leu'); 367 (Pro).
Partial structure of tryptophyllin L 4.1 obtained 'Leu' Pro (Trp + Tyr) (NH_2)			

Table 3.6. The CA MIKES data from tryptophyllin L 4.1. (MH^+ = 577 Da). Format of fragment ions [fragment ion, m/z and (fragment lost)].

Caeridin-type Rubellidin 4.1.

(1) HPLC retention time of the peptide was 24.7 minutes and MH^+ = 1039 Da.

(2) Methylation yielded a (bis)methyl ester (MH^+ = 1068 Da) which identifies that the peptide contains one -$CONH_2$ and one -CO_2H group.

(3) Edman/MS indicated the first two N-terminal residues to be Gly and 'Leu', ([MH^+ - Gly] = 982 Da, [MH^+ - (Gly + 'Leu')] = 869 Da).

(4) CA MIKES only gives the partial sequence Gly 'Leu' -...- 'Leu' 'Leu' (Gly + 'Leu') (NH_2) (data shown in Table 3.7).

(5) The full sequence could not be determined by available MS data. Automated sequencing provided the structure Gly Leu Gly Asp Ile Leu Gly Leu Leu Gly Leu (NH_2).

Precursor Ion	MH^+ (m/z)	Fragment Type	Fragment Ions
Rubellidin 4.1.	1039	'b'	1022 (NH_3); 852, (Gly + 'Leu'); 739, ('Leu'); 626, ('Leu').
		'y'	982, (Gly); 869, ('Leu').
Partial structure of rubellidin 4.1 Gly 'Leu' -...- 'Leu' 'Leu' (Gly + 'Leu') (NH_2)			

Table 3.7. The CA MIKES data from caeridin-type rubellidin 4.1 (MH^+ = 1039 Da). Format of fragment ions [fragment ion, m/z and (fragment lost)].

Caeridin-type Rubellidin 4.2.

(1) HPLC retention time of the peptide is 21.9 minutes and MH^+ = 883 Da.

(2) Methylation gives a (bis)methyl ester (MH^+ = 912 Da) which indicates that the peptide contains one -$CONH_2$ and one -CO_2H group.

(3) Edman/MS shows the first two N-terminal residues to be Ala and Gly, ([MH^+ - Ala] = 812 Da, [MH^+ - (Ala + Gly)] = 755 Da).

(4) CA MIKES determined the final six residues to be ...-'Leu' Asp 'Leu' 'Leu' Gly 'Leu' (NH_2) (see Figure 3.6).

(5) A combination of the above mass spectral data gives the sequence as Ala Gly 'Leu' 'Leu' Asp 'Leu' 'Leu' Gly 'Leu' (NH_2). Automated sequencing gives Ala Gly Leu Leu Asp Ile Leu Gly Leu (NH_2).

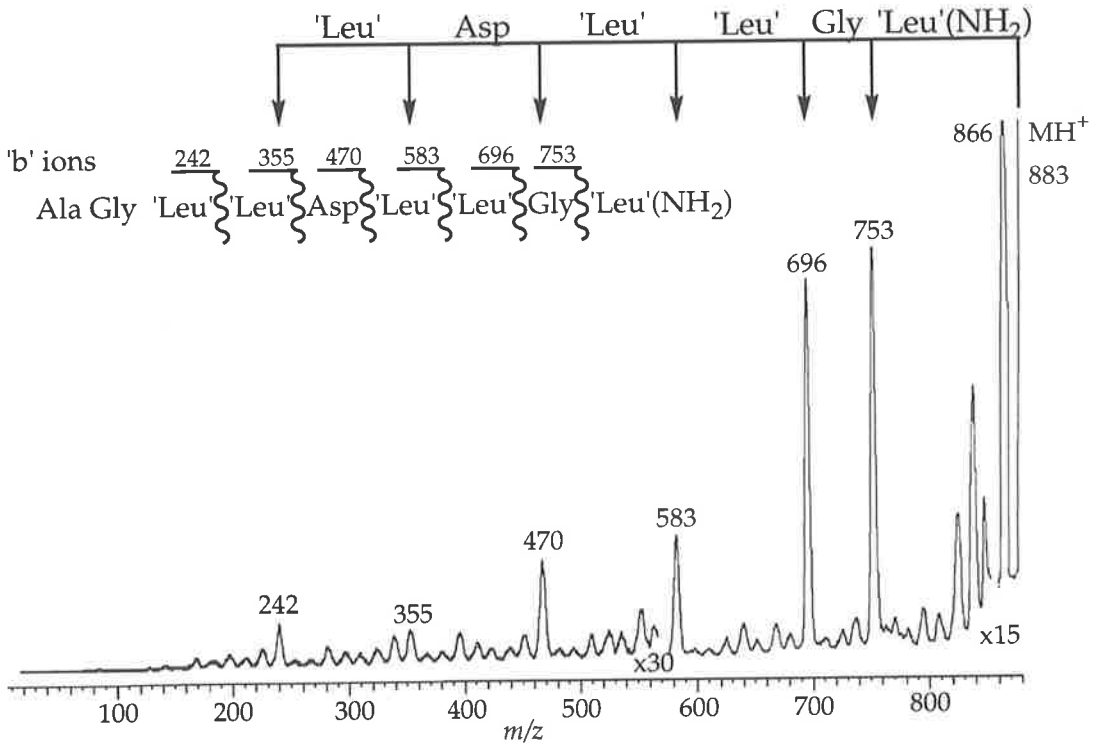


Figure 3.6. CA MIKE spectrum of rubellidin 4.2 (MH^+ = 883 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

3.2.2. Bioactivity of Peptides from *L. rubella.*

The activities of the tryptophyllins L (as introduced in Chapter 3.1) are limited. Antibiotic testing on the synthetic tryptophyllins L 1.2, 1.3 and 5.1, showed no significant activity. Tryptophyllin L 1.3 showed low activity against *Micrococcus luteus* and *Streptococcus uberis* at minimal inhibitory concentration (MIC) values of 50 and 100 µg/ml respectively. No activity was shown against *Escherichia coli, Listeria innocua* or *Staphylococcus aureus*. The tryptophyllins appear to be neuropeptides, however Erspamer was able to find only minimal activity in the tryptophyllins he characterised.[82,144,235] Limited pharmacological activity was observed with the synthetically modified tryptophyllin, Ile Met Phe Pro Trp Leu (NH_2) (MH^+ = 805 Da), which showed

smooth muscle activity in mouse vas deferens in the micromole range.*[248] The particular role of the tryptophyllins in the homeostasis of *L. rubella* is still under investigation.[192]

The rubellidin peptides 1.1, 2.1 and 3.1 are unique to *L. rubella*, but are devoid of antibiotic or smooth muscle activity,[152] as are the caeridin-type rubellidins 4.1 and 4.2. The latter peptides are related to the caeridin peptides found in other *Litoria* species and also show no bioactivity. It is possible that the anionic caeridin and rubellidin peptides may be peptide spacers in tryptophyllin peptide biosynthesis.[155,249]

3.2.3. Evolutionary Significance of Peptides from *L. rubella*.

The predominant peptides characterised from *L. rubella* throughout Australia are not related to the peptides from any other *Litoria* species so far studied. The application of amphibian peptide secretions to identify chemically distinct populations of frogs within a species was introduced in Chapter 2.8. Differences in the peptide profiles of *L. rubella* revealed that different populations were located around Darwin (N.T.) and in the Kimberley Ranges in the north-west of Western Australia.[152] The current analysis of the peptide secretions from specimens of *L. rubella*, collected from locations throughout Australia, when combined with the previous data obtained by Steinborner *et. al.*[152] indicate a unique scenario. These evolutionary trends are seen in both peptide profiles and morphological changes in *L. rubella* collected from specific areas. A complete summary comparing the peptide profile with geographic

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* pGlu Phe Pro Trp Leu (NH_2), with a few other tryptophyllins have also shown pharmacological activity in preliminary tests, but all the activities are in the micromole range, which is considered low.[247] However, given the large amounts of tryptophyllin peptides present in the secretion, activity in the µg/ml range may be sufficient for the needs of the frog.

location is collated in Table 3.3 and in the individual traces shown in Figures 3.3 and 3.4.

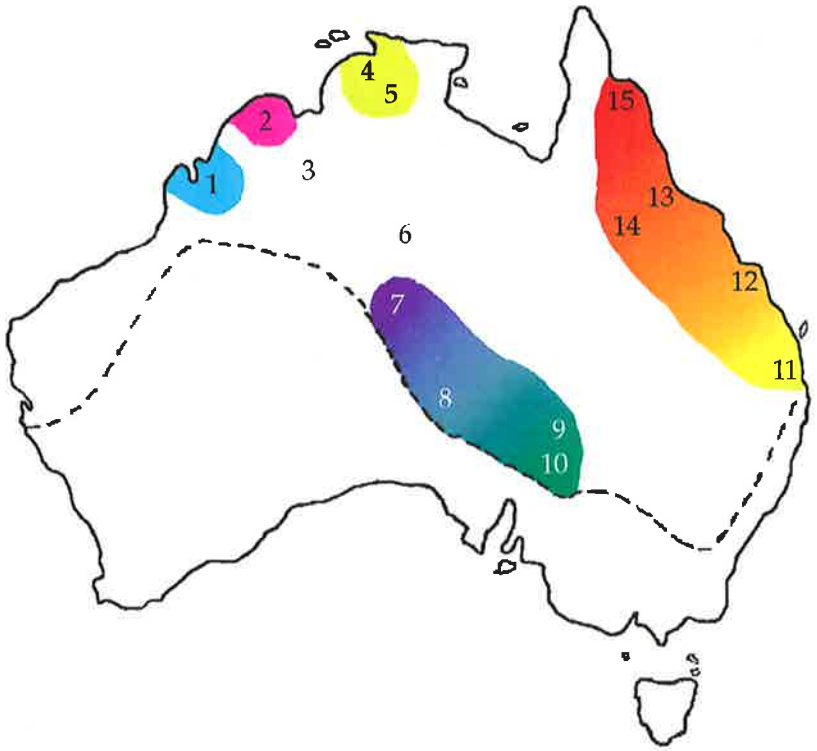

Figure 3.7. A schematic representation of the chemically distinct populations of *L. rubella*. The dotted line represents the geographical distribution of *L. rubella*. The numbers indicate where each specimen was collected, viz. **1**, Derby (W.A.); **2**, Kalumburu (W.A.); **3**, Lake Argyle (W.A.); **4**, Jabiru (N.T.); **5**, Adelaide River (N.T.); **6**, Davenport Ranges (N.T.); **7**, Simpson's Gap near Alice Springs (N.T.); **8**, Dulkaninna (S.A.); **9**, Farina (S.A.); **10**, Arkaroola (S.A.); **11**, Maryborough (Qld.), **12**, Gracemere (Qld.); **13**, Townsville (Qld.); **14**, Ollera Creek (Qld.) and **15**, Mt. Carbine (Qld.). The unique populations are indicated by the solid areas, whereas the evolving populations are indicated by the graded areas.

Conclusions from the presented data, combined with that from Steinborner *et. al.*[152] are shown in Figure 3.7 and are summarised below;

(1) Tryptophyllin L 1.2 is the major peptide in the skin secretions of *L. rubella* collected from the Kimberley region of Western Australia. The situation is complex and frogs from other locations in this area need to be examined before a precise conclusion concerning this area can be determined. However, there are (at least) two isolated populations of *L. rubella* in the

Kimberley region. One is located around Derby and the other is further north near Kalumburu (see Figure 3.7).

(2) A spectacular change in the peptide profile occurs as the location moves from the Kimberley region, west into the Northern Territory. Tryptophyllin L 1.2 is now completely absent and replaced by tryptophyllin L 1.3. This is where *Litoria rubella* was first named by Gray in 1842.[73] This is a unique population occurring in the Kakadu/Adelaide River region of the Northern Territory (see Figure 3.7). Therefore if the present study is correct, only this 'species' should be known as *L. rubella*.

(3) Moving south to central Australia (in particular Simpson's Gap near Alice Springs). This peptide profile is unique and the most complex. The major peptide is tryptophyllin L 1.2, which constitutes 80% by weight of the peptide mixture. As the collection location continues into South Australia, the peptide profile becomes simpler with tryptophyllin L 1.2 becoming more predominant (see Figure 3.3 and 3.7). There is a gradual change in peptide profile between central Australia and southern-central Australia.

(4) The final locations to be studied were those in a 2 000 km transect along the eastern seaboard of Queensland.[192] The five locations from which specimens were collected produced a trend different from anything observed previously. The most southern location where specimens were collected was Maryborough. The peptide profile contained the major peptide tryptophyllin L 1.2. A minor, not previously discovered peptide tryptophyllin L 3.1 was also characterised. In the more northern locations there is an increase in the amount of tryptophyllin L 3.1, with a corresponding decrease in the amount of tryptophyllin L 1.2 (see Figure 3.4). The specimens collected from the north (including Townsville) contained a new tryptophyllin L 1.4 in minor amounts

(see Table 3.2). This trend observed in the peptide profile of *L. rubella* from the Queensland eastern seaboard, occurred in conjunction with a change in the colour and markings of each of the specimens.[192] The colour of *L. rubella* collected from Maryborough is a dark rust colour, with lateral markings that range from the snout right through to the hind leg of the animal, with dark mottling on the dorsal surface. This intense colour and marking decreases markedly as the location becomes more northerly. From Mt. Carbine, the most northerly location, *L. rubella* is a pale brown with minor lateral markings from the snout to just past the eye (see Figure 3.8).

In regions apart from coastal Queensland, the general overall appearance of *L. rubella* is basically the same and colour alone cannot be used to identify different populations. For example, specimens collected from Simpson's Gap and Kalumburu look very similar, yet the peptide profiles are quite different. The gradual change observed on the eastern seaboard of Queensland is unusual. The suspected evolutionary changes that are occurring in the other populations analysed are not accompanied by a morphological change.

3.3. Summary.

In conclusion, the peptide profiles derived from the skins of *L. rubella* collected from different locations throughout Australia, indicate that there are at least five different populations of this frog in Australia (see Figure 3.7). The reasons for the evolutionary divergence of *L. rubella* may be due to changes in climate, predators, vegetation, geographical isolation or some combination of these. The time scale in which these changes have taken place is not known. When we know the precise roles of the tryptophyllins and rubellidins in *L. rubella*, perhaps then we will have a better understanding of this evolutionary trend.

Figure 3.8. Specimens of *L. rubella* from the Queensland locations of Mt. Carbine (15), Townsville (13) and Maryborough (11). The numbers reference the locations in Figure 3.7 (photos courtesy of C. Williams, Department of Zoology, University of Adelaide).

CHAPTER FOUR: *Litoria ewingi.*

4.1. Introduction.

Litoria ewingi belongs to the Anura order and is a member of the Hylidae family. *L. ewingi* was first discovered by Duméril and Bibron in 1841 and was first named *Hyla ewingi*. It was reclassified in 1971 by Tyler as *Litoria ewingi*.[233] It is a slender, small brown frog measuring up to 4.5 cm in length and is commonly known as Ewing's tree frog or the Whistling tree frog. The colour of *L. ewingi* can change dramatically, e.g. in the south-east of South Australia and western Victoria the dorsal surface can be green, whereas in the northern locations of South Australia the dorsal surface is brown (Figure 4.1).[87]

L. ewingi is abundant and widespread throughout southern Australia. The northern boundary is unknown, but it is thought to be near Wilmington in South Australia. The species is found in the south-east of Australia along the Murray River, and extends into south-eastern Victoria and eastern New South Wales (see Figure 4.2). It is typically found in low moist vegetation on the banks of permanent streams or pools and commonly lives in suburban gardens.[233]

L. ewingi is one of the best known and studied tree frogs in southern Australia. Fossils of *L. ewingi* have been found in Victoria Fossil Caves,[250] Tantanoola Caves[251] and Henschke's Cave[252] in the south-east of South Australia. Several of the fossil deposits have been dated from 50 000 to 100 000 years.[251-253]

Figure 4.1. Pictures of *L. ewingi*, (A) brown [L. Redler (1979)] and (B) green colouration (B. Stankovich).

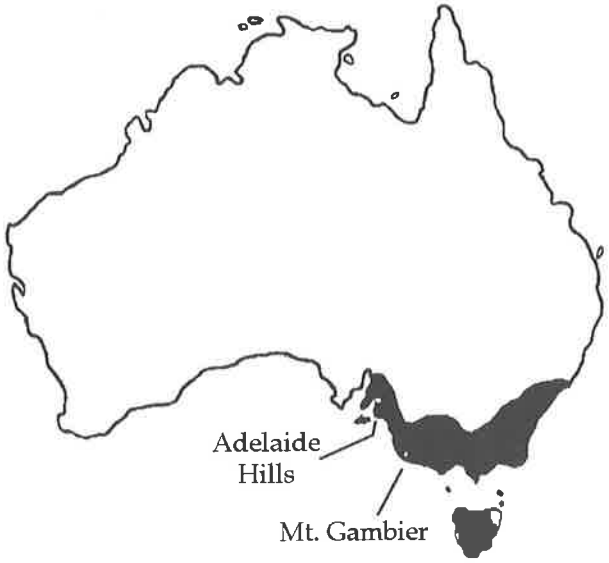

Figure 4.2. Diagram of the geographic distribution of *L. ewingi* (redrawn from Encyclopaedia of Australian Animals: Frogs[231]).

The peptide secretion from *L. ewingi* has been analysed previously by Erspamer and colleagues using bioassays.[127] The presence of litorin (1.3 μg/dry skin) and caerulin (35 μg/dry skin) were identified. Litorin is a neurotransmitter,[254,255] while caerulin is a gastrointestinal and neuropeptide.[112] Therefore the secretion is known to contain biologically active peptides.

This chapter initiates the characterisation of peptides from *L. ewingi* obtained from the Adelaide Hills. The specimen from Mount Gambier was analysed for the major peptide components observed in the Adelaide Hills specimens.

4.2. Results and Discussion.

Specimens of *L. ewingi* were collected live from locations in the Adelaide Hills and from Mt. Gambier.* The amphibians were maintained in captivity for a year and surface electrical stimulation of their granular glands were performed periodically. The glands were allowed at least four weeks to replenish. Each milking on average produced three milligrams of peptide material following lyophilisation of the secretion. The amount obtained is dependent upon the size of the frog and in some cases the smaller frogs yielded less than one milligram of peptide material per milking. The HPLC traces of the secretion have been repeated on numerous occasions with the results being reproducible. The HPLC separation is shown in Figure 4.3. Only the major peptides were characterised with their masses ranging from 670 to 2 582 Da.

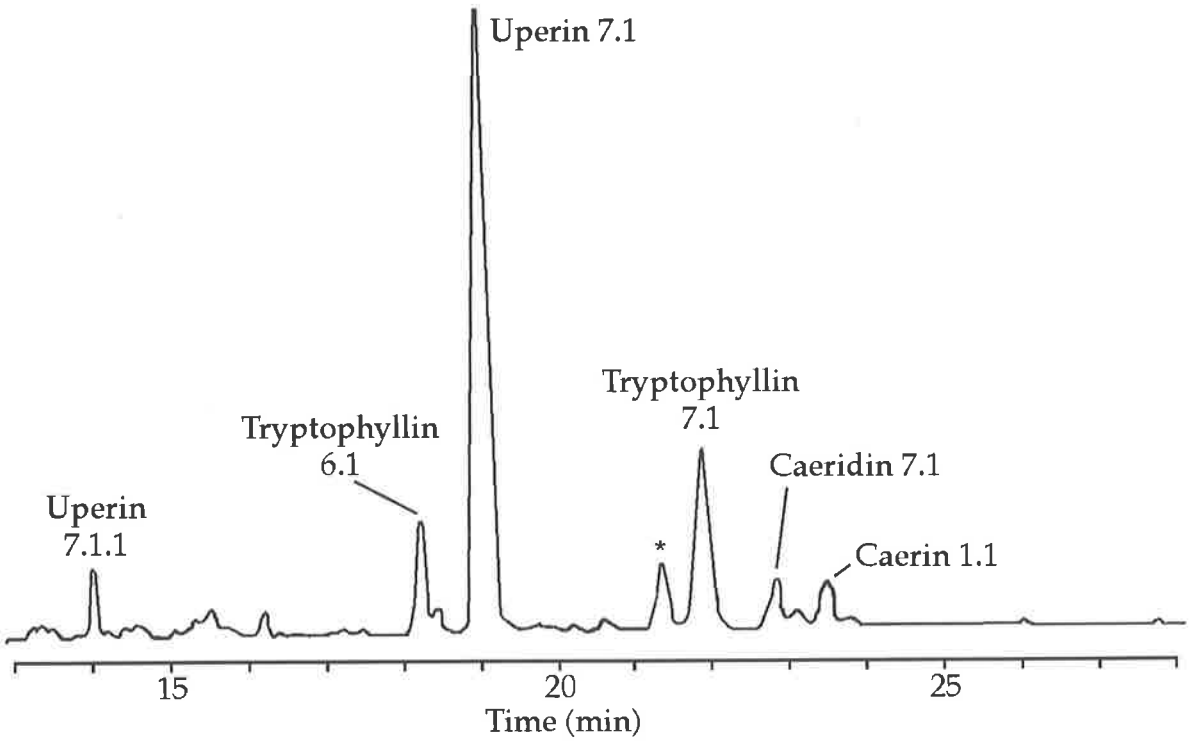

Figure 4.3. The HPLC traces of *L. ewingi* from the Adelaide Hills [(*) indicates that no peptide material was present].

* The specimen of *L. ewingi* collected from Mount Gambier was of a green colouration (see Figure 4.1).

The HPLC trace of *L. ewingi* collected from Mt. Gambier, was similar to that from the Adelaide Hills. The major peaks were obtained and monitored using the MH^+ value.

4.2.1. Structural Determination of Peptides.

A detailed description of the characterisation of the peptides that were isolated by HPLC will be described. The structures of all the peptides are shown in Table 4.1. Six peptides were characterised in total, one of these is known, having been identified previously from *L. caerulea*.[135,191,226,227] Neither litorin or caerulin are present in the secretion, a result that differs with an earlier report of Erspamer.[127] The peptides characterised were obtained from the specimens collected from the Adelaide Hills. Of the six peptides isolated, two are tryptophyllin-like and are designated as tryptophyllin L (L = *Litoria*) peptides, to distinguish them from tryptophyllins found earlier by Erspamer.

The structures of the peptide components shown in the HPLC traces were determined by positive ion FAB MS. FAB MS methods were used to determine the MH^+ values of the underivatised peptide, methyl esters, acetate derivatives (H_2N-R → MeCONH-R = 42 Da) and degradation products produced by the Edman/ MS technique and enzymatic digests.[209]

In addition, CA MIKES of the parent MH^+ ion provides sequencing information from the underivatised peptide. The above sequencing procedures do not allow isomeric Leu and Ile residues to be distinguished.*[217,221,222] This problem was overcome by the use of an

* MS/MS data can, on occasions, differentiate between Leu and Ile by the loss of C_3H_6 from the former.[217,221,222] These fragmentations are not observed in the mass spectra described here.

Peptide	Sequence	MH^+ (Da)#
	Caerin	
1.1*	Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2)	2582
	Caeridin	
7.1	Gly Leu Leu Asp Met Val Thr Gly Leu Leu Gly Asn Leu (NH_2)	1315
	Tryptophyllins	
6.1	Leu Phe Phe Trp Gly (NH_2)	668
7.1	Ile Phe Phe Phe Pro (NH_2)	669
	Uperins	
7.1	Gly Trp Phe Asp Val Val Lys His Ile Ala Ser Ala Val (NH_2)	1427
7.1.1	Phe Asp Val Val Lys His Ile Ala Ser Ala Val (NH_2)	1184

Table 4.1. Sequences of all peptides characterised from *L.ewingi*. Peptides marked (*) have been previously sequenced[135,191,226,227] (# indicates that the masses are nominal, i.e. that obtained by the summation of the intergral masses of all of the constituent amino acids).

automated Edman sequencer,[198] which was also used to confirm the overall structure of the peptide.

The caeridin, tryptophyllin L and uperin peptides are all newly discovered, except caerin 1.1 which has been previously isolated from *L. gilleni*, *L. caerulea* and *L. splendida*.[135,191,226,227] All peptides are post-translationally modified, in that they contain C-terminal amide functionalities. The structure determination of all new peptides is listed below. Both Ile and Leu are designated 'Leu', since the two residues cannot be distinguished using these MS techniques. The full structure is confirmed by the automated Edman technique (*cf.* also Table 4.1).

Caerin 1.1.

(1) HPLC retention time of this peptide is 23.5 minutes* and MH^+ = 2582 Da.

(2) (i) This compound has same mass as the peptide caerin 1.1 (from *Litoria* species[188]) and was sequenced by peptide mapping techniques (Chapter 2.9.3).

(ii) It co-eluted with both natural and synthetic caerin 1.1.

(iii) Lys-C digest produced two digest fragments, MH^+ = 1043 and 1558 Da, identical to those previously isolated from caerin 1.1.[135,188-190,227]

(3) Automated sequencing confirmed the structure to be Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2).

* For experimental conditions, see Chapter 8.

Caeridin 7.1.

(1) HPLC retention time of this peptide is 22.8 minutes and MH^+ = 1315 Da. Only 15 μg of this material was available for structure determination.

(2) Methylation yielded a (bis)methyl ester (MH^+ = 1344 Da) which indicates the presence of -$CONH_2$ and a -CO_2H group. These data do not indicate whether the C-terminal substituent is an acid or an amide.

(3) Acetylation yielded an acetate derivative (MH^+ = 1357 Da) which identified the presence of one free amine.

(4) Automated Edman sequencing gave the sequence as Gly Leu Leu Asp Met Val Thr Gly Leu Leu Gly Asn Leu (NH_2).

Uperin 7.1.

(1) HPLC retention time of this peptide is 19 minutes and MH^+ = 1427 Da.

(2) Methylation yielded a (bis)methyl ester (MH^+ = 1456 Da) which indicates the presence of -$CONH_2$ and a -CO_2H group.

(3) Acetylation yielded an acetate derivative (MH^+ = 1511 Da) which identified the presence of two free amine groups.

(4) Edman/MS revealed the first four amino acids as Gly Trp Phe Asp (see Table 4.2).

Edman Cycle and Precursor Peptide (Da)	Mass Lost (Da)	Amino Acid
- (1427)		
1 (1370)	57	Gly
2 (1184)	186	Trp
3 (1037)	147	Phe
4 (922)	115	Asp

Table 4.2. Four Edman degradation/MS cycles of uperin 7.1.

(5) CA MIKES of the parent ion, MH^+ = 1427 Da (see Table 4.3), identified the partial sequence of Gly Trp-...-'Leu' Ala Ser Ala Val (NH_2)

(6) Lys-C digest of uperin 7.1 yielded two peptides, 7.1.A (MH^+ = 850 Da) and 7.1.B (MH^+ = 596 Da).

(7) CA MIKES data of 7.1.A (MH^+ = 850 Da) determined the structure to be Gly Trp Phe Asp Val Val Lys (OH) (as illustrated in Figure 4.4). The CA MIKE spectrum of 7.1.B (MH^+ = 596 Da) determined the structure to be His 'Leu' Ala Ser Ala Val (NH_2) (data shown in Table 4.3). Edman/MS data (see Table 4.2) shows that the digest fragment 7.1.A is the N-terminal fragment.

(8) Automated sequencing confirmed the structure as Gly Trp Phe Asp Val Val Lys His Ile Ala Ser Ala Val (NH_2).

Precursor Ion	MH^+ (m/z)	Fragment Type	Fragment Ions
Uperin 7.1	1427	'b'	1410, (NH_3); 1311, (Val); 1240, (Ala); 1153, (Ser); 1082, (Ala); 969, ('Leu').
		'y'	1370, (Gly); 1184, (Trp).
Partial structure of uperin 7.1 Gly Trp-...-'Leu' Ala Ser Ala Val (NH_2)			
Uperin 7.1.B	596	'b'	579, (NH_3); 480, (Val); 409, (Ala); 322, (Ser); 251, (Ala).
		'y'	459, (His).
Structure of uperin 7.1.B His 'Leu' Ala Ser Ala Val(NH_2)*			

Table 4.3. The CA MIKES data from uperin 7.1 (MH^+ = 1427 Da) and the Lys-C digest fragment uperin 7.1.B (MH^+ = 596 Da). (*) The 'Leu' residue at position two was inferred from the first 'y' and last 'b' ion. Format of fragment ions [fragment ion, m/z and (fragment lost)].

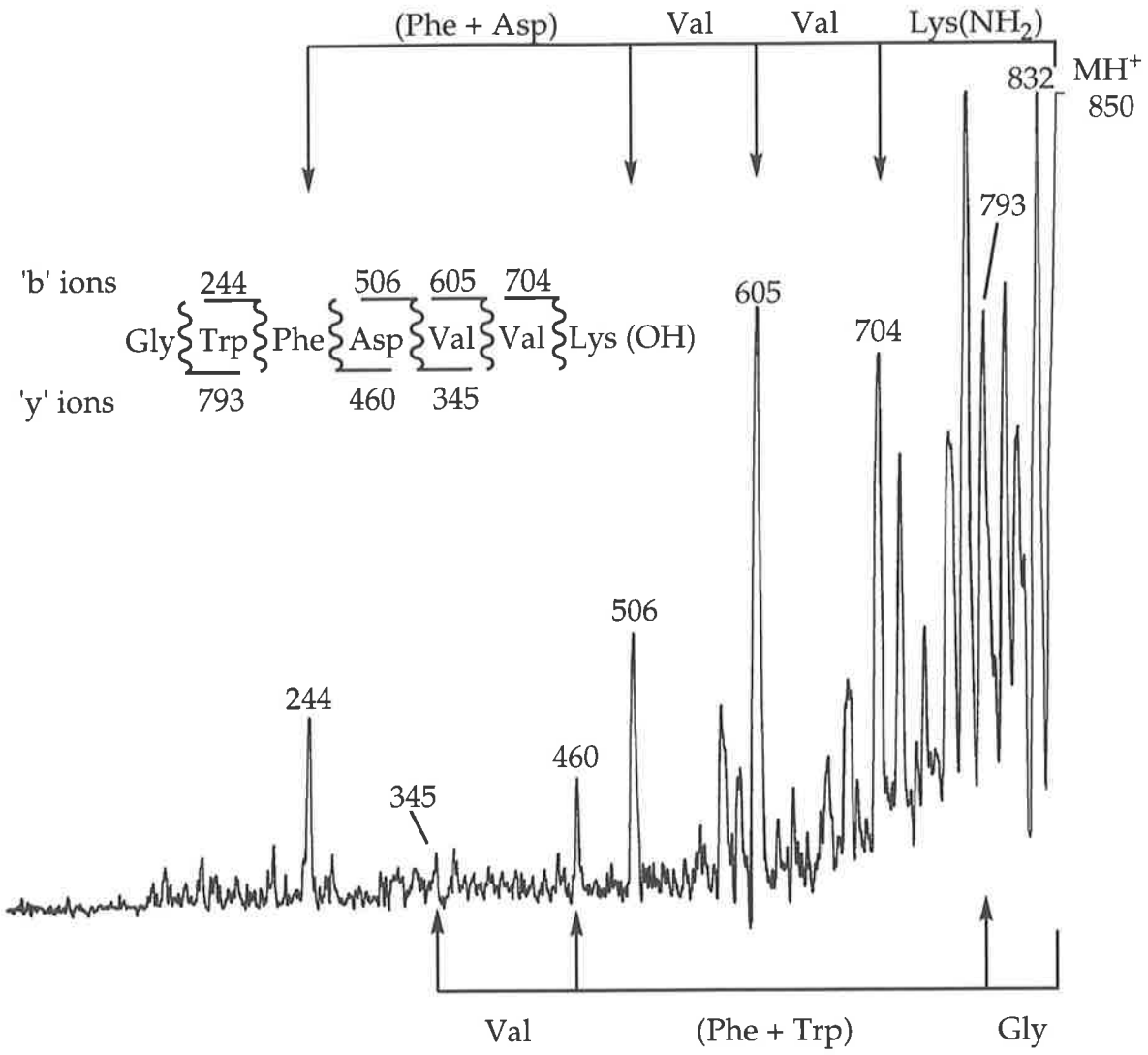

Figure 4.4. CA MIKE spectrum of peptide 7.1.A (MH^+ = 850 Da) formed from the Lys-C digest of uperin 7.1. The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

Uperin 7.1.1.

(1) HPLC retention time of this peptide is 14 minutes and MH^+ = 1184 Da.

(2) Methylation yielded a (bis)methyl ester (MH^+ = 1213 Da) which indicates the presence of one $-CONH_2$ and one $-CO_2H$ group.

(3) Acetylation yielded an acetate derivative (MH^+ = 1268 Da) showing the presence of two free amine groups.

(4) Lys-C digestion of uperin 7.1.1 yielded two peptides, 7.1.1.A (MH^+ = 607 Da) and 7.1.1.B (MH^+ = 596 Da).

(5) The CA MIKE spectrum of 7.1.1.B (MH^+ = 596 Da) was identical to that of 7.1.B. (see Table 4.3), identifying the structure to be His 'Leu' Ala Ser Ala Val(NH_2). The CA MIKE spectrum of 7.1.1.A gave the structure as Phe Asp Val Val Lys (OH) (see Figure 4.5).

(6) Automated Edman sequencing confirmed the structure as Phe Asp Val Val Lys His Ile Ala Ser Ala Val (NH_2).

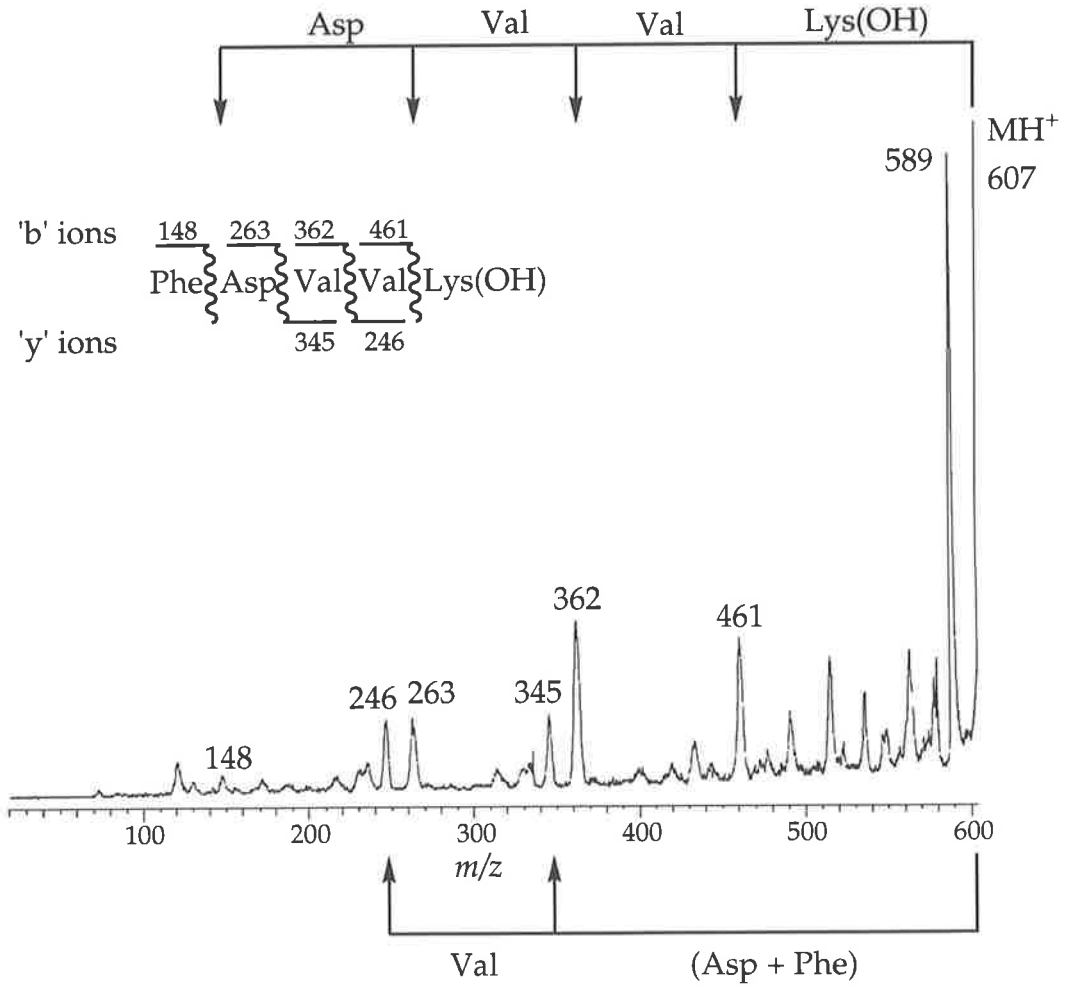

Figure 4.5. CA MIKE spectrum of peptide 7.1.1.A (MH^+ = 607 Da), formed from the Lys-C digest of uperin 7.1.1. The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

Tryptophyllin L 6.1.

