Chemistry and Medical Implications of Novel Amphibian Peptides

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A thesis submitted for the Degree of Doctor of Philosophy

by

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Statement of Originality

This thesis contains no material that 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.

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Paul A. Wabnitz Date

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Abstract

Amphibian skin is like a chemical warehouse, an extraordinary source of biologically active compounds. Amphibians have the ability to generate a number of natural defence compounds onto their integument. Some of these natural compounds that are produced include amines, steroid derivatives, toxic alkaloids and peptides. In particular, the amphibian integument is a rich source of biologically active peptides. With such an astounding diversity of abundant and readily accessible biologically active peptides, amphibians can be looked upon as a rich source of new and novel biologically active chemicals. This includes hormones, neuropeptides and antibacterial peptides. The chemical and pharmacological investigation of these compounds derived from amphibian skin offers a promising pathway towards new medicinal discoveries. The work presented in this thesis is centred on this investigation and isolation of novel amphibian peptides, and further investigates the biological activity of some of the peptides discovered throughout this research.

The research presented in this thesis encompasses three main areas:

(i) The investigation of the skin peptide profiles of three distinct species of Australian amphibians. These include larval *Litoria splendida*, *Litoria citropa* and *Litoria electrica*.

(ii) The investigation of a sex pheromone found in the adult male skin secretion of *Litoria splendida*. Although much is known about the sex pheromones in insects, very little is known of pheromones in amphibians. This research reports the discovery, isolation and characterisation of an aquatic female-attracting pheromone. This is the first anuran pheromone to be identified, and only the second peptide pheromone identified in a vertebrate.

(iii) The investigation of the biological activity of some of the peptides characterised from this research. These biological investigations included testing for antibacterial activity, nitric oxide synthase activity, neuropeptide activity and anticancer activity. Of the 45 new peptides discovered from this research, 14 exhibited antibacterial activity, 3 exhibited inhibition activity on neuronal nitric oxide synthase and 3 exhibited preliminary anticancer activity, with an extra 15 suspected to be potent neuropeptides.

CHAPTER 1. Introduction

1.1. Peptide Pharmacology

1.1a. General

The science of drugs, their properties, uses and effects within the world of pharmacology has been predominantly based on signal molecules of low molecular weight, and non-peptide in nature. With advancing technology it has become clear that peptides are at least as important, and possibly much more so as signal molecules[1]. However, the pharmacological understanding and application of peptide signalling has not reached the same level of development as many other pharmacological systems such as the adrenergic system[1].

Historically, there are two main reasons why pharmacology has had a strong disposition towards non-peptides. One is that medical science has been greatly influenced by the analysis of natural products that demonstrated medicinal importance [2]. Very few if any of these products were peptides. The second reason is related to methodology. Only within the last thirty years have there been significant advances in peptide research, including the development of solid phase peptide synthesis, the application of antibodies for radioimmunoassay and immunocytochemical localisation of peptides, various sequencing and analytical structural determination techniques, three-dimensional molecular modelling and genetic engineering, which includes the use of recombinant deoxyribonucleic acid (DNA) systems.

By the 1930s, a number of uncharacterised peptide mediators had been discovered. For example, substance P, bradykinin and angiotensin had been identified as peptides, but their structures remained unknown for many years[2]. In 1953, the peptide oxytocin* (Figure 1.1) was structurally characterised and synthesised [3]. This was the first peptide for which this was

* Oxytocin is a hormone found within a specific region of the mammalian brain. It stimulates the contraction of the smooth muscle of the uterus to aid the expulsion of the baby during childbirth. It also promotes ejection of milk from the mammary glands during breastfeeding.

achieved and the first to be synthesised commercially for medical applications.

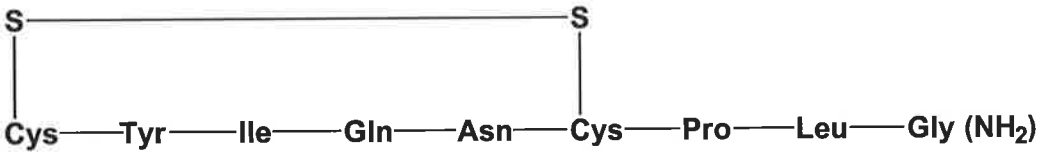

Figure 1.1. Oxytocin.

Today, modern methods allow most peptides to be fully characterised and synthesised. However, the pharmacology of biologically active peptides and their therapeutic utilisation is still relatively sparse[4]. Nevertheless, many researchers have predicted the upcoming therapeutic growth area of peptides[5].

1.1b. Structure and function of peptide mediators

Peptides are compounds comprised of amino acids that are covalently linked together by the formation of an amide bond (Figure 1.2) between α-amino and α-carboxyl groups.

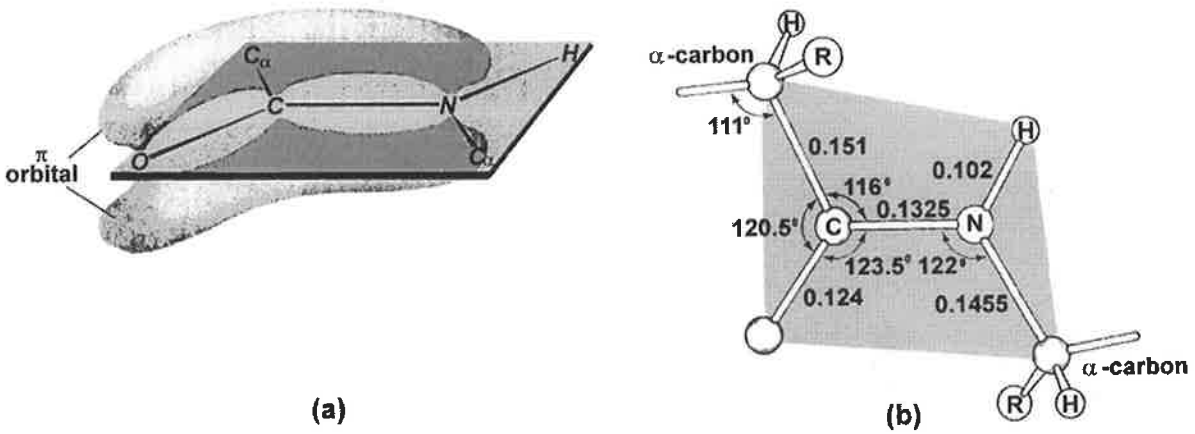

Figure 1.2. Structure of the peptide bond. (**a**) A schematic representation of the electron density around the peptide bond. The –C=O and –N-H bonds are parallel, accounting for the rigidity around the C-N. This is because the peptide bond exhibits partial double bond character due to the delocalisation of the π-electron orbitals over O-C-N, producing a resonance stabilisation. (**b**) Bond lengths and angles within the peptide bond. Bond lengths are in nanometers (nm).

The distinction between peptides and proteins is a subjective one, with the cut off point generally being 50 amino acids. Endogenous peptide mediators characteristically comprise a linear chain of approximately 5-40 amino acids. Usually the carboxy terminus (C-terminus) has been modified into a post-translated amide. Endogenous peptides often occur with other post-translational modifications, such as glycosylation, acetylation, carboxylation, sulphation or phosphorylation of specific residues [1]. They often contain intramolecular disulphide bonds, which allows the molecule to adopt a partially cyclic formation[6]. Peptides of this size are often difficult to crystallise, restricting the use of X-ray diffraction methods for conformational structure studies[7]. Nuclear magnetic resonance (NMR) spectroscopy has been applied successfully for the examination of peptide conformational structure in solution, and has generally shown these molecules to be highly flexible[8]. However, to imagine them fitting into a receptor site in a precise 'lock and key' manner is to imagine unlocking a door with a length of cooked spaghetti. This has restricted the development of non-peptide analogues that mimic or block the actions of peptides, as synthetic chemists simply do not know where to begin[1].

Most known mammalian peptide mediators come from the central nervous system (CNS) and the endocrine glands*. However, some are formed within plasma, and may occur at other sites like the vascular endothelium, heart, cells of the immune system and so on[9]. The same peptide may also be produced in several locations, and serve several different functions. For example, the plasma peptide angiotensin acts on a specific region within the brain to release the peptide hormone vasopressin, which in turn causes water retention. It also acts elsewhere in the brain to stimulate drinking behaviour and to increase blood pressure by activation of the sympathetic system[10]. Furthermore, it also acts directly to constrict blood vessels.

Like other chemical mediators, peptides produce their effects by binding to specific receptors. The transduction mechanisms by which they control cell function generally involve the same second messenger systems as those used by other mediators[4]. Peptides also often act in the CNS as co-transmitters with other peptides or with non-peptide transmitters. The number of known peptide mediators now surpasses that of non-peptides[8].

* Endocrine glands are ductless glands that secrete hormones directly into the surrounding tissue fluids and blood vessels. The hormones are carried to the target cell in other parts of the body by the circulatory system.

As previously mentioned, most known mammalian peptide mediators come from the CNS (neuropeptides) and the endocrine glands (peptide hormones).

1.1c. Neuropeptides

Neuropeptides are chemical messengers that are released from sensory neurons and act on neuronal receptors. In doing so, they usually activate a second messenger system producing intracellular effects that alter the synaptic activity of particular neurons[11]. Neuropeptides can act on either presynaptic or postsynaptic sites, and are usually released from sensory neurons. Neuropeptides can also contribute to the inflammatory reactions in some tissues[12]. For example, the peptide substance P and a particular group of peptides, known as the tachykinins, produce smooth muscle contraction, mucus secretion and various other functions.

Substance P was originally discovered in 1931 during biological activity investigations of extracts of the brain and intestine[13]. It was found that one of the compounds showed activity that was not due to acetylcholine, and which lowered blood pressure and contracted smooth muscle[14]. The chemical properties suggested that it was a peptide, and it was simply named substance P[15]. This was the first neuropeptide to be discovered. In the 1950s, Vittorio Erspamer was investigating bioactive amines in marine animals. He discovered a peptide in a Mediterranean octopus that had similar activity to that of substance P[16]. The peptide was isolated from nearly two tons of octopus and named eledoisin[17]. This encouraged Erspamer to conduct similar experiments in amphibians, and another peptide named physalaemin was discovered[18]. These peptides produced rapid contractions of smooth muscle preparations, and exhibited potent vasodilatation activity. Erspamer named this family of peptides as the tachykinins (fast acting), distinguishing them from the bradykinins that were much slower acting on smooth muscle. In 1970, substance P was purified and characterised as a member of the tachykinin family of peptides, which are characterized by the C-terminal sequence –Phe-X-Gly-Leu-Met-NH_2[19]. Since then more tachykinins and many other neuropeptides have been discovered and characterised. Some examples of tachykinins are shown in Table 1.1.

Tachykinins	Sequence	Origin
Substance P	R P K P Q Q **F** F **G L M** (NH_2)	Mammalian
Substance K	H K T D S **F** V **G L M** (NH_2)	Mammalian
Substance B	D M H D F **F** V **G L M** (NH_2)	Mammalian
Eledoisin	pE P S K D A **F** I **G L M** (NH_2)	Cephalopod
Physalaemin	pE A D P N K **F** F **G L M** (NH_2)	Amphibian

Table 1.1. Structures of some tachykinin peptides, with common amino acids shown underlined.

1.1d. Hormonal peptides

Hormones are chemicals that are secreted (without the advantage of a duct) into the blood stream, usually acting on a distant tissue[20]. Hormones fall into three distinct categories. These are: (i) peptides and proteins, (ii) amines and (iii) steroids. The majority of hormones fall within the protein and peptide category, and are secreted throughout the CNS, pancreas, gastrointestinal tract, kidneys, heart and liver[20]. Peptide hormones are stored in secretory vesicles until they are ready to be released[21]. This storage of peptide hormones, in a readily releasable form, allows the ductless gland to respond rapidly to any demands for increased secretion without the requirement of an increase in hormone synthesis[22]. Upon appropriate stimulation, the secretory vesicles fuse with the plasma membrane and liberate their contents to the outside by a process known as exocytosis*. Such secretion is not continuous, but is triggered only by specific stimuli[20]. Hormonal peptides are generally hydrophilic, making them water-soluble but with low lipid solubility. This allows the secreted peptide hormone to readily dissolve in the plasma and be distributed freely throughout the blood stream, in contrast to the hydrophobic steroid hormones that circulate in the blood to their target cells reversibly bound to plasma proteins[20]. The hydrophilic hormonal peptide cannot pass through the lipid membrane of the target cell, and consequently binds with specific receptors on the outer surface of the target cell. Binding of the hormone activates the membrane-bound

* Exocytosis is the mechanism whereby substances originating within the cell are extruded to the exterior.

enzyme adenylate cyclase that in turn converts intracellular adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), the intracellular second messenger. Most hormonal peptides use cAMP as the second messenger, however a few peptide hormones are known to use intracellular Ca^{2+} as the second messenger. The second messenger activates a second enzyme (protein kinase) that may phosphorylate a number of enzymes[1]. Phosphorylation causes some of the enzymes to be activated and others to be deactivated[23]. It is this alteration in enzyme activity that produces the ultimate physiological response to the hormone[24]. This mechanism is represented in Figure 1.3. Once a hormonal peptide has interacted with a target cell, it is rapidly deactivated or removed so that it is no longer available to interact with another target cell. Once the hormone is removed, cAMP is converted to inactive AMP by the enzyme phosphodiesterase (PDE)[25]. Some examples of known hormonal peptides are vasopressin, gastrin and oxytocin.

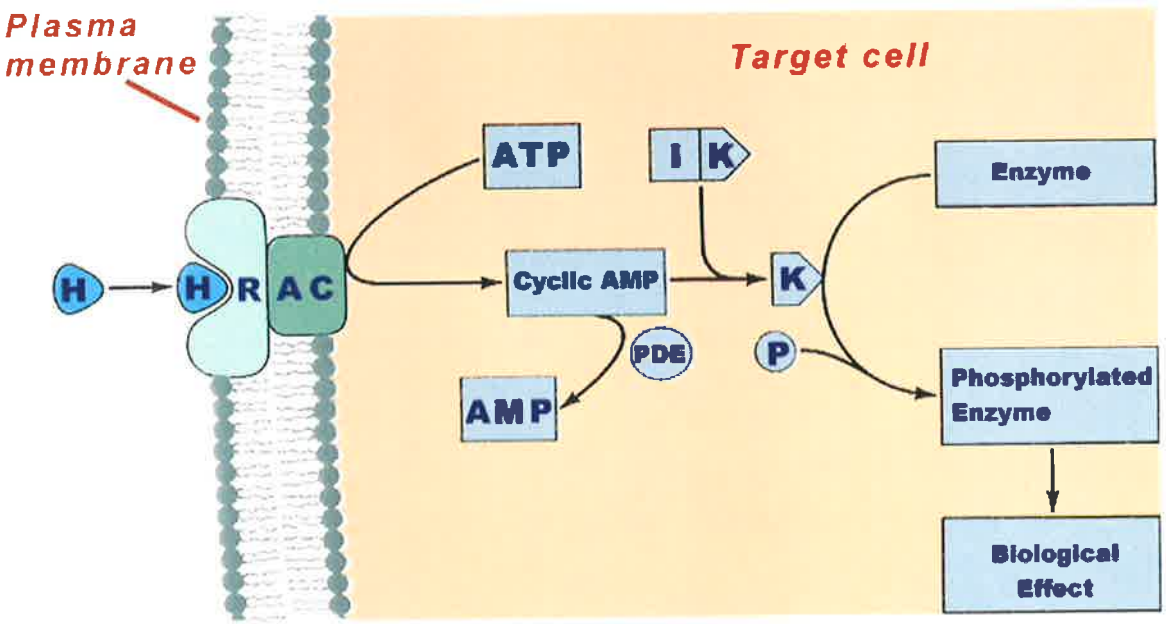

Figure 1.3. Mechanism of action of hormonal peptides on the target cell. H (free hydrophilic hormone), R (surface receptor), AC (adenylate cyclase), ATP (adenosine triphosphate), cAMP (cyclic adenosine monophosphate), PDE (phosphodiesterase), IK (inactive protein kinase), K (active protein kinase), P (phosphate).