(1) HPLC retention time of this peptide is 18.3 minutes and MH^+ = 668 Da.

(2) Methylation yielded a methyl ester (MH^+ = 683 Da) indicating the presence of one -$CONH_2$ group.

(3) Edman/MS indicated that the first two residues are 'Leu' Phe, ([MH^+ - 'Leu'] = 555 Da, [MH^+ - ('Leu' + Phe)] = 408 Da).

(4) The CA MIKE spectrum of tryptophyllin L 6.1 (see Figure 4.6) indicated the sequence 'Leu' Phe Phe Trp Gly (NH_2).

(5) Automated Edman sequencing confirmed the structure as Ile Phe Phe Trp Gly (NH_2).

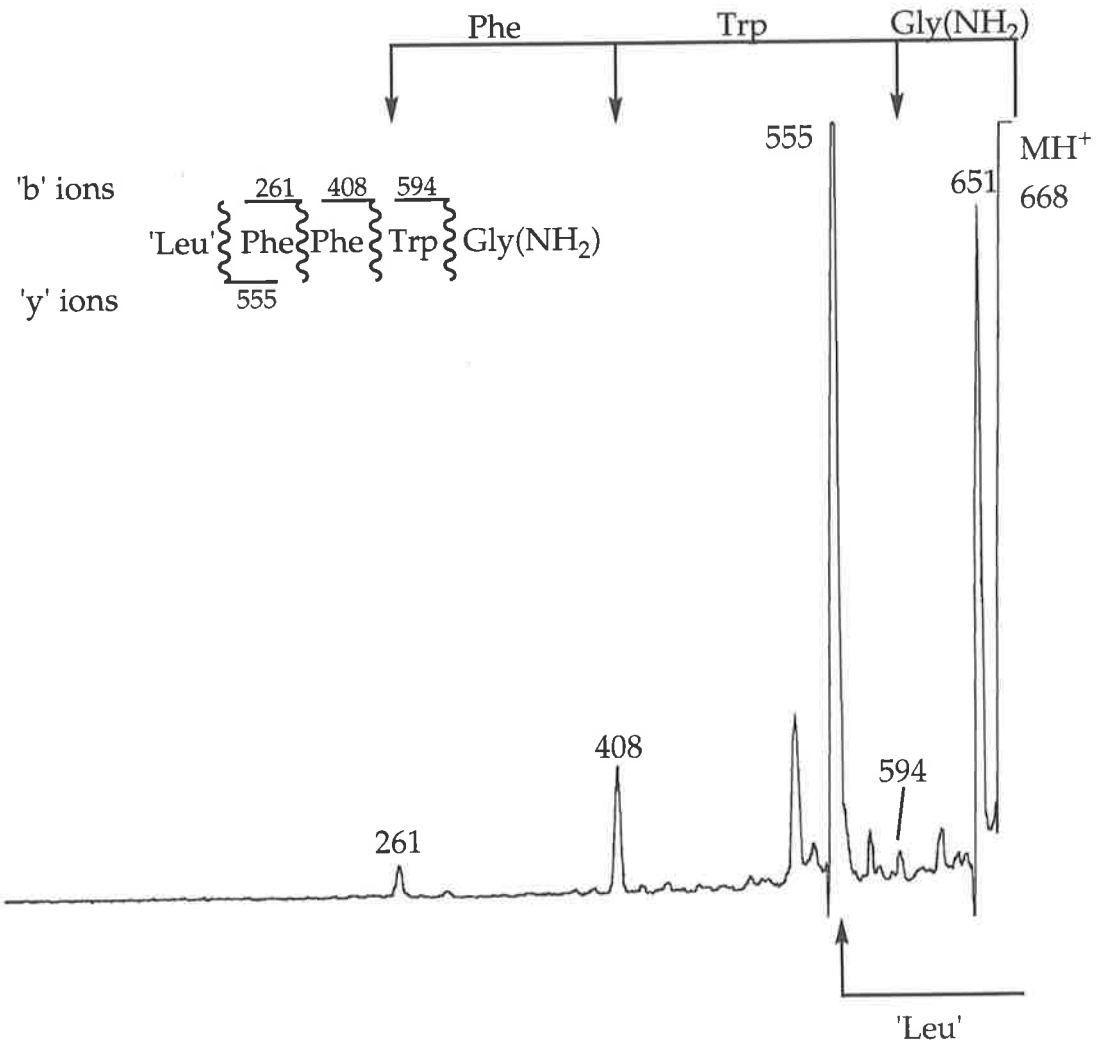

Figure 4.6. CA MIKE spectrum of tryptophyllin L 6.1 (MH^+ = 668 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

Tryptophyllin L 7.1.

(1) HPLC retention time of this peptide is 21.9 minutes and MH^+ = 669 Da.

(2) Methylation yielded a methyl ester (MH^+ = 684 Da) indicating one $-CONH_2$ group.

(3) Edman/MS indicated that the first two residues are 'Leu' Phe, ([MH^+ - 'Leu'] = 556 Da, [MH^+ - ('Leu' + Phe)] = 409 Da).

(4) The CA MIKES of tryptophyllin L 6.1 (see Figure 4.7) gave the partial sequence ...-Phe Phe $Pro(NH_2)$.

(5) Automated Edman sequencing confirmed sequence Ile Phe Phe Phe Pro (NH_2).

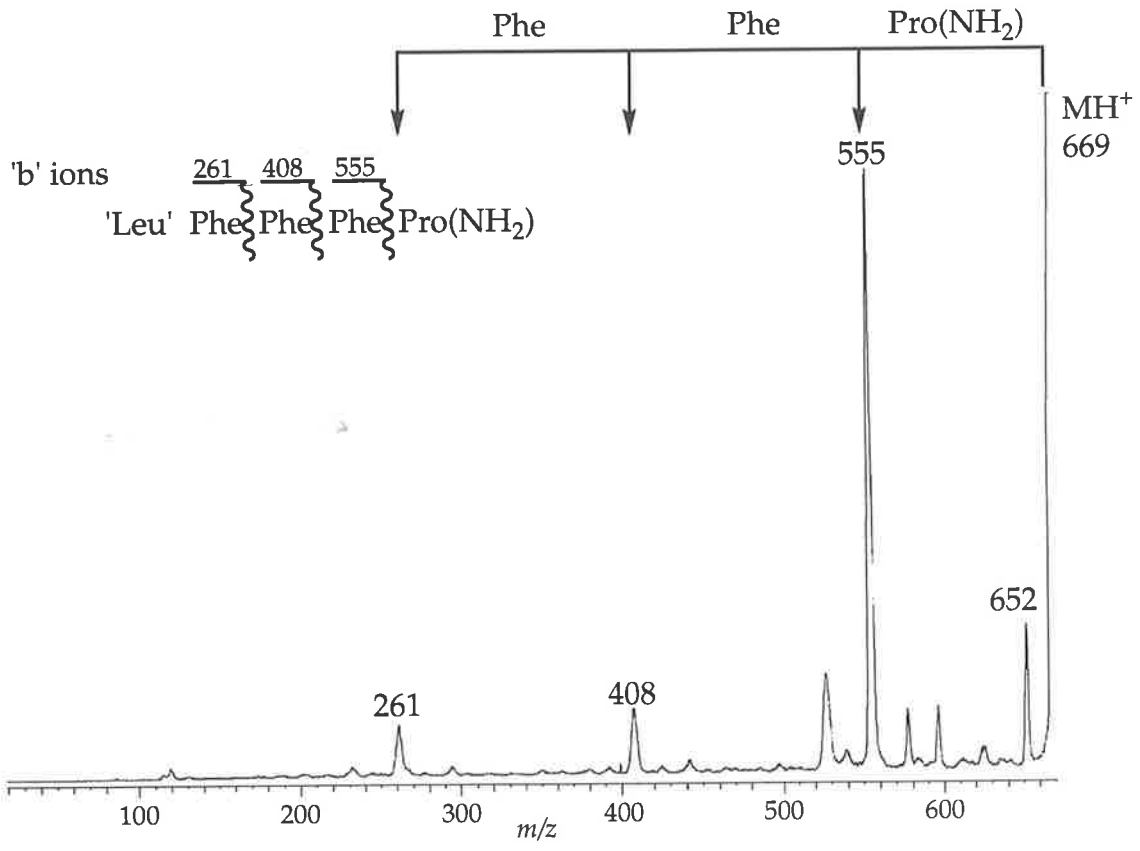

Figure 4.7. CA MIKE spectrum of tryptophyllin L 7.1 (MH^+ = 669 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

4.2.2. Bioactivity of Peptides from *L. ewingi.*

Peptides like the magainins,[150] uperins[145] and caerins[135,189,190,226,227] show antibiotic activity: they form large amphipathic α-helices and are cationic (see Chapter 2.7.2). Caerin 1.1 is found in many of the *Litoria* species so far studied, e.g. *L. caerulea*,[189-191] *L. splendida*[135] and *L. gilleni*,[135,226,227] and is also present in *L. ewingi*. Caerin 1.1. has wide spectrum antibiotic activity (see in Table 4.4). However, *L. ewingi* does not contain the inactive analogue of caerin 1.1 that is present in other species of Australian green tree frogs. [135,188-191] This analogue has two amino acid residues missing from the N-terminal end of the peptide and shows no antibiotic activity less than MIC of 100 μg/ml.[135,188-191] When active peptides are released upon the dorsal surface, an enzyme is often present, which after a suitable period deactivates the peptides so that they do not act upon the host.[136] In *L. ewingi*, uperin 7.1 co-occurs with uperin 7.1.1, in which the first two amino acids (of uperin 7.1 [Gly Trp]), are removed. The co-occurrence of uperin 7.1.1 and 7.1 in the secretion suggests that uperin 7.1 is bioactive. Uperin 7.1 is similar to uperin 3.5[230] (see Table 4.4) except that the last four amino acid residues are missing.* Synthetic uperin 7.1 and uperin 7.1.1 have been tested for antibiotic activity (see Table 4.4). The antibiotic activity of uperin 7.1 is low and shows only minimal activity against *Leuconostoc lactis* and *Streptococcus uberis*, while uperin 7.1.1 shows no antibiotic activity. Pharmacological activity testing is currently in progress for uperin 7.1.[247]

Caeridin 7.1 and the tryptophyllins L 6.1 and L 7.1, have not been tested for any antibiotic activity, yet previous experience[152] has shown that peptides of these families are inactive. Tryptophyllins that have been previously characterised

* Uperins 7.1 and 3.5 have structural similarities as follows, Asp residues (4); uperin 3.5 has Arg-Lys (7,8), with uperin 7.1 has Lys-His (7,8) ,and Ser (11). Other amino acids in the peptide are hydrophobic.

by Erspamer[82,144,235-237] and Steinborner[152,248] show minor hormonal neuropeptide or neuromodulator effects. The tryptophyllin L peptides from *L. ewingi* may show pharmacological effects and are currently being tested for such activity.[247]

	Peptide Sequence
Caerin 1.1	Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val-Pro Val Ile Ala Glu His Leu (NH_2)
Uperin 3.5	Gly Val Gly Asp Leu Ile Arg Lys Ala Val Ser Val Ile Lys Asn Ile Val (NH_2)
Uperin 7.1	Gly Trp Phe Asp Val Val Lys His Ile Ala Ser Ala Val (NH_2)
Uperin 7.1.1.	Phe Asp Val Val Lys His Ile Ala Ser Ala Val (NH_2)

Organism	**MIC (μg/ml)[a]**			
	caerin 1.1	**uperin 3.5**	**uperin 7.1**	**uperin 7.1.1**
Bacillus cereus	50	25		
Escherichia coli				
Leuconostoc lactis	1.5	3	50	
Listeria innocua	25	25		
Micrococcus luteus	12.5	12.5		
Pasteurella multocida	25	100		
Staphylococcus aureus	3-12[b]	50		
Staphylococcus epidermidis	12.5	12.5		
Streptococcus uberis	12.5	12.5	100	

Table 4.4. Comparison of the antibiotic activities of caerin 1.1, and the uperins 3.5, 7.1 and 7.1.1. [([a]) no figure means that the MIC is > 100 μg/ml; ([b]) depends upon the strain used].

4.2.3. Evolutionary Significance of Peptides from *L. ewingi*.

The six peptides characterised from *L. ewingi* are an unusual combination since they belong to three different peptide families. The caerin and caeridin peptides are observed in other Australian green tree frogs,[135,189-191,226,227]

while tryptophyllins have only been observed before in the *Litoria* genus from *L. rubella*.[152] The presence of the uperin peptides is interesting, as there have been no other uperin analogues found in other *Litoria* species. The uperin family of peptides was originally isolated from species in the genus *Uperoleia*.[145,230]

L. ewingi is not associated directly to any of the other green tree frogs or the Red tree frog, *L. rubella*, in the phylogenic groupings of *Litoria* genus.[256] However these species are more closely related to each other than to members of any other genus. Therefore the presence of caerin and caeridin peptides together with the modified tryptophyllins in *L. ewingi*, are probably attributable to a common ancestor of the *Litoria* genus.

The presence of uperin-like compounds in the secretion of *L. ewingi* was not expected. The presence of the uperin peptides cannot be due to a common ancestor between the *Litoria* and *Uperoleia* genus. The *Litoria* genus is a major component of the Hylidae family, which has an extensive global distribution. However *Uperoleia* is a genus of the Leptodactylidae family, which is confined to the southern continents. Any common ancestor must predate the divergence of these two lineages at least 100 million years ago. Such a scenario seems improbable. This may be an example of independent evolutionary origin (arising from structural convergences), rather than a shared ancestor. Such a scenario already exists for anurans. The caerulin peptide family, which contains five members[112,141,257-260] has been observed in frogs of different genera on three continents.*[180]

* For examples of the independent evolutionary origin of certain peptide families see ref [180].

4.3. Summary and Future Work.

The peptide profile from the granular glands of *L. ewingi* contains an unusual combination of peptides. These peptides are related to peptide families found in different species and different genera. This work raises some interesting questions;

(1) *L. ewingi* is also found on Kangaroo Island and Tasmania, populations that have been isolated since the islands were connected to the mainland both 5 000 and 10 000 years ago. Is this reflected in the changes in the peptide profile? (Evolutionary changes have been observed in the Australian green tree frog, *L. caerulea*. The peptide secretions of *L. caerulea* collected from Darwin and from the offshore Melville Island are reproducibly different[189,190]).

(2) There are three other species related phylogenetically to *L. ewingi*. These are located throughout south-eastern Australia and are *L. paraewingi*, *L. verreauxi verreauxi* and *L. v. alpina*.[261-263] The peptide profiles of the granular glands of these four closely related species would provide an interesting comparison.

CHAPTER FIVE: *Litoria xanthomera.*

Chapters 5 and 6 will concentrate upon the similar peptides obtained from the granular glands of the closely related Australian green tree frogs *L. xanthomera* and *L. chloris*.

5.1. Introduction.

Litoria xanthomera (*xanthos* translates to orange and *meros* to thigh in Greek)[264] belongs to the Anura order and is a member of the family Hylidae.[231] *L. xanthomera* was originally classified with *L. chloris*, which is found in rain forest on the eastern seaboard of Australia, from Cairns to Sydney. Differences within '*L. chloris*' were noticed with the northern population having orange eyes and orange thigh colouration (see Figure 5.1), while the southern population has red eyes and blue thighs (see Figure 6.1).[87] Differences in head shape between the northern and southern populations were also observed.[265] In 1986, the northern population was classified as a separate species and called *L. xanthomera*.[264] The geographic distributions of *L. xanthomera* and *L. chloris* populations are shown in Figure 5.2.[87]

L. xanthomera is a medium sized frog, measuring up to 5.6 cm in length and is commonly called the orange thighed beach frog (see Figure 5.1). It is from these brightly coloured markings that *L. xanthomera* may be easily identified.

L. xanthomera is found along a 600 km stretch of the northern Queensland coast (from Cape Melville to Ingham) (see Figure 5.2). It is typically confined to dense rain forest, where it lives in the tree canopy and descends to the ground for breeding.[87]

Figure 5.1. Picture of *L. xanthomera* (B. Stankovich).

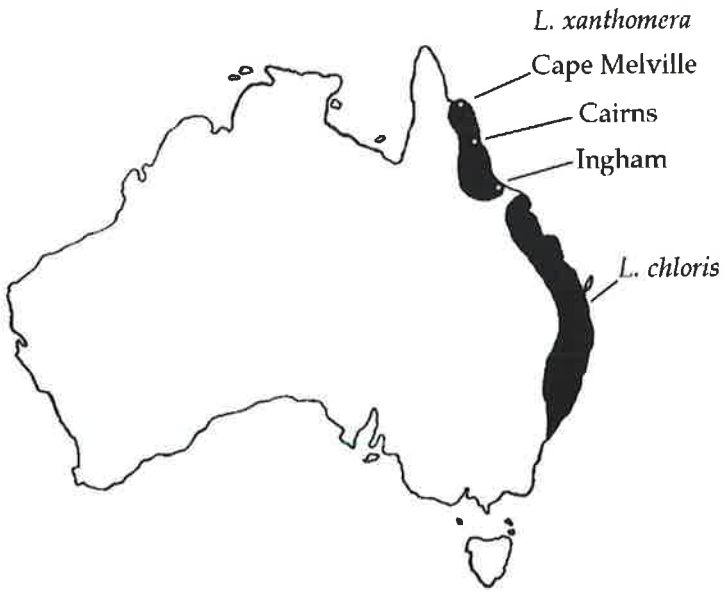

Figure 5.2. Diagram of the geographic distribution of *L. xanthomera* (northern population) and *L. chloris* (southern population). The two species are geographically isolated (redrawn from Encyclopaedia of Australian Animals: Frogs[231]).

L. xanthomera is closely related to the Australian green tree frogs *L. splendida, L. caerulea, L. gilleni* and *L. chloris* with all having a haploid chromosome number of 13.[186] However *L. xanthomera* and *L. chloris* differ from the other Australian green tree frog species, as the granular glands are spread other the entire dorsal surface of the frog. In the other species, the granular glands are hypertrophied in the rostral position.* This close relationship with other Australian green tree frogs suggest that the peptides from *L. xanthomera* (and *L. chloris*) should be similar to those obtained from the other green tree frogs,[135,189-191,226,227] i.e. belong to the caerin and caeridin peptide families (peptide structures (2.13) and (2.16) respectively). The major peptides obtained from the granular gland secretion of *L. xanthomera* are described in this chapter.

5.2. Results and Discussion.

Specimens of *L. xanthomera* were collected live from Cairns (Qld.) (see Figure 5.1). The amphibians were maintained in captivity for at least a year and surface electrical stimulation of their granular glands was performed periodically. On average each 'milking' produced five milligrams of peptide material following lyophilisation of the secretion. The glands were allowed at least four weeks to replenish before another milking was performed. The HPLC traces of the secretion have been repeated on numerous occasions with the results being reproducible. The HPLC trace is shown in Figure 5.3. Seven major peptides were isolated and characterised, their molecular weights range from 1 096 to 2 634 Da.

* For example see Figure 2.1 for the rostral glands of *L. splendida*.

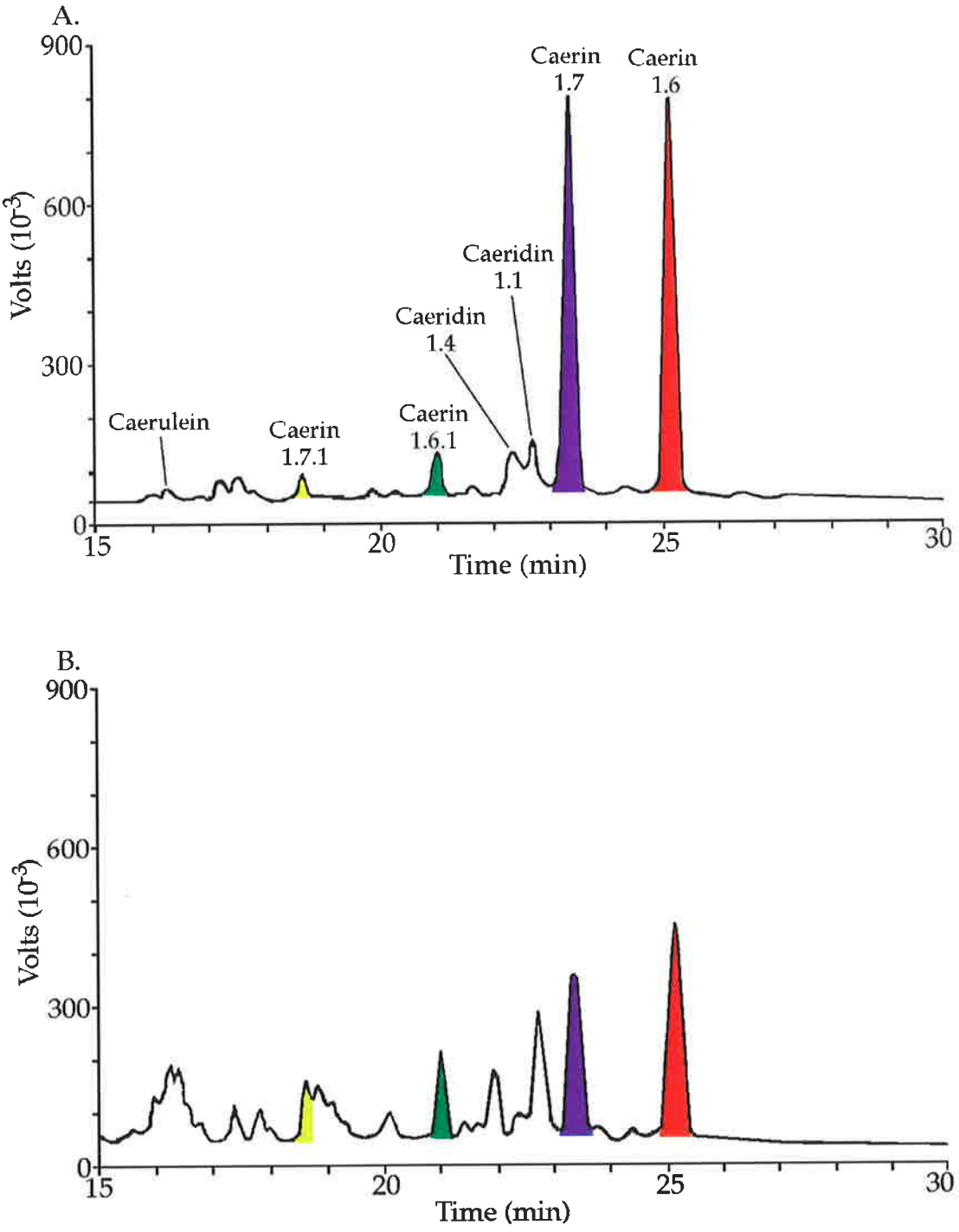

Figure 5.3. The HPLC traces of the granular extract from *L. xanthomera* specimens collected from near Cairns (Qld.). Trace (A) is the HPLC trace from the typical work up procedure. Trace (B) is the HPLC trace without the addition of methanol allowing degradation of the peptides (for experimental conditions, see Chapter 8). The corresponding colours represent the presence of the same peptide.

5.2.1. Structural Determination of Peptides.

A detailed description of the characterisation of the peptides that were isolated by HPLC will be described. Six peptides were characterised in total and the sequences are shown in Table 5.1.

The sequences of the peptide components isolated by HPLC were determined by positive ion FAB MS methods. The molecular weights for the following were determined by FAB MS; (1) the underivatised peptide, (2) peptide methyl esters (described in Chapter 3.2.1), (3) Edman/MS products,[209] and (4) products from enzymatic digestion with endoproteinase Lys-C (Lys-C) or α-chymotrypsin. The CA MIKES data of the enzymatic digest fragments provided sequencing information. The above MS procedures do not distinguish between the isomeric Leu and Ile residues.*[217,221,222] This problem was overcome using automated Edman sequencing[198] which also confirmed the overall structure of the peptide.

Caeridin 1.4. and the caerin peptides are new, but caeridin 1.1 and caerulein have been isolated previously from other *Litoria* species.[135,189-191,226,227] All peptides are post-translationally modified containing C-terminal amide functionality. The structure determination of individual peptides are listed below. The structures are summarised in Table 5.1. Both Ile and Leu will be designated as 'Leu' using MS methods of structure determination.

* MS/MS data can, on occasions, differentiate between Leu and Ile by the loss of C_3H_6 from the former.[217,221,222] These fragmentations are not observed in the mass spectra described here.

Peptide	Sequence	MH^+ (Da)[#]
	Caerulein*	
	pGlu Gln Asp Tyr(SO_3H) Thr Gly Trp Met Asp Phe (NH_2)	1354
	Caeridins	
1.1.*	Gly Leu Leu Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2)	1140
1.4.†	Gly Leu Leu Asp Gly Leu Leu Gly **Gly** Leu Gly Leu (NH_2)	1096
	Caerins	
1.6.	Gly Leu Phe Ser Val Leu Gly Ala Val Ala Lys His Val Leu Pro His Val Val Pro Ile Ala Glu Lys Leu (NH_2)	2591
1.6.1.	Phe Ser Val Leu Gly Ala Val Ala Lys His Val Leu Pro His Val Val Pro Ile Ala Glu Lys Leu (NH_2)	2421
1.7.	Gly Leu Phe **Lys** Val Leu Gly **Ser** Val Ala Lys His **Leu** Leu Pro His Val Ala Pro **Val** Ile Ala Glu Lys Leu (NH_2)	2634
1.7.1.	Phe **Lys** Val Leu Gly **Ser** Val Ala Lys His **Leu** Leu Pro His Val Ala Pro **Val** Ile Ala Glu Lys Leu (NH_2)	2464

Table 5.1. Sequences of peptides characterised from *L. xanthomera*. Peptides marked (*) have been previously isolated from other *Litoria* species.[135,189-191,226,227] (†) Caeridin 1.4 was isolated from from *L. xanthomera*, by then Honors student, S. Ramsay.[266] Residues underlined show the difference in the sequence from that of caeridin 1.1 or caerin 1.6. (#) All masses are nominal, i.e. those obtained by the summation of the integral masses of all of the constituent amino acids.

Caerin 1.6.

(1) HPLC retention time of the peptide is 25.3 minutes and MH^+ = 2591 Da.

(2) Methylation yielded a (bis)methyl ester (MH^+ = 2620 Da) which indicates the presence of one $-CONH_2$ and one $-CO_2H$ group.

(3) Edman/MS identified the first two amino acid residues as Gly 'Leu', ([MH^+ - Gly (57)] = 2534 Da, [MH^+ - (Gly + 'Leu')] = 2421 Da).

(4) Lys-C digestion gave two digest fragments, 1.6.A (MH^+ = 1061 Da) and 1.6.B (MH^+ = 1437 Da). The CA MIKE spectrum of the digest fragment 1.6.A determined the full sequence to be Gly 'Leu' Phe Ser Val 'Leu' Gly Ala Val Ala Lys(OH). The CA MIKE spectrum of the digest fragment 1.6.B provided the partial sequence His Val 'Leu' -...- Glu Lys (OH). The data obtained from both the CA MIKE spectra are shown in Table 5.2.

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.6.A	1061	'b'	1043, (H_2O); 915, (Lys); 844, (Ala); 745, (Val); 674, (Ala); 617, (Gly).
		'y'	1004, (Gly); 891, ('Leu'); 744, (Phe); 657, (Ser); 558, (Val); 445, ('Leu'); 388, (Gly); 317, (Ala).
Structure of caerin 1.6.A Gly 'Leu' Phe Ser Val 'Leu' Gly Ala Val Ala Lys(OH)			
Caerin 1.6.B	1437	'b'	1419, (H_2O); 1291, (Lys); 1162, (Glu).
		'y'	1300, (His); 1201, (Val); 1088, ('Leu').
Partial structure of caerin 1.6.B His Val 'Leu' -...- Glu Lys(OH)			

Table 5.2. The CA MIKES data from caerin 1.6.A (MH^+ = 1061 Da) and caerin 1.6.B (MH^+ = 1437 Da) from the Lys-C digest of caerin 1.6. Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

(5) α–Chymotrypsin digest of the Lys-C digest fragment 1.6.B (MH^+ = 1437 Da) gave the fragments 1.6.B.a (MH^+ = 602 Da), 1.6.B.b (MH^+ = 656 Da) and 1.6.B.c (MH^+ = 854 Da) (see Table 5.3). The CA MIKE spectra of these digest fragments produced the full structure of fragment 1.6.B. CA MIKE spectrum of 1.6.B.a (MH^+ = 602 Da) is shown in Figure 5.4, with the data from 1.6.B.b (MH^+ = 656 Da) and 1.6.B.c (MH^+ = 854 Da) being shown in Table 5.4. The CA MIKES data reveal the structure of caerin 1.6.B as His Val 'Leu' Pro His Val Val Pro Val 'Leu' Ala Glu Lys (OH).

Peptide Fragment (Da)	**Structure Determined**
1.6.B.a (MH^+ = 602)	His Val 'Leu' Pro His (OH)
1.6.B.b (MH^+ = 656)	Pro Val 'Leu' Ala Glu Lys (OH)
1.6.B.c (MH^+ = 854)	Val Val Pro Val 'Leu' Ala Glu Lys (OH)

Table 5.3. The α-chymotrypsin fragments of Lys-C digest fragment of caerin 1.6.B (MH^+ = 1437 Da).

Precursor Ion	**MH^+ (*m/z*)**	**Fragment Type**	**Fragment Ions**
Caerin 1.6.B.b	656	'b'	638, (H_2O); 510, (Lys); 381, (Glu); 310, (Ala); 197, ('Leu').
		'y'	460, (Pro + Val); 347, ('Leu').
Structure of caerin 1.6.B.b (Pro + Val) 'Leu' Ala Glu Lys (OH)			
Caerin 1.6.B.c	854	'b'	836, (H_2O); 708, (Lys); 579, (Glu); 508, (Ala); 395, ('Leu'); 296, (Val); 199, (Pro).
		'y'	755, (Val); 656, (Val); 460, (Pro + Val); 347, ('Leu').
Structure of caerin 1.6.B.c Val Val Pro Val 'Leu'Ala Glu Lys (OH)			

Table 5.4. The CA MIKES data from caerin 1.6.B.b (MH^+ = 656 Da) and caerin 1.6.B.b (MH^+ = 854 Da) from the chymotrypsin digest of caerin 1.6.B. Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

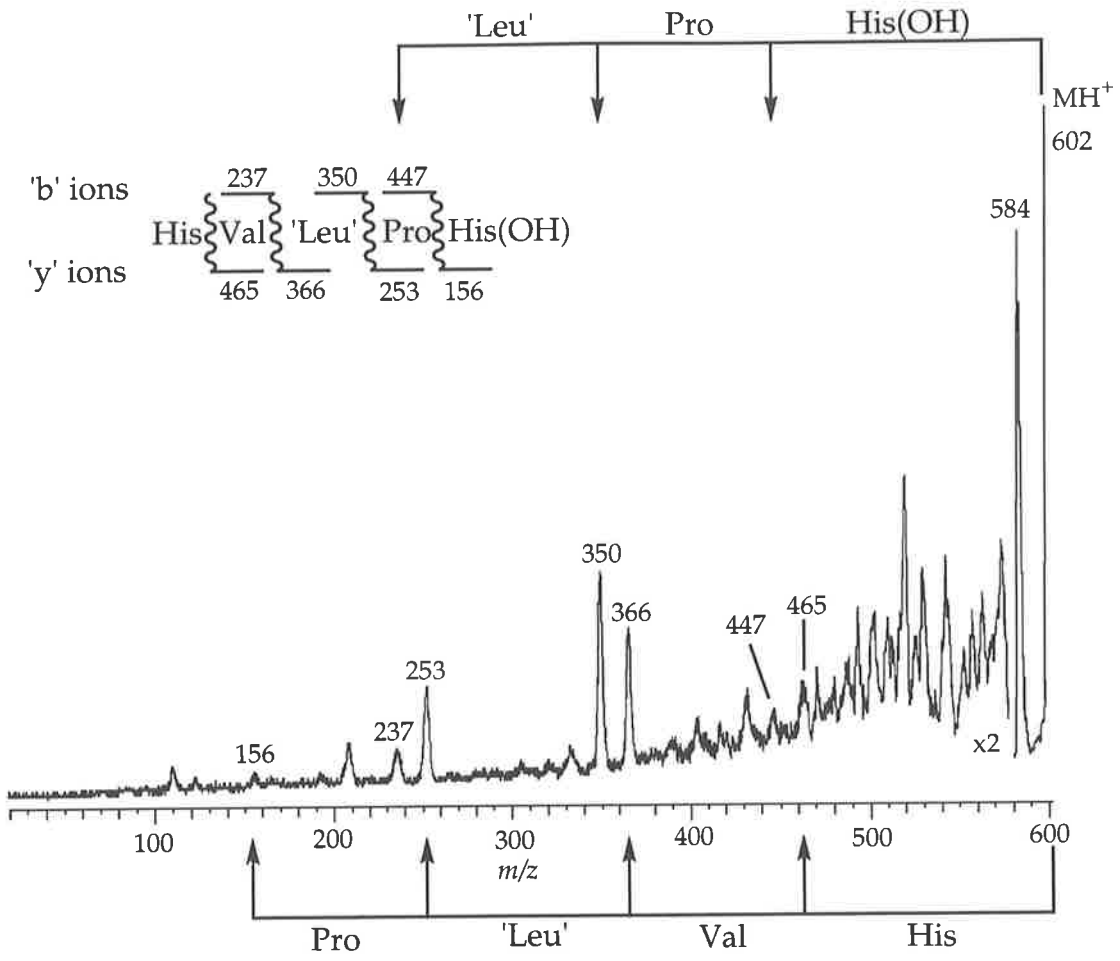

Figure 5.4. CA MIKE spectrum of caerin 1.6.B.a (MH^+ = 602 Da) formed from the Lys-C digest of caerin 1.6.B (MH^+ = 1437 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

(6) The α-chymotrypsin digest of caerin 1.6 yielded numerous digest fragments, including fragment 1.6.C (MH^+ = 768 Da). The CA MIKE spectrum of 1.6.C gave the sequence (Pro + Val) 'Leu' Ala Glu Lys 'Leu'(NH_2) (data is shown in Table 5.5).

(7) Automated sequencing confirmed the structure of caerin 1.6 to be Gly Leu Phe Ser Val Leu Gly Ala Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2).

Precursor Ion	MH+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.6.C	768	'b'	751, (NH_3); 638, ('Leu'); 510, (Lys); 381, (Glu); 310, (Ala); 197, ('Leu').
		'y'	572, (Pro + Val); 459, ('Leu'); 259, (Ala + Glu).
Structure of caerin 1.6.C (Pro + Val) 'Leu' Ala Glu Lys 'Leu'(NH_2)			

Table 5.5. The CA MIKES data from caerin 1.6.C (MH^+ = 768 Da) produced from the chymotrypsin digest of caerin 1.6. Format of fragment ions [fragment ion, *m/z* and (amino acid lost)].

Caerin 1.6.1.

(1) HPLC retention time of the peptide is 21.1 minutes and MH^+ = 2421 Da.

(2) Methylation yielded a (bis)methyl ester (MH^+ = 2450 Da) which indicated the presence of one -$CONH_2$ and one -CO_2H group.

(3) Edman/MS revealed that the first four amino acids were Phe Ser Val 'Leu' (see Table 5.6).

Edman Cycle and Precursor Peptide (Da)	Mass Lost (Da)	Amino Acid
- (2421)		
1 (2274)	147	Phe
2 (2187)	87	Ser
3 (2088)	99	Val
4 (1975)	113	'Leu'

Table 5.6. Four Edman degradation/MS cycles of caerin 1.6.1.

(4) Lys-C digest of caerin 1.6.1 produced two peptide fragments, 1.6.1.A (MH^+ = 891 Da) and 1.6.1.B (MH^+ = 1437 Da). The mass spectrum of digest fragment caerin 1.6.1.B is the same as that of 1.6.B, identifying the structure as His Val 'Leu' Pro His Val Val Pro Val 'Leu' Ala Glu Lys (OH). The CA MIKE spectrum of fragment 1.6.1.A gave the sequence ...-Val 'Leu' Gly Ala Val Ala Lys(OH) (see Figure 5.5).

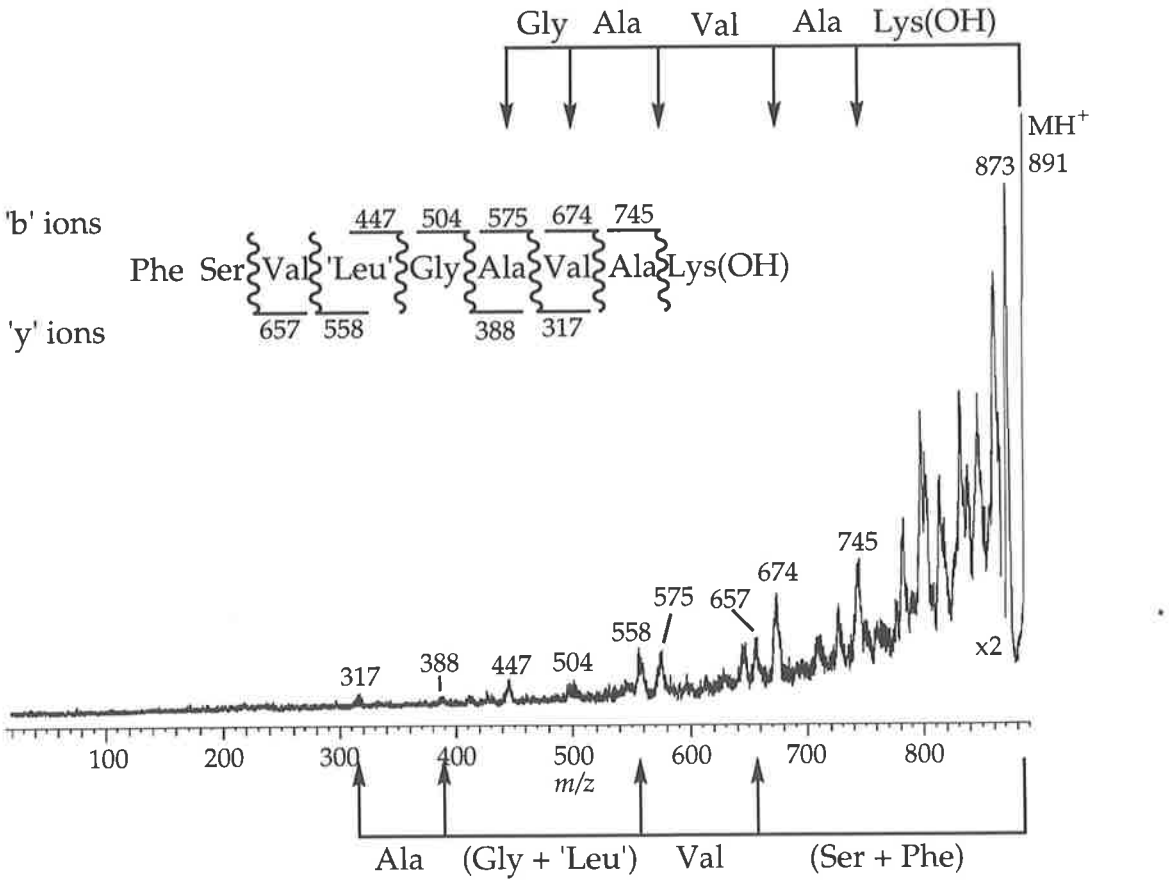

Figure 5.5. CA MIKE spectrum of fragment 1.6.1.B (MH^+ = 891 Da), from the Lys-C digest of caerin 1.6.1. The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

(5) Automated Edman sequencing confirmed the primary structure sequence of caerin 1.6. to be Phe Ser Val Leu Gly Ala Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2).

Caerin 1.7.

(1) HPLC retention time of the peptide is 24.4 minutes and MH^+ = 2634 Da.

(2) Methylation yielded a (bis)methyl ester (MH^+ = 2663 Da) indicating the presence of one -$CONH_2$ and one -CO_2H group.

(3) Edman/MS revealed the first three amino acid residues to be Gly 'Leu' Phe (data shown Table 5.7).

Edman Cycle and Precursor Peptide (Da)	Mass Lost (Da)	Amino Acid
- (2634)		
1 (2577)	57	Gly
2 (2464)	113	'Leu'
3 (2317)	147	Phe

Table 5.7. Three Edman degradation/MS cycles of peptide caerin 1.7.

(4) Lys-C digest of caerin 1.7 produced three peptide fragments, 1.7.A (MH^+ = 464 Da), 1.7.B (MH^+ = 673 Da) and 1.7.C (MH+ = 1423 Da) shown in Table 5.8. The CA MIKE spectrum of fragment 1.7.A is shown in Figure 5.6 and gives the sequence as Gly 'Leu' Phe Lys (OH). The CA MIKE spectrum of fragment 1.7.B gave the sequence Val 'Leu' Gly Ser Val Ala Lys (OH) (Table 5.10). Manual Edman/MS showed that the first three amino acid residues of 1.7.C to be His 'Leu' 'Leu' (Table 5.9). The CA MIKE spectrum of 1.7.C partially determined the structure to be His 'Leu' (347 Da) Val Ala (Pro + Val) 'Leu' Ala Glu Lys(OH) (data shown Table 5.10).

Peptide Fragment (Da)	Structure Determined
1.7.A (MH^+ = 464)	Gly 'Leu' Phe Lys (OH)
1.7.B (MH^+ = 673)	Val 'Leu' Gly Ser Val Ala Lys(OH)
1.7.C (MH^+ = 1423)	His 'Leu' 'Leu' Pro His Val Ala Pro Val 'Leu' Ala Glu Lys(OH)

Table 5.8. The Lys-C digest fragments of caerin 1.7 (MH^+ = 2634 Da).

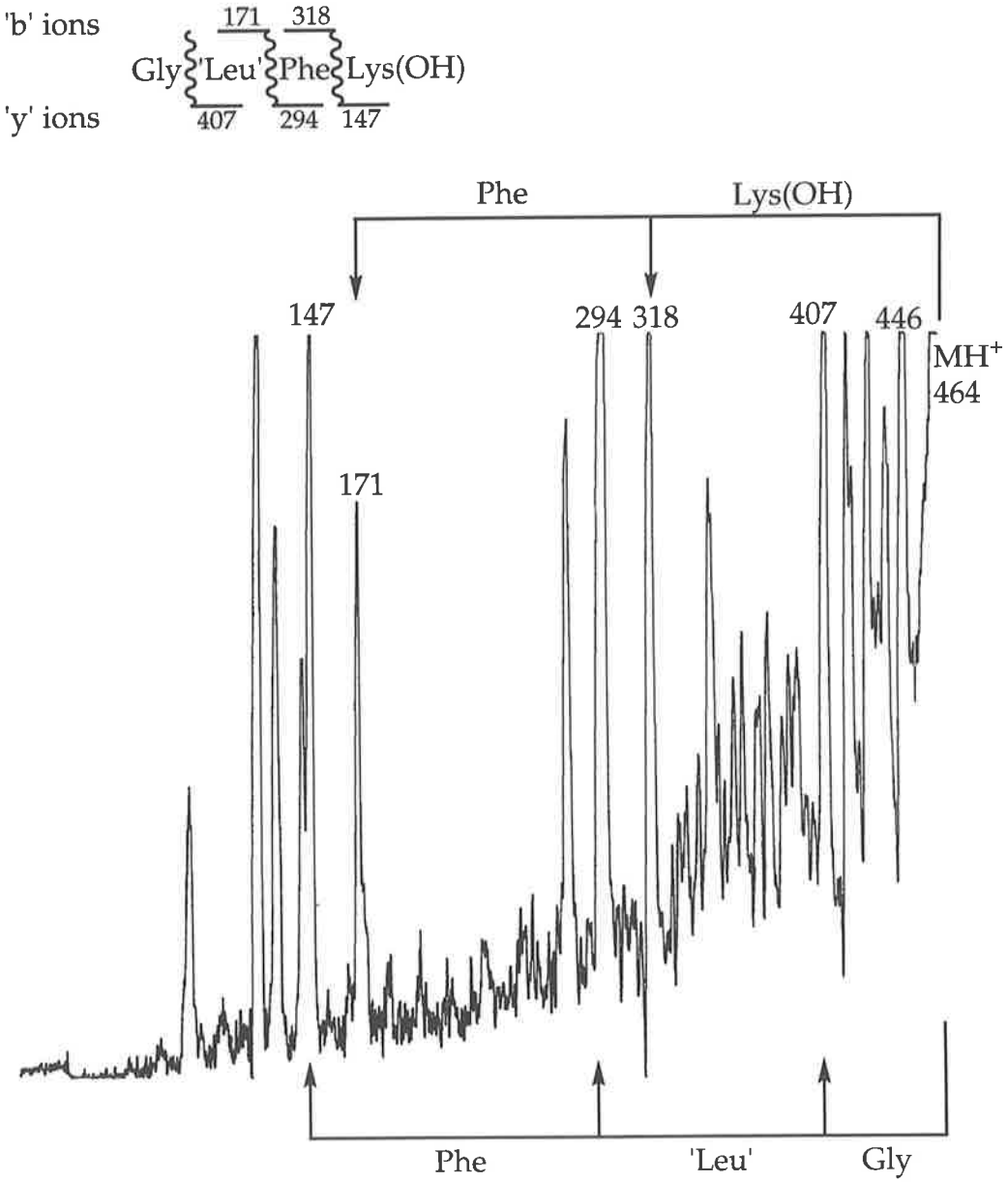

Figure 5.6. CA MIKE spectrum of digest fragment 1.7.A (MH^+ = 464 Da), formed by the Lys-C digest of caerin 1.7. The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

Edman Cycle and Precursor Peptide (Da)	Mass Lost (Da)	Amino Acid
- (1423)		
1 (1286)	137	His
2 (1173)	113	'Leu'
3 (1060)	113	'Leu'

Table 5.9. The Edman degradation/MS cycles of peptide caerin 1.7.C.

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.7.B	673	'b'	655, (H_2O); 527, (Lys); 456, (Ala); 357, (Val); 270, (Ser); 213, (Gly).
		'y'	574, (Val); 461, ('Leu'); 404, (Gly); 317, (Ser).
Structure of caerin 1.7.B Val 'Leu' Gly Ser Val Ala Lys(OH)			
Caerin 1.7.C	1423	'b'	1405, (H_2O); 1277, (Lys); 1148, (Glu); 1077, (Ala); 964, ('Leu'); 768, (Val + Pro); 697, (Ala).
		'y'	1268, (His); 1173, ('Leu'); 826, (347 Da); 727, (Val); 460 (267 Da).
Partial structure of caerin 1.7.C. His 'Leu' (347 Da) Val Ala (Pro + Val) 'Leu' Ala Glu Lys(OH)			

Table 5.10. The CA MIKES data from caerin 1.7.B (MH^+ = 673 Da) and caerin 1.7.C (MH^+ = 1423 Da) from the Lys-C digest of caerin 1.7. Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

(5) The α-chymotrypsin digest of peptide 1.7.C gave two peptide fragments 1.7.C.a (MH^+ = 616 Da) and 1.7.C.b (MH^+ = 826 Da). The CA MIKE spectrum of 1.7.C.a gave the sequence His 'Leu' 'Leu' Pro His (OH) (see Table 5.11), while that of 1.7.C.b gave Val Ala Pro Val 'Leu' Ala Gly Lys (OH) (see Figure 5.7).

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.7.C.a	616	'b'	598, (H_2O); 461, (His); 364, (Pro); 251, ('Leu').
		'y'	497, (His); 366, ('Leu'); 253, ('Leu'); 156, (Pro).
Structure of caerin 1.7.C.a His 'Leu' 'Leu' Pro His (OH)			

Table 5.11. The CA MIKES data from caerin 1.7.C.a (MH^+ = 616 Da) from the α-chymotrypsin digest of caerin 1.7.C. Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

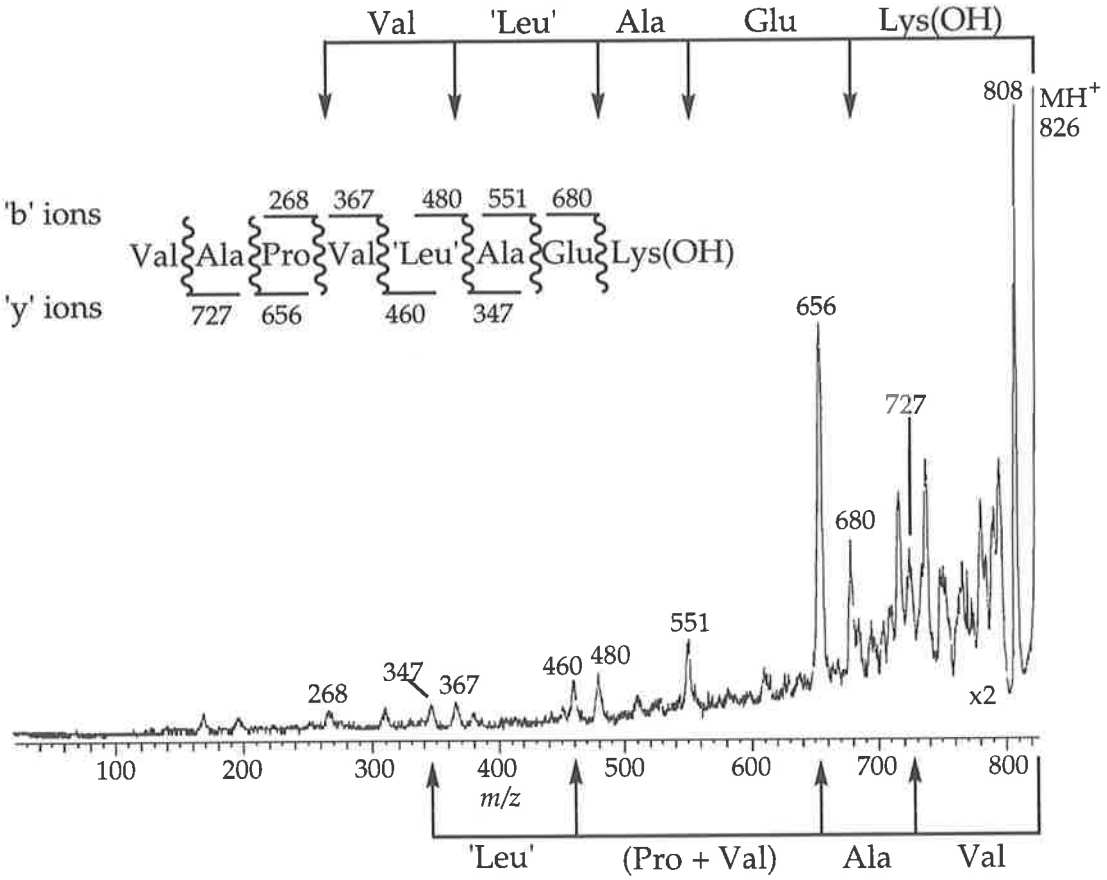

Figure 5.7. CA MIKE spectrum of digest fragment 1.7.C.b (MH^+ = 826 Da) formed by the α-chymotrypsin digest of caerin peptide 1.7.C. The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure.

(6) The α-chymotrypsin digest of caerin 1.7 yielded numerous digest fragments, including fragment 1.7.D (MH^+ = 1398 Da). The CA MIKE spectrum of 1.7.D gave the partial sequence 'Leu' 'Leu' (333 Da) Ala -...- Ala Glu Lys Leu (NH_2) (data is shown in Table 5.12).

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.7.D	1398	'b'	1381, (NH_3); 1268, ('Leu'); 1140, (Lys); 1011, (Glu); 940, (Ala); 827, ('Leu').
		'y'	1172, ('Leu' + 'Leu'); 839, (333 Da); 768, (Ala).
Partial structure of caerin 1.7.D			'Leu' 'Leu' (333 Da) Ala -...- Leu' Ala Glu Lys 'Leu' (NH_2).

Table 5.12. The CA MIKES data from caerin 1.7.D (MH^+ = 1398 Da) from the Lys-C digest of caerin 1.7.1. Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

(7) Combination of the mass spectrometric data together with automated sequencing gives the sequence of caerin 1.7 as Gly Leu Phe Lys Val Leu Gly Ser Val Ala Lys His Leu Leu Pro His Val Ala Pro Val Ile Ala Glu Lys Leu (NH_2).

Caerin 1.7.1.

(1) HPLC retention time of the peptide is 18.6 minutes and MH^+ = 2464 Da.

(2) The structure determination of caerin 1.7.1 is identical to that of caerin 1.7 except that the Lys-C digest yielded a fragment 1.7.1.A (MH^+ = 294 Da). CA MIKE spectrum of 1.7.1.A gave the sequence Phe Lys (OH) (data shown in Table 5.13).

(3) Automated Edman sequencing of caerin 1.7.1 determined the sequence Phe Lys Val Leu Gly Ser Val Ala Lys His Leu Leu Pro His Val Ala Pro Val Ile Ala Glu Lys Leu (NH_2).

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.7.1.A	294	'b'	276, (H_2O); 148, (Lys).
		'y'	147, (Phe).
Structure of caerin 1.7.1.A Phe Lys(OH)			

Table 5.13. The CA MIKES data from caerin 1.7.1.A (MH^+ = 294 Da) from the Lys-C digest of caerin 1.7.1. Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

5.2.2. Bioactivity of Peptides from *L. xanthomera.*

The caerin 1 peptides are an unusual class of antibiotic peptides since they do not form ideal α-helices (see Chapter 2.7.2). The sequences contain two proline residues that break the α-helix after residue 14 and the α-helix is reformed after residue 20. The antibiotic testing of the caerins from *L. xanthomera* showed significant results* (see Table 5.14).

Both caerins 1.6 and 1.7 exhibit significant antibiotic activity, but not as good as that of caerin 1.1. The ratio of caerin 1.6 to 1.7 in the secretion is approximately 1:1. When a 1:1 mixture of synthetic caerins 1.6 and 1.7 was tested for antibiotic activity, it was observed that there is a synergistic effect in certain cases (see Table 5.14).

* The activity tests were done on synthetic caerins since not enough natural peptide material was available for testing.

	Peptide Sequence
Caerin 1.1	Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2)
Caerin 1.6	Gly Leu **Phe** Ser Val Leu Gly **Ala** Val Ala Lys His Val Leu Pro His Val Val Pro **Ile** **Ala** **Glu** **Lys** Leu (NH_2)
Caerin 1.6.1	**Phe** Ser Val Leu Gly **Ala** Val Ala Lys His Val Leu Pro His Val Val Pro **Ile** **Ala** **Glu** **Lys** Leu (NH_2)
Caerin 1.7	Gly Leu **Phe** **Lys** Val Leu Gly Ser Val Ala Lys His **Leu** Leu Pro His Val Ala Pro Val Ile Ala Glu **Lys** Leu (NH_2)
Caerin 1.7.1	**Phe** **Lys** Val Leu Gly Ser Val Ala Lys His **Leu** Leu Pro His Val Ala Pro Val Ile Ala Glu **Lys** Leu (NH_2)

	MIC (µg/ml)[a]					
Organism	**caerin 1.1**	**1.6**	**1.6.1**	**1.7**	**1.7.1**	**[1.6 + 1.7]**
Bacillus cereus	50	100		100		25
Escherichia coli						
Leuconostoc lactis	1.5	3		3		3
Listeria innocua	25	50		50		12
Micrococcus luteus	12.5	12.5		12.5		12.5
Pasteurella multocida	25	25		25		50
Staphylococcus aureus	3-12[b]	12.5-50[b]		12.5-50[b]		50
Staphylococcus epidermidis	12.5	50		50		12.5
Streptococcus uberis	12.5	50		50		12.5

Table 5.14. Comparison of the antimicrobial activities of the synthetic caerins 1.1, 1.6, 1.6.1, 1.7 and 1.7.1 [([a]) no figure means that the MIC is > 100 µg/ml; ([b]) depends upon the strain used; ratio of (1.6. + 1.7) was (1:1)].

Caerins 1.6.1 and 1.7.1 have the first two amino acids of caerin 1.6 and 1.7 removed: they show no antibiotic activity in the test regime. Such a scenario is also observed for the caerins 1 of *L. caerulea*, *L. gilleni* and *L. splendida*.[135,188-191] The assumption that *L. xanthomera* is able to deactivate caerins 1.6 and 1.7 is confirmed by a simple experiment. Upon milking of *L. xanthomera*, half the secretion was worked up as per normal (addition of methanol), while the other half was worked up without the addition of methanol. The resultant HPLC traces are compared in Figure 5.3. The relative abundances of the caerins 1.6, 1.6.1, 1.7 and 1.7.1 differ significantly in the two traces. An increase in the inactive analogues occurs when there is no deactivation of enzymes in the work up. This experiment indicates that *L. xanthomera*, together with other frogs of the genus *Litoria*, are able to deactivate their active peptides by the removal of the first two amino acid residues.

CHAPTER SIX: *Litoria chloris*.

6.1. Introduction.

Litoria chloris belongs to the order Anura and is a member of the family Hylidae.[231] *L. chloris* was first identified by Boulenger in 1893.[87] It is a medium sized frog measuring up to 6.5 cm in length and is commonly known as the green beach frog (Figure 6.1).[87,231] It is easily identified and distinguished from *L. xanthomera* by its red eyes and purple colouration on the rear of the thighs.[87] *L. chloris* is closely related to *L. xanthomera* (as detailed in Chapter 5.1), with *L. xanthomera* being classified as a separate species in 1986.[264] The relationship between *L. chloris* and *L. xanthomera* is so close that when hybridisation occurs between these two species: some of the hybrid frogs develop abnormalities which supports the premise of the genetic differentiation between the two species. However, there was also the development of normal froglets, which supports the premise that the two species are closely related.[267]

L. chloris is distributed along a 2 000 km stretch of the eastern Australian coastline from Townsville (Qld.) to Sydney (N.S.W.) (see Figure 5.2), but is rarely encountered since it spends the majority of its life in trees, descending only to breed.[87]

L. chloris is closely related to *L. xanthomera* (introduced in Chapter 5.1) and to a lesser extent the other members of the Australian green tree frogs.[186] The granular glands of *L. chloris* and *L. xanthomera* differ from the other green tree frogs as they are spread over the entire dorsal surface of the frog. The close relationship between *L. chloris* and *L. xanthomera* is further emphasised by the similarities in the skin peptides. These peptides also demonstrate the relationship of *L. chloris* and *L.*

xanthomera to the other Australian green tree frogs *L. splendida, L. caerulea* and *L. gilleni*.[135,189-191,226,227]

Figure 6.1. Picture of *L. chloris*. (Diagram copied from Barker *et.al.*[87] with permission from M.J. Tyler, Department of Zoology, University of Adelaide).

Figure 6.2. Diagram of the geographic distribution of *L. chloris* (redrawn from Encyclopaedia of Australian Animals: Frogs[231]).

6.2. Results and Discussion.

One specimen of *L. chloris* was opportunistically obtained from a shipment of ferns from Brisbane (Qld.) into South Australia. The specimen was maintained in captivity for a few weeks to regain its health before its granular glands were stimulated. Surface electrical stimulation of the granular glands of *L. chloris* was performed when needed, with the glands being allowed at least four weeks to replenish. Each 'milking' on average produced four milligrams of peptide material following lyophilisation of the secretion. The HPLC trace with the peptide fractions identified is shown in Figure 6.3. The purpose of the characterisation of peptides from *L. chloris* is to observe the evolutionary relationship between *L. chloris* and *L. xanthomera*. A total of eight peptides were characterised, with four being major constituents of the peptide profile. The molecular weights of the peptides range between 1 096 and 2 662 Da.

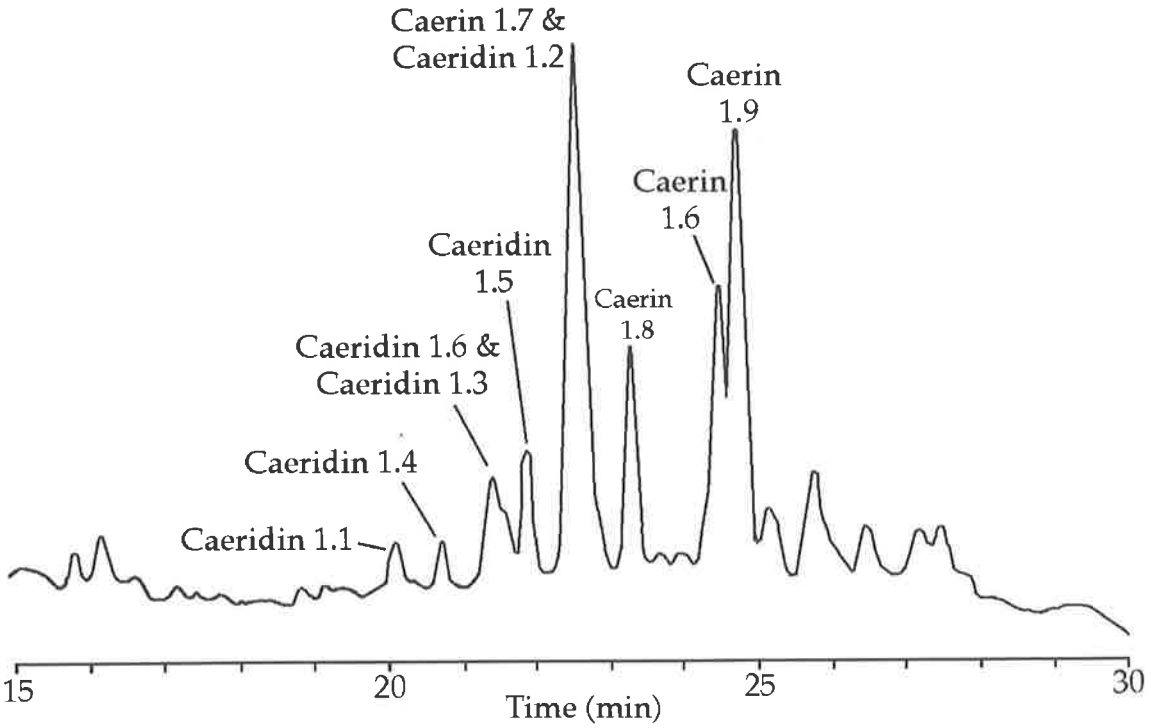

Figure 6.3. The HPLC trace from *L. chloris* specimens collected from Brisbane (Qld.).

6.2.1. Structural Determination of the Peptides.

A detailed description of the peptide characterisation from fractions isolated by HPLC will be described. The structure of all the peptides are shown in Table 6.1. Ten peptides were characterised in total, with six identical to peptides isolated previously from other *Litoria* species.[135,189-191,226,227,268]

The structures of the peptide components shown in the HPLC traces were determined by positive ion electrospray using the Finnigan LCQ (LCQ) with parent ion scanning and MS^2 and MS^3 techniques.* No peptide derivatisations by enzymatic digests were necessary for caerin peptides, due to the MS^n capability of the LCQ. The LCQ allowed the sequencing of approximately 80% of the peptide structure from the $(M+2H)^{2+}$ parent ion. Only members of the caerin and caeridin peptide families were isolated from the granular glands from *L. chloris*. All the peptides were post-translationally modified containing C-terminal amide functionality. The MS sequencing procedure does not distinguish between the isomeric Leu and Ile residues.#[217,221,222] Automated Edman sequencing[198] overcomes this problem and also confirms the overall structure of the peptide.

Caeridin 1.1.

(1) HLPC retention time is 20.2 minutes** and MH^+ = 1140 Da.

(2) This compound was determined to have the same molecular weight as a previously identified peptide, caeridin 1.1, from other *Litoria* species.[135,141,188-190] It co-elutes with synthetic caeridin 1.1, therefore the amino acid sequence is Gly Leu Leu Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2).

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* Spectra obatined through courtesy of Dr. G. Currie, from the Department of Botany, University of Melbourne, Melbourne, Victoria.

MS/MS data can, on occasions, differentiate between Leu and Ile by the loss of C_3H_6 from the former.[217,221,222] These fragmentations are not observed in the mass spectra obtained.

** The experimental conditions for all of the retention times, see Chapter 8.

Peptide	Sequence	MH^+ (Da)#
	Caeridins	
1.1*†	Gly Leu Leu Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2)	1140
1.2*	Gly Leu Leu (β)Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2)	1140
1.3*		1122
1.4*†	Gly Leu Leu Asp Gly Leu Leu Gly **Gly** Leu Gly Leu (NH_2)	1096
1.5	Gly Leu Leu (β)Asp Gly Leu Leu Gly **Gly** Leu Gly Leu (NH_2)	1096
1.6		1078
	Caerins	
1.6†	Gly Leu Phe Ser Val Leu Gly Ala Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2)	2591
1.7†	Gly Leu Phe **Lys** Val Leu Gly **Ser** Val Ala Lys His **Leu** Leu Pro His Val **Ala** Pro Val Ile Ala Glu Lys Leu (NH_2)	2634
1.8	Gly Leu Phe **Lys** Val Leu Gly **Ser** Val Ala Lys His **Leu** Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2)	2664
1.9	Gly Leu Phe **Gly** Val Leu Gly **Ser Ile** Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2)	2591

Table 6.1. Sequences of all peptides characterised from *L. chloris*. Peptides marked (*) have been sequenced previously.[135,189-191,226,227,268] Peptides marked (†) have been isolated in *L. xanthomera*. Residues underlined indicate the difference in the sequence compared to caeridin 1.1 (for the caeridin peptides) and caerin 1.6 (for the caerin peptides) (# indicates that the masses are nominal, i.e. are obtained by the summation of the intergral masses of all of the constituent amino acids).

Caeridin 1.2.

(1) HPLC retention time is 22.6 minutes and MH^+ = 1140 Da.

(2) MS^2 gave the partial sequence ...- Asp Gly 'Leu' 'Leu' Gly Thr 'Leu' Gly 'Leu' (NH_2) (see Figure 6.4).

(3) Caeridin 1.2 has been observed in *L. gilleni*[268] and co-elutes with synthetic caeridin 1.2. Therefore the amino acid sequence is Gly Leu Leu (β)Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2).

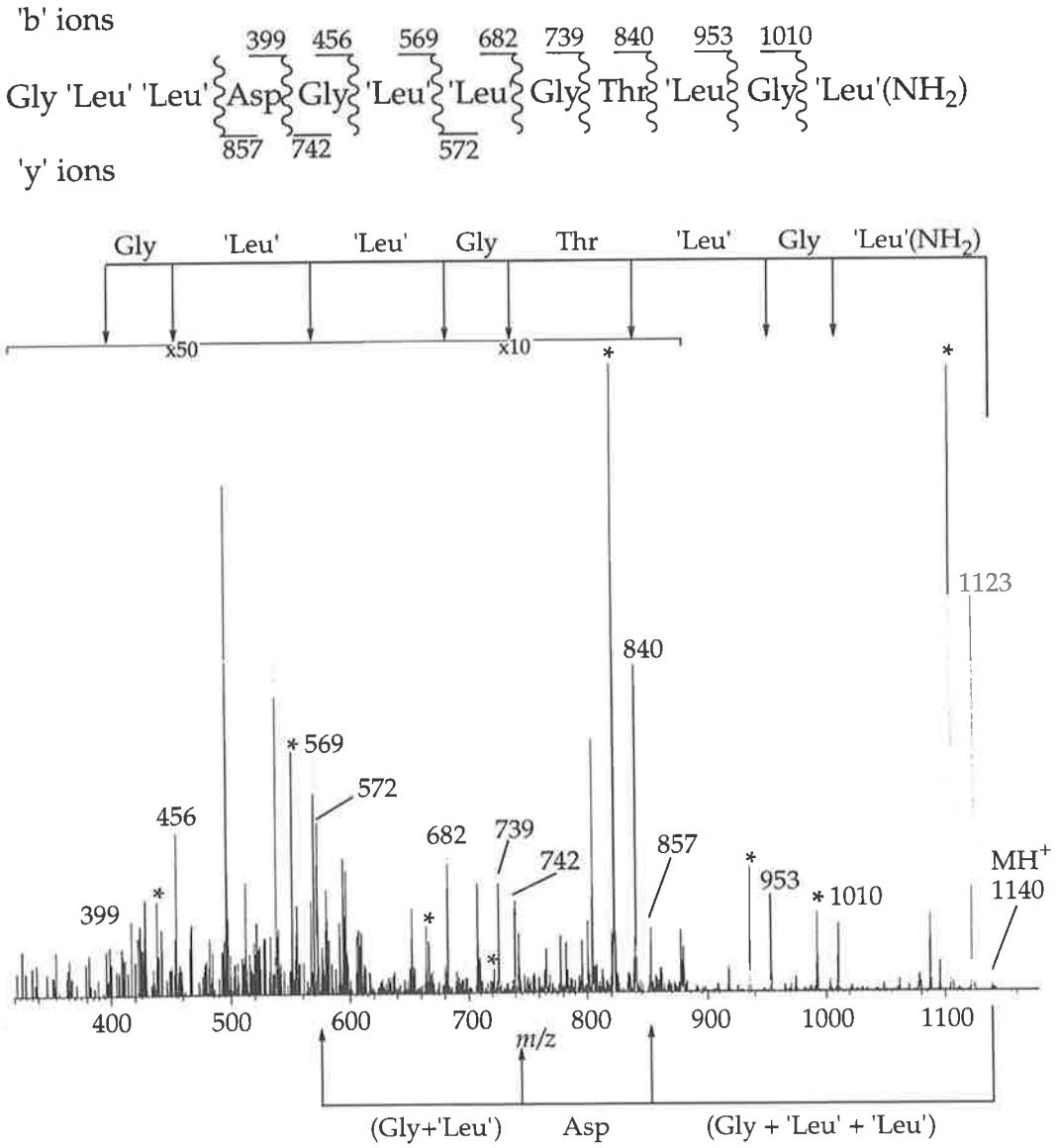


Figure 6.4. MS^2 spectrum of caeridin 1.2 (MH^+ = 1140 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised above the figure. (*) indicates a peak of 18 Da lower than immediate 'b' ions. The loss of 18 Da from the 'b' ions aided in the sequencing and is produced by the loss of H_2O from the Asp amino acid residue.

Caeridin 1.3.

(1) HPLC retention time is 21.4 minutes and MH^+ = 1122 Da.

(2) MS^2 of the peptide determined the structure to be (Gly + 'Leu') 'Leu' -...- 'Leu' 'Leu' Gly Thr 'Leu' Gly 'Leu' (NH_2). The MS^2 spectrum and the sequence is shown in Figure 6.5.