1.1e. Peptide biosynthesis

Peptide structure is directly encoded within the genome. Peptide production (Figure 1.4) involves the formation of a precursor protein, known as a prepropeptide, which has the peptide sequence embedded within it, along with specific proteolytic enzymes that excise the active peptide[20]. The prepropeptide is packaged into vesicles at the point of synthesis, and the active peptide is produced intracellularly by selective enzymic cleavage[25]. The genetically coded prepropeptide is a relatively large protein comprising a signal sequence and a propeptide. The signal sequence is involved in the transfer of the protein across the cell membrane[26]. The propeptide contains the embedded sequences of one or more active peptides[27]. The signal sequence is cleaved off at an early stage, forming the propeptide[28]. The active peptides are usually clearly confined within the propeptide sequence by pairs of basic amino acids (Lys-Lys or Lys-Arg), which are the cleavage points for the various trypsin-like proteases that act to release the active peptides[25]. This endoproteolytic cleavage generally transpires before the peptides have been stored in secretory vesicles. Little is known about the enzymes involved, or what governs the process[22].

Cloning techniques have allowed the characterisation of peptide precursors. Once the peptide has been purified and its amino acid sequence determined, the corresponding complementary deoxyribonucleic acid (cDNA) can be synthesised and used to extract the complete DNA segment encoding for the protein in which the peptide sequence is embedded[1]. Generally it has been observed that the precursor protein (prepropeptide), usually 100-250 residues in length, consists of a N-terminal signal sequence, followed by a variable stretch of unknown function (spacer peptide), further followed by a peptide containing regions in which several copies of active peptide fragments are contained[29]. The peptide 'scraps', that are left over (spacer peptides) as the large prepropeptide molecule is cleaved, are often stored and co-secreted along with the active peptides[25]. This raises the possibility that these other peptides may also exert biological effects that differ from the traditional peptide products; that is, the cell may actually be secreting multiple active peptides, but the functions of the other peptide products are usually unknown. Often, several different peptides are found in one precursor, but sometimes there is only one in multiple copies[30]. The signal peptide is usually strongly hydrophobic, and is important for insertion of the protein into the endoplasmic reticulum[20].

A single precursor gene may give rise to several peptides, either by selective DNA splicing before transcription by selective cleavage of the propeptide, or by post-translational modification[31]. Many biologically active peptides are converted to amides, by amidation at the C-terminus[7]. This conversion is vital for the biological activity of numerous peptides, such as tachykinins and peptides related to corticotrophin[10]. Another common observation is the generation of peptides of varying length from the same primary sequence, through the activity of specific peptidases that cut the chain at different points[32]. For example, pro-cholecystokinin (pro-CCK) contains the sequences of at least 5 CCK-like peptides ranging in length from 58 to 4 amino acids, all with the same C-terminal sequence[1]. There are also many examples of closely related peptides with different origins of location and physiological functions, likely to be produced by divergent adaptations from a single gene[19].

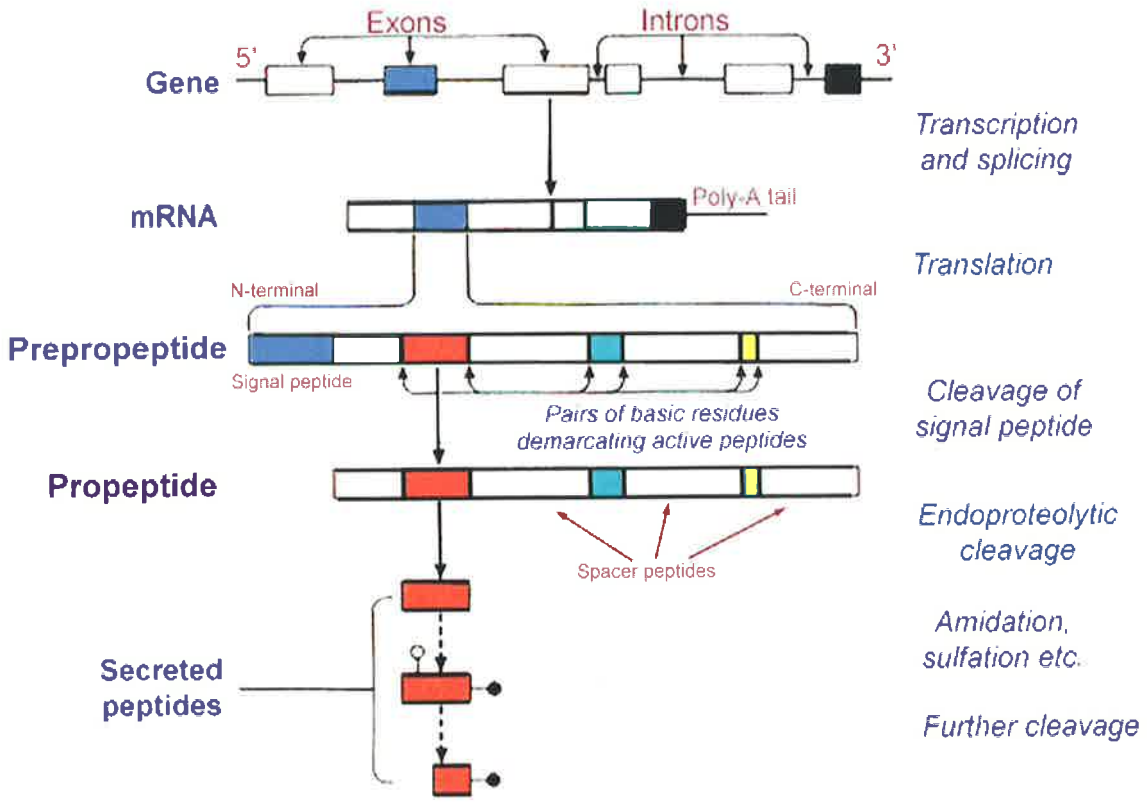

Figure 1.4. Production of a peptide mediator. The gene contains the coding regions (exons) interspersed with non-coding regions (introns). The coding regions of the gene (exons) are transcribed and spliced, which removes the introns and some of the exons, to give rise to the messenger ribonucleic acid (mRNA). The mRNA segments are translated to produce the prepropeptide. Cleavage of the N-terminal signal peptide produces the propeptide, from which endopeptidases excise the peptide fragments. The resulting fragments may be active or may further undergo post-translational processing (amidation, etc.).

1.1f. Peptides as pharmaceuticals

In spite of the relatively large number of known active peptide mediators, few peptides to date are useful as drugs. Generally, peptides produce unfavourable drugs for several reasons:

(i) Peptides cannot be given orally. This is because they are either hydrolysed in the stomach, or poorly absorbed when given orally. An exception is the fungal peptide drug cyclosporin, which is not affected by peptidase activity[1].

(ii) Peptides are usually expensive to manufacture on a commercial basis.

(iii) Peptides are usually rapidly degraded by plasma and tissue peptidases, resulting in a short biological half-life, although there are exceptions to this.

(iv) Peptides do not penetrate the blood-brain barrier.

Nevertheless, with the advances of biotechnology and research development in this area of study, the clinical applications of peptide mediators should markedly increase. Some examples of peptides that are being used clinically are listed in Table 1.2[33].

Peptide Drug	Application	Route
Captopril/Enalapril	Hypertension, heart failure	Oral
Vasopressin/Desmopressin/lypressin	Diabetes insipidus	Intra-nasal, Injection
Oxytocin	Prevention of post-partum bleeding	Injection
Saralasin	Hypertension	Injection
GnRH* and analogues	Infertility/suppression of ovulation	Injection
LHRH† antagonists	Prostatic and other tumours	Injection
ACTH‡	Diagnosis of adrenal insufficiency	Injection
TSH§/TRH**	Diagnosis of thyroid disease	Injection
Calcitonin	Paget's disease of bone	Injection
Somatostatin	Acromegaly††	Injection
Cyclosporin	Immunosuppression	Oral

Table 1.2. Peptides as drugs.

* GnRH; Gonadotropin-releasing hormone.
† LHRH; Luteinising hormone-releasing hormone.
‡ ACTH; Adrenocorticotropic hormone.
§ TSH; Thyroid-stimulating hormone.
** TRH; Thyrotropin-releasing hormone.
†† Acromegaly is the excessive secretion of growth hormone in adults.

1.2. Amphibian Peptides

1.2a. Amphibians

Amphibians are a class of vertebrate that, with a few exceptions, generally live on land but breed in water, with the young metamorphosing (changing) into adult form from an early fish-like (tadpole) stage. There are three orders of amphibia[34]:

(i) Anura (frogs and toads).

(ii) Urodela (salamanders and newts).

(iii) Apoda (worm-like burrowers).

The anurans alone have been classified into some 24 families and more than 3,800 species, and are the essence of this research.

1.2b. Why study anurans?

Numerous organisms utilise toxic chemicals to protect themselves from predators and microbial pathogens, therefore increasing their chances of survival in the wild. However, of the many examples, none can be compared to that of anuran skin in regard to variety, and even more, concentration of these active compounds. Anuran skin is like a chemical warehouse, an extraordinary source of biologically active compounds. Anurans, and particularly the skins of anurans, have been used traditionally for medicinal purposes for more than 2000 years[35]. In ancient times powdered anuran skins were used to develop medicines such as heart stimulants and diuretics, and more recently in some parts of South America an anuran is simply strapped to an injury to treat any bacterial infections[36]. One example out of many where the chemically active properties of an anuran has been exploited is that of the poison dart frog (Figure 1.5). The native Indians from Central and South America use the skin secretion, from a number of anuran species known as poison dart frogs, as a killing agent[37]. The Indians poison the tips of their darts with the secretion of the poison dart frog. They hang the frogs by their legs over the fire, which stresses the frog and causes it to secrete a poison over its back. A dart is then wiped over the back, which transfers the secretion onto the tip of the dart. The Indians then use these darts to kill animals. The poison from the poison dart

frogs contains toxins that dull the nerves and can cause heart and respiratory failure. As little as 2 micrograms of the poison in some species can kill an adult human[38]. Each poison dart frog contains nearly 200 micrograms of the particular active substance. The poison from one of these anurans could kill 100 adult humans. Medical researchers have taken advantage of these toxins and are currently finding ways to make pharmaceutical agents such as muscle relaxants, heart stimulants, heart regulators and anaesthetics from the poisons[39]. An example is the analgaesic dermorphin (listed in Table 1.5), which was isolated from the poison dart frog *Phyllomedusa bicolour*[40].

Figure 1.5. Two different species of poison dart frogs from the genus *Phyllobates*.

Anurans live and breed in environments where there are numerous microbial pathogens (Figure 1.6). However, they appear to be essentially immune to infection[41]. This natural immunity is primarily due to their ability to generate a defence mechanism involving various host defence compounds. Some of the natural defence compounds that anurans produce include amines, steroid derivatives, peptides and toxic alkaloids[42]. The skin secretion contains a mixture of host defence compounds that exhibit a striking display of biological activity.

Figure 1.6. The typical environment of an anuran.

The host defence compounds within the skin secretions originate from specialised glands within the dermal layers. There are essentially two types of glands within the skins of most anurans, the granular glands and the mucosal glands[43]. The active components are exuded from the granular glands. These glands are dispersed throughout the dermal regions of the anuran. In some species the glands are localised and enlarged. The glands are strategically concentrated in areas most exposed to predatory attack. There are several specific types of hypertrophied glands occurring in anuran species[44]. These forms are classified according to their anatomical position, and are listed in Table 1.3.

Type of Hypertrophied Glands	Anatomical Position
Rostral	Dorsal surface of the head
Supralibial	Upper lip region extending beyond the jaw
Parotoid	Shoulder region
Submandibular	Region adjacent to lower jaw
Coccygeal	Flanks of each side of the coccyx
Inguinal	Each side of the groin region
Femoral	Postural surface of each femur
Tibial	Dorsal surface of each calf
Dorsal	Distributed throughout the surface of the back
Submental	Underneath the chin

Table 1.3. Classifications of the various hypertrophied glands present in anurans.

The action of the glands is under the control of the CNS, with the release of secretion occurring in response to a variety of stimuli including stress[45]. For example, when predatory snakes attack the South African clawed frog, it can secrete particular chemicals that induce a yawning reflex on the snake, allowing the anuran to escape unharmed[46]. Besides protecting the anuran from potential predators, many of the secreted chemicals from the skin function as antibacterial agents. These agents, mainly peptides, are released onto the skin either by adrenergic stimulation or in response to direct physical injury[47]. Many of these antibacterial agents have shown to be considerably potent, killing many strains of bacteria, protozoans and even cancerous cells[48]. These peptides are responsible for the extraordinary freedom from infection experienced by anurans.

Active peptides, including the antibacterial peptides, are stored as inactive, processed peptides within the granular glands of the skin[49]. The granular syncytial gland* is located directly below

* Syncytial gland is a gland composed of numerous types of cells fused together, producing a giant multinucleated cell or gland.

the epidermis gland (Figure 1.7). This type of gland consists of a myoepithelial cell envelope (the outer wall) on the periphery, with the nuclei*, endoplasmic reticulum† and Golgi complexes‡ located inside the cell. The lumen (tubular cavity) of the gland contains the secretory granules, where the peptides are stored[46].

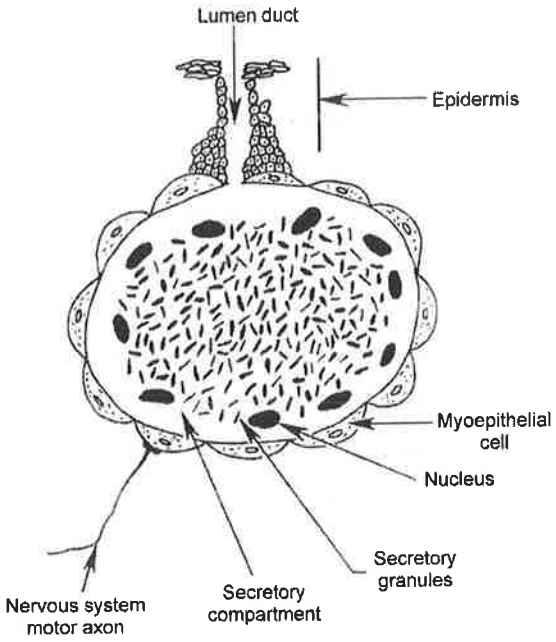

Figure 1.7. Representation of a granular gland found in anuran skin.

The production of the mature peptide occurs by the same fundamental process as described in section 6.1e. Transcription from the DNA to the mRNA transpires within the nucleus. This is followed by translation of the mRNA to produce the prepropeptide. The signal peptide (pre-segment) facilitates transportation within the cell and is subsequently cleaved[39]. The storage of the mature peptide is assisted by the spacer peptide (pro-segment). The spacer peptide counteracts the activity of the mature peptide by equalising the positive charges and assisting with the folding of the peptide to inhibit any enzymatic degradation[50, 51]. The mature peptide is

* Nucleus is a membrane bound structure containing the genetic information in the form of DNA organised into chromosomes.
† 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 involved in sorting proteins for transportation through the cell.
‡ Golgi complexes are individual stacks of membranes near the endoplasmic reticulum. They are involved in both the modifications of peptides, and in arranging them for transport to different cellular locations.

inactive in the propeptide form. On stimulation of the granular gland by the CNS, the propeptide is subjected to a series of specific enzymatic cleavages, which ultimately produces the active mature peptide[30]. In many cases the skin peptides are post-translationally modified in order for them to be active[52]. This includes specific endoproteolytic cleavage, carboxy-terminal alpha-amidation, tyrosine sulfation and amino acid isomerisation, suggesting that an array of processing enzymes is present in the granular gland[53]. The peptide is then released from the granule by a process known as the holocrine mechanism* and secreted onto the exterior surface of the skin[54]. The peptides can be readily synthesised at a low metabolic cost due to their relatively small size (as compared to specific antibodies or phagocytic cells) and they can be stored in large quantities that are rapidly available when required[55]. An interesting compositional characteristic of the majority of anuran skin peptides is their lack of cysteine residues, although such residues occur in many other biologically active polypeptides and proteins derived from other living systems[35]. Many of the antibacterial peptides are specific only to pathogenic microorganisms, making them ideal models for the development of therapeutic agents[56].

The search for therapeutic agents from anuran skins commenced in the 1960s. The pioneering work of Vittorio Erspamer and colleagues established the anuran integument as a rich source of biologically active peptides[57]. Since these initial studies that were conducted nearly four decades ago, many families of peptides have been identified, including angiotensins, bombesins, bradykinins, caeruleins, dermorphins, tachykinins, magainins and bombinins[35]. Some of the interesting features that distinguish anuran skin from that of other living systems include: (i) the abundant concentration of peptides present within the skin, and (ii) different species of anurans commonly store different types of pharmacologically active peptides within their skin glands[58]. With such an astounding diversity of abundant and readily accessible biologically active peptides, amphibians can be looked upon as a rich source of new and novel biologically active chemicals. This includes hormones, neuropeptides and antibacterial peptides that often are not found in mammalian systems. The chemical and pharmacological investigation of these compounds derived from anuran skin offers a promising pathway towards new medicinal discoveries.

* Holocrine mechanism is a process that involves the disintegration of a cell, resulting in the release of the cellular contents.

1.2c. Anuran antibacterial peptides

In mid 1980, several peptides within the skin of a particular anuran species (*Xenopus laevis*) were found to exhibit broad-spectrum antibacterial activity[59]. Their presence was revealed during an investigation for local sterilising agents, which had been initiated in order to explain the absence of local inflammation during the healing of surgical wounds to this particular vertebrate[60]. The new antibacterial peptides were named magainins 1 and 2, and their antibacterial activity could be demonstrated specifically against bacteria, fungi and protozoa at molar concentrations comparable to commercial antibacterial agents[61]. This generated significant interest into the examination of antibacterial peptides from anuran skin secretions.

The structures of antibacterial peptides have certain features in common. While the primary structures may vary significantly, the secondary structures of antibacterial peptides are crucial for their activity[62]. They usually have an overall positive charge due to the presence of basic residues (for example His, Arg and Lys) and possess the ability to form amphiphilic* structures (alpha helices)[63]. As stated earlier, research has shown that many of the antibacterial peptides are specific only to pathogenic microorganisms. The antibacterial peptides specifically target bacterial membranes due to the differences between erythrocyte (red blood cell) and bacterial membrane designs[64]. Bacterial membranes contain a greater concentration of anionic phospholipids, which enhance the affinity of the bacterial membrane for basic (cationic) antibacterial peptides[65]. In contrast, the erythrocyte membrane contains an insignificant amount of anionic phospholipids and those present are usually localised on the inner bilayer surface, orientated towards the cytoplasm† and away from the binding actions of any antibacterial peptides[66]. Furthermore, bacterial membranes lack cholesterol, a steroid that has the ability to decrease the affinity of most amphiphilic antibacterial peptides for the phospholipid bilayer[67]. In contrast, vertebrate cells contain a substantial amount of cholesterol[37]. The favoured binding of these basic peptides to negatively charged phospholipids might explain their capability to lyse cancerous cells, which essentially express anionic phospholipids on their membrane surface.

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* Amphiphilic molecules are molecules that contain both hydrophobic and hydrophilic moieties.

† Cytoplasm includes the organelles and fluid portion of the eukaryotic cell.

The exact mechanism of bactericidal action by antibacterial peptides is currently uncertain, but it is believed to involve the modification of the bacterial membrane by the formation of membrane channels or pores. These formations disrupt the normal functionality of the membrane, disrupting the osmotic regulation and ion transport functions, which ultimately lead to lysis and destruction of the cell[68]. Some peptides have shown selectivity for a specific bacterial membrane; this may be related to the number and distribution of positive charges on the antibacterial peptide. Experiments involving the synthetic D-enantiomers of these active L-peptides have shown that the D-enantiomers exhibit similar, if not identical, antibacterial activities as their native L counterparts[69]. Therefore, chiral receptors or enzymes do not play a role in their membrane activity. For example, the known antibacterial peptides L- and D-magainins, L- and D-caerins, and L- and D-cecropins have all shown to have identical activities with their isomeric counterparts[70,71,72].

Various structural experiments have shown that antibacterial peptides adopt alpha (α)-helical conformations in the vicinity of a membrane[63,73]. Further investigation of these helical antibacterial peptides indicated that there was a distinct segregation of hydrophobic and hydrophilic residues along the length of the helix (Figure 1.8). For example, the hydrophobic residues showed to aggregate on one side of the helix and the hydrophilic residues on the other. Peptides that exhibit these characteristics are known as amphiphilic peptides.

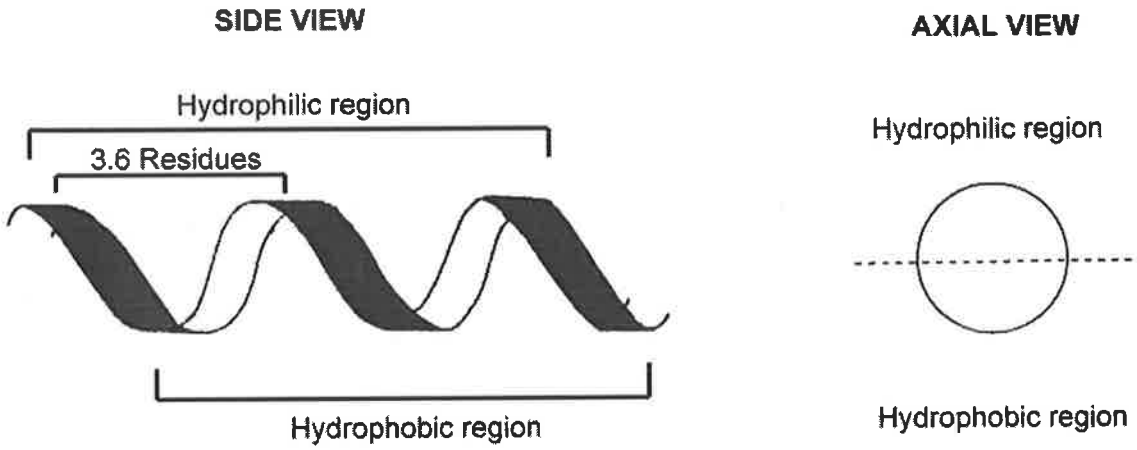


Figure 1.8. Representations of an amphiphilic α-helix.

The likelihood that a peptide may adopt an amphiphilic α-helix conformation can be predicted by using an Edmundson helical wheel projection. This is a two-dimensional representation of the three-dimensional structure of an α-helix. The perimeter of the wheel corresponds to the backbone of the polypeptide chain. The amino acid residues are projected onto a plane perpendicular to the axis of the helix. One turn of an α–helix is 3.6 residues long, therefore each adjacent residue can be spaced 100° apart around the circumference of the circle representation, as shown in Figure 1.9. This corresponds to 18 residues per entire revolution of helix. The residues are numbered from the N-terminus[74].

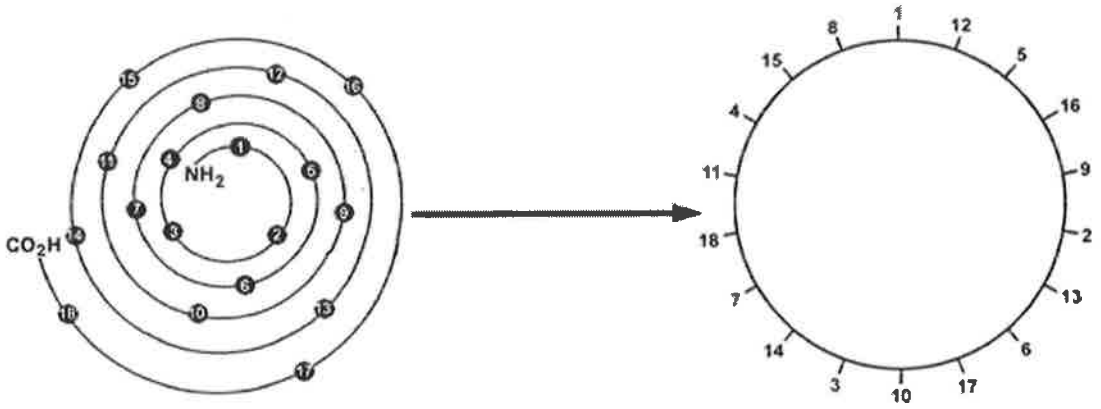


Figure 1.9. Edmundson helical wheel projection.

The projection of an amphiphilic peptide onto an Edmundson helical wheel generally shows distinct hydrophobic and hydrophilic zones.

An antibacterial peptide is usually initially in a random conformational state within the extracellular fluid[75]. The positively charged peptide then binds electrostatically to the bacterial membrane, which exhibits accessible negatively charged phospholipid head groups[67]. During binding, the lipid environment induces the amphiphilic peptides to undertake an α–helical structure[30]. The peptides arrange themselves in a parallel manner to the membrane with the hydrophobic regions buried within the lipid environment and the hydrophilic region exposed

to the aqueous environment. Formation of the amphiphilic α–helix is thought to promote this peptide absorption onto the surface of the bacterial lipid bilayer membrane[76].

The bacterial cell membrane is formed from a phospholipid bilayer, which is a double layer of phospholipid molecules (Figure 1.10) arranged with their hydrophobic fatty acid tails pointing inward. Many protein molecules are also embedded in the hydrophobic interior of the bilayer, in which numerous proteins are also suspended[40]. The protein molecules are involved in a number of functions including the regulation of ions and solutes throughout the cell[20]. These proteins, known as integral proteins, generally span the bilayer and protrude on either side. The portions embedded in the bilayer have hydrophobic surfaces, whereas the surfaces of those portions that extend beyond the bilayer are hydrophilic. The lipid bilayer functions as a natural barrier, and confines the flow of ions and other solutes throughout the cell. Any disruption to this membrane barrier will ultimately cause cell death.

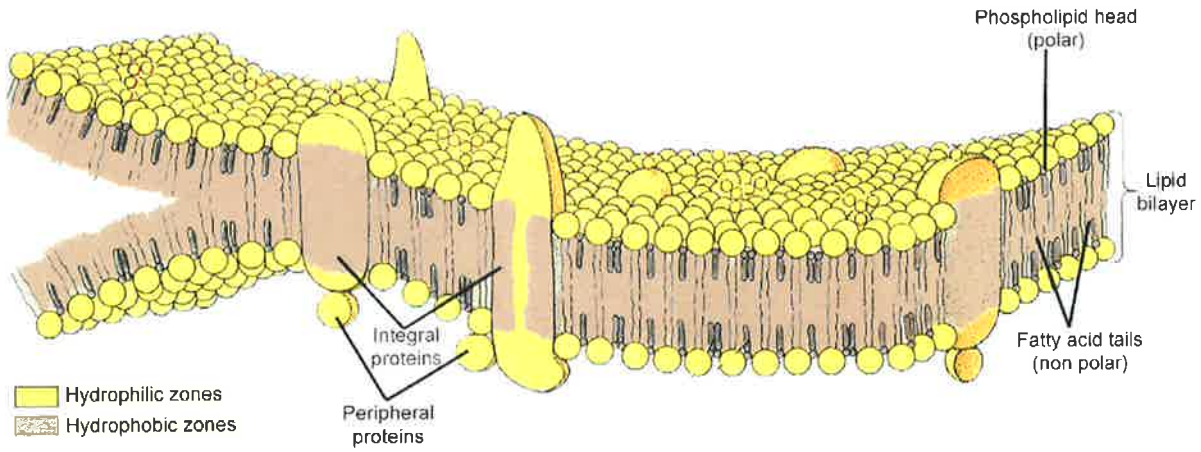


Figure 1.10. Bacterial cell membrane model.

Antibacterial testing for purposes of this research has been conducted using an assay known as a minimum inhibitory (MIC) test[77]. This assay involves the measurement of inhibition of the applied test substance (test peptide) against a range of microorganisms. The activities are then recorded as MIC values. The values therefore represent the minimum concentration of the test substance required to totally inhibit the growth of a particular microorganism. The lower the MIC value, the more potent the antibacterial activity.

An example of an antibacterial peptide is maculatin 1.1. Maculatin 1.1, from the skin secretion of the Australian tree frog *Litoria genimaculata*, exhibits wide spectrum antibacterial activity[78]. The MIC results are recorded in Table 1.4.

Peptide	**Sequence**
Maculatin 1.1	GLFGVLAKVAAHVVPAIAEHF (NH_2)
	MIC (μg/ml)
Bacillus cereus	25
Escherichia coli	
Leuconostoc lactis	3
Listeria innocua	100
Micrococcus luteus	12
Pasteurella multocida	50
Staphylococcus aureus	6
Staphylococcus epidermidis	12
Streptococcus faecalis	25
Streptococcus uberis	3

Table 1.4. Antibacterial activity of maculatin 1.1. Where there is no figure listed the MIC value is > 100 μg/ml.

The three-dimensional structure of maculatin 1.1 has been determined by nuclear magnetic resonance (NMR) studies. The NMR experiments have shown the solution structure of this peptide to be that shown in Figure 1.11.

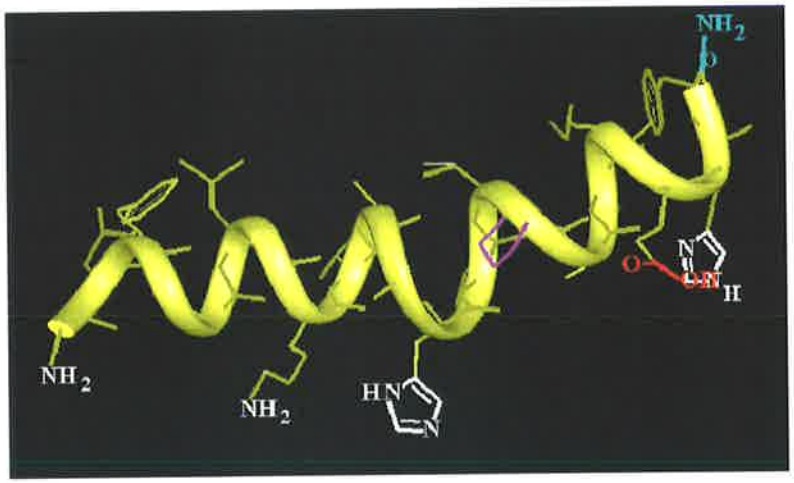


Figure 1.11. Three-dimensional structure of maculatin 1.1.