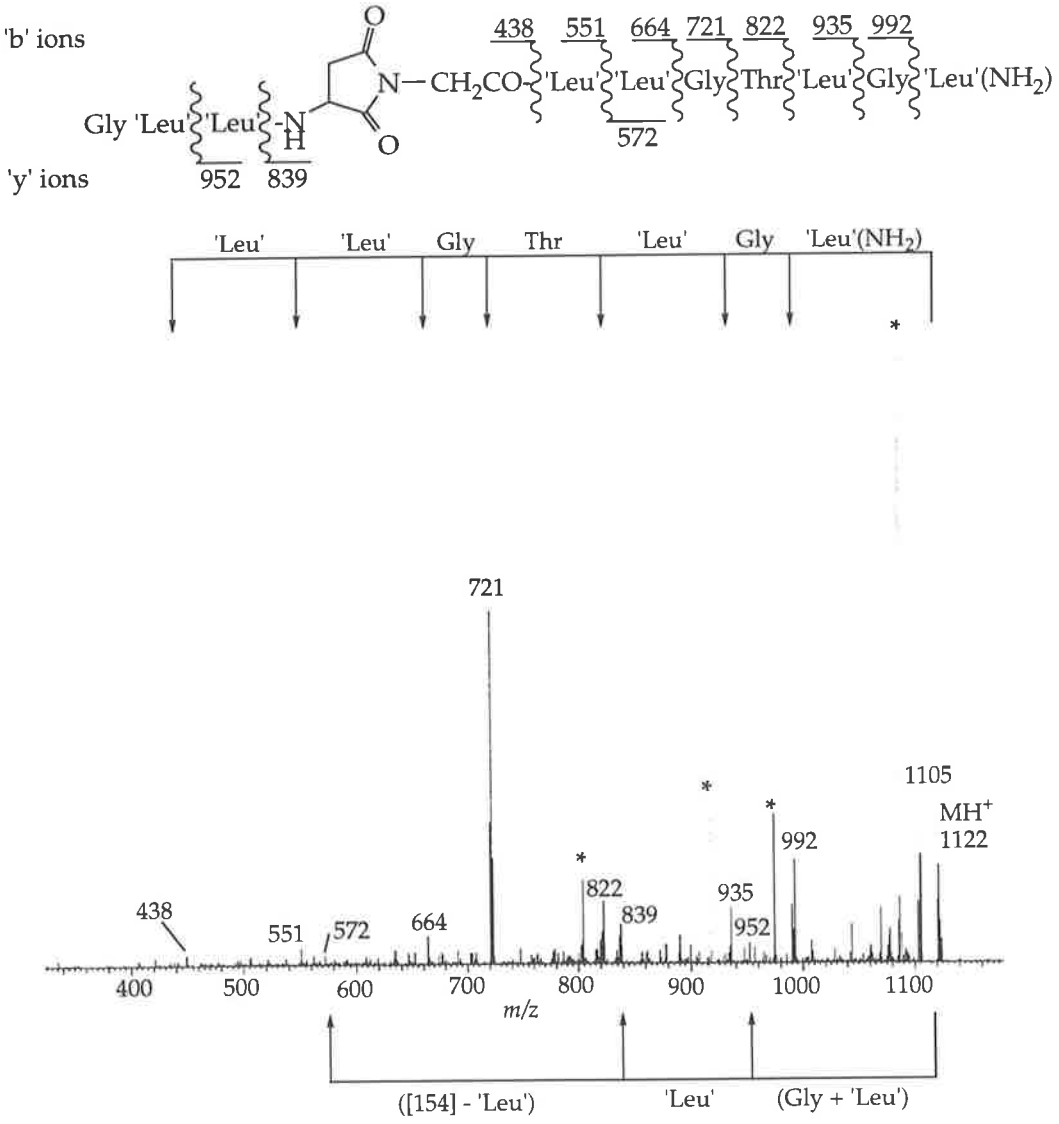


Figure 6.5. MS^2 spectrum of caeridin 1.3 (MH^+ = 1122 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised above the figure. (*) indicates a peak of 18 Da lower than immediate 'b' ions.

The presence of caeridins 1.1-1.3 has been observed before in *L. gilleni*[268] and investigated in detail. The main difference between caeridin 1.1 and 1.2 is that caeridin 1.1 has the usual α-Asp residue at position four (4), whereas caeridin 1.2 contains the isomer β-Asp (see Figure 6.6). Caeridin 1.3 is the corresponding cyclic aminosuccinyl derivative produced by cyclisation between the Asp (4) and Gly (5). It is the intermediate between caeridin 1.1 and 1.2.

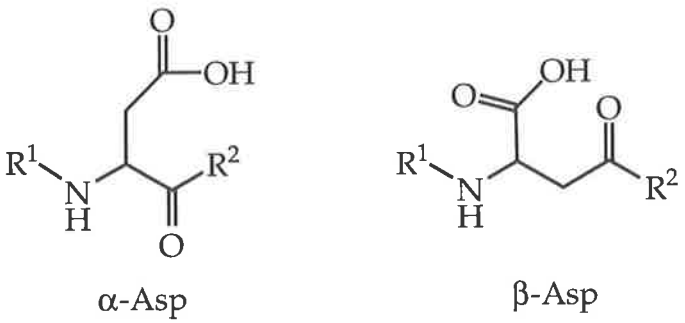


Figure 6.6. The difference between the α-Asp and the β-Asp, in caeridins 1.1 and 1.2 respectively. α-amino acid residues are named because the amide bond linking it to the adjacent C-terminal amino acid is bound to the α-carbon. The β-amino acid residues occur when the adjacent amino acid is bound to the β–carbon.[269]

Caerin 1.4.

(1) HPLC retention time is 20.7 minutes and MH^+ = 1096 Da.

(2) MS^2 of this compound determined the structure to be (Gly + 'Leu') 'Leu' Asp Gly 'Leu' 'Leu' Gly Gly 'Leu' Gly 'Leu'(NH_2) (see Figure 6.7). Caeridin 1.4 has been previously identified in the analogous secretion from *L. xanthomera*. The structure is Gly Leu Leu Asp Gly Leu Leu Gly Gly Leu Gly Leu (NH_2).

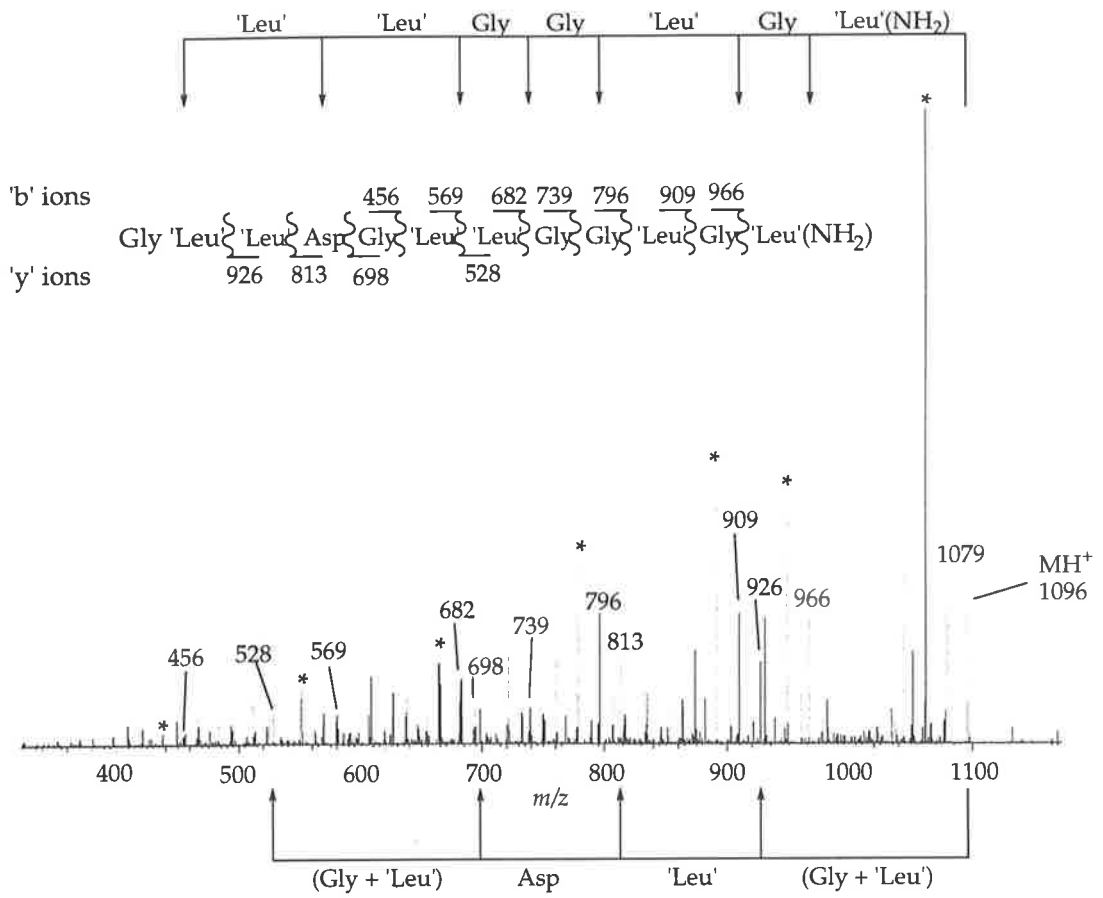


Figure 6.7. MS^2 spectrum of caeridin 1.4 (MH^+ = 1096 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum, and summarised in the top left hand corner of the figure. * indicates a peak of 18 Da lower than immediate 'b' ions. The loss of 18 Da aided in the sequencing and is produced by the loss of H_2O from the Asp amino acid residue.

Caeridin 1.5.

(1) HPLC retention time is 21.9 minutes and MH^+ = 1096 Da.

(2) MS^2 of this compound determined the structure to be ..-'Leu' Asp Gly 'Leu' 'Leu' Gly Gly 'Leu' Gly 'Leu' (NH_2) (see Table 6.2).

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caeridin 1.5.	1096	'b'	1079, (NH_3); 966, ('Leu'); 909, (Gly); 796, ('Leu'); 739, (Gly); 682, (Gly); 569, ('Leu'); 456, ('Leu').
		'y'	926, (Gly + 'Leu'); 813, ('Leu').
Structure of caeridin 1.5.			(Gly + 'Leu') 'Leu' Asp Gly 'Leu' 'Leu' Gly Gly 'Leu' Gly 'Leu' (NH_2).

Table 6.2. The CA MIKES data from caeridin 1.5 (MH^+ = 1096 Da). Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

Caeridin 1.6.

(1) HPLC retention time is 21.4 minutes and MH^+ = 1078 Da.

(2) MS^2 of this compound determined the structure to be ...-'Leu'-...-'Leu' 'Leu' Gly Gly 'Leu' Gly 'Leu' (NH_2). The MS^2 data is shown in Table 6.3.

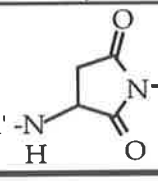
Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caeridin 1.6.	1078	'b'	1061, (NH_3); 948, ('Leu'); 891, (Gly); 778, ('Leu'); 721, (Gly); 664, (Gly); 551, ('Leu'); 438, ('Leu').
		'y'	908, (Gly + 'Leu'); 795, ('Leu').
Structure of caeridin 1.6.			(Gly + 'Leu') 'Leu'  CH_2CO-'Leu' 'Leu' Gly Gly 'Leu' Gly 'Leu' (NH_2)

Table 6.3. The CA MIKES data from caeridin 1.6 (MH^+ = 1078 Da). Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

Caeridins 1.4-1.6 are similar in structure to the caeridin peptides 1.1-1.3, except that Gly (9) replaces Thr (9). We have not carried out experiments to confirm the interconversion of caeridins 1.4 and 1.5, but have assumed that the chemistry is similar to that of caeridin 1.1 and 1.2.[268] The difference between caeridin 1.4 and 1.5 is that caeridin 1.4 has the usual α-Asp (4) and caeridin 1.5 has the unusual β-Asp (see Figure 6.6). Caeridin 1.6 is the corresponding succinimide derivative produced by cyclisation between the Asp (4) and Gly (5).

Caerin 1.6.

(1) HPLC retention time of the peptide is 24.5 minutes and MH^+ = 2591 Da [$(M+2H)^{2+}$ = 1296 Da and $(M+3H)^{3+}$ = 864 Da].

(2) MS^2 of the doubly charged parent ion [$(M+2H)^{2+}$ = 1296 Da] revealed the partial structure to be ...- Gly Ala Val Ala Lys His Val 'Leu' Pro His Val Val Pro -... (see Table 6.4).

(3) MS^3 of the daughter ion (*m/z* 768) was performed to establish part of the missing C-terminal sequence. The spectrum determined the C-terminal end to be ...-Glu Lys 'Leu' (NH_2) (see Table 6.7). Due to the mass range of the Finnigan LCQ MS^n (*m/z* 300-2 000 Da) and producing predominantly 'b' ions, the sequencing of the N-terminal end of the peptide was limited. The combined MS data gave the partial sequence of caerin 1.6 as ...- Gly Ala Val Ala Lys His Val 'Leu' Pro His Val Val Pro -...-Glu Lys 'Leu' (NH_2).

(4) The molecular weight of the peptide was identical to that of caerin 1.6 isolated from *L. xanthomera*. Synthetic caerin 1.6 co-eluted with the natural peptide from *L. chloris*. The full structure of caerin 1.6 is Gly Leu Phe Ser Val Leu Gly Ala Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2).

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.6.	$(M+2H)^{2+}$ 1296	'b'	1921, (671 Da); 1824, (Pro); 1725, (Val); 1626, (Val); 1489, (His); 1392, (Pro); 1279, ('Leu'); 1180, (Val); 1043, (His); 915, (Lys); 844, (Ala); 745, (Val).
		'y'	1975, (617 Da); 1918, (Gly); 1847, (Ala); 1748, (Val); 1677, (Ala); 1549, (Lys); 1412, (His); 1313, (Val); 1200, ('Leu'); 1103, (Pro); 966, (His); 867, (Val); 768, (Val).
Partial structure obtained ...-Gly Ala Val Ala Lys His Val 'Leu' Pro His Val Val Pro-...			
Daughter ion	768	'b'	751, (NH_3); 638, ('Leu'); 510, (Lys); 381, (Glu).
Partial structure of *m/z* 768 ...-Glu Lys 'Leu'(NH_2)			
Structure of caerin 1.6 obtained ...-Gly Ala Val Ala Lys His Val 'Leu' Pro His Val Val Pro-...-Glu Lys 'Leu'(NH_2)			

Table 6.4. The MS^2 data from caerin 1.6. (MH^+ = 2591 Da) and the MS^3 of the daughter ion *m/z* 768. Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

Caerin 1.7.

(1) HPLC retention time of the peptide is 22.6 minutes and MH^+ = 2634 Da [$(M+2H)^{2+}$ = 1318 Da and $(M+3H)^{3+}$ = 879 Da].

(2) MS^2 of the doubly charged parent ion [$(M+2H)^{2+}$ = 1317 Da] revealed the partial structure to be ...-'Leu' Gly Ser Val Ala Lys His 'Leu' 'Leu' Pro His Val Ala Pro -... (see Table 6.5).

Precursor Ion	MH^+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.7.	$(M+2H)^{2+}$ 1318	'b'	1964, (671 Da); 1867, (Pro); 1796, (Ala); 1697, (Val); 1560, (His); 1463, (Pro); 1350, ('Leu'); 1100, (His + 'Leu'); 972, (Lys); 901, (Ala); 802, (Val); 715, (Ser); 658, (Gly); 545, (Leu).
		'y'	1977, (658 Da); 1920, (Gly); 1833, (Ser); 1734, (Val); 1663, (Ala); 1535, (Lys); 1398, (His); 1285, ('Leu'); 1172, ('Leu'); 1075, (Pro); 938, (His); 839, (Val); 768, (Ala).
Partial structure of caerin 1.7	...-'Leu' Gly Ser Val Ala Lys His 'Leu' 'Leu' Pro His Val Ala Pro-...		

Table 6.5. The MS^2 data from caerin 1.7 (MH^+ = 2634 Da). Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

(3) The molecular weight of this peptide is the same as that of caerin 1.7 (isolated from *L. xanthomera*). Synthetic caerin 1.7 co-eluted with the natural peptide from *L. chloris*. The full structure of caerin 1.7 is Gly Leu Phe Lys Val Leu Gly Ser Val Ala Lys His Leu Leu Pro His Val Ala Pro Val Ile Ala Glu Lys Leu (NH_2).

Caerin 1.8.

(1) HPLC retention time of the peptide is 23.3 minutes and MH^+ = 2662 Da [$(M+2H)^{2+}$ = 1332 Da and $(M+3H)^{3+}$ = 888 Da].

(2) MS^2 of the doubly charged parent ion [$(M+2H)^{2+}$ = 1332 Da] revealed the partial structure to be ...-Val 'Leu' Gly Ser Val Ala Lys His 'Leu' 'Leu' Pro His

Val Val Pro -... (see Figure 6.8). The partial sequence obtained is similar to that of caerin 1.7, except that Ala (18) is exchanged for Val (18). The difference in the molecular weights of caerin 1.8 and 1.7 suggested that the only difference between the two structure is at this position.

(3) MS^3 of the daughter ion (*m/z* 768) was performed to establish the C-terminal end of the structure. MS^3 spectrum gave the sequence ...-Ala Glu Lys 'Leu'(NH_2) (see Figure 6.9). The partial sequence deduced was ..-Val 'Leu' Gly Ser Val Ala Lys His 'Leu' 'Leu' Pro His Val Val Pro Val 'Leu' Ala Glu Lys 'Leu' (NH_2).

(4) Automated Edman sequencing gave the structure Gly Leu Phe Lys Val Leu Gly Ser Val Ala Lys His Leu Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2).

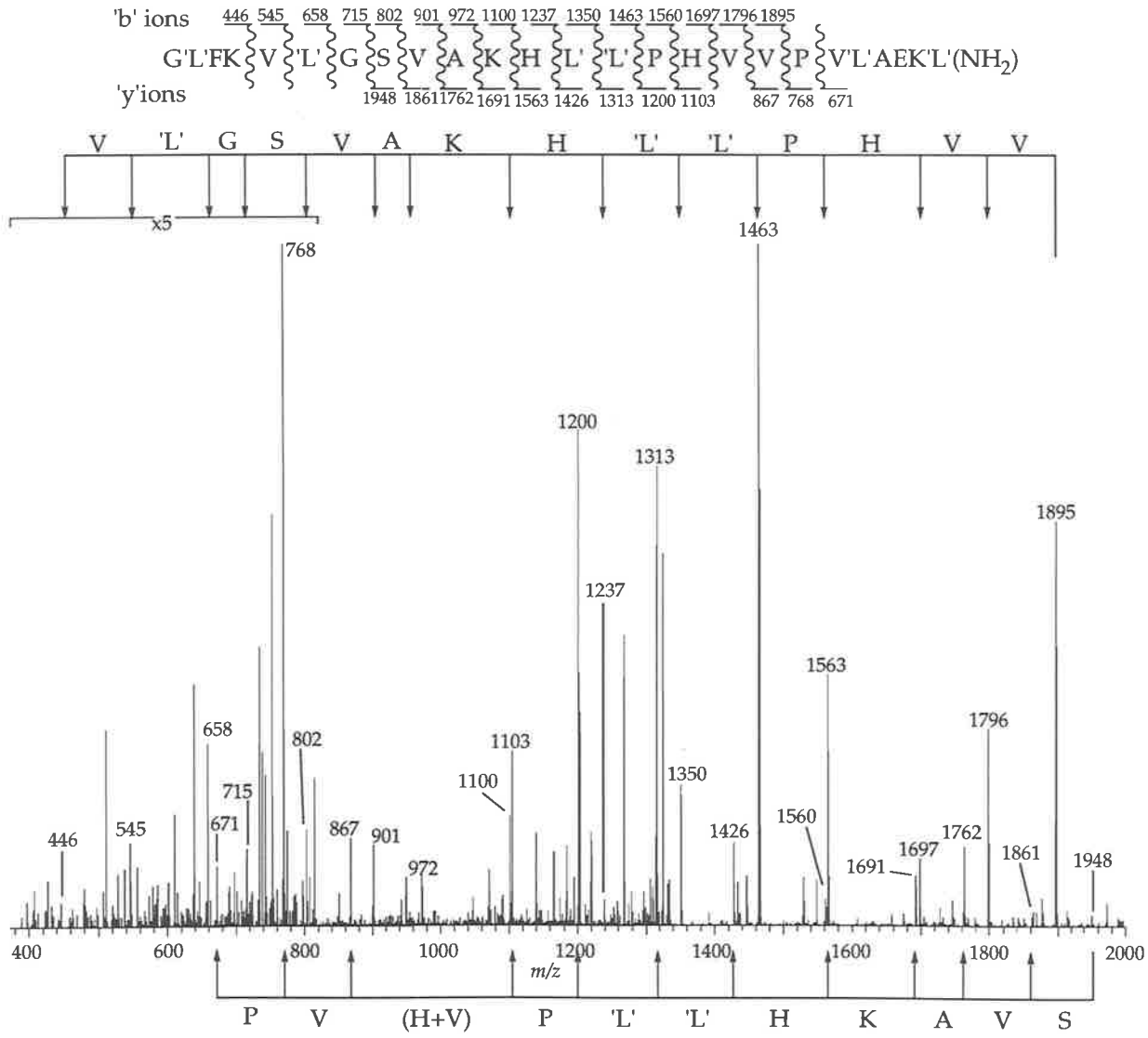


Figure 6.8. MS^2 spectrum $(M+2H)^{2+}$ = 1332 Da of caerin 1.8. (MH^+ = 2662 Da). The 'b' and 'y' ions produced are schematically shown above ('b' ions) and below ('y' ions) the spectrum. The fragmentations are summarised at the top of the figure.

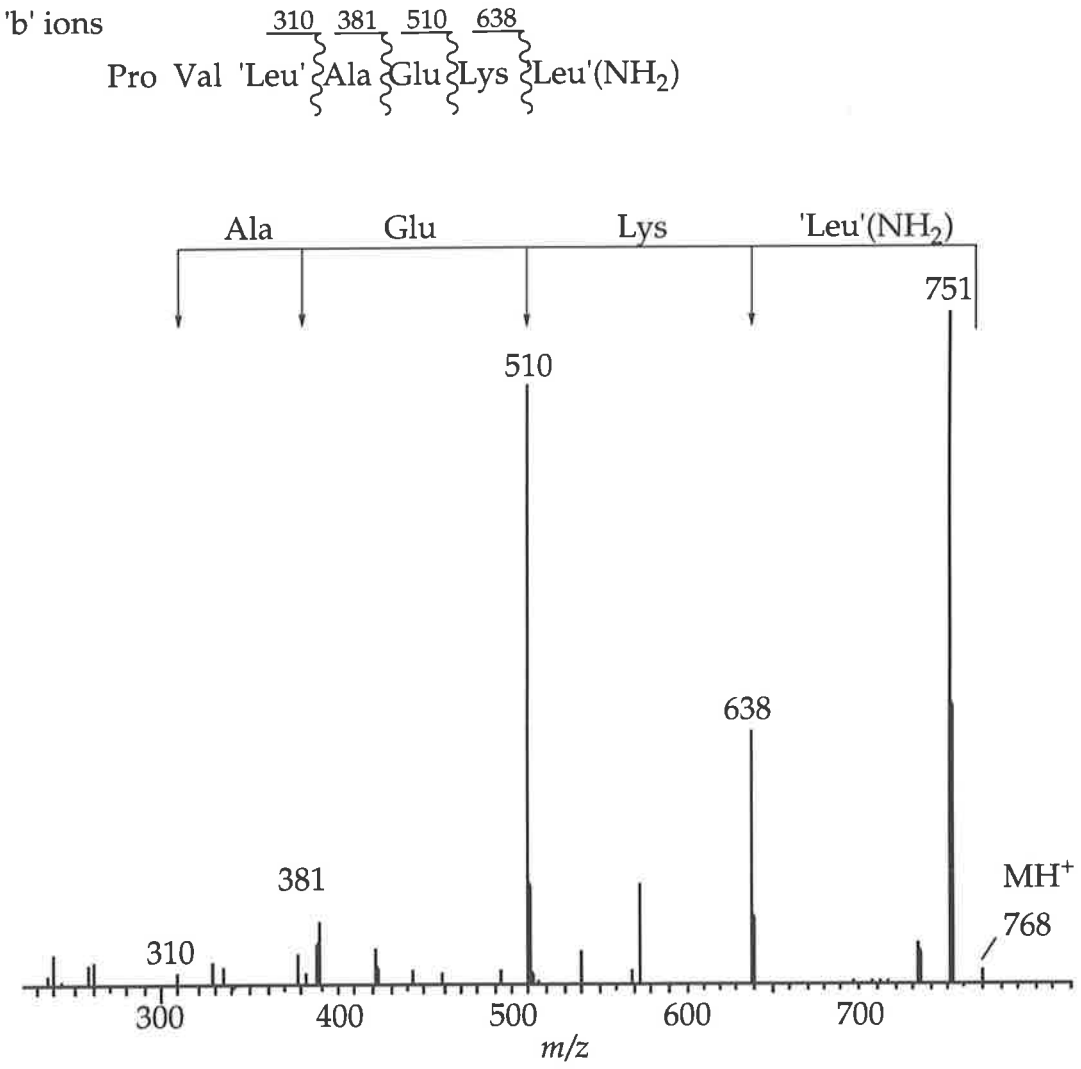


Figure 6.9. MS^3 spectrum of the daughter ion *m/z* 768 from caerin 1.8. The 'b' ions are shown above the spectrum. The fragmentations are summarised at the top of the figure.

Caerin 1.9.

(1) HPLC retention time is 24.8 minutes and MH^+ = 2591 Da [$(M+2H)^{2+}$ = 1296 Da and $(M+3H)^{3+}$ = 864 Da].

(2) MS^2 of the doubly charged parent ion [$(M+2H)^{2+}$ = 1296] gave ...-Gly Ser 'Leu' Ala Lys His Val 'Leu' Pro His Val Val -... (*cf* Table 6.6).

(3) MS^3 of the daughter ion (*m/z* 768) gave the same sequence as previously shown in Figure 6.8 [...-Ala Glu Lys 'Leu'(NH_2)]. The combined MS data gave

the partial sequence of caerin 1.9 as ...-Gly Ser 'Leu' Ala Lys His Val 'Leu' Pro His Val Val-...-Ala Glu Lys 'Leu' (NH_2).

Precursor Ion	MH+ (*m/z*)	Fragment Type	Fragment Ions
Caerin 1.9.	$(M+2H)^{2+}$ 1296	'b'	1824, (768 Da); 1725, (Val); 1626, (Val); 1489, (His); 1392, (Pro); 1279, ('Leu'); 1180, (Val); 1043, (His); 915, (Lys); 844, (Ala); 731, ('Leu'); 644, (Ser); 587, (Gly).
		'y'	1861, (731 Da); 1748, ('Leu); 1677, (Ala); 1549, (Lys); 1412, (His); 1313, (Val); 1200, ('Leu'); 1103, (Pro); 966, (His); 867, (Val); 768, (Val).
Partial structure obtained			...-Gly Ser 'Leu' Ala Lys His Val 'Leu' Pro His Val Val -...
Daughter ion	768	'b'	751, (NH_3); 638, ('Leu'); 510, (Lys); 381, (Glu); 310, (Ala).
Partial structure of *m/z* 768			...-Ala Glu Lys 'Leu'(NH_2)
Partial structure of caerin 1.9			...-Gly Ser 'Leu' Ala Lys His Val 'Leu' Pro His Val Val -...-Ala Glu Lys 'Leu'(NH_2)

Table 6.6. The MS^2 data from caerin 1.9. (MH^+ = 2662 Da) and the MS^3 of the daughter ion *m/z* 768. Format of fragment ions [fragment ion, *m/z* and (fragment lost)].

(4) Automated Edman sequencing of caerin 1.9 determined the structure to be Gly Leu Phe Gly Val Leu Gly Ser Ile Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2).

6.2.2. Bioactivity of Peptides from *L. chloris.*

The caerin peptide family is an unusual class of antibiotic peptides as they do not form an ideal α-helix. The activities of caerin 1.6 and 1.7 are shown in Table 5.5, as well as in Table 6.7 to compare the activities against caerins 1.8 and 1.9. The antibiotic activity of caerin 1.8 was expected to be similar to that of caerin 1.7, as the major difference between the two peptides is that a Val (18) is exchanged for an Ala (18). However with such a small structure change, caerin 1.8 is twice as potent as caerin 1.7 in the majority of cases.

Caerin 1.9 contains a Gly (4), a feature seen before only in caerin 1.2 (see Table 6.7). The antibiotic activity of caerin 1.2 is considerably less than that of caerin 1.1. Gly is a known helix breaker,[169] will affect the secondary structure of the peptide and reduce the activity (*cf* Chapter 2.6.2)]. The structure of caerin 1.9 also compares very closely to that of caerin 1.7 and it is therefore assumed that the antibiotic activity will be less than that of caerin 1.7. However, the activity is comparable to that of the caerins 1.6 and 1.7. This example shows that it is difficult to predict the activity of the caerin peptides with respect to the primary structure.

The caeridins 1.1-1.3 have been tested for bio-activity and are found to be devoid of both antibiotic and smooth muscle activity.[268] We have not tested caeridins 1.4-1.6 for bio-activity, because of the lack of activity seen in other caeridins. Caeridins 1.2, 1.3, 1.5 and 1.6 are present because they are rearrangement products of the caeridins 1.1 and 1.4. The rerrangement occurs through cyclisation of the Asp side chain with the nitrogen in the amide bond belonging to the adjacent Gly (see Figure 6.10). In larger systems the formation of the (β) Asp residue can cause considerable disruption of the normal secondary and tertiary protein structures, thus affecting the activity.[269]

	Peptide Sequence
Caerin 1.1.	Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2)
Caerin 1.2.	Gly Leu Leu **Gly** Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu(NH_2)
Caerin 1.6.	Gly Leu **Phe** Ser Val Leu Gly **Ala** Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu **Lys** Leu(NH_2)
Caerin 1.7.	Gly Leu **Phe Lys** Val Leu Gly Ser Val Ala Lys His **Leu** Leu Pro His Val **Ala** Pro Val Ile Ala Glu **Lys** Leu (NH_2)
Caerin 1.8.	Gly Leu **Phe Lys** Val Leu Gly Ser Val Ala Lys His **Leu** Leu Pro His Val Val Pro Val Ile Ala Glu **Lys** Leu (NH_2)
Caerin 1.9.	Gly Leu **Phe Gly** Val Leu Gly Ser **Ile** Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu **Lys** Leu (NH_2)

	MIC (μg/ml)[a]					
Organism	**caerin 1.1**	**caerin 1.2**	**caerin 1.6**	**caerin 1.7**	**caerin 1.8**	**caerin 1.9**
Bascillus cereus	50	50			50	50
Escherichia coli			50		100	
Leuconostoc lactis	1.5	25	3	3	-	-
Listeria innocua	25	100	50	50	25	25
Micrococcus luteus	12.5		25	12.5	6	12
Pasteurella multocida	25		25	25	50	100
Staphylococcus aureus	3-12[b]		6-12	12-50	6-12	12
Staphylococcus epidermidis	12.5	50	12.5	50	12	25
Streptococcus uberis	12.5		25	50	25	25

Table 6.7. Comparison of the antibiotic activities of the synthetic caerins 1.1, 1.2, 1.6, 1.7, 1.8 and 1.9. Residues underlined indicate the differences in structure compared to caerin 1.1. [([a]) no figure means that the MIC is > 100 μg/ml; ([b]) depends upon the strain used; (-) means not tested]

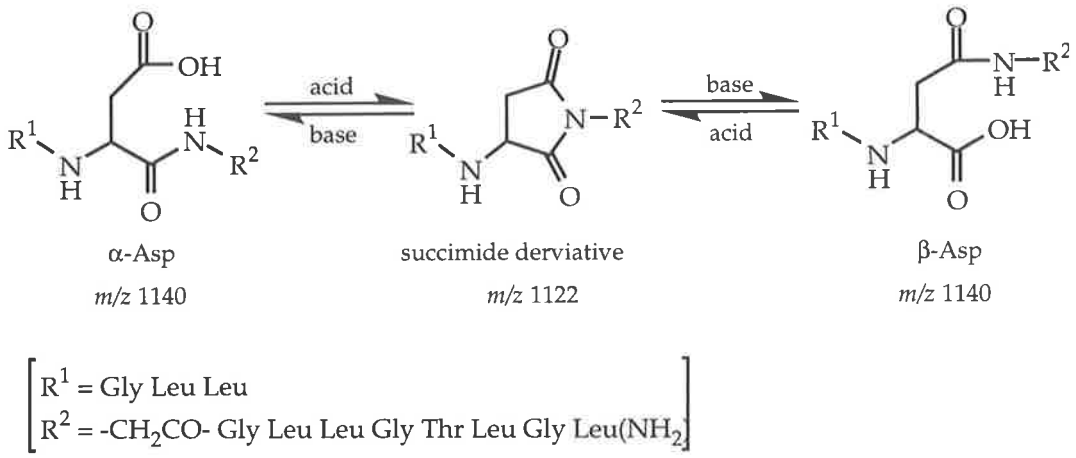


Figure 6.10. Schematic representation of the cyclisation of the Asp amino acid residue in caeridin 1.1 (MH^+ = 1140 Da).[268,270]

The role of the caeridin peptides in the granular gland secretion is still unknown and it is believed that they may be spacer peptides involved in the biosynthesis of the mature caerin peptides. However the sequences are highly conserved (see Table 6.1) and they are also post-translationally modified with C-terminal amide groups. These two observations raise some interesting points;

(1) Sequence conservation is observed in all amphibian peptides that have smooth muscle and antibiotic activity. Therefore, why is the caeridin sequence conserved when there is no obvious activity?

(2) Why would the Australian green tree frogs put energy into post-translationally modifying these peptides when they are not active?

These questions may be answered when the role of the caeridin peptides are determined.

6.2.3. Evolutionary Significance of Peptides from *L. chloris* and *L. xanthomera.*

The two Australian green tree frogs *L. chloris* and *L. xanthomera* are very closely related and are visually similar except for the colour of the eyes and thighs. The expectation was that both species of frog would have similar peptides and this was realised. Although all peptides in both species come from the caeridin and caerin families, there are distinct differences observed in the peptide content of the two secretions. These are summarised in Table 6.8.

Peptide	*L. chloris*	*L. xanthomera.*
Caerulein	no	yes
Caeridin 1.1	yes	yes
Caeridin 1.2	yes	no
Caeridin 1.3	yes	no
Caeridin 1.4	yes	yes
Caeridin 1.5	yes	no
Caeridin 1.6	yes	no
Caerin 1.6	yes	yes
Caerin 1.6.1	no	yes
Caerin 1.7	yes	yes
Caerin 1.7.1	no	yes
Caerin 1.8	yes	no
Caerin 1.9	yes	no

Table 6.8. Comparison of the peptides obtained from the granular glands of *L. chloris* and *L. xanthomera.*

The first difference between the two secretions is the absence of caerulein in *L. chloris* (caerulein is a known neuropeptide showing powerful smooth muscle, analgesic and hormone activty[74,112]). This is unusual as the secretion from the other members of the Australian green tree frogs contain significant amounts of

caerulein.[135,189,190,226,227] The second difference is the presence of six caeridin peptides in *L. chloris*, with only caeridins 1.1 and 1.4 observed in *L. xanthomera*. The third difference concerns the caerin 1 peptides. Both species contain the caerins 1.6 and 1.7. *L. xanthomera* contains caerins 1.6.1 and 1.7.1 (the inactive analogues of caerins 1.6 and 1.7), while *L. chloris*, in addition contains caerins 1.8 and 1.9, but no inactive analogues of the caerins 1.6-1.9. It is interesting that *L. chloris* is the only green tree frog (so far studied) that does not have the ability to deactivate its antibiotic skin peptides by removing the first two amino acids residues.