Maculatin 1.1 is essentially an α–helix with well-defined hydrophobic and hydrophilic faces, therefore making it amphiphilic in structure. The Pro^{15} residue distorts the structure from being a pure α–helix. A synthetic modification, where the Pro^{15} is replaced with Ala^{15} achieves a pure α–helix, but the antibacterial activity is lost. This indicates the importance of the proline residue, which functions like a 'hinge', allowing for considerable flexibility within the structure that is crucial for its antibacterial activity.

The exact mechanism for peptide antibacterial activity is currently unknown, but one accepted model that has been postulated is the 'Barrel-Stave' mechanism[79,80] (Figure 1.12). This mechanism suggests the peptides in solution are initially unstructured (random state), and that the positive charge distributions of these peptides cause them to attract to the polar head groups of the phospholipids in the bacterial cell membrane. This therefore initiates peptide association with the membrane surface through electrostatic interactions. On interaction with the membrane surface, the peptide adopts an amphiphilic α–helix that accommodates an energetically favoured low energy conformation when in close vicinity to the bacterial membrane. Solid state NMR has shown that these peptides are then orientated parallel to the membrane surface[81]. The helical amphiphilic 'stave-like' peptides then aggregate, bind and insert into the membrane, forming ion-sized pores. The pores increase in diameter through successive inducement of additional peptides. The pore is formed by the lateral arrangement of the peptides such that the hydrophobic face of the peptide is exposed to the membrane lipid region, and the hydrophilic face lines the pore. This results in the formation of a

transmembrane pore, which allows the movement of ions and small solutes across the bilayer. The flux of ions causes a disruption of the osmotic gradient, causing the cell to rapidly decompose.

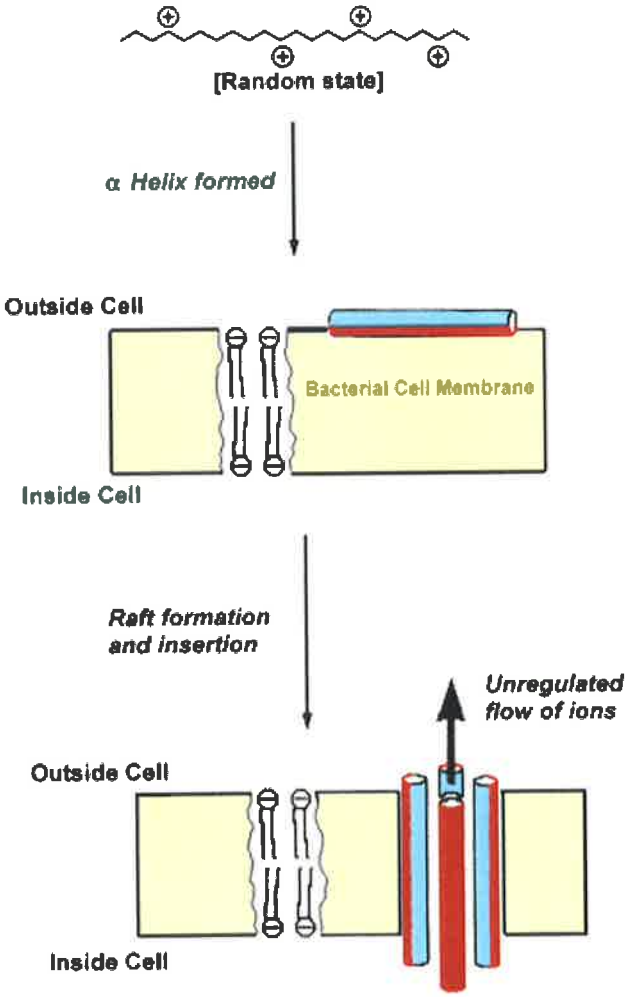


Figure 1.12. 'Barrel-Stave' model.

The 'Barrel-Stave' mechanism requires the peptide to be of an adequate length in order to span the entire bilayer. Experimental evidence has indicated that each amino acid residue in an α–helix is spaced from its adjacent residue by a distance of approximately 1.5 Å. The bacterial membrane is generally 30 Å in thickness. A peptide must therefore have a minimum of 20 residues to effectively span the bacterial membrane in order to produce the transmembrane pore. Antibacterial peptides smaller than this required length are therefore not supported by this postulation[82].

1.2d. Anuran neuropeptides

Various powerful neuropeptides have been found in anuran skin. For example, the majority of Australian anurans of the genus *Uperoleia* and *Litoria* contain a potent neuropeptide within their skin secretions. *Uperoleia* species usually produce the neuropeptide, uperolein, which is a hypotensive toxin and a member of the tachykinin family. Uperolein produces rapid smooth muscle contraction, vasodilatation and hypotensive action[83].

With the exception of a small Australian tree frog, known as *Litoria rubella*, all of the *Litoria* species studied to date contain the neuropeptide caerulein[84]. Caerulein is characterised by a post-translated tyrosine sulfate residue, and exhibits activity that modifies thermoregulation, pain reception and induces sedation[85]. Caerulein is several thousand times more potent as an analgaesic than morphine, and is often the major component of the skin secretions of the *Litoria* genus[35]. Some additional examples of neuropeptides derived from specific anurans are listed in Table 1.5.

Peptide	Anuran	Sequence	Function
Bombesin	*Bombina bombina*	pEQRLGNQWAVGHLM (NH_2)	Smooth muscle stimulant
Bradykinin	*Rana temporaria*	RPPGFSPFR (OH)	Vasodilator, Hypotensive
Crinia Angiotensin II	*Crinia georgiana*	APGDRIYVHPF (OH)	Hypotensive
Dermorphin	*Phyllomedusa bicolour*	YAFGYPS (NH_2)	Analgaesic
Tachykinin	*Physalaemus fuscumaculatis*	pEADPNKFYGLM (NH_2)	Vasodilator, Hypotensive
Uperin 1.1	*Uperoleia inundata*	pEADPNAFYGLM (NH_2)	Vasodilator, Hypotensive
Caerulein	*Litoria caerulea*	pEQDY(SO3)TGWMDF (NH_2)	Vasodilator, Hypotensive Analgaesic
Uperolein	*Uperoleia rugosa*	pEPDPNAFYGLM (NH_2)	Vasodilator, Hypotensive

Table 1.5. Examples of some neuropeptides, and the anuran from which the peptide was originally derived.

1.2e. The *Litoria* genus

The anuran order within Australia can be divided into five distinct families. These include Hylidae, Lepodactylae, Microhylidae, Ranidae and Bufonidae. Together these families comprise of twenty-nine genera. For example, the family Hylidae contains three genera, *Litoria*, *Nyctimystes* and *Cyclorana*[44]. The genus *Litoria* is one of the most diverse genera of anurans throughout Australia, consisting of some sixty species. The geographical populations of *Litoria* are also greatly diverse as they cover the majority of the Australian continent (Figure 1.13). Erspamer initiated the research of peptides from the genus *Litoria*, with the isolation of caerulein from the tree frog *Litoria caerulea*[86]. Arboreal anurans (tree frogs) are characterised by their flattened disc shaped fingers and toes. These digits secrete a sticky substance that aids climbing. Tree frogs usually spend the majority of their time in the high canopy of trees, generally only coming down to an aquatic environment to breed. The tree frogs *Litoria splendida*, *Litoria citropa* and *Litoria electrica* of the *Litoria* genus, are the primary subjects of the research presented in this thesis.

Figure 1.13. Distribution (indicated by the black region) of the genus *Litoria* throughout Australia.

1.3. Methodology

1.3a. Surface electrical stimulation

In the past, researchers that studied amphibian peptides often killed large numbers of anurans. The skins were removed, dried and the peptides extracted using methanol. Sometimes more than 1000 specimens were sacrificed in order to obtain enough material for identification[87]. The need for a non-harmful collection was therefore required since many anuran species are currently risking extinction. A method developed in 1981 involved the injection of noradrenaline into the dorsal surface of the anuran, inducing discharge of peptide material without harm to the anuran[88]. The method relevant to this research, developed in 1992, involves a surface electrical stimulation (SES) method[89]. The anuran is held while its skin is moistened by distilled water. A platinum electrode, which is attached to an electrical stimulator, is massaged over the glandular region (Figure 1.14). The mild electrical stimulation induces the release of the skin secretion.

Figure 1.14. SES method.

The stimulus strength varies in relation to the thickness and conductivity of the skin. After a short delay, the secretion is discharged and the entire milking process is completed in about 30 seconds. This SES method is simple, quick, harmless and repeatable, allowing research to be conducted by means of a single anuran rather than the extreme quantities sacrificed in earlier research. After stimulation, the crude secretion is rinsed off the skin with distilled water. The glands in some species can release up to 70-100 mg of peptide material. For example, *Litoria splendida* produces about 70 mg of peptide material for each secretion (Figure 1.15). The secretion is immediately worked on as the active peptides risk being degraded by any enzymes present within the secretion. This deactivation process is believed to be a safety mechanism in order to prevent any toxic peptides from affecting the host.

Figure 1.15. Crude aqueous secretion taken from *Litoria splendida*.

The contents of the glands are usually replenished within several days, so successive secretions can be readily obtained from the same anuran. The resulting crude secretion is separated and purified by HPLC.

1.3b. Analysis by HPLC

The crude skin secretions that are obtained are injected into a HPLC machine, where the peptides within the secretion are separated and purified. A reverse phase column is used for the chromatography. The peptides absorb onto the hydrophobic surface from the mobile phase and remain there until a sufficiently high concentration of organic solvent reaches the peptide and displaces it from the hydrophobic surface. Only one part or 'face' of the peptide molecule interacts with the hydrophobic surface of the reverse phase column. The remainder of the peptide is in contact with the mobile phase. Desorption of the peptide from the hydrophobic surface occurs when the concentration of the organic modifier reaches a particular concentration.

The ability to separate peptides by reverse phase HPLC is dependent on the subtle differences in the hydrophobic face of the peptide resulting from differences in the amino acid sequences. The organic modifier used in this research is acetonitrile. It is used because of its high ultraviolet transparency at low wavelengths, low viscosity resulting in low column back pressure and high column efficiency, and high volatility, which allows the easy removal of this solvent from peptide containing fractions. The aqueous solvent, which contains an ionic modifier to adjust the pH, solubilises the peptide. The ionic modifier used is trifluoroacetic acid (TFA) as it gives high-resolution separations and is volatile and easily removed. The appropriate wavelength used throughout the chromatography experiments to detect the peptide bonds ranged between 214 and 215nm.

Two HPLC systems were used in this research.

(i) Waters Millipore Lambda Max 481 LC spectrophotometer
Waters Millipore 510 HPLC pump
Waters Millipore 501 HPLC pump
ICI DP 800 data interface
ICI DP 800 data station

(ii) ICI 1200 UV/VIS detector LC spectrophotometer
ICI LC 1100 HPLC pumps (X2)
ICI DP 800 data interface
ICI DP 800 data station

1.3c. Mass spectrometry

In its simplest form, mass spectrometry is a technique that allows measurement of the molecular weight of a molecule, and is designed to perform three basic functions:

(i) To produce ions from vaporised compounds (ionisation).
(ii) To separate these ions according to their mass to charge (*m/z*) ratios (analysis).
(iii) To subsequently distinguish and record the resulting separated ions (detection).

The discovery of positive rays in 1886 led to the birth of mass spectrometry[90]. Since then mass spectrometry has greatly developed. The single sector (single focussing) mass spectrometers developed in the early 1900s were replaced by the double sector mass spectrometers and tandem double focussing mass spectrometers[91]. The search for greater resolution and sensitivity resulted in the development of the three and four sector mass spectrometers. With the advancement of biotechnology many other types of mass spectrometry techniques and machines were produced. Some of these included the reverse geometry, flowing afterglow, time of flight, ion cyclotron, Fourier ion cyclotron resonance and ion trap mass spectrometers. Along with the mass analysers, the techniques of ion generation also evolved with time. From the early emission of positive ions from the discharge tube, to electron impact ionisation, chemical ionisation and field desorption. The progressions of biological analysis led to further developments of desorption ionisation techniques. These included secondary ion mass spectrometry, fast atom bombardment, plasma desorption ionisation, matrix assisted laser desorption and electrospray ionisation techniques. Developments in these areas of ion production, analysis and detection have expanded the powerful analytical applications of mass spectrometry into many fields of research.

The structural determination of all peptide fractions isolated in this research was predominantly characterised by mass spectrometry experiments. The research involved the use of two mass spectrometers. Early research involved analysis by a Vacuum Generators ZAB 2HF mass spectrometer, which includes all work described in chapter 2. Further into the period of research a Finnigan LCQ electrospray mass spectrometer was acquired and used in the work described in chapter 3 and thereafter.

1.3d. Vacuum Generators ZAB 2HF mass spectrometer

The Vacuum Generators ZAB 2HF mass spectrometer (ZAB) is a two-sector reverse geometry instrument[92]. The reverse geometry refers to the arrangement of the sectors, with the magnetic (B) sector preceding the electric (E) sector. This arrangement has several advantages over the conventional electric followed by magnetic sector geometry. The major advantage is the ability of the reverse geometry instrument to mass select a parent ion and record the spectrum of its fragment ions by scanning the voltage across the electric sector. This technique is known as mass analysed ion kinetic energy (MIKE) spectrometry. A schematic diagram of the ZAB mass spectrometer is shown in Figure 1.16.

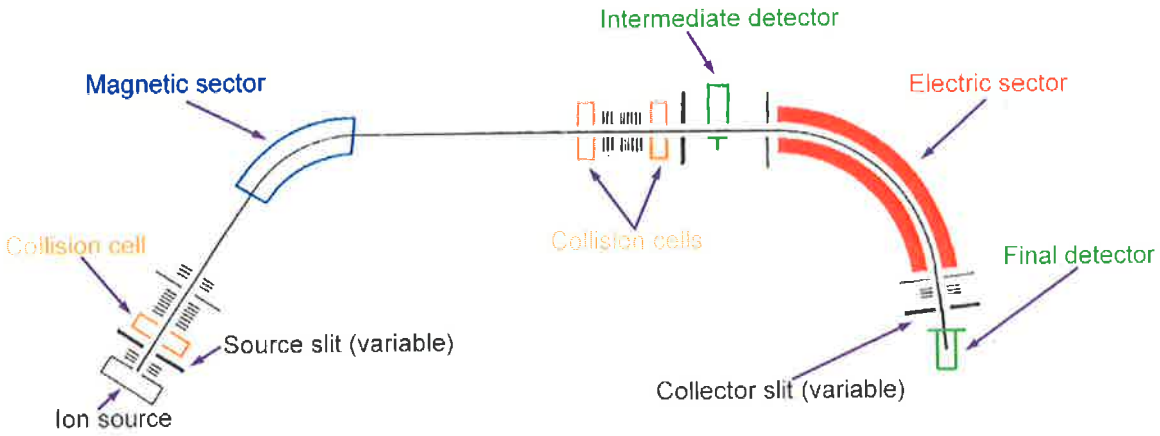


Figure 1.16. Schematic of the VG ZAB 2HF double focussing reverse sector mass spectrometer.