6.3. Summary.

In conclusion, the peptide profiles from *L. xanthomera* and *L. chloris* show the close relationship of these two species. Four peptides are common to both secretions (see Table 6.8), the other peptides only differ by minor structural or amino acid alterations. It would be interesting to compare the peptide profiles between the hybrid offspring of *L. chloris* and *L. xanthomera* (both normal and abnormal) to those peptides profiles outlined in Chapters 5 and 6.

6.4. Amphibian Peptide Summary.

The first few chapters of this thesis have dealt with the isolation and characterisation of peptides from Australian tree frogs of the *Litoria* genus. These characterised peptides have not only shown antibiotic and pharmacological activity, but has also allowed us to track evolutionary trends of these frogs. The previous work done on the Australian green tree frogs *L. splendida*, *L. caerulea* and *L. gilleni* demonstrated that the peptides produced from the granular glands

mirrored the relationship between the species as shown by phylogenic studies.[135,189-191,226,227,256] Surprising trends can be observed. For example the peptides obtained from the granular glands of *L. rubella* from various locations around Australia have suggested that there are at least four chemically distinct populations, indicating the separate evolution of *L. rubella* in different locations. This evolution is illustrated by dramatic changes in colouration of *L. rubella* as the location becomes more northerly along the Queensland coastline. The cause of such change is unknown, but possible reasons include a change in climate, geographical variation (i.e. diet), predation (from both micro- and macroscopic predators) or some combination of these. A knowledge of the role of the tryptophyllin peptides may provide an insight into the reason for the evolutionary change.

The peptides obtained from *L. ewingi* are very interesting from the evolutionary aspect, as it contains peptides associated with two different frog genera, *Litoria* and *Uperoleia*. The peptides associated with the *Litoria* genus are probably the result of a common ancestor: whereas those associated to the *Uperoleia* genus may be due to an independent evolutionary occurrence.

Finally, the comparison of the peptides from the granular gland secretion of *L. chloris* and *L. xanthomera* identify the close relationship between the two species. The peptides also show the lesser relationship of *L. chloris* and *L. xanthomera* to the other Australian green tree frogs.

The characterisation of amphibian peptides is a benign, reliable and simple method to aid in the classification, or analysis of evolutionary trends in frogs.

CHAPTER SEVEN. NEGATIVE ION COLLISIONAL ACTIVATED MASS ANALYSED ION KINETIC ENERGY SPECTRA OF PEPTIDES.

7.1. Introduction.

The advent of FAB and other soft ionisation techniques has allowed the mass spectrometric investigation of large biologically important systems. The method of analysis of these samples has predominantly utilised positive ion techniques coupled with CA MIKES, to provide sequencing information. Historically, the development of the negative ion methods has been slower. The initial use of negative ions was for molecular weight determination via the $(M-H)^-$ ion.[271-275] Production of the $(M-H)^-$ ions (even electron species) were formed by fast atom bombardment using a Vacuum Generator ZAB 2HF mass spectrometer (see Chapter 1.4). Such anions may undergo a variety of fragmentations;[276]

(1) fragmentations involving loss of a radical.

(2) fragmentations initiated by formation of an ion complex.

(3) fragmentations following proton transfer to the initial site of deprotonation.

(4) fragmentations involving rearrangement reactions.

(5) charge remote fragmentations.

7.1.1. Fragmentations Involving a Loss of a Radical.

One of the simplest fragmentations is the homolytic cleavage reaction that occurs when there is a loss of a radical to produce a stable radical anion. Typical

examples include the loss of H$^{\bullet}$ and alkyl$^{\bullet}$ from an (M-H)$^{-}$ ion [an example is shown in equation (7.1)].[277]

$$Ph^{-}CHOMe \longrightarrow PhCHO^{-\bullet} + Me^{\bullet} \qquad (7.1)$$

7.1.2. Fragmentations Initiated by Formation of an Anion Complex.

Many fragmentations are initiated from the site of deprotonation via an anion complex. This may either involve an ion/neutral or a radical/radical anion complex, which can then fragment in a number of ways, e.g.

(1) dissociation of the complex [equation (7.2)].[278]

$$^{-}CH_2COCOMe \longrightarrow [MeCO^{-}(CH_2CO)] \longrightarrow Me^{-}CO + CH_2CO$$
$$[MeCO^{-}(CH_2CO)] \longrightarrow HC_2O^{-} + MeCHO \qquad (7.2)$$

(2) the 'bound' anion effects an S_N2 reaction with the 'neutral' component of the complex [equation (7.3)].[276]

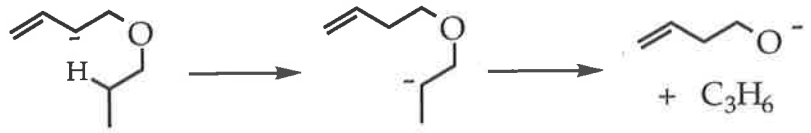
$$MeCON^{-}CH_2CD_3 \longrightarrow [Me^{-}(CD_3CH_2NCO)] \longrightarrow NCO^{-} + C_3H_5D_3 \qquad (7.3)$$

7.1.3. Fragmentations Following Proton Transfer to the Initial Site of Deprotonation.

The fragmentations which follow proton transfer to the initial site of deprotonation may occur in a variety of ways including,

(1) proton transfer to yield an anion which forms an ion complex that can fragment as described in the previous section 7.1.2.

(2) fragmentations where the proton transfer is followed by direct elimination of a neutral [equation (7.4)].[279]

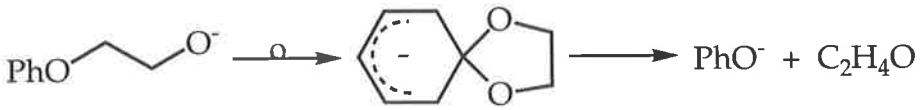


(7.4)

7.1.4. Fragmentations Involving Rearrangement Reactions.

Rearrangement reactions commonly occur in the fragmentation pathways of $(M-H)^-$ ions when other reactions are energetically unfavourable in comparison. These may be classified as follows,

(1) cyclisation reactions where nucleophilic attack by the initial anion occurs intramolecularly. Such an example is shown in equation (7.5).[276,280,281]



(7.5)

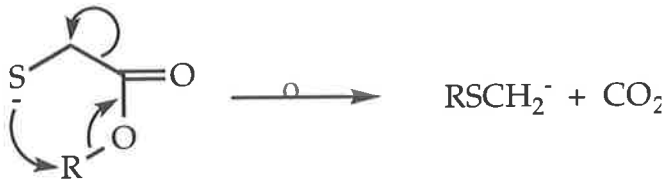
(2) 1,2-anionic rearrangements, e.g. Wittig rearrangements,[277] as shown in equation (7.6).

(7.6)

(3) six-centre rearrangements, i.e. oxy-Cope rearrangements[282,283] [equation (7.7)].

(7.7)

(4) miscellaneous rearrangements [*cf.* equation (7.8)].[276]

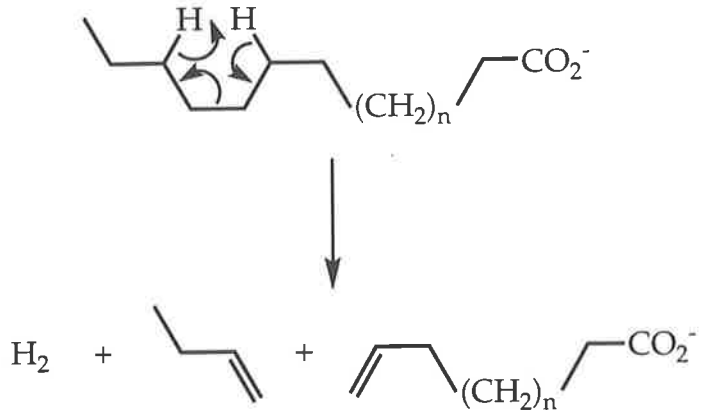


(7.8)

7.1.5. Charge Remote Fragmentations.[284,285]

These fragmentations are classified as 'charge remote' for two reasons; (1) bond cleavage takes place at a position in the molecule that is removed from the charged centre, and (2) the charged centre is not involved in the fragmentation. Support for the charge remote mechanism has been provided by identifying the neutral using neutral fragment reionisation mass spectrometry[286] or when the identical reaction occurs when the charge of the molecule is reversed.[284,285] The 'classical' negative ion charge remote reaction is shown in equation (7.9).[287]

The activation barriers for charge remote fragmentations are generally high. Charge remote fragmentations have been proposed for $(M-H)^-$ ions of fatty acids, steroids, prostaglandins, complex lipids, peptides, carbohydrates and certain antibiotics.[285]



(7.9)

Work on negative ion fragmentations of peptides commenced in the early 1980's.[288-292] The Adelaide research group has reported the basic fragmentations of $(M-H)^-$ ions of amino acids,[293] dipeptides,[294-301] tripeptides[302,303] and tetrapeptides.[304] This work is summarised below.

7.2. CA MIKES of Underivatised Amino Acids.

The analysis into the CA MIKES of underivatised and deuterated amino acids, revealed that fragmentations through the amino acid side chain were dominant.[293,305,306] For example Leu and Ile lost *tert*-Bu$^\bullet$ and *sec*-Bu$^\bullet$ respectively (both 57 Da).[293] Other losses are shown in Table 7.1.

7.3. CA MIKES of Di-, Tri- and Tetrapeptides.

The negative ion fragmentation analysis of di- and tripeptides were studied systematically with amino acids containing the following side chains, H or alkyl (Gly, Ala, Val, Leu, Ile),[302] hydroxyl (Ser, Thr),[295] carboxyl (Asp, Glu),[296] amide (Asn),[297] aryl (Phe, Tyr), heterocyclic (His, Trp),[294] amine (Arg, Lys)[297] and

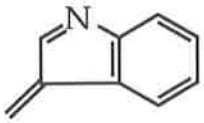
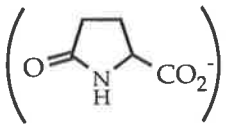
Amino Acid Residue	Loss (or formation)	Mass (Da)	Di-peptides	Tetra-peptides
Ala	$Me^{\bullet}$	15	Yes	*a*
Val	$Pr^{\bullet}$	43	Yes	No
Leu (Ile)	$Bu^{\bullet}$	57	Yes	No
Phe	$PhCH^{-}$	91	Yes	No
Tyr	*p*-$HOC_6H_4CH_2^{-}$	107	Yes	Yes
	$O{=}C_6H_4{=}CH_2$	106	Yes	Yes
Trp		129	Yes	Yes
Ser	CH_2O	30	Yes	Yes
Thr	MeCHO	44	Yes	Yes
Cys	H_2S	34	Yes	Yes
Met	MeSH	48	Yes	Yes
	MeSMe	62	Yes	Yes
	$^{\bullet}CH_2CH_2SMe$	75	Yes	Yes
Asp	H_2O	18	Yes	Yes
Glu	H_2O	18	Yes	Yes
		128	Yes	No
Asn	NH_3	17	Yes	Yes
Arg	$NH{=}C{=}NH$	42	Yes	No

Table 7.1. Characteristic negative ion fragmentations of side chains of amino acid residues from $(M\text{-}H)^{-}$ ions of peptide observed in di- and tetrapeptides (*a* only observed in tetrapeptide Ala Ala Ala Ala). Redrawn from Bradford.[304]

organosulfur groups (Cys, Met).*[298] The negative ion fragmentations of peptides containing Pro residues have also been studied.[301] These papers have revealed three major categories of fragmentations that can be used for structural determination;

(1) backbone cleavages which can be used for primary sequence determination.

(2) side chain fragmentations which identify the presence of a particular amino acid residue and are *dependent* upon the position in the peptide.

(3) side chain fragmentations which identify the presence of a particular amino acid residue but are *independent* upon the position of the residue in the peptide.

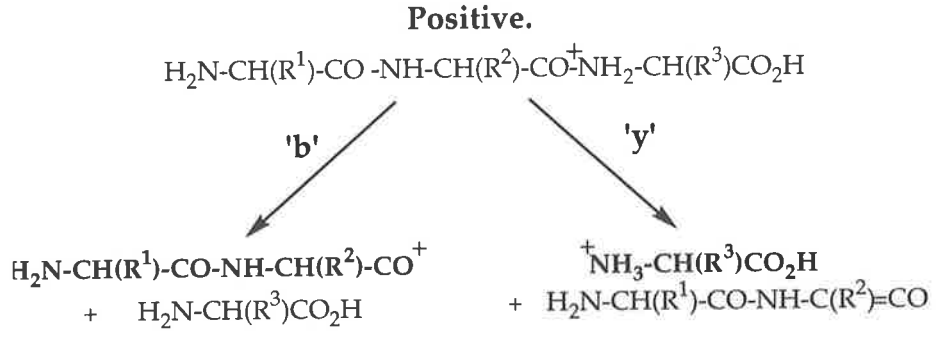
The analysis of the negative ion fragmentations of tetrapeptides[304] showed that sequencing information could be obtained in many cases by the backbone cleavages. However, there were exceptions involving peptides containing the amino acid residues Thr[295] and Asp[296] when they occupy the C-terminal position. The fragmentations through the C-terminal residue often proceed to the exclusion of the backbone cleavages.

7.3.1. Backbone Cleavages Which can be Used for Primary Sequence Determination.

In this thesis, the 'b' and 'y' fragmentation ions were used for sequence determination in positive ion spectra as seen in scheme (7.1), equation (7.10) (the fragmentations are explained in more detail in Chapter 2.9.3). Negative ion spectra show two types of backbone cleavages, named α and β cleavages [see

* For a general review see references (303,304).

scheme (7.1), equation (7.11)].# The backbone cleavages of peptides are considered to be initiated and proceed through an intermediate enolate anion formed by both proton transfer to the carboxylate anion site,* or by direct formation of the enolate anion.[303]



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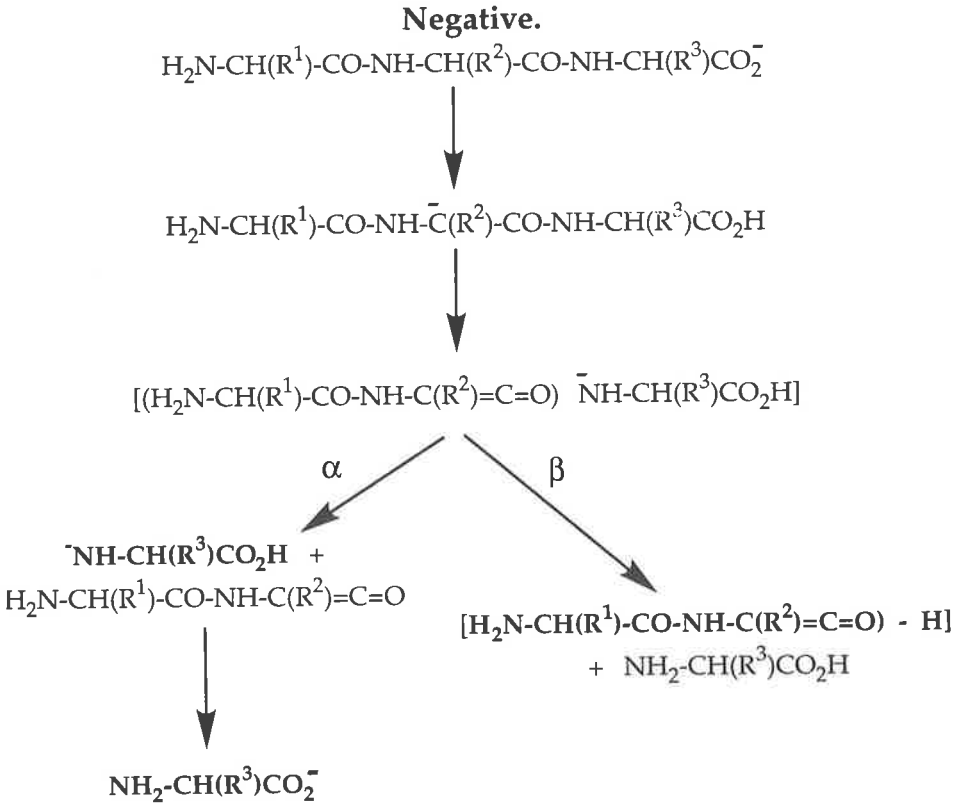


(7.11)

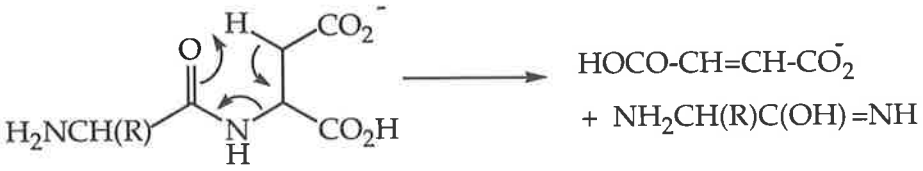
Scheme (7.1)

The negative ion α and β cleavages are named as such as to avoid confusion with respective 'b' and 'y' positive ion fragmentations.

* The feature has been observed in simple alkyl carboxylic acids.[307] The prototypical case is with acetic acid, deprotonation mainly forms $MeCO_2^-$, but $^-CH_2CO_2H$ is also produced.[308] $\Delta G°_{acid}$ ($C\underline{H}_3CO_2H$) = 363 kcal/mol,[308,309] $\Delta G°_{acid}$ ($MeCO_2\underline{H}$) =341.3 kcal/mol,[310] with both ions being interconvertible upon collisional activation.

However with the number of acidic sites in peptides, e.g. $CO_2\underline{H}$, N<u>H</u>CO and C<u>H</u>NHCO, it is difficult to determine the deprotonation sites of a peptide in a quantitative fashion, even when deuterium labelling is used.[296] Deprotonation at the carboxylate site is favoured, but some deprotonation also occurs at other sites. In addition, it has been shown that extensive proton transfer reactions commonly precede or accompany the collisional activated fragmentations of the peptide $(M-H)^-$ ions.[293]

It has been proposed that some negative ion fragmentations can occur by charge remote fragmentations rather than anionic fragmentations. An example is shown in equation (7.12), here a six centred remote mechanism is invoked.[284,285] While such a fragmentation is possible, we prefer to use mechanisms which involve charged sites because of the ready equilibration of such sites within the peptide $(M-H)^-$ species following collisional activation [*cf.* equation (7.13)].[296,302]



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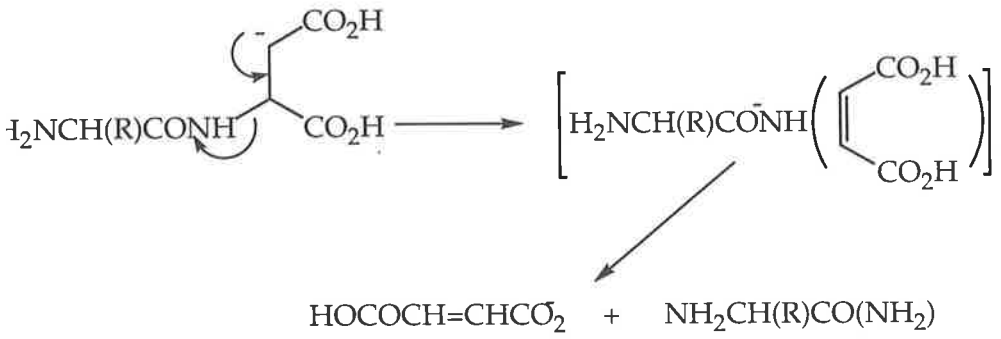


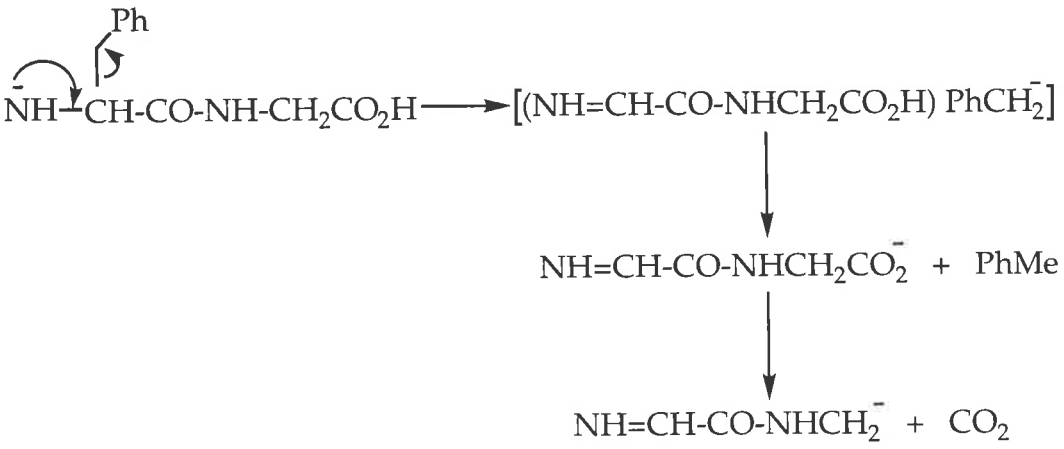
(7.13)

7.3.2. Side Chain Fragmentations Which Identify the Presence of a Particular Amino Acid Residue, *Dependent* Upon the Position in the Peptide.

The main advantage of negative ions in this context is that structural information can be derived from the side chain fragmentations. Numerous examples occur which identify the presence of a particular amino acid residue *dependent* upon the position in the peptide. These examples are most prevalent in dipeptide spectra.

The C-terminal position is where the majority of the 'position dependent' fragmentations occur, especially involving the aromatic or heterocyclic amino acid residues. C-terminal Phe, Tyr or His lose $R^1CH{=}CH\text{-}CO_2H$ (where R^1 = C_6H_5, C_6H_5O [Phe and Tyr respectively] and $C_3H_3N_2$ [His]).[294] The Asp and Glu amino acids show pronounced fragmentation through the side chain losing water and carbon dioxide.[296] However, when Asp is C-terminal the characteristic fragmentation results in the production of a maleate anion [see equation (7.13)].[296]

Characteristic fragmentations of amino acids residing in the N-terminal position occur to a lesser extent. For example Phe and Tyr both lose CO_2 and RC_6H_4Me (where R = H and HO respectively).[294,303] An example is shown for the dipeptide Phe-Gly in equation (7.14).

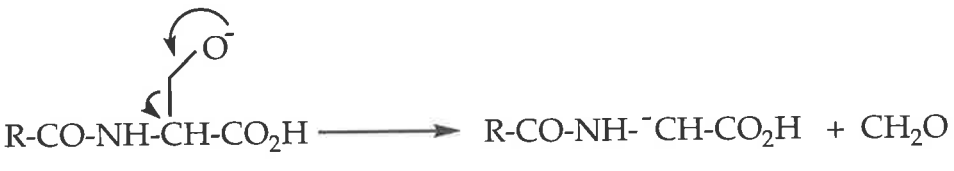


(7.14)

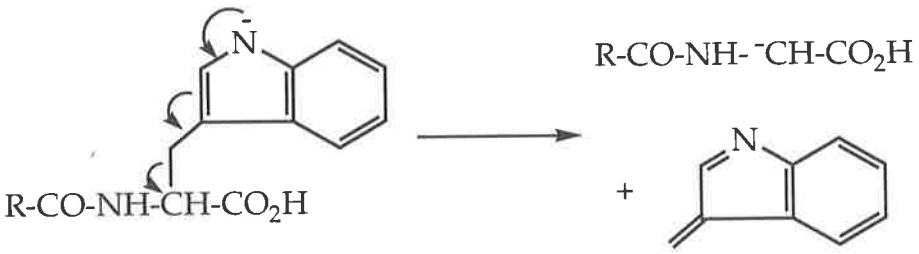
7.3.3. Side Chain Fragmentations that Identify the Presence of a Particular Amino Acid Residue *Independent* Upon the Position in the Peptide.

Side chain fragmentations that are *independent* of the position of the residue in the peptide are typically facile and dominate the negative ion fragmentation spectra. Table 7.1 indicates the side chain fragmentations that identify the presence of various amino acids in tetrapeptides.[304]

Two of the most facile side chain cleavages occur through the amino acid residues Ser and Thr, which lose formaldehyde [see equation (7.15)] and acetaldehyde respectively.[303,304] Trp is another example where the major loss is C_9H_7N (129 Da) [see equation (7.16)].[294] One drawback with facile side chain cleavages is that in some instances, these fragmentations dominate the negative ion spectra at the expense of backbone cleavages. This is particularly true of Ser and Thr side chains, in some cases backbone cleavages are absent for small peptides containing these residues.[303,304]



(7.15)



(7.16)

This chapter will address the negative ion collisionally activated fragmentation spectra of larger peptide systems.* From this analysis, the following questions are to be investigated;

(1) Is it possible to observe the full set of backbone cleavages in order to deduce the sequence of larger peptides?

(2) Will side chain fragmentations through the amino acid residues Asn, Asp, Ser, Thr and Trp dominate the spectra? If so, what amount of sequencing information can be obtained?

(3) It has been suggested that negative ion CA MIKE spectra will provide sequence information, at least complementary to that of positive ion spectra. Will a direct comparison of positive and negative ion CA MIKE spectra support this proposal?

7.4. Negative Ion CA MIKE Spectra of Larger Peptides.

To date the analysis of negative ion peptide fragmentations has dealt with systems up to four amino acid residues in length. The next logical step is to analyse more complex peptide systems. The characterisation of peptides from the granular glands of Australian frogs from the *Litoria* and *Limnodynastes* genera have resulted in numerous peptides. The peptide families that have been characterised include the rubellidin, tryptophyllin[152,192] and caeridin[191,192,311] families from the genus *Litoria* and the dynastin[151,228] family from the genus *Limnodynastes* . Peptides from these families have been used in this negative ion study and their structures are shown in Tables 7.2 and 7.4 (peptides used in the comparison of the positive and negative ion CA MIKE spectra are shown in Table 7.7). The masses of the $(M-H)^-$ ions range from 525 to 1 502 Da. The peptides contain one or two amino acid residues which are known to undergo facile side chain cleavage, i.e. Asp, Asn, Ser,

* The $(M-H)^-$ and fragment ions are all designated using nominal masses.

Thr and Trp. The positive ion CA MIKE spectra identified at least 80% of the sequence in all of the peptides analysed.[151,191,228,268]

7.4.1. Negative Ion Fragmentations of Tryptophyllin and Rubellidin Peptides.

The tryptophyllin peptides used for the negative ion analysis are shown in Table 7.2. $(M-H)^-$ ions range from 525 to 803 Da in mass and contain the amino acid residue Trp which is known to undergo facile side fragmentation.[295,303,304] One exception is the rubellidin peptide (7.6) which contains one Glu and one Thr residue, each of which should show facile side chain cleavage.

	Tryptophyllins	**$(M-H)^-$ (m/z)**
7.1	Ile Pro Trp Leu (NH_2)	525
7.2	Phe Pro Trp Pro (NH_2)	543
7.3	pGlu Phe Pro Trp Leu (NH_2)	670
7.4	Phe Pro Trp Leu (NH_2)	559
7.5	Ile Met Pro Trp Leu (NH_2)	803
	Rubellidin	
7.6	Ile Glu Phe Phe Thr (NH_2)	653

Table 7.2. Structures of the tryptophyllin and the rubellidin peptides used for the analysis of negative ion fragmentations. Peptide (7.5) is a synthetically modified tryptophyllin.

Three of negative ion CA MIKE spectra of the tryptophyllins, together with that of the rubellidin peptide are analysed in detail and shown in Figures 7.1-7.3. The fragmentation data from the remaining peptides are tabulated in Table 7.3. Even

though the structures of the peptides are simple, the fragmentation data can be complex.

Precursor Ion	$(M-H)^-$ (*m/z*)	Fragment Ions
7.1	525	508, (NH_3), 86; 412, (α_1), 32; 396, (C_9H_7N), 100; 379, [$C_9H_7N + NH_3$], 18; 315, (α_2), 27.
7.2	543	526, (NH_3), 22; 429, (β_1), 9; 414, (C_9H_7N), 100; 396, (α_1), 12; 299, (α_2), 9.
7.3	670	653, (NH_3), 46; 627,($(M-H)^-$ - CONH), 36; 559, (α_1), 14; 9; 541, (C_9H_7N), 100; 498, (*m/z* 541 - CONH), 9; 412, (α_2), 94; 369, (*m/z* 412 - CONH), 17; 315, (α_3), 4.

Table 7.3. Negative ion CA MIKES data for the $(M-H)^-$ ions of the tryptophyllins 7.1 [Ile Pro Trp Leu (NH_2)], 7.2 [Phe Pro Trp Pro (NH_2)] and 7.3 [pGlu Phe Pro Trp Leu (NH_2)]. Format of fragment ions [fragment ion, *m/z*, (group lost or specified cleavage) and percentage compared to base peak].

The negative ion CA MIKE spectrum of tryptophyllin 7.4, [$(M-H)^-$ = 559 Da] is shown in Figure 7.1. There are three backbone cleavages. Two α-cleavages from the $(M-H)^-$ ion identify the first two N-terminal amino acids (the third will be discussed later). The base peak of the spectrum, as with all of the tryptophyllins, is due to the loss 129 Da (C_9H_7N) from the Trp side chain, that yields the fragment ion *m/z* 430 [Phe Pro Gly Leu(NH_2), *cf.* equation (7.16)]. The *m/z* 430 ion then undergoes β cleavage (the only β cleavage observed) to lose 130 Da [Leu(NH_2)] and yield *m/z* 300. The initial loss of 129 Da has been confirmed by B/E linked scan* in tryptophyllin 7.4, as well as in the other tryptophyllins containing the C-

* β cleavage involving loss of Leu(NH_2) would produce loss of 130 Da and the resolution of the MIKE scan is not sufficient to resolve peaks at *m/z* 429 and 430. The B/E scan gives better resolution, confirming the major peak at *m/z* 430. If there is any peak at *m/z* 429 it is less than 10% of the abundance of *m/z* 430.

terminal sequence ...-Trp-Leu(NH_2).* In this context, tryptophyllin 7.2 [$(M-H)^-$ = 543 Da, Phe Pro Trp Pro(NH_2)] does lose Pro(NH_2) from the $(M-H)^-$ ion by β cleavage, but the loss is only 9% of that compared with the loss of 129 Da (data is shown in Table 7.2). This confirms that β cleavage does occur in these systems but is minor in comparison to the loss of the Trp side chain. The negative ion CA MIKE spectrum of tryptophyllin 7.4 [$(M-H)^-$ = 559 Da] reveals the full sequence Phe Pro Trp Leu(NH_2). The N-terminal Phe Pro sequence is determined directly by α cleavages. The presence of the Trp residue is determined by the loss of 129 Da from the $(M-H)^-$ ion, which is then followed by the loss of Leu(NH_2) (130 Da).

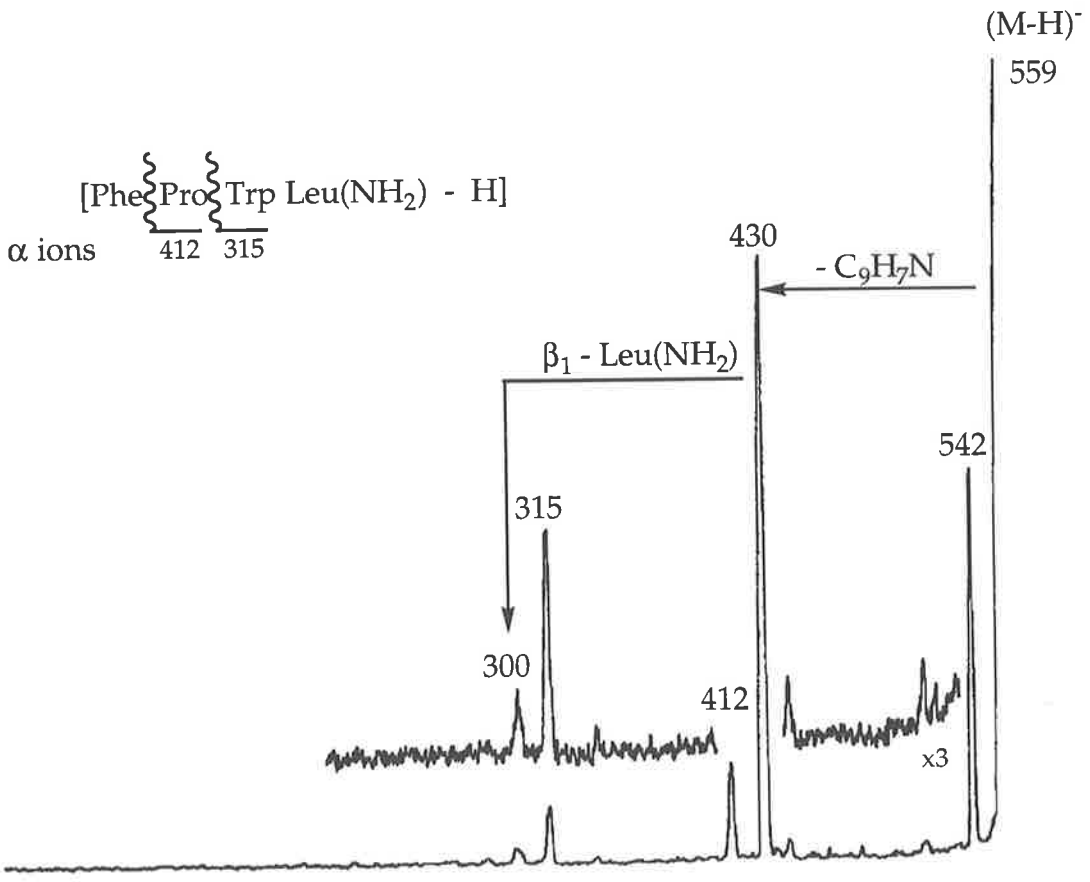


Figure 7.1. The negative ion CA MIKE spectrum of the $(M-H)^-$ ion of tryptophyllin 7.4 [Phe Pro Trp Leu(NH_2)]. Backbone cleavages are summarised in the top left hand corner of the figure.

* The B/E linked scan data of tryptophyllins 7.1, 7.3, 7.4 and 7.5 is shown in Appendix 1.

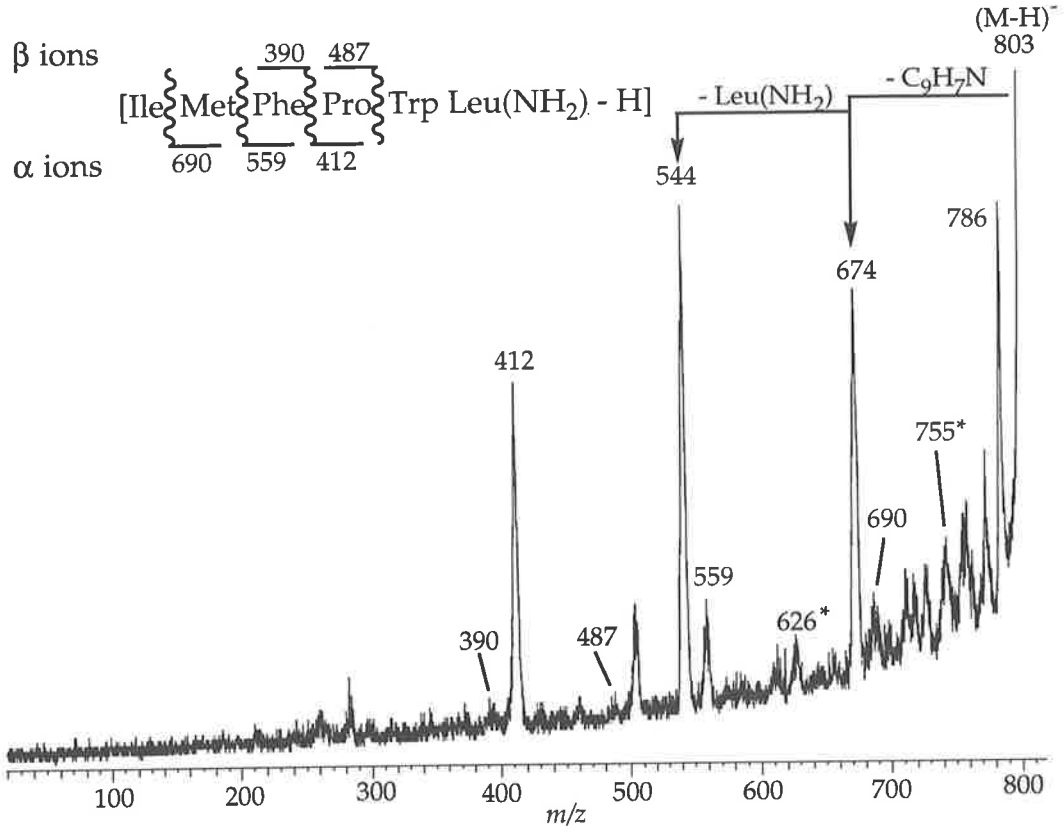


Figure 7.2. The negative ion CA MIKE spectrum of the $(M-H)^-$ ion of tryptophyllin 7.5 [Ile Met Phe Pro Trp Leu(NH_2)]. Backbone cleavages are summarised in the top left hand corner of the figure. (*) indicates that *m/z* 755 and 626 are formed by the loss of MeSH from $(M-H)^-$ and *m/z* 674 respectively.

The negative ion CA MIKE spectrum of tryptophyllin 7.5 [$(M-H)^-$ = 803 Da] is shown in Figure 7.2. Five backbone cleavages are observed, three α and two β fragment ions, which provide most of the required sequencing information. The loss of the Trp side chain (129 Da) to yield the ion *m/z* 674, is followed by the (secondary) β cleavage of Leu(NH_2). Loss of MeSH (48 Da) is characteristic of Met. This loss is observed from the $(M-H)^-$ ion to yield *m/z* 755 and from the fragment ion *m/z* 674 to produce *m/z* 626. This negative ion CA MIKE spectrum thus reveals the full sequence of tryptophyllin 7.5.

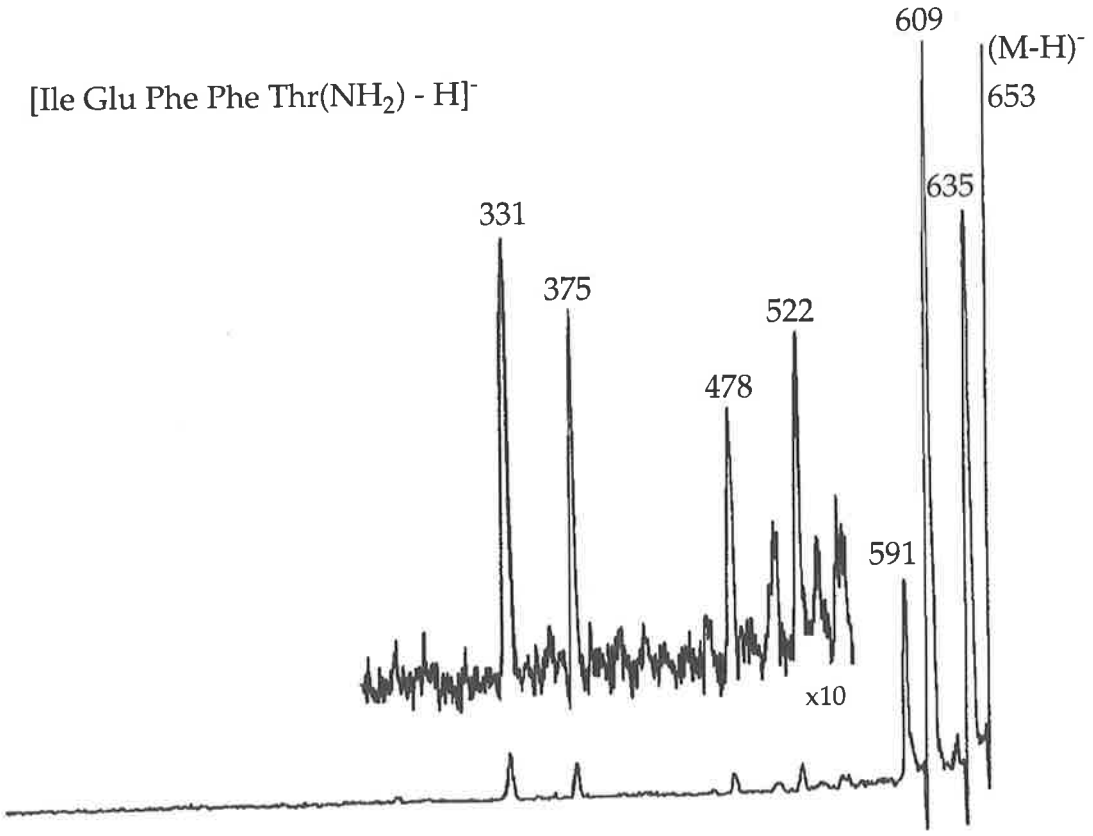
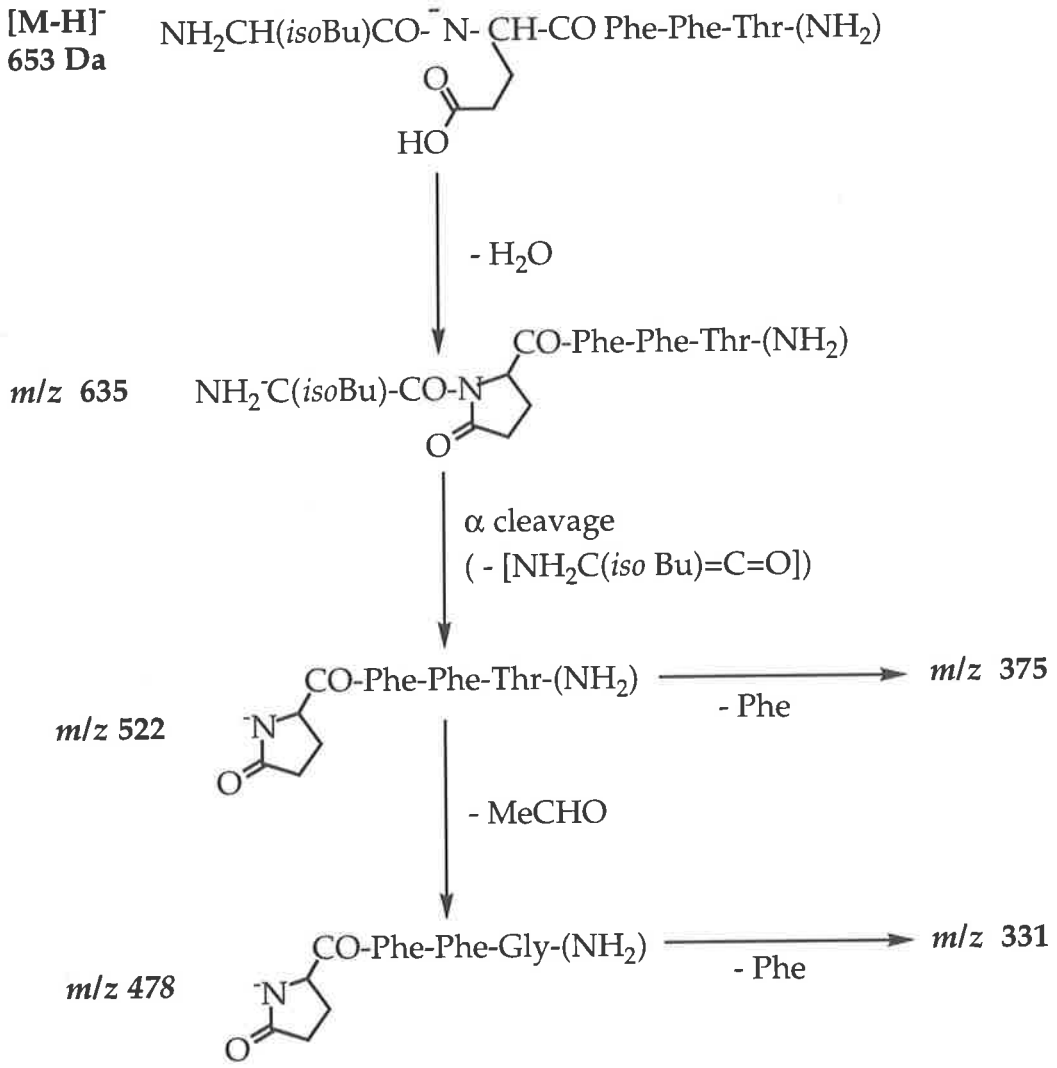


Figure 7.3. The negative ion CA MIKE spectrum of $(M-H)^-$ ion of the rubellidin 7.6 [Ile Glu Phe Phe Thr (NH_2)].

The negative ion CA MIKE spectrum of rubellidin 7.6 [$(M-H)^-$ = 563 Da] is shown in Figure 7.3. The peptide was chosen for study because it contains Glu and Thr residues which are known to undergo facile side chain fragmentation. It was expected that the CA MIKE spectrum would show no useful sequencing information, as fragmentation through the side chains would be dominant. The base peak (*m/z* 609) is formed by the side chain loss of MeCHO [(44 Da) from Thr]. Subsequent loss of water [(18 Da) from Glu] yields the fragment ion *m/z* 591. No other information can be obtained directly from the spectrum as there are no normal backbone cleavage ions. The remaining fragmentations are unusual and are due to internal rearrangements. These are summarised in Scheme (7.2).



Scheme (7.2)

Loss of water occurs through the cyclisation of the Glu side chain with the peptide backbone to produce the ion *m/z* 635. This then undergoes α cleavage to lose $NH_2C(isoBu)=C=O$ (Ile) and form *m/z* 522. The α cleavage ion (*m/z* 522) then undergoes internal rearrangement to lose Phe and give the ion *m/z* 375, or may lose acetaldehyde from Thr (*m/z* 478) and undergo internal rearrangement to lose Phe and yield *m/z* 331. The mechanism for the internal rearrangements involving the loss of Phe is unknown, but it is presumed to require formation of the pyroglutamate structure. Internal rearrangements are observed in positive ion CA MIKE spectra of peptides,[52,218] but this is the first time they have been observed in negative ion CA MIKE spectra.

7.4.2. Negative Ion Fragmentations of Caeridin and Dynastin Peptides.

The next step in this study is to determine the amount of information that can be obtained from negative ion CA MIKE spectra of larger peptide systems. The peptides used were characterised from Australian tree frogs of the *Litoria* and *Limnodynastes* genera. They are listed in Table 7.4.

	Caeridins	**$(M-H)^-$ (m/z)**
7.7	Ala Gly Leu Leu **Asp** Ile Leu Gly Leu (NH_2)	881
7.8	Gly Leu Leu **Asp** Val Val Gly **Asn** Leu Leu Gly Gly Leu Gly Leu (NH_2)	1406
7.9	Gly Leu Phe **Asp** Ala Ile Gly **Asn** Leu Leu Gly Gly Leu Gly Leu (NH_2)	1426
7.10	Gly Leu Leu Gly **Met** Val Gly **Ser** Leu Leu Gly Gly Leu Gly Leu (NH_2)	1353
7.11	Gly Leu Leu **Asp** Val Val Gly **Asn** Val Leu His **Ser** Leu Gly Leu (NH_2)	1502
7.12	Gly Leu Leu **Asp** Gly Leu Leu Gly **Thr** Leu Gly Leu (NH_2)	1138
7.13		1122
7.14	Gly Leu Leu **Glu** Gly Leu Leu Gly **Thr** Leu Gly Leu (NH_2)	1154
	Dynastins	
7.15	Gly Ala Val **Ser** Gly Leu Leu **Thr** **Asn** Leu (OH)	942
7.16	Gly Ala Val **Ser** Gly Leu Leu **Thr** **Asn** Leu Gly Leu (OH)	1112
7.17	Gly Leu Leu **Ser Ser** Leu Gly Leu **Asn** Leu (OH)	984
7.18	Gly Leu Val Pro **Asn** Leu Leu **Asn Asn** Leu Gly Leu (OH)	1234

Tables 7.4. Structures of the caeridin and dynastin peptides for the analysis of negative ion fragmentations. The amino acids underlined contain side chains that undergo facile side chain cleavage.

The masses of $(M-H)^-$ ions of the listed peptides are between 881 and 1502 Da. Each peptide contains one to three amino acid residues which undergo facile side chain cleavage from the $(M-H)^-$ ion. These include Ser, Thr, Asp and Asn, and their side chain fragmentation were expected to dominate the CA MIKE spectra. Fragmentations through the Asp and Asn side chains are of most interest in these spectra.

The negative ion CA MIKE spectrum of the caeridin 7.7 [$(M-H)^-$ = 881 Da] is shown in Figure 7.4. Numerous α and β cleavage ions, albeit minor, are present and are summarised in Figure 7.4.

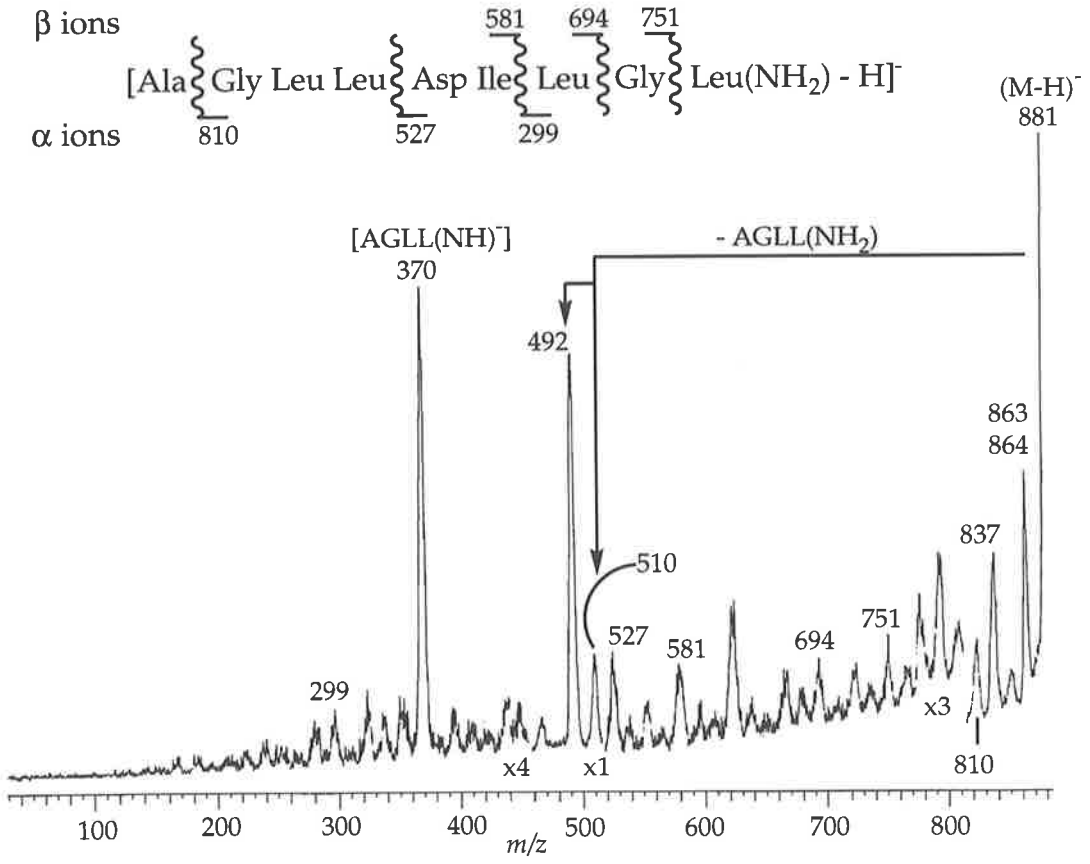
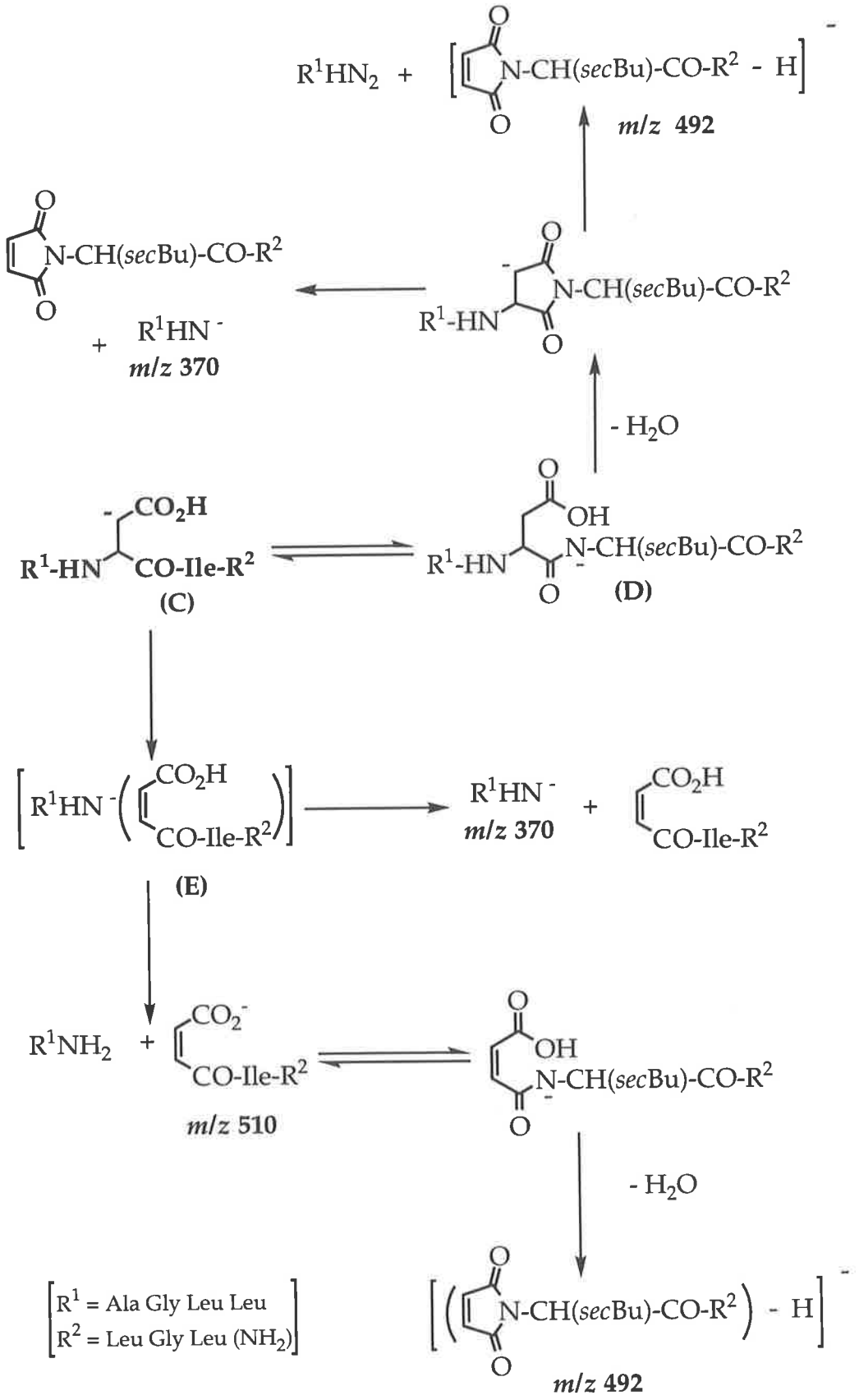


Figure 7.4. The negative ion CA MIKE spectrum of the $(M-H)^-$ ion of caeridin 7.6 [Ala Gly Leu Leu Asp Ile Leu Gly Leu (NH_2)]. Backbone cleavages are summarised in the top left hand corner of the figure.

Collisional activation of the $(M-H)^-$ ion induces the loss of water to yield the ion *m/z* 863 via cyclisation of the Asp side chain as shown in Scheme (7.3). Loss of NH_3 (17 Da) from the C-terminal amide to yield the ion *m/z* 864 also occurs. However the resolution of the CA MIKE spectrum does not enable these two peaks to be resolved. Fragment ions of interest are those at *m/z* 510, 492 and 370. These ions are also formed in the source of the mass spectrometer and B^2/E linked scanning* shows; (1) that *m/z* 370 and 510 come predominantly from the parent $(M-H)^-$ ion, but are also produced from the $[(M-H)^- - H_2O]$ species, and (2) *m/z* 492 is produced mainly from *m/z* 510, but to a lesser extent also from the $[(M-H)^- - H_2O]$. These fragmentations are directed through the Asp side chain and are summarised in Scheme (7.3).

The pathway resembles that shown in equation (7.13). The enolate anion (C) fragments through the anion/neutral complex (E) which can dissociate to produce $R^1(NH)^-$ (*m/z* 370), or $R^1(NH)^-$ can deprotonate the neutral component of the complex to yield the carboxylate species (*m/z* 510). Proton transfer occurs within *m/z* 510 ion (proton transfer within peptides is known to be facile[296,303,304]) which then undergoes cyclisation to lose water and produce a maleimide ion [*m/z* 492 (base peak of the spectrum)]. A minor pathway also produces the *m/z* 370 and 492 [Scheme (7.3)]. The enolate anion (C) can undergo an internal proton transfer[296,303,304] to form the isomer (D) which cyclises producing the $[(M-H)^- - H_2O]$ species which fragments further to yield the ions *m/z* 370 and 492.

* B^2/E linked scanning provides the mass of the parent(s) from source formed daughter ions (see Chapter 1.3.3 [page 10]).


R1HN2 +
N-CH(secBu)-CO-R2 - H
m/z 492
N-CH(secBu)-CO-R2
R1-HN
+ R1HN -
m/z 370
- H2O
- CO2H
R1-HN
CO-Ile-R2
(C)
OH
N-CH(secBu)-CO-R2
R1-HN
(D)
R1HN -
CO2H
CO-Ile-R2
(E)
m/z 370
R1NH2 +
CO2-
CO-Ile-R2
m/z 510
OH
N-CH(secBu)-CO-R2
- H2O
R1 = Ala Gly Leu Leu
R2 = Leu Gly Leu (NH2)
N-CH(secBu)-CO-R2 - H
m/z 492

Scheme (7.3)

CA MIKE spectra of the caeridins 7.8 [$(M-H)^-$ = 1406 Da] and 7.9 [$(M-H)^-$ = 1426 Da] show similar fragmentations as both contain Asp (4) and Asn (8). The spectrum of caeridin 7.9 is shown in Figure 7.5 and data from the spectrum of caeridin 7.8 are shown in Table 7.5. The main difference between the spectra of the peptides 7.9 and 7.8 are that the fragment ions *m/z* 1074 and 1092 (see Figure 7.5) shift to *m/z* 1088 and 1106 (see Table 7.5) respectively, which consistent with replacement of Leu(3) by Phe (3). The base peak is produced by the unresolvable losses of water and ammonia from the $(M-H)^-$ ion.

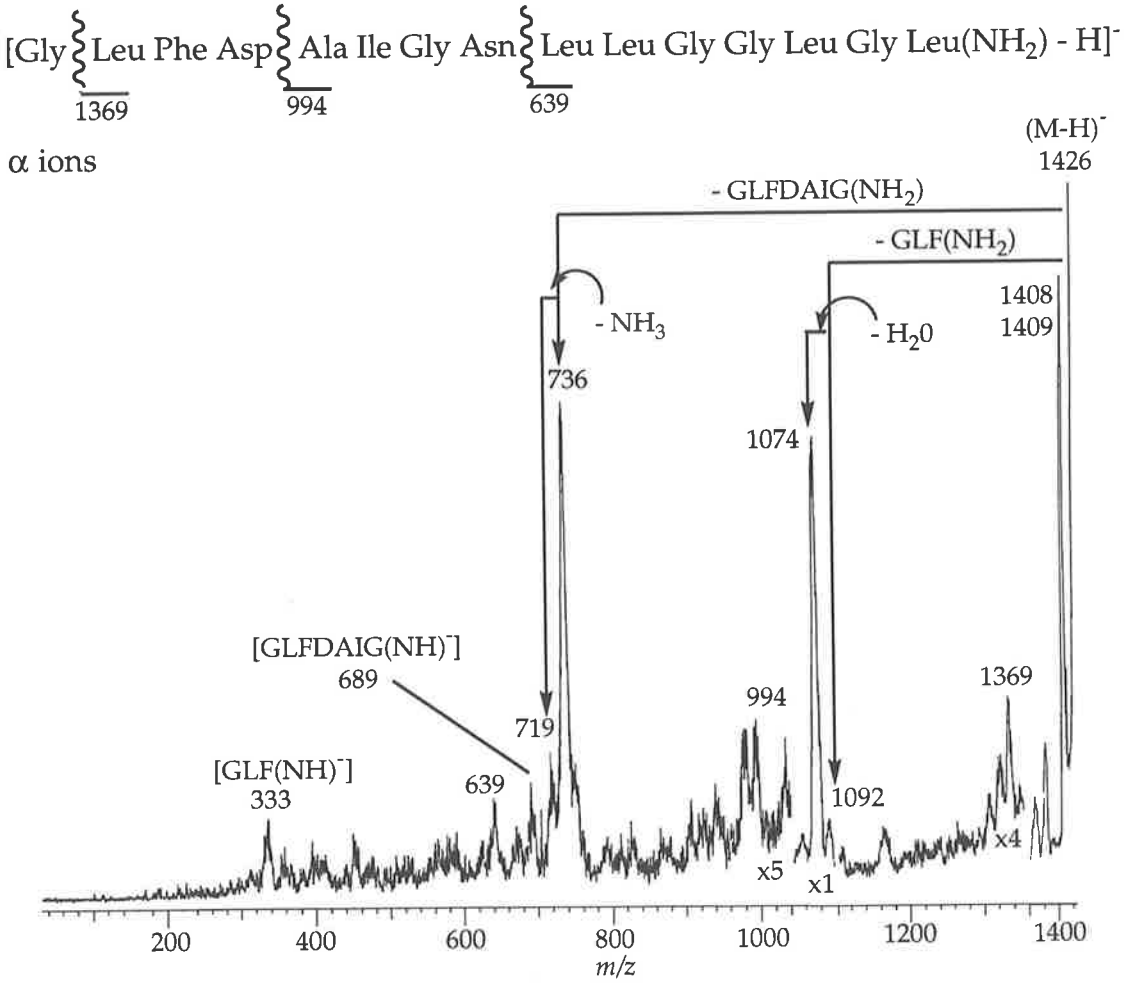


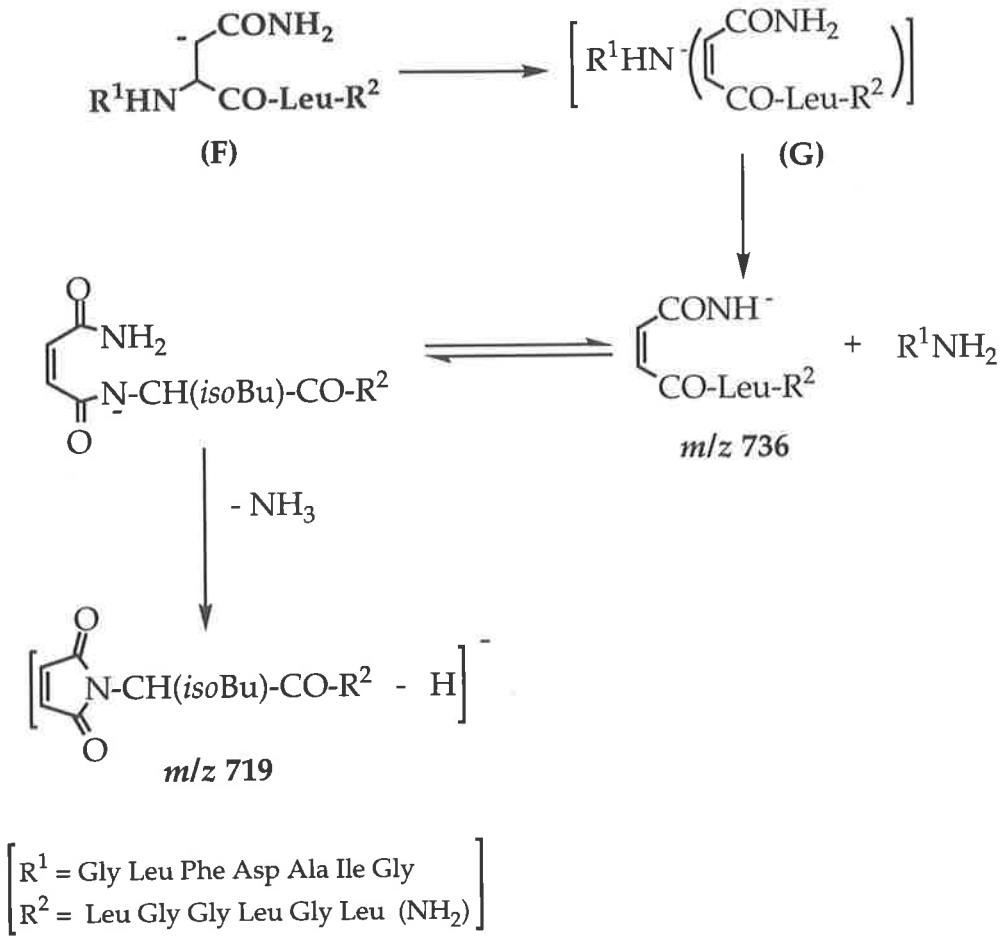
Figure 7.5. The negative ion CA MIKE spectrum of the $(M-H)^-$ ion of caeridin 7.9 [Gly Leu Phe Asp Ala Ile Gly Asn Leu Leu Gly Gly Leu Glu Leu (NH_2)]. Backbone cleavages are summarised at the top left hand corner of the figure.

Precursor Ion	$(M-H)^-$ (*m/z*)	Fragment Ions
7.8	1406	1389/1388, (NH_3/H_2O), 100; 1349, (α_1), 17; 1106, [$(M-H)^-$ - GLL(NH_2)], 10; 1088, (*m/z* 1106 - H_2O), 32; 992, (β_5), 7; 736, [$(M-H)^-$ - GLLDVVG(NH_2)], 28; 719, (*m/z* 736 - NH_3), 8.

Table 7.5. Negative ion CA MIKES data of caeridin 7.8 [Gly Leu Leu Asp Val Val Gly Asn Leu Leu Gly Gly Leu Gly Leu (NH_2)]. Format of fragment ion [fragment ion, *m/z*, (group lost or specified cleavage) and percentage compared to base peak].

There is a decrease in the number of observed backbone cleavages with an increase in the number of amino acid residues which undergo facile cleavage. The spectrum of caeridin 7.9 [$(M-H)^-$ = 1426 Da] shows three α cleavage ions but no β cleavage ions. In addition, ions characteristic of fragmentation through the Asp side chain [similar to those shown in Scheme (7.3)] are the formation of GLF$(NH)^-$ (*m/z* 333) together with loss of GLF(NH_2) from the $(M-H)^-$ ion to yield *m/z* 1092. The latter ion cyclises to yield *m/z* 1074 and water.

Characteristic fragmentations through Asn yield *m/z* 689, 719 and 736 (see Scheme 7.4). The major fragmentation through the Asn side chain involves loss of R^1NH_2 from the $(M-H)^-$ ion to produce *m/z* 736 (which has been confirmed by B^2/E linked scanning). Fragmentation is initiated through the enolate anion (F). This converts to the anion/neutral complex (G) which dissociates to lose R^1NH_2 and form *m/z* 736. The R^1NH^- ion is also produced (*m/z* 689). The cyclisation of *m/z* 736 to *m/z* 719 does occur, but is minor in comparison to the corresponding loss of H_2O from Asp (*m/z* 1092 to 1074). This provides a means to distinguish Asp and Asn residues in a peptide.



Scheme (7.4)

The side chain fragmentations of Ser and Thr dominate normal backbone cleavages.[296,303,304] Caeridin 7.10 [$(M-H)^-$ = 1353 Da] contains Met and Ser but no Asp or Asn residues. The negative ion spectrum of caeridin 7.10 (shown in Figure 7.6) is dominated by the loss of CH_2O (30 Da) from Ser. Minor loss of MeSH (48 Da) from Met is also observed. Ions resulting from α and β backbone cleavage are greatly diminished.

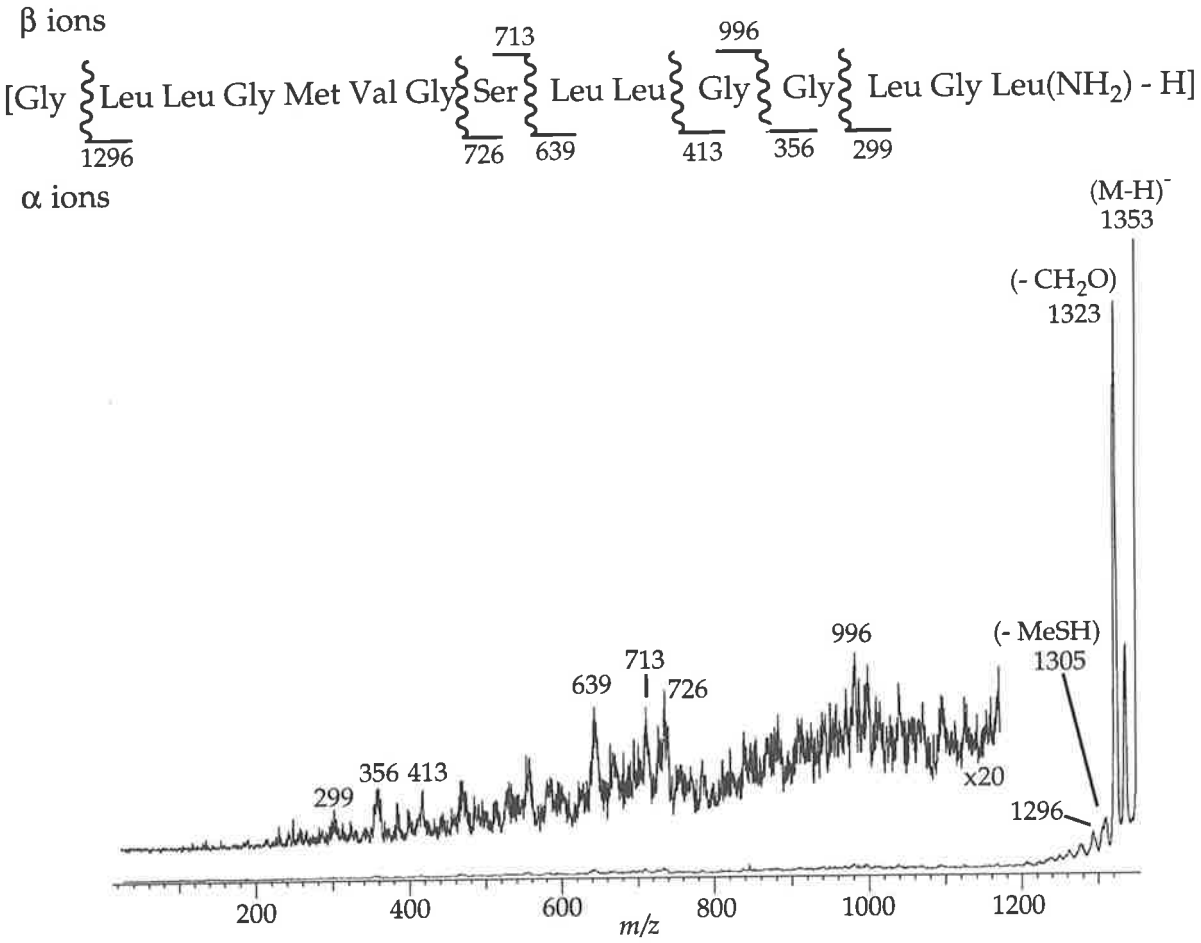


Figure 7.6. The negative ion CA MIKE spectrum of the $(M-H)^-$ ion of caeridin 7.10 [Gly Leu Leu Gly Met Val Gly Ser Leu Leu Gly Gly Leu Gly Leu (NH_2)]. Backbone cleavages are summarised at the top of the figure.