Ionisation was achieved for this research by fast atom bombardment (FAB) ionisation. The FAB technique is a variation of secondary ion mass spectrometry using a liquid matrix[93]. The sample is dissolved in a liquid matrix and then bombarded by a high-energy beam of atoms. The high-energy beam is generated from an inert gas like argon or xenon. In this research argon was used as the inert gas. Argon atoms are released from the FAB gun (Figure 1.17) and enter a high potential field that ionises the atoms producing argon ions (Ar^+). The Ar^+ ions are then accelerated over a range of 8-10keV and passed through a gas of neutral argon atoms. Electron transfer then occurs between the Ar^+ ions and the stationary Ar atoms (Equation 1.1),

which results in a beam containing a high proportion of fast atoms. Fast ions that escape the charge transfer process are removed by electrostatic deflection.

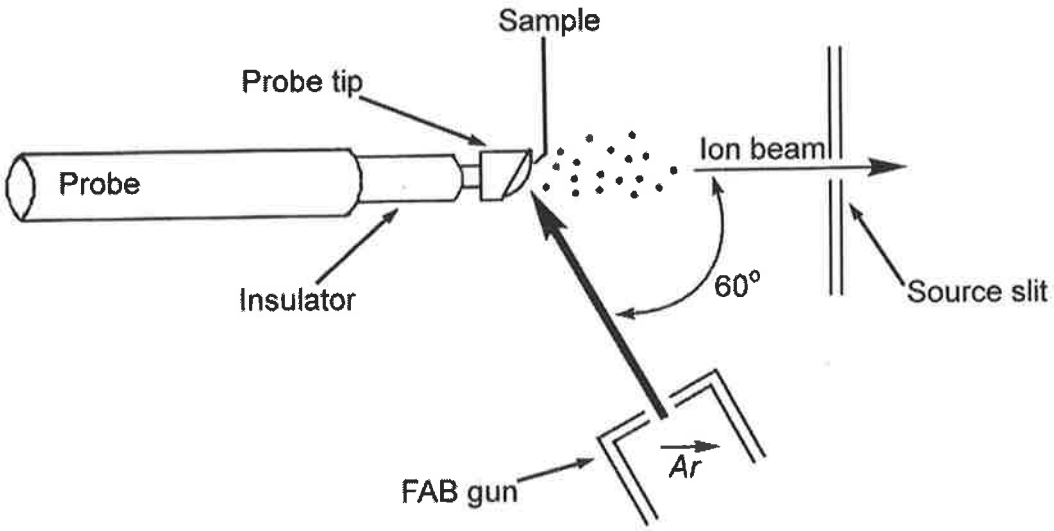


Figure 1.17. Schematic representation of the FAB source.

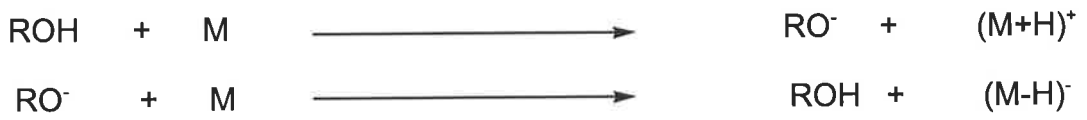
$$\overrightarrow{Ar^{+}}_{\alpha} + Ar_{\beta} \longrightarrow \overrightarrow{Ar}_{\alpha} + Ar^{+}_{\beta}$$

Fast ions — Fast atoms

Equation 1.1.

The fast atoms impact the sample/matrix mixture, which creates a dispersing effect. The dispersion results by momentum transfer from the impacting atoms to the target, producing random collision chains. Some of the dispersed material consists of sample molecules that have crossed the surface into the gas phase as neutrals. Abundant positively or negatively charged ions, which have interacted with the matrix before entering the gas phase, constitute the remainder. The resulting gas phase ions can then be accelerated and mass analysed in the spectrometer.

The key attribute to the success of FAB is the liquid matrix, which enhances the production and duration of the secondary ion beam[94]. The matrix promotes the production of a well-defined and relatively long lasting spectrum from the moment the primary beam is switched on. The matrix softens the impact of the fast atoms as it absorbs some of the momentum and therefore reduces sample damage. An ideal matrix is one that is polar and of low volatility. The most commonly used matrix is glycerol. The formation of ions occurs by a number of processes including those shown in scheme 1.1.



Scheme 1.1.

After the ions are produced in the source, they are accelerated by a strong electrostatic field potential V (volts) and focussed through the source slit into the analyser region. The ions are focussed through both the magnetic and electric sectors. The kinetic energy ($1/2mv^2$) of the ion is given by Equation 1.2, 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)[95].

$$1/2mv^2 = zV \qquad \text{Equation 1.2.}$$

The flight path of the ion when entering the magnetic sector is perpendicular to the lines of the magnetic field B (tesla). The ion is then exposed to a centripetal force (BzV) that is perpendicular to both the magnetic field and path of the ion. This is further balanced by a centrifugal force (mv^2/r) that causes the ions to transverse the magnetic sector, where r is the radius of the curved path. The motion of this path is outlined by Equation 1.3.

$$Bzv = mv^2/r \qquad \text{Equation 1.3.}$$

Rearrangement of Equation 1.3 indicates that the magnetic sector disperses the ions according to their momentum (mv) to charge (z) ratios as outlined in Equation 1.4[96].

$$mv/z = B/r \qquad \text{Equation 1.4.}$$

When a fixed magnetic field strength is focussed on a specific mass, the ions of different mass but same velocity that are entering the magnetic field are defocussed by their mass to charge ratios (m/z). Only the ions with the correct mass to charge ratios follow the trajectory through the central radius (r_c) of the magnetic sector. The m/z value of the ions that emulate this trajectory are outlined in Equation 1.5, where $r_c = r$.

$$m/z = B^2r^2/2V \qquad \text{Equation 1.5.}$$

Scanning the magnetic field results in the separation of different m/z ions and a routine spectrum is obtained. The electric sector can focus the ion beam as a function of kinetic energy. The ions that are focussed enter the electric field where the lines of force are perpendicular to the path of the ion. Because of this perpendicular force, the ion pathway becomes circular as outlined in Equation 1.6.

$$mv^2/r = zE \qquad \text{Equation 1.6.}$$

Equation 1.6 is further arranged to give Equation 1.7.

$$mv^2/z = Er \qquad \text{Equation 1.7.}$$

The field strength controls the passage of ions, with particular energies and velocities, through the electric sector. As r is fixed, ion kinetic energy (IKE) spectra of ions containing different energies can be obtained by scanning the electric field strength. The resulting ions are detected after the electric sector by a photon multiplier. The modes of scanning that were applied for the acquisition of data in this research were primarily low-resolution scans and MIKE spectroscopy.

Low-resolution scans* were used to determine routine molecular weight information of the peptide samples. Generally the magnet sector was linearly scanned from *m/z* 3000 to 300, using a mass resolution of 1500 (10% valley definition) as shown in Figure 1.18. The resolution between two adjacent peaks of equal height, m and $m + \Delta m$, is obtained from the ratio of $m/\Delta m$.

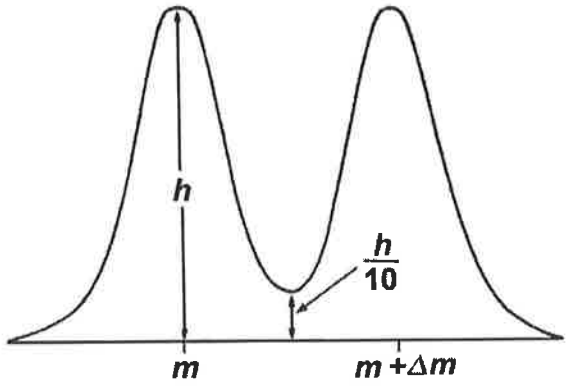


Figure 1.18. Representation of 10% valley definition.

The collisional activation mass analysed ion kinetic energy spectroscopy (CA MIKES) technique applied to peptides generates a number of fragments, which often lead to the identification of at least a portion of the amino acid sequence of the peptide[97]. Since FAB is a relatively soft ionisation technique, the resulting parent ions have little excess internal energy, and therefore produce little fragmentation. Collisional activation is then used to induce fragmentation of the parent ion. The metastable spectrum of the peptide fragment ions are initiated by focussing and accelerating ions of a particular mass into the second collision cell that contains the inert gas. The pressure is controlled so that some of the ions undergo a single collision with an inert gas atom. In these instances, a small proportion of translational energy (normally <5eV) of the ion is converted into internal (vibrational, electronic and rotational) energy, resulting in fragmentation. The daughter ions produced from the fragmentation of the parent peptide ion are then analysed by scanning the electric field, according to the kinetic energies of the daughter ions. This technique, used in accordance with other classical methods of peptide and protein sequencing, leads to an efficient method of peptide sequence analysis.

* Low-resolution scans are scans that obtain data with accuracy to at least unit resolution.

1.3e. Finnigan LCQ electrospray mass spectrometer

The Finnigan LCQ electrospray mass spectrometer used in this research is an octapole ion trap mass spectrometer equipped with a liquid chromatography system (Figure 1.19).

Figure 1.19. Finnigan LCQ mass spectrometer.

The internal schematic arrangement of the Finnigan LCQ mass spectrometer (LCQ) is represented in Figure 1.20. The LCQ is considerably more sensitive (low-picomole range) and easier to use when compared with the ZAB. The mass range for analysis is also higher (50-4000 Da) with a mass accuracy of about 0.01%. This capability of analysing molecules of considerable size is also due to the fact that the electrospray technique can exhibit multiply charged states, which then can be interpreted. Other capabilities of the LCQ are its MS^n function, zoom scanning and on line liquid chromatography options. This makes the LCQ an ideal tool for the analysis of large biological molecules at minimal quantities.

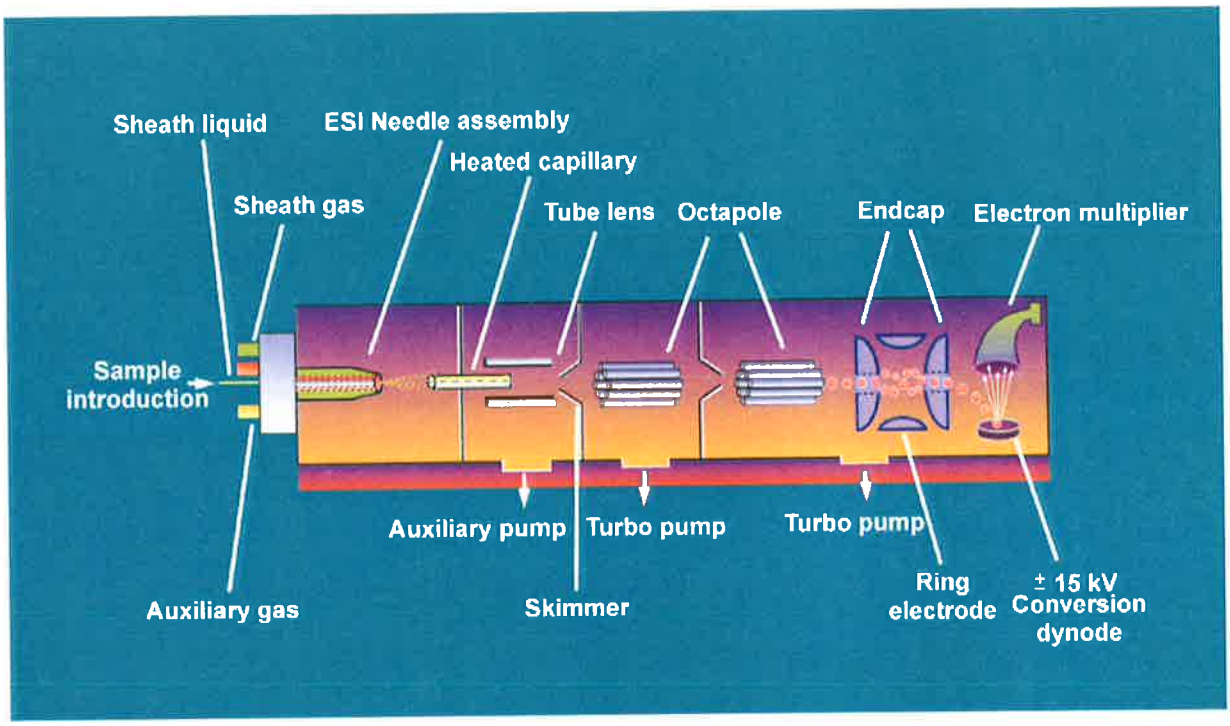


Figure 1.20. Schematic diagram of the LCQ.

Electrospray (ES) ionisation is a soft ionisation process that is capable of producing intact ions, with multiple charges, from remarkably large, complex, and fragile parent species[98]. The ES technique involves the application of a high voltage electric field to a flow of sample liquid within a heated capillary. The capillary is generally held at 4000V, resulting in a spray of charged droplets. As the droplets evaporate, their surface density increases until the forces due to electrostatic repulsion approach equality with those due to surface tension. Extreme instability results, sometimes called a 'Coulomb explosion', which produces an array of daughter droplets that also evaporate until they too 'explode'. This process continues until the ultimate droplets contain only one solute molecule. As the last of the solvent evaporates from the droplets, the solute molecules produce a series of free ions (Figure 1.21) that retain the charge. The free ions are then passed through the octapole region into the ion trap.

Multiply charged ions often result from peptides using the ES ionisation technique. Multiply

charged ions are resolved through a process known as a ZoomScan*. This technique allows the *m/z* values of the isotopes of the multiply charged ions to be determined. The molecular difference between the isotope peaks is inversely proportional to the charge state of that particular envelope. For example, for an isotopic difference of 0.5 Da the charge state of that envelope would be +2. For a difference of 0.25 Da the charge state would be +4, and so on.

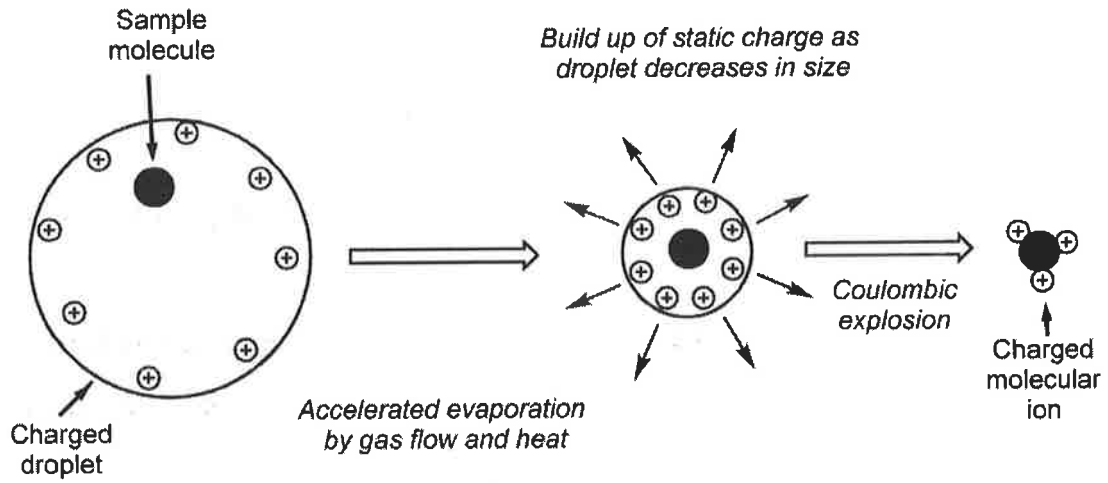


Figure 1.21. Electrospray ionisation mechanism.