Are the characteristic side chain fragmentations of Asp and Asn still present when a Ser or Thr residues are also present in the peptide? Examples of such systems are shown in Figures 7.7 and 7.8 for caeridins 7.11 [$(M-H)^-$ = 1502 Da] and 7.12 [$(M-H)^-$ = 1138 Da] respectively.

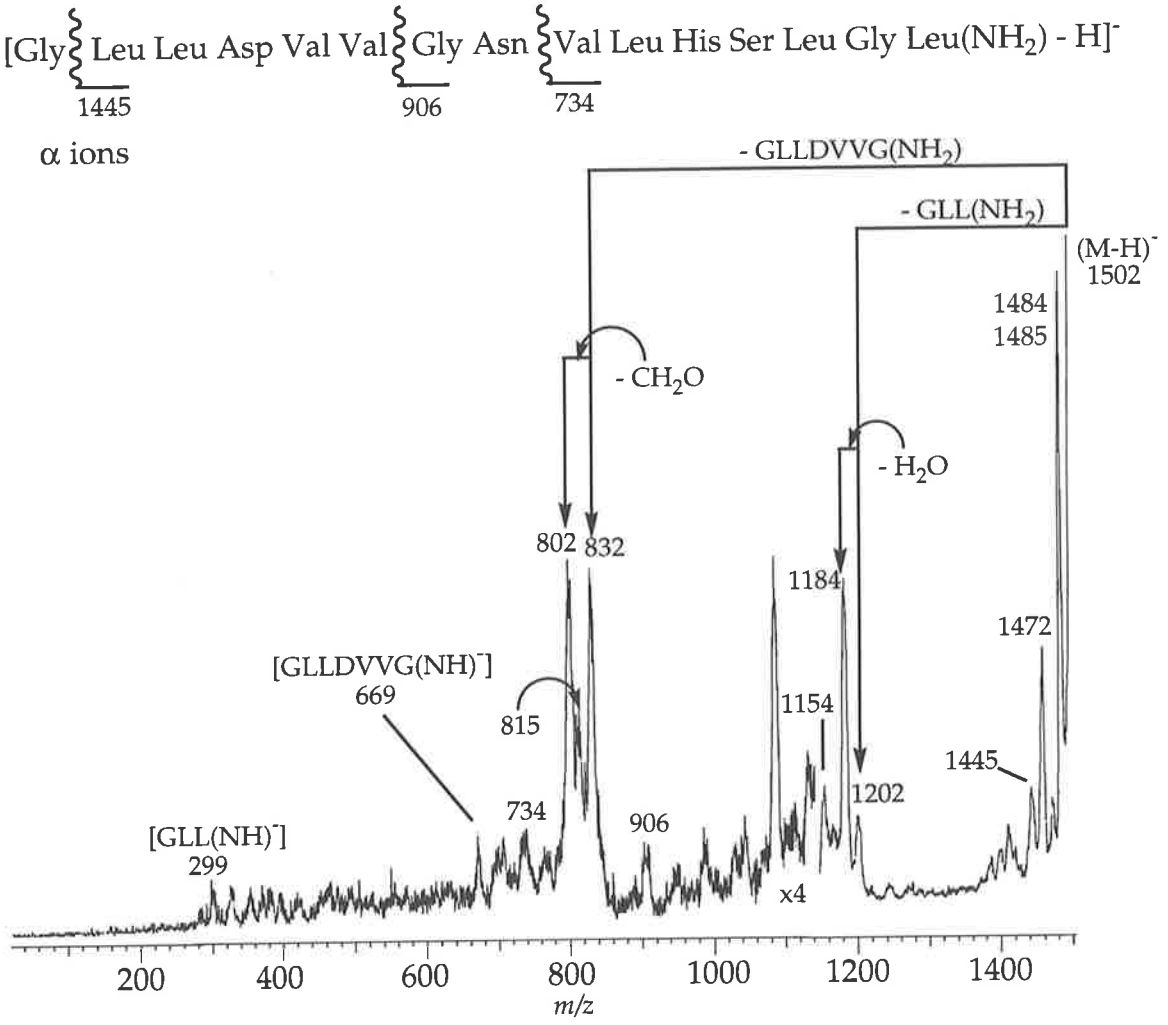


Figure 7.7. The negative ion CA MIKE spectrum of the $(M-H)^-$ ion of caeridin 7.11 [Gly Leu Leu Asp Val Val Gly Asn Val Leu His Ser Leu Gly Leu (NH_2)]. Backbone cleavages are summarised at the top of the figure.

Peptide 7.11 [$(M-H)^-$ = 1502 Da] contains Asp (4), Asn (8) and Ser (12). The major losses in the spectrum are due to water and ammonia giving *m/z* 1484 and 1485 respectively and CH_2O (*m/z* 1472) from the $(M-H)^-$ ion. Some α backbone fragment ions are observed giving only minimal sequencing information. The characteristic fragmentations that occur through Asp (*m/z* 1184 and 1202) and Asn (*m/z* 815 and 832) are observed in the spectrum and their origin confirmed by B^2/E linked scanning. The difference between the cyclisations of Asp and Asn is observed, i.e. the pronounced loss of H_2O from Asp (*m/z* 1202 to 1184), compared

to the smaller loss of NH_3 from Asn (*m/z* 832 to 815). The loss of CH_2O is also observed from the *m/z* 1184 and 832 to produce *m/z* 1154 and 802 respectively.

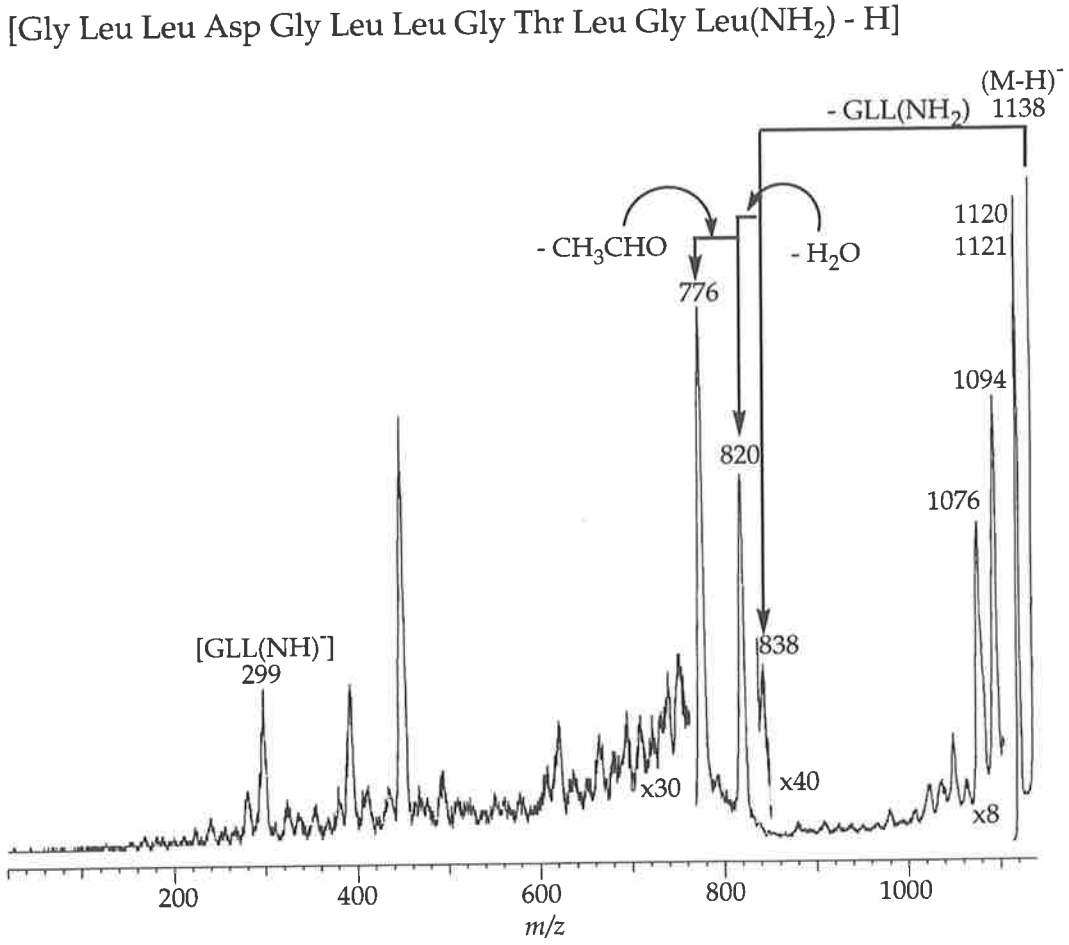


Figure 7.8. The negative ion CA MIKE spectrum of the $(M-H)^-$ ion of caeridin 7.12 [Gly Leu Leu Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2)].

Similar fragmentations are observed in the CA MIKE spectrum of caeridin 7.12 [$(M-H)^-$ = 1138 Da] (see Figure 7.8), which contains one Asp (4) and one Thr (9). The base peak is due to the unresolvable loss of water and ammonia from the $(M-H)^-$ ion. The presence of Thr is confirmed by the loss of 44 Da from the $(M-H)^-$ ion. No α or β backbone fragmentations are observed in this spectrum. Fragmentations that occur through Asp yield the ions of *m/z* 838 and 820. The ion

m/z 820 undergoes further fragmentation of MeCHO from the Thr side chain to produce the ion *m/z* 776. These pathways have been confirmed by B^2/E linked scans of source formed ions.

Caeridin 7.13 [$(M-H)^-$ = 1120 Da] occurs naturally in the granular secretion of *Litoria gilleni* and *L. chloris* and is a degradation product from caeridin 1.1 (see Chapter 6.2.2 and referenced as caeridin 7.12 in this chapter).[151,222] The succinimide 7.13 is produced by the cyclisation of the acid on the Asp side chain and the amide nitrogen of the adjacent C-terminal Gly. The spectrum of peptide 7.13 is simple with no α or β backbone cleavages observed (see Figure 7.9).

The fragmentation pathway is summarised in Scheme (7.5). The $(M-H)^-$ ion (H) is able to fragment in two ways; (1) produce the anion/neutral complex (I) that dissociates to yield *m/z* 820, which can then lose MeCHO to produce *m/z* 776, or (2) lose MeCHO from the $(M-H)^-$ ion to form (J), which can then dissociate to form *m/z* 776. These pathways support the succinimide representation of [$(M-H)^-$ - H_2O] ions derived from Asp containing peptides.

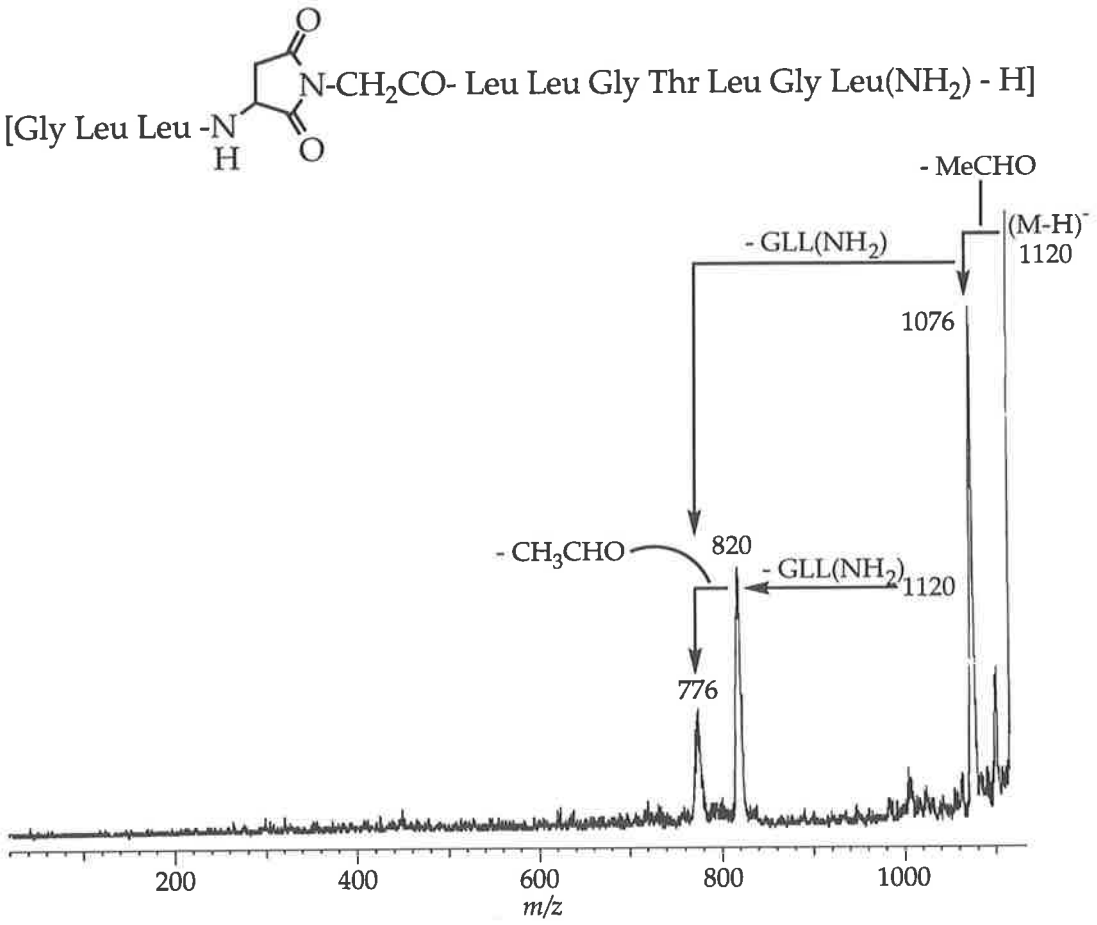
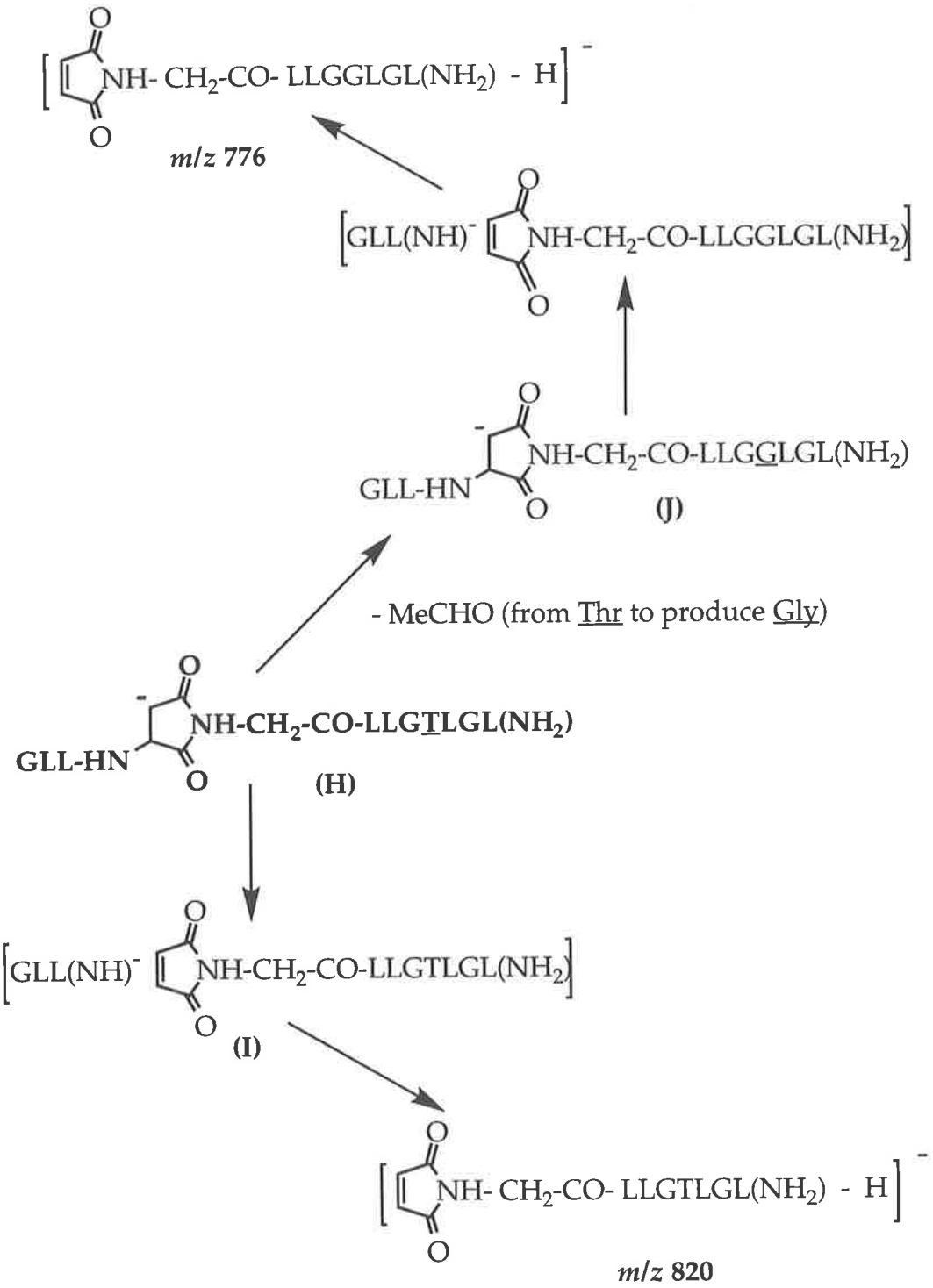


Figure 7.9. The negative ion CA MIKE spectrum of the $(M-H)^-$ ion of caeridin 7.13.



Scheme (7.5)

Three dynastin peptides 7.15-7.17 all contain Asn residues together with one or two Ser and Thr residues. The base peaks in all spectra are formed by losses of CH_2O and MeCHO from Ser and Thr respectively. Losses of water and ammonia

from Asp and Asn occur respectively, but are minor in comparison. Fragmentation data from the dynastins 7.15-7.17 are recorded in Table 7.6.

Precursor Ion	$(M-H)^-$ (*m/z*)	Fragment Ions
7.15	942	925/924, (NH_3/H_2O), 26; 912, (CH_2O), 74; 898, (MeCHO), 100; 885, (α_1), 7; 811, (β_1), 6.
7.16	1112	1095/1094, (NH_3/H_2O), 25; 1082, (CH_2O), 48; 1068, (MeCHO), 100; 1055, (α_1), 6; 1038, (*m/z* 1068 - CH_2O or *m/z* 1082 - MeCHO), 16; 798, (α_4), 1; 741, (α_5), 2; 697, (β_4), 1;
7.17	984	967/966, (NH_3/H_2O), 27; 954, (CH_2O), 100; 924, (*m/z* 954 - CH_2O), 11; 853, (β_1), 4; 756, [$(M-H)^-$ - ($C_4H_4O_2N$)Leu(OH)]; 2; 614, (α_4), 1; 527, (α_5), 0.6; 414, (α_6), 0.5; 357, (α_7), 0.4.

Table 7.6. Negative ion CA MIKES data of dynastins 7.15 [Gly Ala Val Ser Gly Leu Leu Thr Asn Leu (OH)], 7.16 [Gly Ala Val Ser Gly Leu Leu Thr Asn Leu Gly Leu (OH)] and 7.17 [Gly Leu Leu Ser Ser Leu Gly Leu Asn Leu (OH)]. Format of fragment ion, [fragment ion, *m/z*, (group lost or specified cleavage) and percentage compared to base peak].

Dynastin 7.18 [$(M-H)^-$ = 1234 Da] contains three Asn residues with no Ser or Thr residues. The spectrum is shown in Figure 7.10. There is only one α backbone cleavage ion observed. The base peak (unresolved) is due to the losses of ammonia and water from the $(M-H)^-$ ion. The spectrum shows internal cleavages through all three Asn residues. The fragmentation through Asn (5) gives the most pronounced fragmentation (to *m/z* 851), while those from Asn (8) and (9) (to *m/z* 551 and 397 respectively) are minor in comparison.

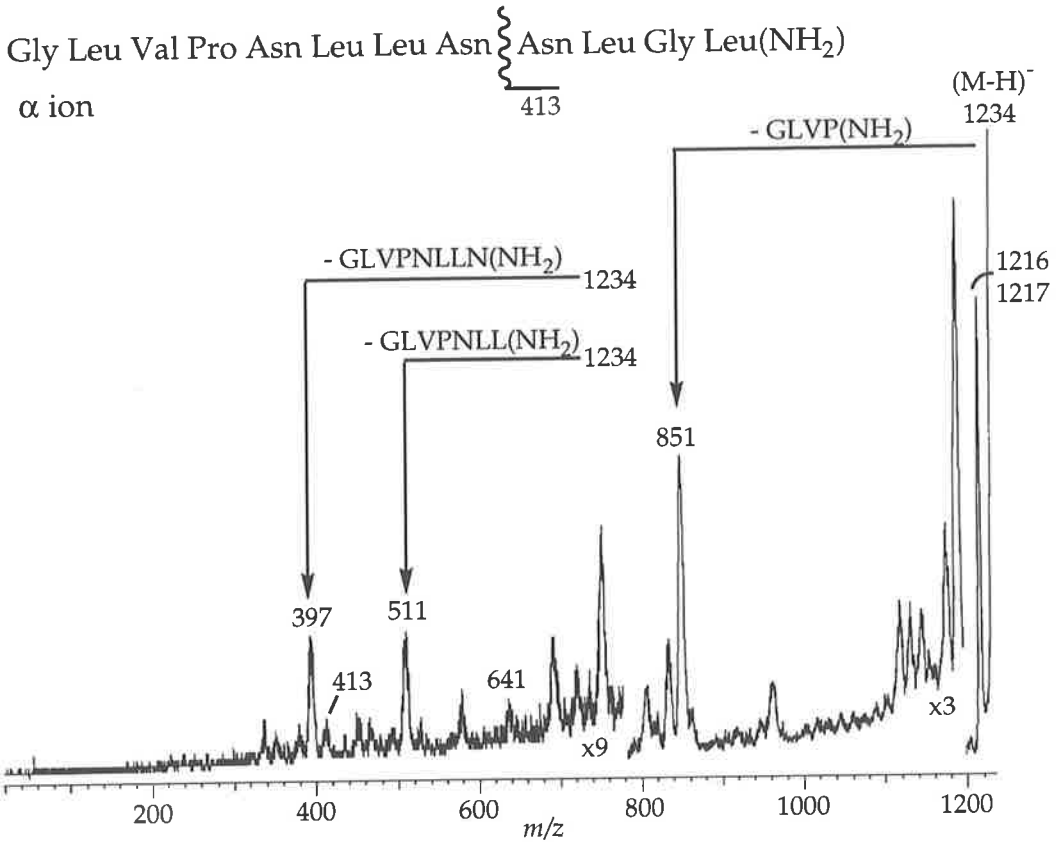


Figure 7.10. The negative ion spectrum of the $(M-H)^-$ ion of dynastin 7.18 [Gly Leu Val Pro Asn Leu Leu Asn Asn Leu Gly Leu(NH_2)]. The backbone cleavage is observed at the top of the figure.

The characteristic fragmentations that occur through Asp and Asn are initiated from enolate anions on the side chains yielding anion/neutral complexes containing RCO-CH=CH-CONHR' functionality [where R = NH_2 or OH for Asn and Asp respectively (e.g. see intermediates (E) and (G) from Schemes (7.3) and (7.4) respectively)]. The residues Glu and Gln should not be able to undergo this type of fragmentation, as the presence of the extra CH_2 group in the side chain will block fragmentation of the enolate anion on the side chain. No naturally occurring peptides of this type have been obtained with Glu or Gln that would enable the investigation of this hypothesis. Caeridin 7.14 [$(M-H)^-$ = 1152 Da] is a synthetic modification of caeridin 7.12, where the Asp (4) is replaced by Glu (4).

The negative ion spectrum of the $(M-H)^-$ ion of peptide 7.14 ion is shown in Figure 7.11.

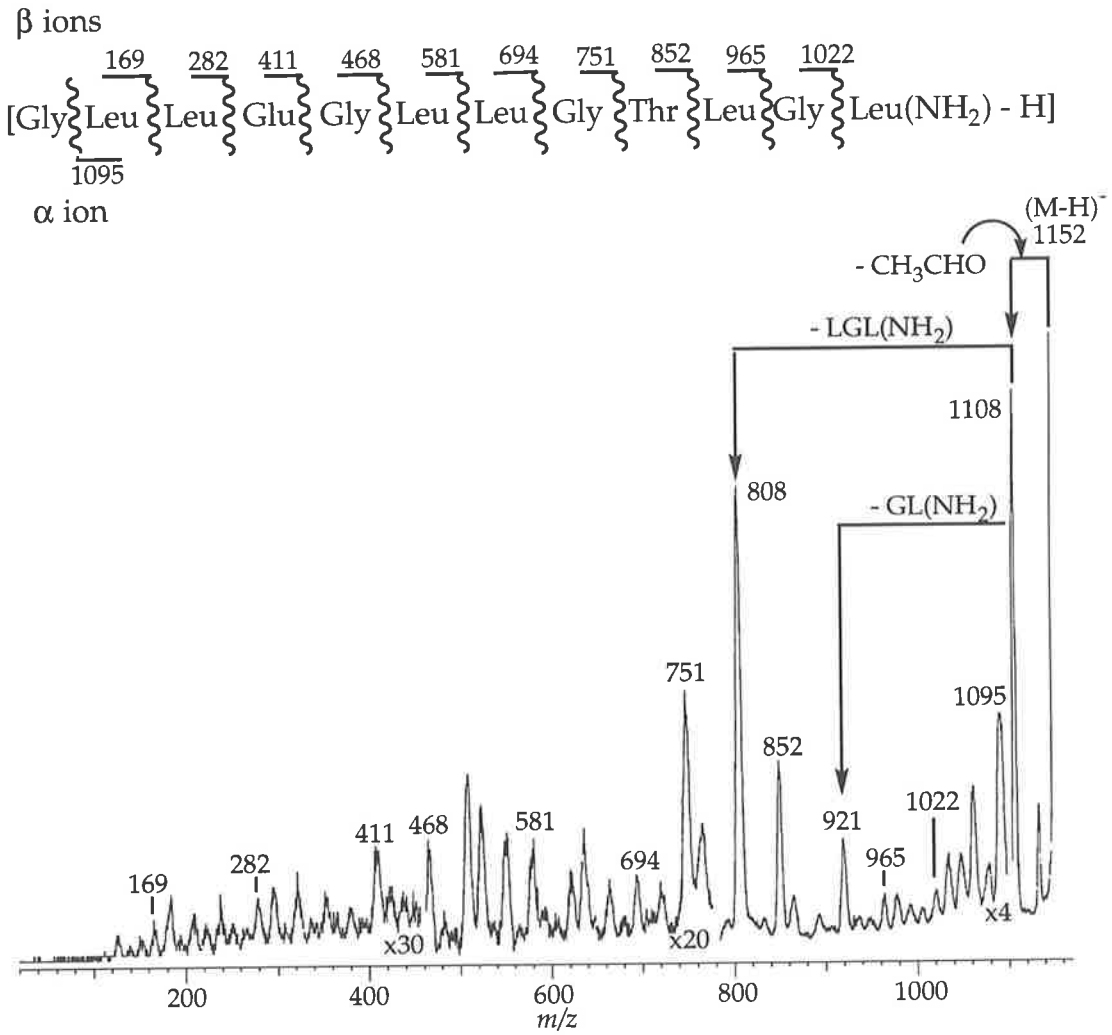
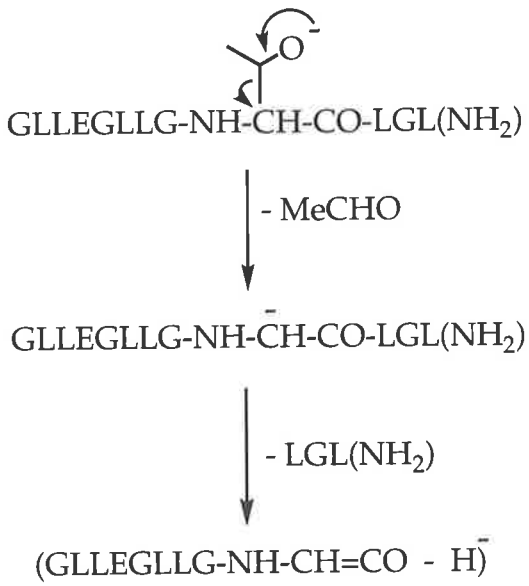


Figure 7.11. The negative ion spectrum of the $(M-H)^-$ ion of caeridin 7.14 [Gly Leu Leu Glu Gly Leu Leu Gly Thr Leu Gly Leu(NH_2)]. The backbone cleavages are summarised at the top of the figure.

No fragmentations were observed corresponding to the characteristic cleavages of Asp or Asn. There is now an abundance of backbone cleavages that give the complete sequence of the peptide. The base peak of the spectrum (*m/z* 1108) arises from the loss of MeCHO from the $(M-H)^-$ ion. This ion then undergoes α cleavage:

loss of Gly Leu(NH_2) (to *m/z* 921) and Leu Gly Leu(NH_2) (to *m/z* 808) [see Scheme 7.6].



Scheme (7.6)

In summary;

(1) The fragmentation patterns of negative ion CA MIKE spectra are dependent upon the nature of the amino acid residues within the peptide. Amino acids that undergo facile side chain fragmentations dominate the spectra at the expense of the backbone cleavages which give sequence information.

(2) Trp undergoes characteristic fragmentation losing 129 Da (C_9H_7N). The C-terminal sequence of ...-Trp Leu(NH_2) can be determined by the loss of 129 Da, followed by the β cleavage of Leu(NH_2) (130 Da).

(3) Peptides containing the residues Asp and Asn show characteristic side chain cleavage which result in rupture of the backbone. These fragmentations may be distinguished as the cyclisation of the Asp side chain to lose water is more pronounced than the corresponding cyclisation through Asn side chain to eliminate ammonia.

(4) Ser and Thr undergo the characteristic losses of CH_2O and MeCHO respectively. In the majority of cases these fragmentations dominate the spectra.

(5) The characteristic fragmentations of Asp and Asn still occur in the presence of Ser or Thr in the peptide.

7.5. Comparison of the Negative and Positive Ion CA MIKE Spectra.

The information obtained from the CA MIKE spectra of the $(M-H)^-$ ion provides both sequence information through backbone cleavages together with residue identification via characteristic side chain cleavage. The information obtained from the negative ion spectrum is at least complementary to that provided by the corresponding positive ion spectrum.[304] The minimum information obtained will identify of the presence of a particular amino acid from its side chain fragmentations (see Chapters 7.3 and 7.4). This section deals with the direct comparison of negative and positive ion CA MIKE spectra of selected members of the rubellidin, tryptophyllin and caeridin peptide families (see Table 7.7).

	Peptide	Da
	Tryptophyllin	
7.4	Phe Pro Trp Leu(NH_2)	560
	Rubellidin	
7.6	Ile Glu Phe Phe Thr(NH_2)	654
	Caeridin	
7.7	Ala Gly Leu Asp Ile Leu Gly Leu (NH_2)	882
7.10	Gly Leu Leu Gly Met Val Gly Ser Leu Leu Gly Gly Leu Gly Leu (NH_2)	1355
7.14	Gly Leu Leu Glu Gly Leu Leu Gly Thr Leu Gly Leu (NH_2)	1153

Table 7.7. Peptides used for the direct comparison of positive and negative ion CA MIKES.

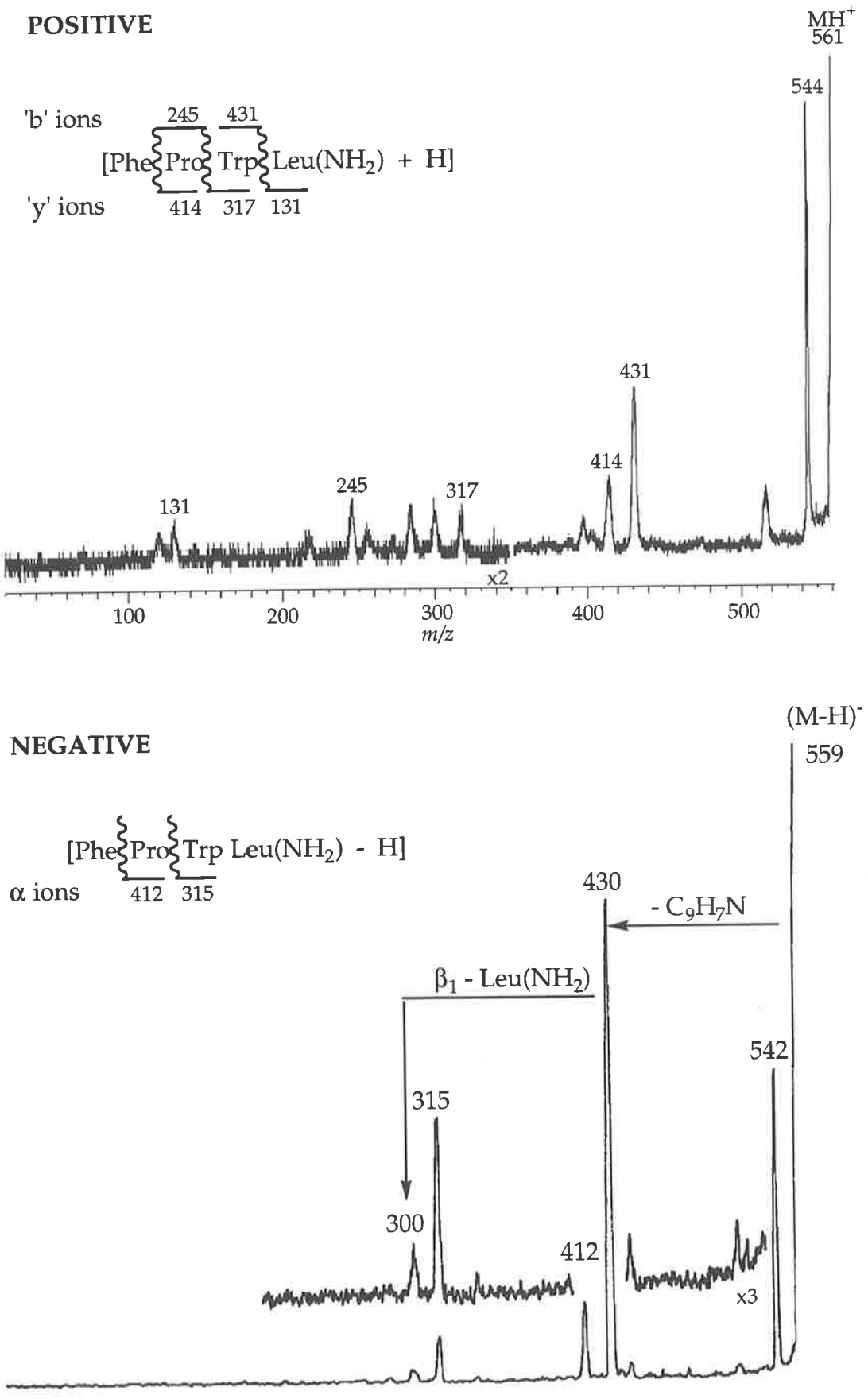


Figure 7.12. The CA MIKE spectra for the MH^+ and $(M-H)^-$ ions from tryptophyllin 7.4 [Phe Pro Trp Leu(NH_2)].