The ion trap is the mass analyser of the LCQ. The ion trap is comprised of three electrodes and a central ring electrode, each of a hyperbolic cross section, forming a chamber in which the ions are confined (Figure 1.22)[99]. Application of a radio frequency (rf) voltage to the ring electrode establishes an electric field in which the force of an ion is proportional to its distance from the centre of the trap. This allows ions of appropriate *m/z* to have stable trajectories within the device and to be trapped for many seconds. For the recording of a mass spectrum, the amplitude of the rf voltage is increased. This causes ions of increasing *m/z* to become unstable. At this point, they are ejected in mass sequence from the trap through perforations in the end cap and recorded by the detector as a mass spectrum.

* ZoomScan is a feature of the LCQ that allows a high resolution scan over a narrow mass window (~10 amu). With this high resolution scan it is possible to resolve quadruply-charged ion clusters with molecular weights of up to 8000 Da. ZoomScan is particularly useful to cleanly resolve multiply charged peptide ions obtained from electrospray ionisation analysis.

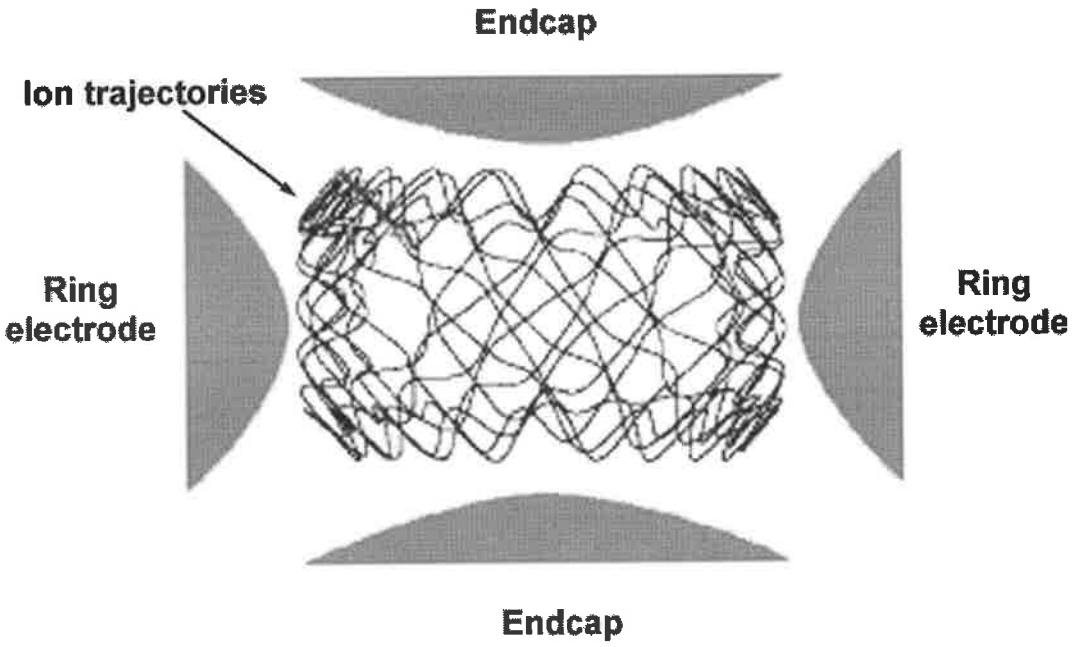


Figure 1.22. Representation of an Ion trap and the simulation of ion trajectories within the trap.

Fragmentation of the chosen ions is carried out by collisional activated dissociation (CAD)[100]. The ion trap contains helium at a pressure of $1x10^{-3}$ Torr. The purpose of this gas is to act as a damping gas for ions injected into the trap, act as the collision gas when performing CAD by resonance excitation of a precursor ion, and to improve the general performance of the trap. An ion of interest is firstly isolated within the ion trap, with the internal energy increased such that it undergoes a series of rapid collisions with the helium gas. A succession of fragmentations result[101]. The MS^2 spectrum is obtained as the resulting daughter ions are then sequentially ejected from the ion trap and detected. MS^n experiments involve the same process except the CID is repeated. For example, one of the resulting daughter ions of interest is further isolated and collisionally activated and the granddaughter ions are then scanned, and so on. Theoretically, this process can continue until there are no more ions to collisionally activate.

In most instances, the experiments in this research were carried out under automatic gain control (AGC), where the instrument performs a short prescan before each analytical scan. This is so the appropriate ionisation time is set, and the trap filled with the appropriate number of ions for each scan regardless of the concentration of the eluting analyte.

1.3f. Peptide sequencing

The sequences of all the peptides presented in this thesis were characterised predominantly by analysing their characteristic mass spectral fragmentations in the positive mode. The peptide fragmentation is initiated by the collision activation of the chosen peptide, as described earlier. In the positive mode, several characteristic fragmentations of linear peptides are possible. For sequencing purposes however, simple B and Y+2 cleavage ions are used[102]. The B and Y+2 cleavage ions are used, as they generally are the most abundant fragmentations observed within the applied conditions. Simple B cleavage ions give sequence information from the C-terminal end of the peptide, and Y+2 cleavage ions (which include proton transfer) give sequence information from the N-terminal end (Figure 1.23). This sequencing technique applies for experiments conducted both on the ZAB and the LCQ mass spectrometers. The foremost limitation of this technique is that it cannot distinguish certain isomeric and isobaric amino acid residues within the peptide sequence. These residues include the isomers Ile (113 Da) and Leu (113 Da), and the isobars Lys (128 Da) and Gln (128 Da). The isomers Ile and Leu are distinguished using automated Edman sequencing techniques, while the isobars Lys and Gln are distinguished by enzyme digestion (specifically Lys-C digestion) experiments and/or automated Edman sequencing.

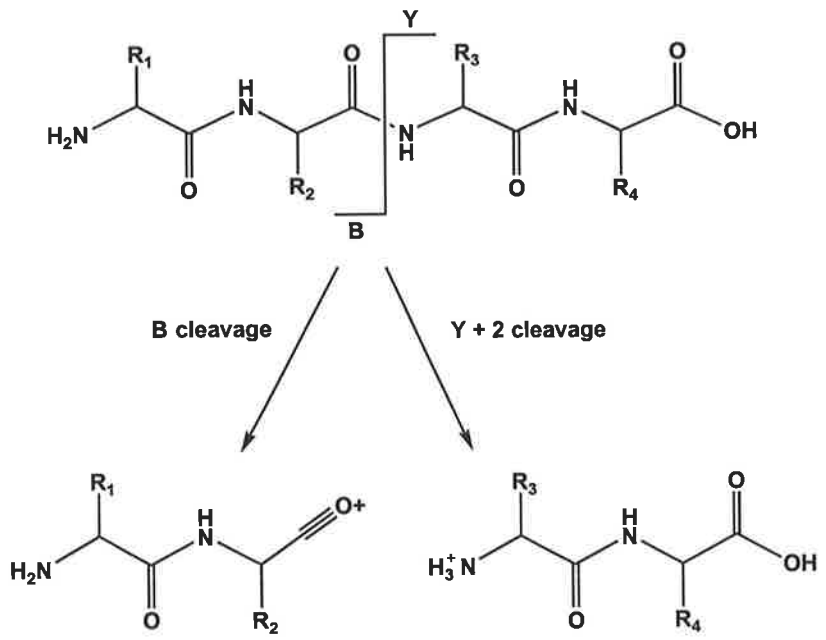


Figure 1.23. Characteristic fragmentations of linear peptides in the positive mode.

All automated Edman sequencing was performed by a standard procedure using an applied Biosystem 470A sequencer equipped with a 900A data analysis module[103]. The peptides were dissolved in aqueous acetonitrile and absorbed onto a disc of immobilon film treated with bioprene in ethanol. The disc was perforated to aid the flow of solvent. Automated Edman sequencing allows the peptide primary sequence to be determined by the sequential removal of amino acids from the N-terminal end of the peptide. There are some limitations to this technique. Peptides with blocked N-terminal ends, for example a peptide containing pyroglutamate as the first residue, cannot be sequenced in this manner, since the blocked group prevents coupling with one of the reagents. Uncommon or modified amino acids are also unable to be detected by automated Edman sequencing. Furthermore, as the peptide is sequentially cleaved, its size decreases and it becomes more soluble and risks being washed from the solid support, losing the remaining portion of the peptide before full sequence information is gained.

Enzyme digestion experiments together with mass spectrometry have also been employed in the research described in this thesis in order to obtain further sequence information. Enzyme digestion is particularly useful when the peptide is quite large and difficult to sequence by collision activation alone. Enzyme digestion of the peptide results in smaller fragments that are then sequenced by mass spectrometry. The primary digest used for the research described in this thesis is Lys-C, since the majority of the larger peptides studied generally contain at least one lysine residue. Cleavage occurs at the carboxyl side of the lysine residue (Figure 1.24).

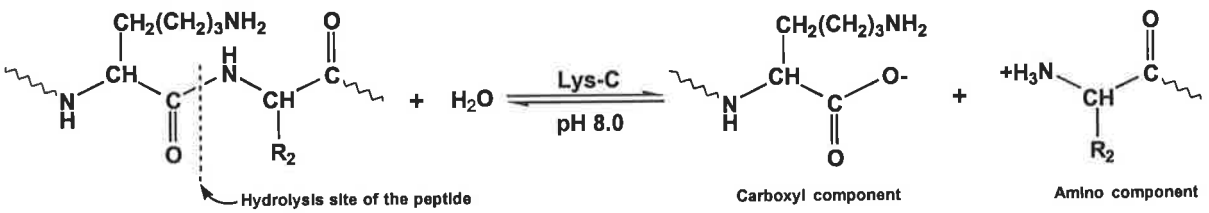


Figure 1.24. Lys-C digestion.

1.3g. C-terminal end group determination

The C-terminal end groups of the peptides were identified following their conversion into methyl esters. The resulting methyl esters were then analysed by mass spectrometry. The mass of the parent ion of the methyl ester minus that of the mass of the original peptide allows the determination of the number of CO_2H and $CONH_2$ groups in the peptide. For example, an amide group will show an increase of 15 Da on methylation, and an acid group will show an increase of 14 Da as outlined in Figure 1.25.

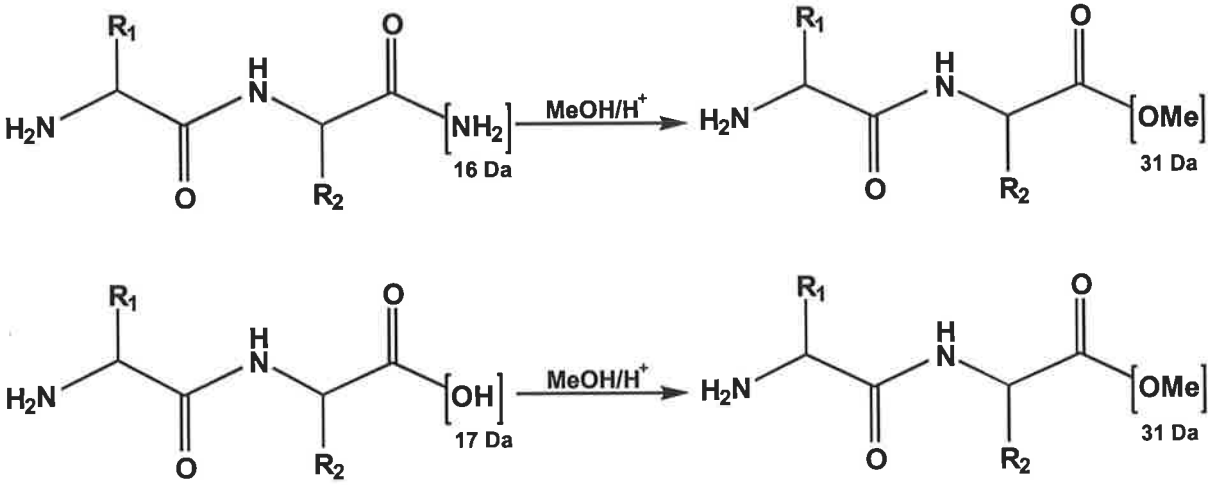


Figure 1.25. Determination of the C-terminal end groups by methylation.

1.3h. Preparation of synthetic peptides

Many of the new peptides described in this thesis were synthesised in order to have sufficient material available for biological testing. Peptide synthesis was undertaken by Chiron Mimotopes (Clayton, Victoria) using L-amino acids through the standard N-α-Fmoc procedure[104]. Each synthetic peptide was checked that it was identical with the native peptide by both HPLC analysis and mass spectrometry.

The masses of all peptides and their corresponding fragments are recorded as nominal* masses.

* The nominal mass of a peptide is equal to the sum of the integral masses of the amino acid residues.

1.4. References

[1] Rang, H.P. and Dale, M.M., in *Pharmacology*, 243-280 (Churchill Livingstone, London, UK, 1992).

[2] Milner-White, E.J., *Trends in Pharmacol. Sci.*, **9**, 290-295 (1989).

[3] Martinez, J. and Potier, P., *Trends in Pharmacol. Sci.*, **7**, 139-147 (1986).

[4] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y. and Kobayashi, M., *Nature*, **332**, 411-415 (1988).

[5] Burger, E., *Trends in Pharmacol. Sci.*, **38**, 754-761 (1994).

[6] Arseniev, A.S., Barsukov, V.F. and Ovchinnikov, Y.A., *FEBS Lett.*, **186**, 168-170 (1985).

[7] Foreman, J.C. and Jordan, C.C., *Trends in Pharmacol. Sci.*, **5**, 116-119 (1984).

[8] Lynch, D.R. and Snyder, S.H., *Annu. Rev. Biochem.*, **55**, 373-399 (1986).

[9] Maggio, J.E., *Annu. Rev. Neurosci.*, **11**, 13-28 (1988).

[10] Swanson, L.W., *Trends in Neurosci.*, **6**, 294-295 (1983).

[11] Iversen, L.L., *Trends in Neurosci.*, **6**, 291-293 (1983).

[12] Bonta, I.L. and Parnham, M.J., *Int. J. Immunopharmac.*, **4**, 103-109 (1982).

[13] Jordan, C.C., in *Substance P: metabolism and biological actions* (ed. Oehme, P.) 113-156 (Taylor and Francis, London, 1985).

[14] Srinivasa, R.N., Gibson, B.W., Tang, D. and Spindel, E.R., *J. Biol. Chem.*, **267**, 6916-6922 (1992).

[15] Carraway, R.E., Cochrane, D.E. and Ruane, S.E., *J. Biol. Chem.*, **262**, 15886-15889 (1987).

[16] Dimaline, R., *Peptides*, **4**, 457-462 (1983).

[17] Erspamer, V. and Anastasi, A., *Experientia*, **18**, 58-59 (1962).

[18] Erspamer, V., Anastasi, A., Bertaccini, G. and Cei, J.M., *Experientia*, **20**, 489-490 (1964).

[19] Buck, S.H. and Burcher, E., *Trends in Pharmacol. Sci.*, **7**, 65-68 (1986).

[20] Curtis, H. and Barnes, N.S., in *Biology* (ed. Anderson, S.) 150-207 (Worth, New York, NY, 1990).

[21] Bladier, D and Herkovitz, J., *FEBS Lett.*, **223**, 330-334 (1987).

[22] Coleman, R.A., Humphrey, P.A. and Kennedy, I., *Trends in Pharmacol. Sci.*, **5**, 303-306 (1984).

[23] Corrigan, J.J., *Science*, **164**, 142-149 (1969).

[24] Blondelle, S.E. and Houghten, R.A., *Biochemistry*, **31**, 12688-12694 (1992).

[25] Sherwood, L., in *Human physiology* (ed. Marshall P.) 93-112 (West, St. Paul, MN, 1989).

[26] Fisher, G.H., Vetere, A., Padula, L. and Man, E.H., *Brain Res. Bull.*, **26**, 983-985 (1991).

[27] Guglietta, A., Irons, B.J., Lazarus, L.H. and Melchiorri, P., *Endocrinology*, **120**, 2137-2143 (1987).

[28] Feurle, G.E., Hamscher, G., Meyer, H.E. and Metzger, J.W., *J. Biol. Chem.*, **267**, 22305-22309 (1992).

[29] Johnson, A.H. and Rehfeld, J.F., *Eur. J. Biochem.*, **207**, 419-428 (1992).

[30] Hauser, F. and Hoffman, W., *J. Biol. Chem.*, **34**, 24620-24624 (1992).

[31] Kera, Y., Nagasaki, H., Iwashima, A. and Yamada, R.H., *Comp. Biochem. Physiol.*, **103**, 345-348 (1989).

[32] Kuks, P.M., Creminon, C., Leseney, A. and Cohen, P., *J. Biol. Chem.*, **264**, 14609-14612 (1989).

[33] Campbell, G., *Annu. Rev. Pharmacol. Toxicol.*, **27**, 51-70 (1987).

[34] Barker, J., Grigg, G. and Tyler, M.J., in *A field guide to Australian frogs* (Surrey Beatty, Norton, NSW, 1995).

[35] Lazarus, L.H. and Attila, M., *Prog. Neurobiol.*, **41**, 473-507 (1993).

[36] Daly, J.W., Caceres, J., Moni, R.W, Gusovsky, F., Moos, M., Seamon, K.B., Milton, K. and Myers, C.W., *Proc. Natl. Acad. Sci. USA*, **89**, 10960-10963 (1992).

[37] Carraway, R.E., and Cochrane, D.E., *J. Biol. Chem.*, **261**, 15886-15889 (1987).

[38] Labler, L., Keilova, H., Sorm, F. and Styblova, S., *Toxicon*, **5**, 247-251 (1968).

[39] Negri, L., Lattanzi, R. and Melchiorri, P., *Brit. J. Pharm.*, **114**, 57-66 (1995).

[40] Mignogna, G., Severini, C., Simmaco, M., Negri, L., Falconeri Erspamer, G., Kreil, G. and Barra, D., *FEBS Lett.*, **302**, 151-158 (1992).

[41] Kreil, G., in *Antimicrobial Peptides: Ciba Foundation Symposium 186*, 72-92 (Wiley and Sons, New York, NY, 1994).

[42] Montecucchi, P.C., De Castiglone, R., Piani, S., Gozzini, L. and Erspamer, V., *Int. J. Pept. Protein. Res.*, **17**, 275-277 (1981).

[43] Erspamer, V., in *Amphibian biology: the integument*, **1**, 178-350 (Surrey Beatty, Norton, NSW, 1994).

[44] Tyler, M.J., in *Encyclopaedia of Australian animals* (ed. Strahan, R.) 3-42 (Harper Collins, London, UK, 1992).

[45] Jackson, I. M. and Reichlin, S., *Science*, **198**, 414-415 (1977).

[46] Barthalmus, G.T. and Zielinski, W.J., *Pharmacol. Biochem. Behav.*, **30**, 957-959 (1988).

[47] Tyler, M.J., in *Toxic plants and animals: a guide for Australia* (ed. Strahan, R.) 329-338 (Queensland Museum, Brisbane, QLD, 1987).

[48] Boman, H.G., *Cell*, **65**, 205-207 (1991).

[49] Zasloff, M., *Proc. Natl. Acad. Sci. USA*, **84**, 5449-5453 (1987).

[50] Terry, A.S., Poulters, L., Williams, J.C., Hutkins, M. and Giovannini, C.H., *J. Biol. Chem.*, **263**, 5745-5749 (1988).

[51] Dockary, G.J. and Hopkins, C.R., *J. Cell Biol.*, **64**, 724-731 (1975).

[52] Flucher, B.E., Lenglachner, K., and Mollay, C., *J. Cell Biol.*, **64**, 724-726 (1985).

[53] Erspamer, V., Falconeri Erspamer, G., Mazzanti, G. and Endean, R., *Comp. Biochem. Physiol.*, **77**, 99-105 (1984).

[54] Hernandez, C., Mor, A. and Dunia, I., *Eur. J. Cell Biol.*, **59**, 414-424 (1992).

[55] Moore, K.S., Bevins, C.L., Brasseur, M.M. and Zasloff, M., *J. Biol. Chem.*, **29**, 19851-19857 (1991).

[56] Maloy, W.L. and Kari, U.P., *J. Peptide Sci.*, **37**, 105-122 (1995).

[57] Bevins, C.L. and Zasloff, M., *Annu. Rev. Biochem.*, **59**, 395-414 (1990).

[58] Zasloff, M., *Proc. Natl. Acad. Sci. USA*, **84**, 5549-5551 (1987).

[59] Zasloff, M., Martin, B. and Chen, H.C., *Proc. Natl.Acad. Sci. USA*, **85**, 910-913 (1988).

[60] Soravia, E., Martin, B. and Zasloff, M., *FEBS Lett.*, **228**, 337-340 (1988).

[61] Giovannini, M.G., Poulter, L., Gibson, B.W. and Williams, D.H., *J. Biochem.*, **243**, 113-120 (1987).

[62] Saberwal, G. and Nagaraj, R., *Biochem. et Biophys. acta.*, **1197**, 109-131 (1994).

[63] Marion, D., Zasloff, M. and Bax, A., *FEBS Lett.*, **227**, 21-25 (1988).

[64] Hoffmann, W., *J. Biol. Chem.*, **263**, 7686-7690 (1988).

[65] Jacob, L. and Zasloff, M., in *Antimicrobial peptides: Ciba Foundation Symposium 186,* 197-223 (Wiley and Sons, New York, NY, 1994).

[66] Williams, R.W., Starman, R., Taylor, K.P., Beeler, T., Covell, D. and Zasloff, M., *Biochemistry*, **29**, 4490-4496 (1990).

[67] Nakajima, Y., Qu, X.M. and Natori, S., *J. Biol. Chem.*, **262**, 1665-1669 (1987).

[68] Waal, A., Gomes, A.V., Mensick, A. and Grootegoed, J.A., *FEBS Lett.*, **293**, 219-223 (1991).

[69] Wade, D., Boman, A., Wahlin, B., Drain, C.M., Andreu, D., Boman, H.G. and Merrifield, R.B., *Proc. Natl. Acad. Sci. USA*, **87**, 4761-4765 (1990).

[70] Wade, D., Boman, A., Wahlen, B., Drain, C.M., Andreu, D., Boman, H.G. and Merrifield, R.B., *Proc. Natl. Acad. USA*, **87**, 4761-4764 (1990).

[71] Wong, H., Bowie, J.H. and Carver, J.A., *Eur. J. Biochem.*, **247**, 545-557 (1997).

[72] Merrifield, R.B., Merrifield, E.L., Juvvadi, P., Andreu, D. and Boman, H.G., in *Antimicrobial peptides: Ciba Foundation Symposium 186,* 5-17 (Wiley and Sons, New York, NY, 1994).

[73] Eisenberg, D., *Annu. Rev. Biochem.*, **53**, 595-623 (1984).

[74] Schiffer, M. and Edmundson, A.B., *J. Biophys.*, **7**, 121-135 (1967).

[75] Amodeo, P., Motta, A., Tancredi, T., Salvadori, S., Tomatis, R. and Temussi, P.A., *Peptide Res.*, **5**, 48-55 (1992).

[76] Westerhoff, H.V., Juretic, D., Hendler, R.W. and Zasloff, M., *Proc. Natl. Acad. Sci. USA*, **86**, 6597-6599 (1989).

[77] Jorgensen, J.H., Clealand, W.A., Craig, G., Doern, M., Ferraro, J., Finegold, C.M., Hansen, S.L., Jenkins, S.G., Novick, W.J., Pfaller, M.S., Preston, D.A., Reller, L.B. and Swanson, J.M., *National committee for clinical laboratory standards Doc. M7-A3*, **13**, 1-12 (1993).

[78] Rozek, T., Waugh, R.J., Steinborner, S.T., Bowie, J.H., Tyler, M.J. and Wallace, J.C., *J. Peptide. Sci.*, **4**, 111-115 (1998).

[79] Ehrenstein, G. and Lecar, H., *Rev. Biophys.*, **10**, 1-34 (1977).

[80] Ojcius, D.M. and Youbg, J.D., *Trends Biochem. Sci.*, **16**, 225-229 (1991).

[81] Ramamoorthy, A., Marassi, F.M., Zasloff, M. and Opella, S.J., *J. Biomol. NMR*, **6**, 329-334 (1995).

[82] Eisenberg, D., *Annu. Rev. Biochem.*, **53**, 595-603 (1984).

[83] Erspamer, V., Negri, L., Falconeri Erspamer, G. and Endean, R., *Arch, Pharmac.*, **289**, 41-54 (1975).

[84] Bowie, J.H., Chia, B.S.C. and Tyler, M.J., *Pharm. News*, **5**, 16-21 (1998).

[85] Erspamer, V. and Melchiorri, P., *Pure Appl. Chem.*, **35**, 463-494 (1973).

[86] Anastasi, A., Erspamer, V. and Endean, R., *Arch. Biochem. Biophys.*, **125**, 57-68 (1968).

[87] Roseghini, M., Erspamer, W. and Endean, R., *Comp. Biochem. Physiol.*, **54**, 31-33 (1976).

[88] Nakajima, T., *Trends Pharmacol. Sci.*, **2**, 202-205 (1981).

[89] Tyler, M.J., Stone, D.J. and Bowie, J.H., *J. Pharm. Toxicol. Methods*, **28**, 199-200 (1992).

[90] Thompson, J.J., *Philos. Mag.*, **13**, 561-564 (1907).

[91] Beynon, J.H., Cooks, R.G., Amy, J.W., Baitinger, W.E. and Ridley, T.Y., *Anal. Chem.*, **45**, 1023-1035 (1973).

[92] Morgan, R.P., Beynon, J.H., Bateman, R.H. and Green, B.N., *Int. J. Mass Spectrom. Ion Physics*, **28**, 171-178 (1978).

[93] Barber, M., Bordoli, R.S., Sedgwick, R.D. and Tyler, A.N., *Anal. Chem.*, **54**, 645-658 (1982).

[94] Biemann, K. and Martin, S.A., *Mass Spectrom. Rev.*, **6**, 2-14 (1987).

[95] Boyd, R.K. and Beynon, J.H., *Org. Mass Spectrom.*, **12**, 163-170 (1977).

[96] Cooks, R.G., Beynon, J.H., Caprioli, R.M. and Lester, R.G., in *Metastable ions* (Elsevier, Amsterdam, 1973).

[97] Busch, K.L., Glish, G.L. and McLuckey, S.A., in *Mass spectrometry: Techniques and applications* (VCN, New York, NY,1988).

[98] Fenn, J.B., Mann, M., Meng, C.K. and Wong, S.F., *Mass Spectrom. Rev.*, **9**, 37-70 (1990).

[99] Cooks, R.G. and Kaiser, R.E., *Acc. Chem. Res.*, **23**, 213-219 (1990).

[100] March, R.E., *Int. J. Mass Spectrom. Ion Processes*, **118**, 71-135 (1992).

[101] Jonscher, K.R. and Yates, J.R., *Anal. Biochem.*, **244**, 1-15 (1997).

[102] Roepstrorff, P. and Fohlman, J., *J. Biomed. Mass Spectrom.*, **11**, 601-607 (1984).

[103] Hunkapiller, M.W., Hewick, R.M., Drewer, W.J. and Hood, L.E., *Methods Enzymol.*, **91**, 399-406 (1983).

[104] Maeji, N.J., Bray, R.M., Valerio, R.M. and Wang, W., *Peptide Res.*, **8**, 33-38 (1995).

CHAPTER 2. Investigation of Bioactive Peptide Development in the Tadpole Cycle of *Litoria splendida*

2.1. Introduction

2.1a. General

Adult anurans have well-developed defence mechanisms that protect them against host invasions by microorganisms. Some of these mechanisms include non-specific physical and chemical barriers, highly specific cell mediated responses, and immune mechanisms involving antibacterial peptides[1]. Of these various defence mechanisms, the skin and the secretions released from the skin glands are vital in protecting the anuran from bacterial and fungal growth*. As described in section 1.2b, there are two types of skin glands in anurans: mucosal and granular. Each type of gland is located near the surface and connected to it via narrow ducts, with both glands being under the control of sympathetic nerves[2].

As indicated previously, anuran granular glands are a rich source of biologically active peptides. Structurally, the glands are made up of large multi nucleated cells containing a cytoplasm filled with large rice shaped granules[3]. These granules store many biologically active peptides, usually antibiotic peptides and neuropeptides. These are discharged on breakdown of the plasma membrane and delivered to the surface epithelium through a duct. Discharge occurs following stressful stimuli or direct injury to the skin[4]. The granules containing the biologically active peptides also undergo osmotic lysis on contact with the water bathing the skin surface[5]. A hydrophobic gel interspersed with peptides and hormones is therefore produced covering the skin, along with an endopeptidase that inactivates the peptides some time after secretion[6]. A system such as this, which produces abundant antibiotics from the epithelium covering the external skin, demonstrates a vertebrate that

* The skins of amphibians that had been detoxified died from skin infections within a few days.

invests its epithelial surfaces with cellular equipment designed to deliver highly-localised concentrations of broad-spectrum antibiotics. Almost every microbe that is exposed to the concentration of antibiotic released will be killed [7]. The epithelium not only serves as a physical barrier, but as an actively defensive barrier capable of killing potent microbial agents.

Anurans in their larval stages (tadpoles) lack the presence of glands within their skin prior to metamorphosis[8, 9]. Their larval skin consists of a thin doubled layered epidermis and a thin dermis. This is replaced in adult skin by granular glands formed during metamorphosis*. The epithelium in the tadpole therefore appears to be very important in serving as a physical barrier to predators, but with the glands absent, the actively defensive properties are unknown[2]. This is interesting, as tadpoles appear to be resistant to pathogenic microorganisms and fungal growth at virtually all stages of development[10]. However, the source of this acquired immunity in tadpoles is currently unknown[5]. Are peptides with antibacterial properties involved in the immunity observed in larval premetamorphic and postmetamorphic tadpoles? Past studies by various researchers have indicated that peptides are present during and after metamorphosis[11]. However, to date there has been no evidence indicating the presence of any active peptide material before metamorphosis. The following examples summarise the type of research carried out previously to investigate this phenomenon.

Seki et al. studied the development of glands producing caerulein in tadpoles of the African anuran *Xenopus laevis*, using immunohistochemistry, HPLC fluorometric systems and radioimmunoassay[9]. Immunohistochemistry, a technique that involves the specific immunoreactivity of antibodies targeted towards a particular compound, was used to observe the initial appearance of caerulein in the developing granular glands. HPLC fluorometry and radioimmunoassay were used to determine the concentration of caerulein in the extracts of *Xenopus* skin during metamorphosis. The results indicated that caerulein was first detected at the beginning of metamorphosis, during the development of the granular glands. The concentration of caerulein increased from that point onwards as the overall development of the tadpole proceeded. No trace of caerulein was found prior to metamorphosis.