Tryptophyllin 7.4 is a good example, as both the positive and negative ion spectra reveal the full sequence of the peptide (see Figure 7.12). The positive ion spectrum shows 'b' and 'y' type cleavage ions which determine the primary sequence. The negative ion spectrum identifies the first two amino acid residues from the N-terminal end via two α cleavage ions. The C-terminal sequence is determined by the loss of 129 Da [from the Trp side chain (C_9H_7N)] followed by (secondary) β cleavage to lose 130 Da [Leu(NH_2)].

Rubellidin 7.6 is a rare example where the full primary sequence could not be obtained from a combination of the positive and negative ion spectra (see Figure 7.13). The positive ion CA MIKE spectrum provides most of the sequence as shown in Figure 7.13. As mentioned in detail in Chapter 7.2, the negative ion spectrum of the rubellidin 7.6 only identifies the presence of Thr by the loss of 44 Da (MeCHO) and Glu by the loss of 18 Da (H_2O) from the $(M-H)^-$ ion. No sequence information could be obtained, but the presence a Phe residue is indicated by internal rearrangement.

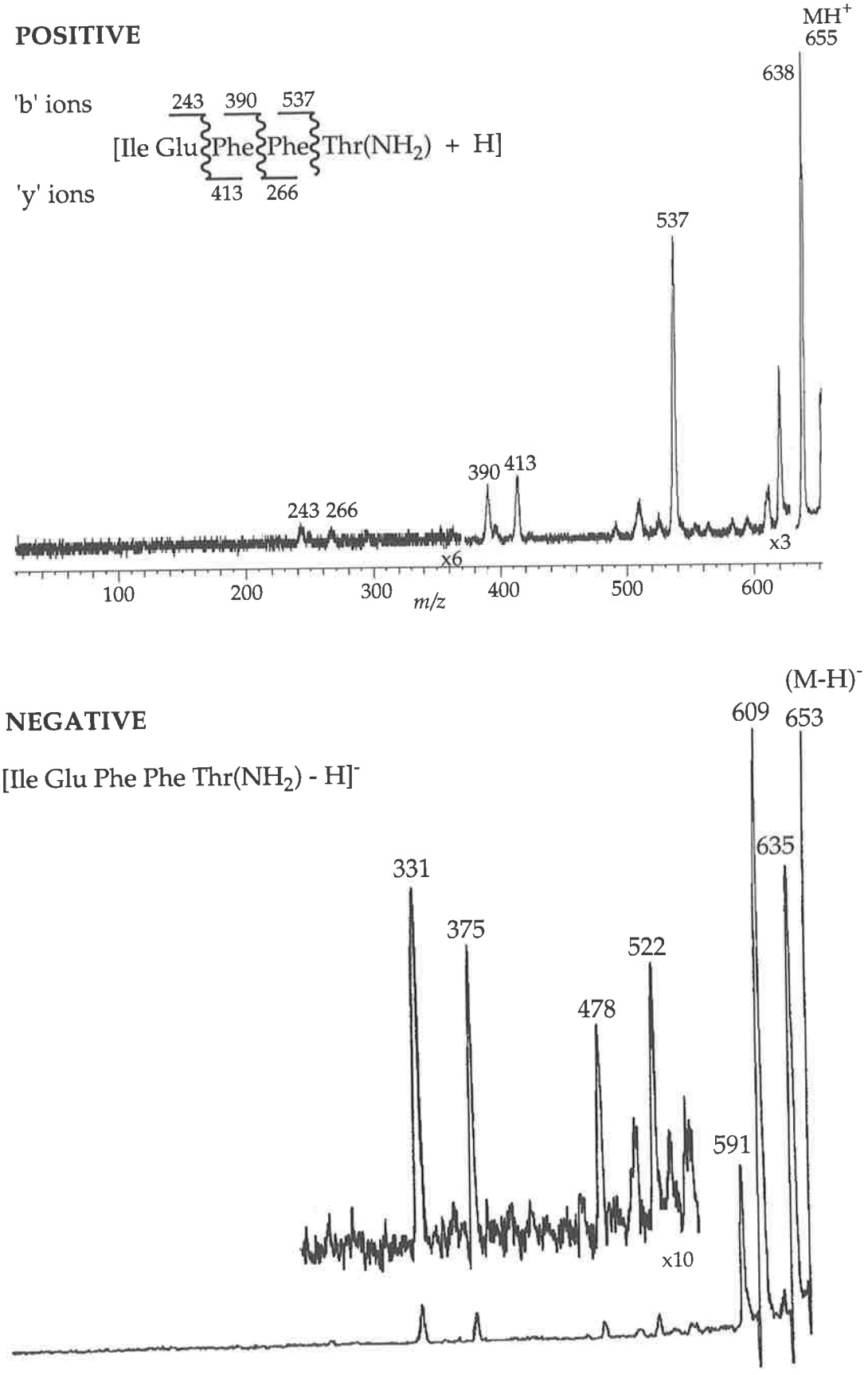


Figure 7.13. The CA MIKE spectra for the MH^+ and $(M-H)^-$ ions from rubellidin 7.6 [Ile Glu Phe Phe Thr(NH_2)].

Caeridin 7.7 is an example of a peptide containing one amino acid residue which undergoes facile side chain fragmentation in the negative ion mode. The positive ion spectrum (see Figure 7.14) reveals most of the sequence except for the first two N-terminal amino acid residues. The negative ion spectrum (mentioned in detail in the previous chapter) determines the partial sequence with the backbone cleavages. The α and β cleavage ions give a partial sequence and the position of the Asp is confirmed by the characteristic fragmentations to *m/z* 510 and 492. The full sequence of the peptide, except for two residues, was able to be determined using a combination of the positive and negative ion spectra.

Comparison of the positive and negative ion spectra of the caeridin 7.10 can be made in Figure 7.15. The positive ion spectrum reveals the full sequence with the majority of backbone fragmentations being observed. The negative ion spectrum of caeridin 7.10 shows completely different structural information. The presence of the Ser and Met residues are identified by the losses of 30 Da (CH_2O) and 48 Da (MeSH) from the $(M-H)^-$ ion to yield *m/z* 1323 and 1305 respectively. The backbone cleavages are significantly diminished in comparison. In this example, the positive ion spectrum give significantly more information.

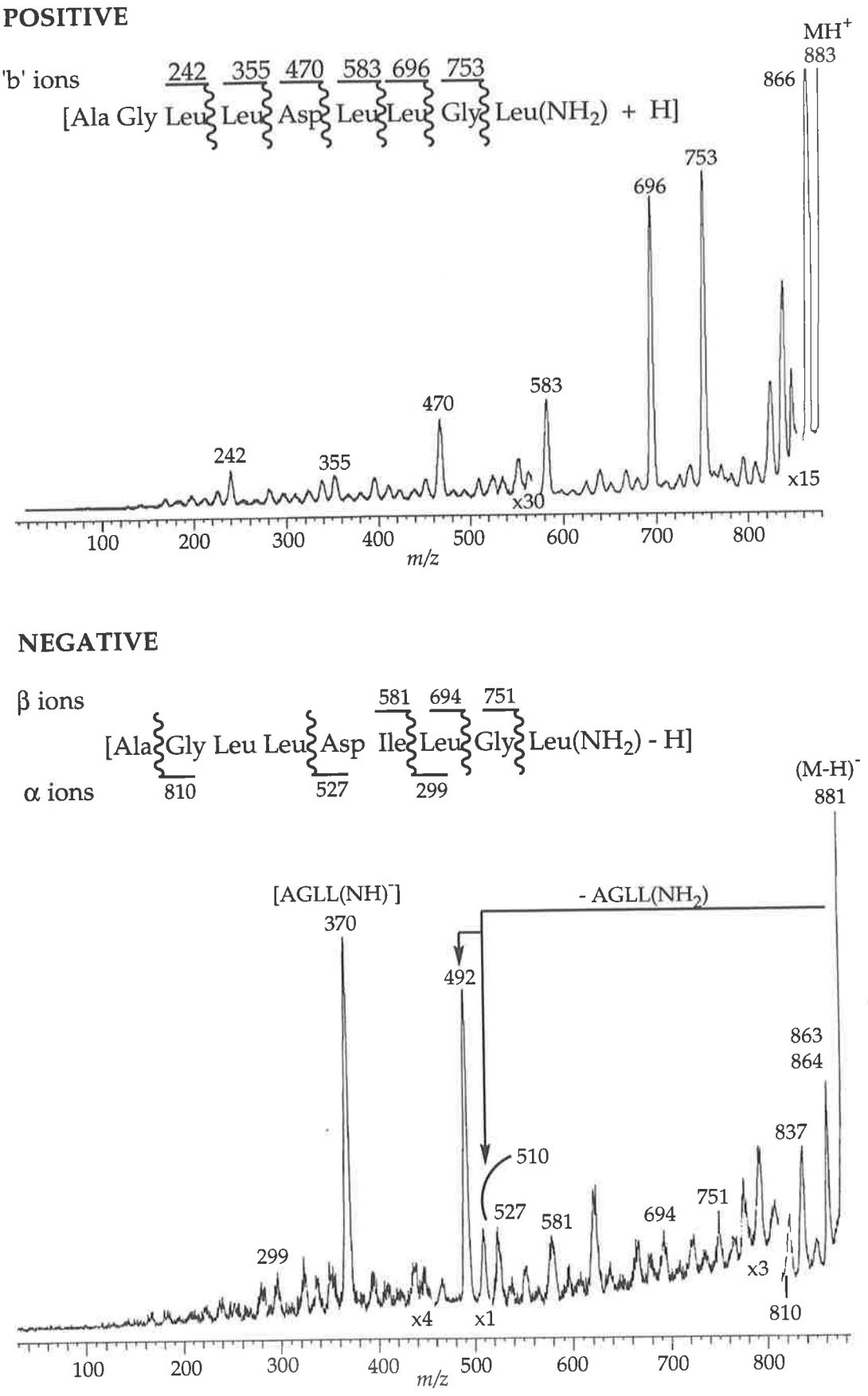


Figure 7.14. The CA MIKE spectra for the MH^+ and $(M-H)^-$ ions from caeridin 7.7 [Ala Gly Leu Leu Asp Ile Leu Gly Leu(NH_2)].

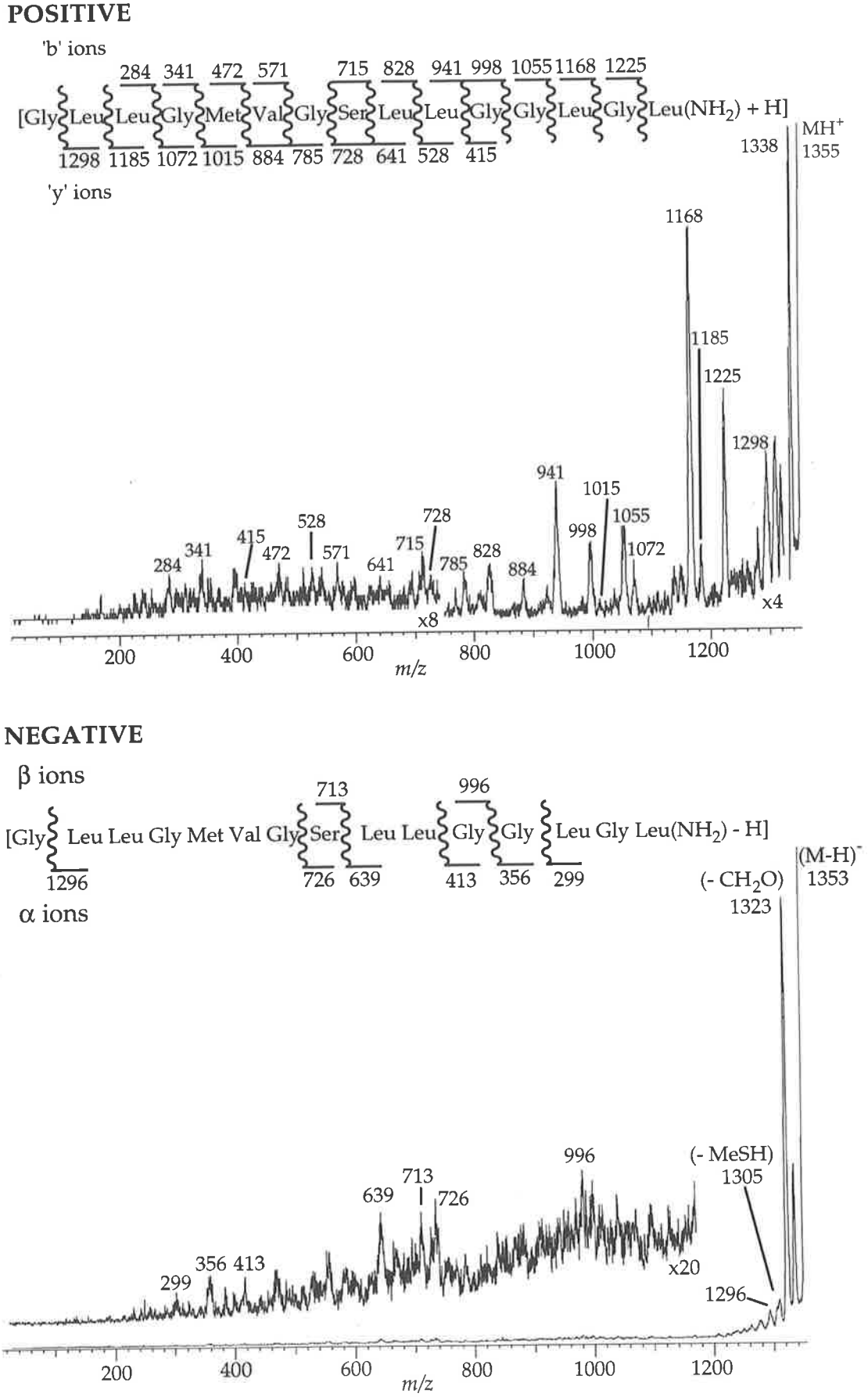
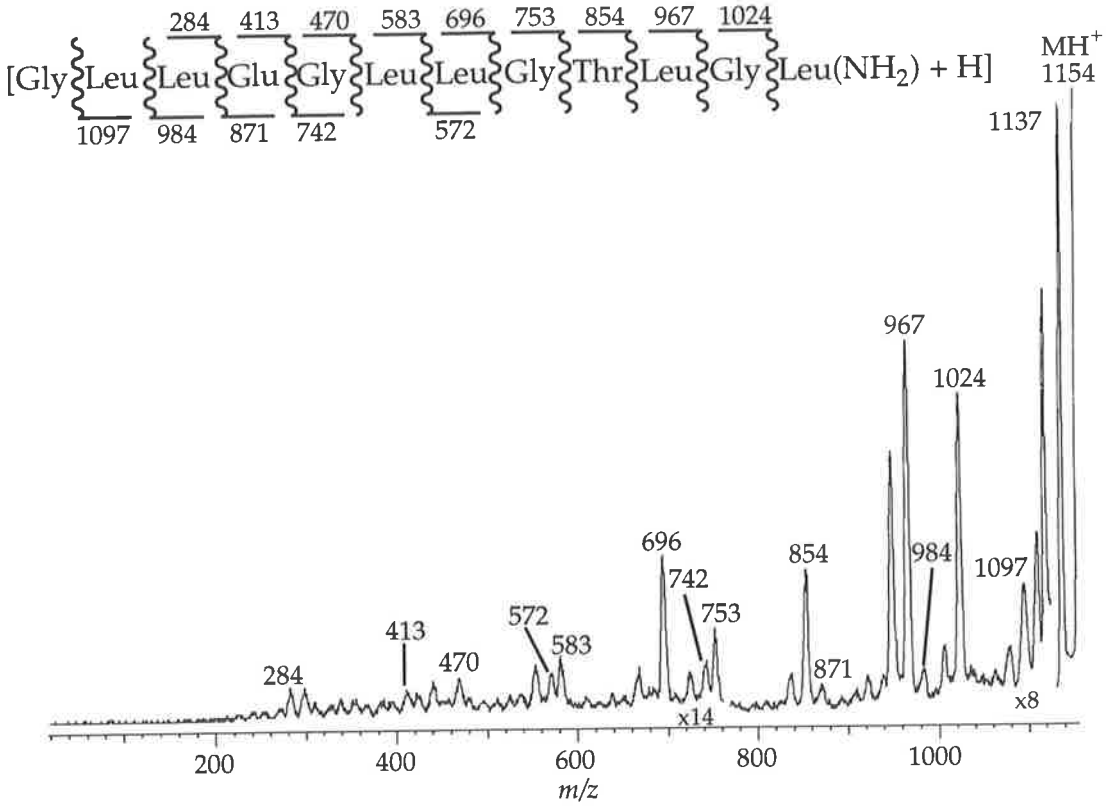


Figure 7.15. The CA MIKE spectra for the MH^+ and $(M-H)^-$ ions from caeridin 7.10 [Gly Leu Leu Gly Met Val Gly Ser Leu Leu Gly Gly Leu Gly Leu(NH_2)].

POSITIVE



NEGATIVE

β ions

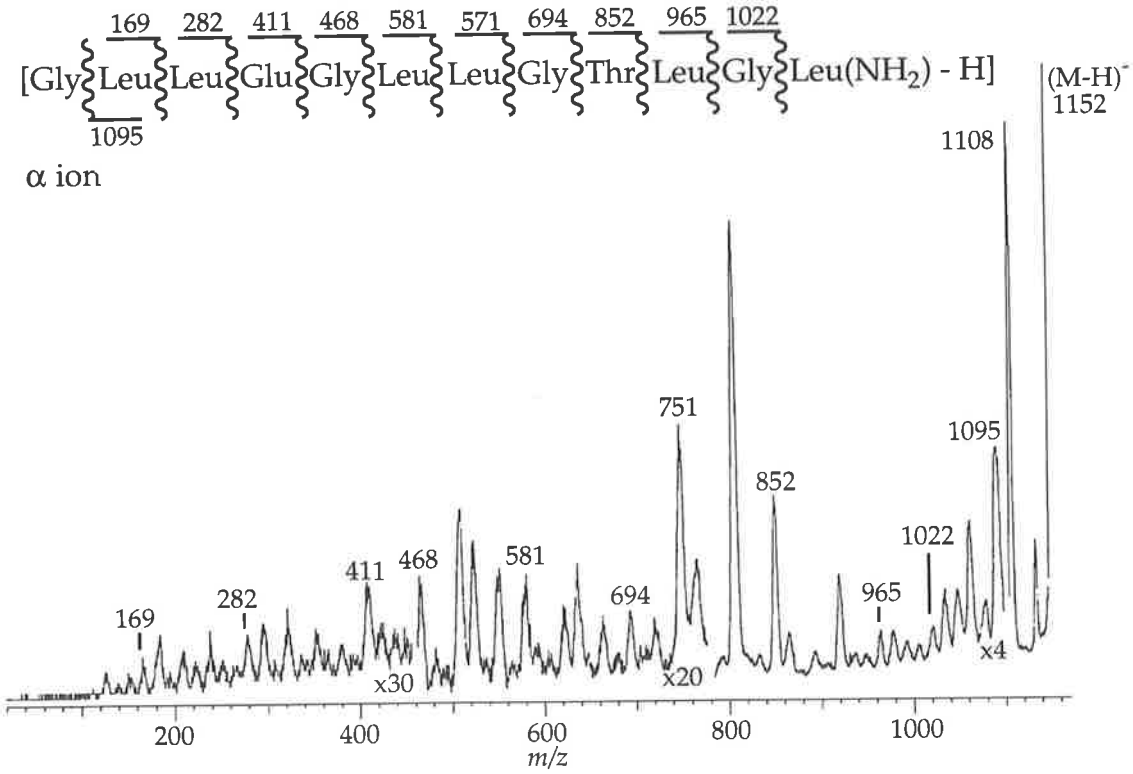


Figure 7.16. The CA MIKE spectra for the MH^+ and $(M-H)^-$ ions from caeridin 7.14 [Gly Leu Leu Glu Gly Leu Leu Gly Thr Leu Gly Leu(NH_2)].

The positive and negative ion CA MIKE spectra of caeridin 7.14 are seen in Figure 7.16. The positive ion spectrum reveals the full sequence of the peptide, by almost showing the full complement of 'b' cleavage ions, with the 'y' cleavage ions sequencing the N-terminal end of the peptide. The same result is seen with the negative ion spectrum. The β cleavage ions determine the full sequence, except for the first two amino acid residues. The single α cleavage ion reveals Gly (1) and Leu (2) can then be determined indirectly.

In summary, the positive ion CA MIKE spectra provide more sequencing information than the corresponding negative ion spectra. However we have deliberately chosen peptides that contain residues which undergo facile side chain fragmentation in the negative ion mode, resulting in a diminution of sequencing information. As a result, the only consistent information that was obtained from the negative ion spectra was the identification of specific amino acid residues, from the side chain cleavages. Backbone cleavages that were observed were typically diminished in comparison.

7.6. Summary.

The examples given in this chapter show that the information obtained from the negative ion CA MIKE spectra of larger peptides is at least complementary to those of the positive ions. As the peptides become larger the amount of information that can be obtained from the negative ion spectra is dependent upon the amino acid composition of the peptide. In contrast the positive ion spectra reveal the majority of the sequence in most cases.

CHAPTER EIGHT: EXPERIMENTAL.

8.1. Mass Spectrometric Analysis.

8.1.1. Vacuum Generators ZAB 2HF Reverse Sector Mass Spectrometer.

All fast atom bombardment mass spectra were obtained using a Vacuum Generators ZAB 2HF reverse sector mass spectrometer[39] at the Department of Chemistry, University of Adelaide. It is equipped with an Ion Tech FAB gun operating with argon gas with an acceleration potential of 7 kV and a current of 1 mA.[64] The ZAB has been discussed in Chapters 1.2 and 1.3.

Routine FAB spectra were required for the detection of protonated molecular ions, including those from Edman and enzymatic degradations and peptide derivatisation. The spectra were obtained by scanning the magnet over a total *m/z* range of 3 000 to 300 Da with a mass resolution between 1 000-1 500 (10% valley definition) at a scanning rate of ≈ 20 sec/decade. Data was collected on a PC data station with Maspec Data System for Windows® software (Mass Spectrometry Services software Ltd., Manchester, England).

Collisionally activated mass analysed ion kinetic spectroscopy (CA MIKES) was performed by focussing the ion of interest through the magnet and into the collision cell in the second field free region. The collision cell is filled with argon to a pressure of $2x10^{-6}$ to $5x10^{-6}$ Torr, which is measured by an ion gauge situated between the electric sector and the collision cell. This resulted in a reduction of the main beam of some 30-50%. The electric sector is then scanned downward at a rate of 20 seconds/decade. All slits were fully open to maximise sensivity, which minimised resolution to 500.[312] Data was collected

on either the PC data station with Maspec Data System for Windows® software, or on paper using a Hewlett-Packard, 7035B X-Y chart recorder. The CA MIKE spectra acquired using the Maspec Data System for Windows® software have a x-axis indicating the *m/z* values. The other CA MIKE spectra were acquired using the Hewlett-Packard, 7035B X-Y chart recorder.

Collisional activated B/E linked scan spectra were generated by filling the collision cell in the first field free region. The pressure inside the cell was ≈ $1x10^{-5}$ Torr (the pressure inside the collision cell could not be directly measured as the ion gauge used measures the source pressure). The magnet and electric sectors are set to allow detection of the ion of interest. Both sectors are then scanned downward at a constant ratio of B/E. Data was collected on paper using a Hewlett-Packard, 7035B X-Y chart recorder.

B^2/E linked scanned spectra were generated by allowing detection of a source formed daughter ion of interest. The magnetic and electric sectors are then scanned at a constant ratio of B^2/E. The data was collected on paper using a Hewlett-Packard, 7035B X-Y chart recorder.

Sample Preparation.

Samples for FAB analysis were dissolved in a minimal amount of water with 2 µl lyophilised onto the probe tip in the pre-vacuum system of the mass spectrometer. The matrix (usually glycerol or thioglycerol/glycerol) was added to the probe tip and mixed thoroughly with the sample before the mixture was inserted into the FAB source. On occasions when performing positive ion CA MIKES, trace amounts of trichloroacetic acid or acetic acid were added to the matrix to increase ion protonation and therefore ion beam strength. When

performing negative ion CA MIKES, glycerol or diethanolamine was used as the matrix. No additives were necessary.

8.1.2. Finnigan LCQ Mass Spectrometer.

Electrospray mass spectra were acquired on a Finnigan LCQ ion trap mass spectrometer (LCQ) through courtesy of Dr. Graeme Currie at the Department of Botany, University of Melbourne. The LCQ and electrospray ionisation have been discussed in detail in Chapters 1.5 and 1.6 respectively.

The samples were dissolved in 50% acetonitrile in water (w/w) and infused into the electrospray source through a rheodyne injector with a 5µl loop at 3 µl/min. The electrospray conditions were, needle potential 4.5 kV; tube lense 60 V; heated capillary 190°C and 30 V potential; sheath gas flow 30 psi.

Mass spectra were acquired with the automatic gain control on a maximum ion time of 1 000 milliseconds and using 1 microscan. MS^n spectra were run for an average of 8 scans and acquired using the same conditions as with the mass spectra: the maximum ion time was 1 500 millisecond and the collisional energy was set at 40% of the maximum arbitrary value.

8.2. Collection and Preparation of Frog Skin Secretions.

All amphibian granular secretions were obtained through the courtesy of Associate Professor Michael Tyler from the Department of Zoology, University of Adelaide. The specimens of interest were held by the hind legs and the skin moistened with distilled water. Surface electric stimulation[115] of the granular

glands was performed with a 21G platinum bipolar electrode attached to a C.F. Palmer Student Model electrical stimulator. The electrode was rubbed in a circular manner over entire the dorsal surface of the frog. The operating conditions used were a pulse duration 2.5 milliseconds; pulse repetition of 50 Hz and the stimulus strength of 2 V. The expelled secretion was washed off the frog with deionised water and collected into a sterile container. The volume was immediately doubled with methanol and stored at -10°C for at least twelve hours. The secretion was then centrifuged to remove any particulate matter or insoluble mucus at 3 000 rpm for 5 min using a Clements GS 100 centrifuge. The supernatant was decanted off and lyophilised to a volume of ≈ 1 cm^3 using a Savant SC 100A Speedvac® Concentrator. The resulting extract was filtered using a 0.45 µm Millex filter. This extract can be stored for up to 12 months at -10°C without any alteration in the peptide composition. The amount of crude peptide material yielded varied with the size of the frog. 3 mg of crude peptide secretion was obtained from *Litoria rubella* and *L. ewingi*, with 5 mg from *L. xanthomera* and *L. chloris*.

8.3. Analytical and Preparative HPLC.

Analytical HPLC traces were obtained using a VYDAC 218TP54 C_{18} Protein and Peptide reverse phase column (5 µ, 300 Å particle size, 4.6 mm i.d. x 250 mm), with a VYDAC 218TP 300 Å guard column preceding the analytical column. The column was equilibrated with acetonitrile:water [1:9], containing 0.1% trifluoroacetic acid (TFA, spectroscopic grade, Aldrich) as the ion-pairing agent. The flow rate through the column was 1 ml/min. The typical gradient used for obtaining a 'peptide profile' was operating a linear gradient of 10-75% acetonitrile over a 30 min period, injecting 1% of the crude unknown secretion. The analytical column was also used for purifying individual

peptide fractions and separating enzyme digest fragments.* The solvent gradient was adjusted accordingly.

Preparative HPLC separations were performed using a VYDAC 218TP510 C_{18} Protein and Peptide reverse phase column (5 μ, 300 Å particle size, 10 mm i.d. x 250 mm). The solvent system and operating procedure is similar to those used for analytical HPLC operation, except that the solvent flow was 4 ml/min.

The elution profiles were generated using two HPLC systems,

(1) The elutant was monitored by an ICI LC 1200 UV/VIS detector (214 nm). The data was processed through ICI DP800 Interface by an ICI DP800 Data Station. The gradient was programmed through the ICI DP800 Data Station that controlled two ICI LC 1100 HPLC pumps.

(2) The elutant was monitored by a Lambda Max, Model 481, LC spectrophotometer (214 nm). The data was processed through an ICI DP800 Data Interface, by ICI DP700 Data Station. The gradient was programmed through an automated Waters Gradient Controller, which controlled Waters 501 and 510 pumps. Samples were injected into a Rheodyne injector fitted with a 1 ml injection loop.

8.4. Automated Edman Degradation.

Automated Edman degradations were carried out through courtesy of Professor John Wallace and Denise Turner at the Department of Biochemistry, University of Adelaide. Automated Edman sequencing[198] of the peptides was performed using an applied Biosystem 470 A sequencer, equipped with a 900A data analysis module. Good results are obtained by using a disc of

* Before separating enzymic digests, the peptide material was cleaned using a C_{18} Sep-pak cartridge to remove the salts contained in the buffer used for the digests.

polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore) treated with Bioprene Plus TM (Applied Biosystems, Inc.) in ethanol, onto which the peptide was absorbed from aqueous acetonitrile (90%). The disc was pierced several times with a razor blade in order to aid flow of the solvent.

8.5. Manual Edman Degradation.

The manual Edman degradation is based on the procedure by Bradley and Williams.[209]

In a 1.5 ml Eppingdörf test tube a peptide sample (≈ 50μg) was dissolved in aqueous redistilled pyridine (50%) (40 μl), followed by the addition of phenylisothiocyanate in heptane (5%, protein sequencing grade, Applied Biosystems) (150 μl). The test tube was purged with oxygen free nitrogen, capped and the mixture incubated at 45°C for 15 minutes. The aqueous solution was then washed with a mixture of hexane:ethyl acetate (4:1) (2 x 100 μl) and lyophilised to dryness by a stream of nitrogen. TFA (spectroscopic grade, Aldrich) (50 μl) was added, the test tube was purged with oxygen free nitrogen, capped and the mixture incubated at 45°C for 10 minutes. The TFA was then removed by a stream of nitrogen. The lyophilised product was resuspended in water (30 μl) and the phenylthiocarbodiimide amino acid derivative extracted into *n*-butyl acetate. The two layers were separated with the aqueous layer monitored by FAB MS to measure the mass of the residual peptide and identify the mass of the eliminated amino acid.

8.6. Enzymatic Digests.

The following enzymatic digests were conducted on peptides that had a molecular weight of 1 000 Da and above. All enzymes were diluted to a factor of 1 unit/ 50 µl.

Endoprotease Lys-C.

The peptide dissolved in 5 µl water, had ammonium hydrogen carbonate (0.1 M, 40 µl, pH = 8) and Endoprotease Lys-C (from *Lysobacter enzymogenes*, Sigma Chemical Company) (1 µl) added. The solution was heated at 45°C for 2 hours, with the products analysed directly by FAB MS.

α-Chymotrypsin.

The peptide dissolved in 5 µl water, had TRIS buffer (0.1 M, 40 µl, pH 8.0) and α-chymotrypsin (Type II, from Bovine Pancreas. Sigma Chemical Company) (1 µl) added. The solution was heated at 45°C for 2 hours, with the products analysed directly by FAB MS.

8.7. Methylation.[313]

The majority of the peptides obtained were converted into the corresponding methyl esters by the following procedure.

'Acidified methanol' (2M, 50 µl) was added to the dry peptide (≈ 5-10 µg) in a

1.5 ml Eppingdörf test tube. The test tube was flushed with oxygen free nitrogen and heated at 45°C for 2 hours. The sample was then lyophilised to dryness by a stream of nitrogen and the methyl ester was then dissolved in 5 µl water and analysed by FAB MS. This procedure resulted in ≈ 70% conversion of the peptide to the methyl ester.

2M 'acidified methanol' was made by adding methanol (HiPerSolv™ for HPLC, BDH) (858 µl) into a 10 ml screw top test tube and cooling it with dry ice for 5 minutes. Acetyl chloride (Aldrich) (142 µl) was added with the test tube flushed with nitrogen, capped and cooled in dry ice for 5 minutes. The solution was then allowed to warm up to room temperature for 1 hour, with the tube being flushed with nitrogen every 15 minutes to remove HCl gas.

8.8. Acetylations.[313]

Redistilled acetic anhydride:acetic acid [1:1] (40 µl) was added to the dry peptide (≈ 50 µg). The mixture was flushed with nitrogen and then left at room temperature for 1 hr. The mixture was then blown dry with nitrogen and the residue redissolved in 5 µl of water and then analysed by FAB MS.

8.9. Partial Acid Hydrolysis.[246]

6N HCl (75 µl) was added to the dry peptide (≈ 75 µg) and the mixture heated at 110°C. 6 µl of the mixture were removed at time intervals of 2, 5, 10, 20 and 40 mins. Each aliquot was blown to dryness with nitrogen. The samples were then dissolved in 5 µl of water and analysed by FAB MS.

8.10. Synthesis of Peptides.

Peptides used were synthesised commercially by Chiron Mimotopes, Clayton, Victoria, Australia). The procedure applied was the standard N-α-Fmoc method[314] using L-amino acids. Each synthetic peptide was shown to be identical to the natural peptide by FAB MS and co-elution of the synthetic and natural peptides by HPLC. (see p 170 for details).

8.11. Bioactivity Testing.

8.11.1. Pharmacological Activity.

Ongoing activity tests of the tryptophyllins from *L. rubella* and *L. ewingi*, and the uperins from *L. ewingi* are being carried out by Dr. C. Severini in the Rome laboratories of Professor V. Erspamer. The test systems were two isolated smooth muscle preparations, the guinea pig ileum and rabbit terminal, colon and rabbit blood pressure.

The smooth muscle preparations were suspended in a bath of tyrode solution (10 ml maintained at 37°C and oxygenated [O_2 and CO_2 (95:5)]). Contractions of the smooth muscle preparations were recorded isometrically by a strain-gauge transducer [DY2, force to 10g and displayed on a recording microdynamometer (Basile, Varese, Milan)]. Carotid blood pressure of the anaesthetised rabbit [urethane (1.5 g/kg. intraperitoneally)] was recorded from a carotid artery by a Trantee pressure transducer (model 880, Bently, Irvine, CA) connected to a microdynamometer. Injections of peptides were given to a polyethylene insertion into the jugular vein.

8.11.2. Antibiotic Activity.

Antibiotic testing of the synthetic peptides was carried out by Dr. Bruce Winter of the Microbiology Unit of the Institute of Medical and Veterinary Science (Frome Rd., Adelaide, South Australia). The method involved the measurement of inhibition zones produced by the applied peptide on a thin agarose plate containing the micro-organism under study. The procedures are standard and have been fully documented.[315] The micro-organisms used are shown in Chapter 3.2.2, and Tables 4.4, 5.14 and 6.7. The activities are recorded as MIC values, i.e. the minimal inhibitory concentration of peptide per ml required to totally inhibit the growth of the named micro-organism.

APPENDIX 1.

Presursor Ion	$[M-H]^-$ (*m/z*)	Fragment Ions
7.1	525	508, (NH_3), 100; 412, (α_1), 40; 396, (C_9H_7N), 75.
7.3	670	653, (NH_3), 36; 559, (α_1), 14; 9; 541, (C_9H_7N), 64; 412, (α_2), 100; 354, (β_2), 5; 315, (α_3), 4; 257, (β_3), 20.
7.4	559	542, (NH_3), 70; 430, (C_9H_7N), 100; 412, (α_1), 61; 315, (α_2), 46.
7.5	803	786, (NH_3), 100; 690, (α_1), 100; 674, (C_9H_7N), 34; 544, (*m/z* 674 - Leu(NH_2), 487, (β_2), 8; 412, (α_3), 26.

Table 1. B/E linked scanning data of the tryptophyllins 7.1 [Ile Pro Trp Leu(NH_2)], 7.3 [pGlu Phe Pro Trp Leu(NH_2)], 7.4 [Phe Pro Trp Leu(NH_2)] and 7.5 [Ile Met Phe Pro Trp Leu(NH_2)]. Format of fragment ions, [fragment ion, *m/z*, (group lost or specific cleavage), percentage compared to base peak].

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