* Metamorphosis involves the remodeling of almost every tissue within the amphibian in response to thyroid hormone

Clark et al. investigated the expression of an antibacterial peptide, named ranalexin, from the skin of an American bullfrog *Rana catesbeiana*[12]. This research was carried out by a screening technique directed towards the messenger ribonucleic acid (mRNA) sequence, encoding for a particular form of ranalexin. Ranalexin mRNA was targeted as it encodes the amino acid sequence for the production of the peptide ranalexin. If ranalexin is present within the larval tissue, the mRNA will also be present. The mRNA screening technique is quite sensitive and is known as northern blot analysis (Figure 2.1).

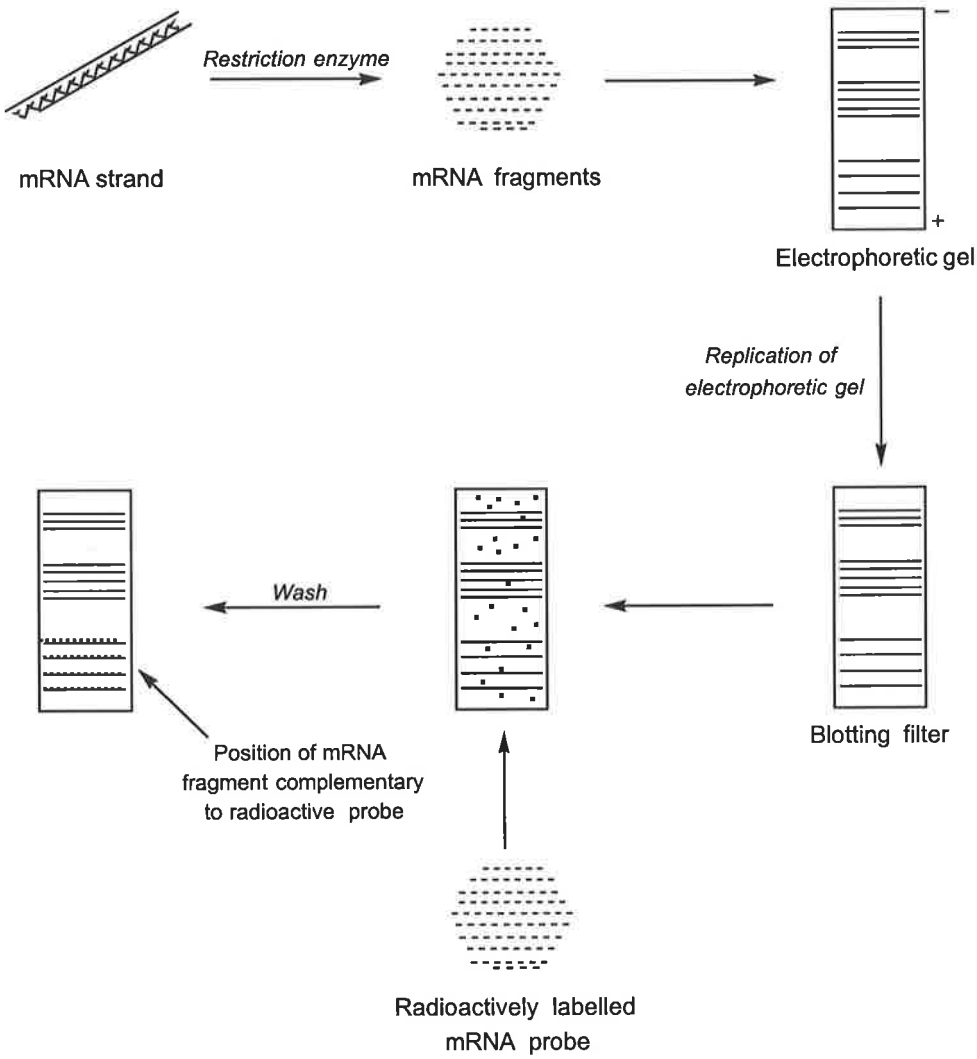


Figure 2.1. Northern Blotting. The mRNA from the sample is isolated and digested by one or more restriction enzymes. Resulting fragments are separated by size on an electrophoretic gel and then blotted onto a specially prepared filter. Therefore, the filter contains a replica of the fragments on the gel. The mRNA fragments are then deactivated, attached and exposed to a radioactive probe. The radioactive labelled probe is a short segment of mRNA labelled with a radioactive isotope. The filter is then treated and washed by a special process, which then reveals the location of the fragments complementary to the probe. The fragments are then purified ready for further analysis.

The results showed that ranalexin mRNA could not be detected in premetamorphic tadpoles, but it was present in the skin of tadpoles undergoing metamorphosis. It was concluded that ranalexin mRNA appeared as an abundant gene product in tadpole skin at metamorphosis, and continued to be expressed within the skin into the adult stage of development. This implied the presence of the peptide ranalexin during metamorphosis and in those stages following metamorphosis. The analytical technique used in this research was effective, but it was only targeted towards one specific amino acid sequence. No analysis was directed towards the detection of a broad range of peptide material before metamorphosis. Therefore no information is available as to the presence of other peptides before metamorphosis in this particular species.

Reily et al. conducted research using similar principles and techniques to that of *Clark et al.* *Reily et al.* investigated the expression of magainin and PGLa* peptides from the skin of *Xenopus laevis* [2]. Again, the results indicated that the peptides were only present during and after metamorphosis. However, the research was also only targeted towards a specific form of peptides (in this case two specific forms) for this particular species. To date, no broad screening for peptide material in premetamorphic tadpoles has been carried out. This raises the question as to how the tadpole is protected from microbial pathogens prior to metamorphosis.

The primary aspect of this research was to consider the possibility that biologically active peptides may be present within the larval skin and contribute to the immunity of the tadpole. This research was intended to investigate this possibility, and to examine whether peptide development could be monitored throughout the developing stages of a particular species of anuran. *Litoria splendida* was chosen for this study.

* PGLa is an abbreviation for peptide with amino-terminal glycine and carboxy-terminal leucinamide.

2.1b. *Litoria splendida*

Litoria splendida (Figure 2.2) is one of the largest tree frogs in Australia, measuring about 12 cm in length[13], and is located in the Kimberley region of Western Australia (Figure 2.3). Secretions containing peptide material can be obtained from the large glands distributed around the front and back of the head. These enlarged (hypertrophied) glands are known as the rostral and parotoid glands. *Litoria splendida* was chosen for this study as the glandular secretion has already been investigated and the skin peptides have been identified[14]. The skin peptide profile of *Litoria splendida* is also the simplest observed for the genus *Litoria* studied to date. All of the peptides isolated from this species have been tested for biological activity.

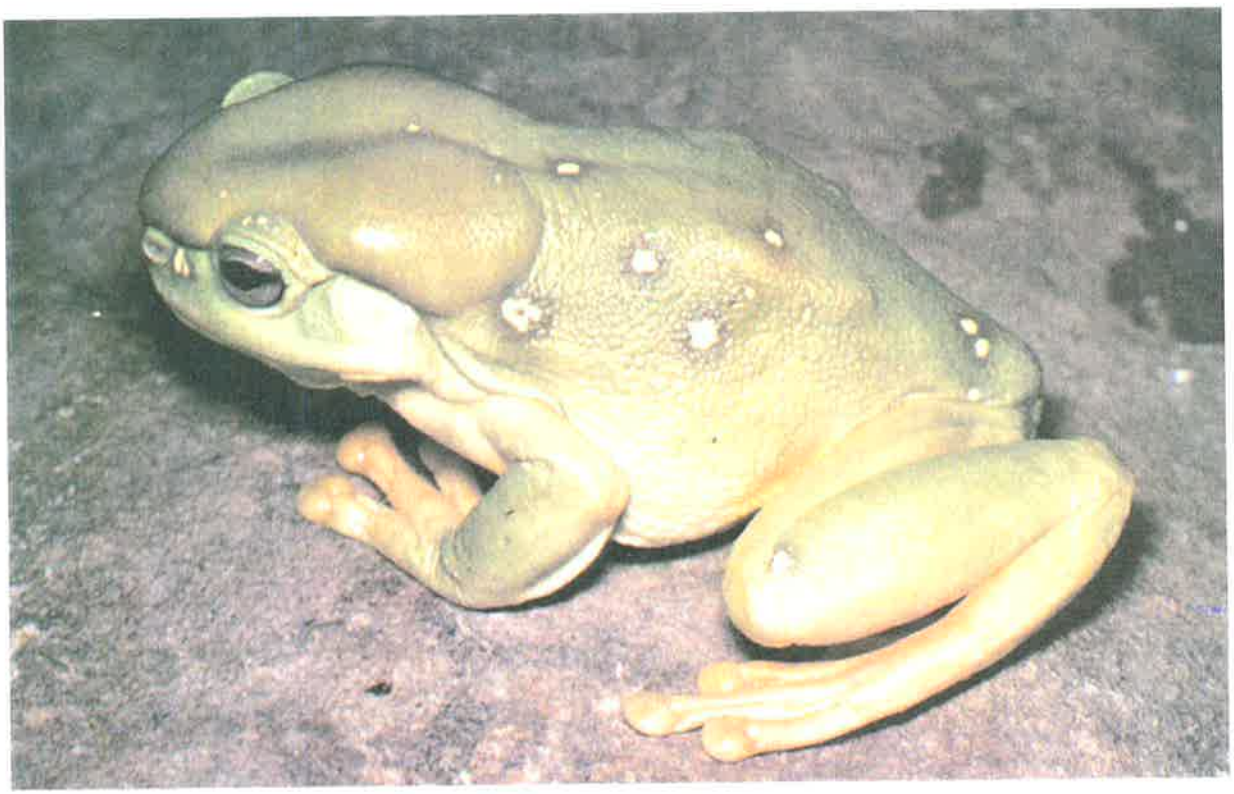


Figure 2.2. *Litoria splendida*.

Figure 2.3. Map of Australia indicating the location of *Litoria splendida*. Distribution indicated by the black region.

Previous analysis of the crude skin secretion of *Litoria splendida* by HPLC, indicated 5 major peptide fractions (A, B, C, D, and E) and 2 minor peptide fractions (F, G) to be present within the secretion (Figure 2.4).

The structures of the peptides isolated from the adult secretion were determined by classical mass spectrometry methods. The structural details of these peptides are listed in Table 2.1. One of the major peptide fractions (A) was identified as the known neuropeptide caerulein. As described in section 6.2d, caerulein is a known hypotensive toxin already isolated from the closely related species *Litoria caerulea*[15]. Caerulein is a very active analgaesic with an activity several thousand times higher than that of morphine[16]. Caerulein also has an extremely powerful action on the muscular wall of the gall bladder and demonstrates a very strong stimulus to the gastrointestinal tract. The properties of caerulein have allowed it to be used clinically for procedures such as treating cholecystography and postoperative paralysis of the gastrointestinal tract[17]. The other peptides were named as indicated in Table 2.1.

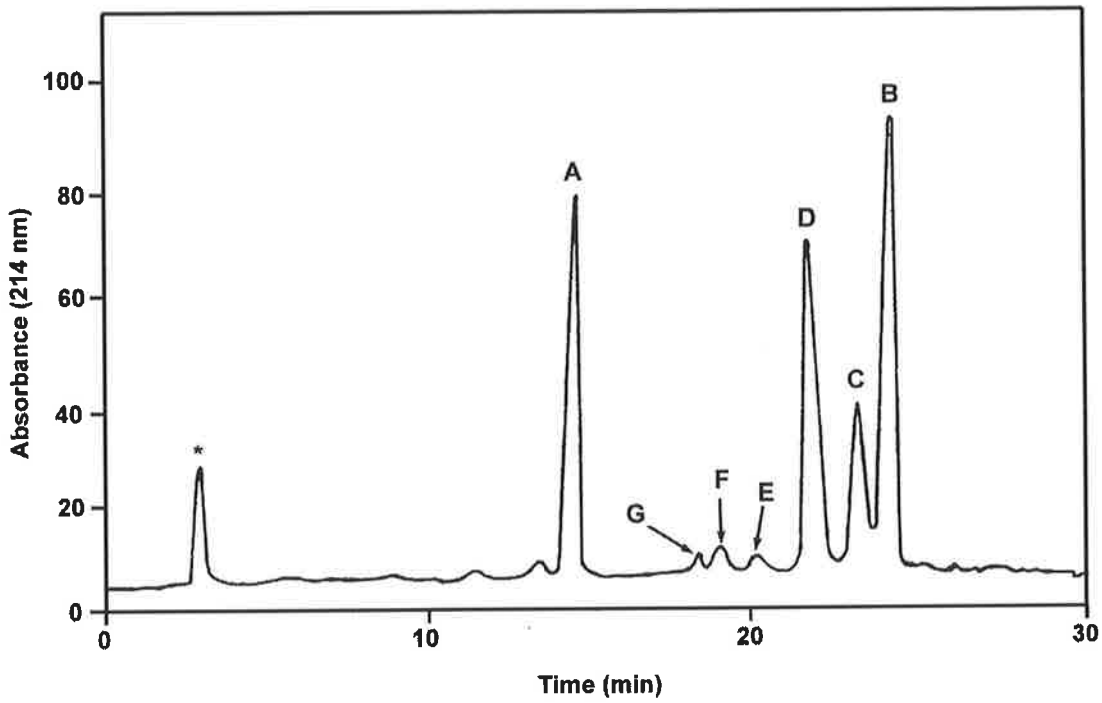


Figure 2.4. HPLC chromatogram from the crude skin secretions of *Litoria splendida* (As taken in 1991 by *Stone et al.* [14]). * Non peptide material.

Peptide	MW	Sequence	HPLC fraction
Caerulein	1354	pEQDY(SO_3)TGWMDF(NH_2)	A
Caerin 1.1	2582	GLLSVLGSVAKHVLPHVVPVIAEHL(NH_2)	B
Caerin 2.1	2392	GLVSSIGRALGGLLADVVKSKGQPA(OH)	C
Caerin 3.1	2382	GLWQKIKDKASELVSGIVEGVK(NH_2)	D
Caeridin 1.1	1140	GLLDGLLGTLGL(NH_2)	E
Caerin 1.1.1	2412	LSVLGSVAKHVLPHVVPVIAEHL(NH_2)	F
Caerin 1.1.2	2299	SVLGSVAKHVLPHVVPVIAEHL(NH_2)	G

Table 2.1. Primary structures of the skin peptides isolated from *Litoria splendida*.

Some of the caerin peptides listed in Table 2.1 exhibit potent antibacterial activity. For example, caerin 1.1 was tested against the microorganisms listed in Table 2.2. The results showed caerin 1.1 to be a wide spectrum antibiotic. The MIC results are recorded in Table 2.2.

	MIC (μg/ml)		
	Caerin 1.1	Caerin 2.1	Caerin 3.1
Bacillus cereus	50		
Escherichia coli			
Leuconostoc lactis	1.5		
Listeria innocua	25		
Micrococcus luteus	12		<0.4
Pasteurella haemolytica	25	25	
Pasteurella multocida	25		
Staphylococcus aureus	3		
Staphylococcus epidermidis	12		
Streptococcus faecalis	25		
Streptococcus uberis	12		

Table 2.2. Antibacterial activity of caerins 1.1, 2.1 and 3.1. Where there is no figure listed the MIC value is > 100 μg/ml.

The three-dimensional structure of caerin 1.1 has been determined by nuclear magnetic resonance (NMR) studies[18]. The NMR experiments have shown the solution structure of caerin 1.1 to be that displayed in Figure 2.5, which indicates the amphiphilic nature of this peptide. Caerin 1.1 is a membrane active antibacterial peptide, with the hydrophobic and hydrophilic regions being clearly defined within the three-dimensional representation. This peptide shows to have two helices separated by a complex central hinge around Pro[15]. The effect of proline in forming hinges or kinks in α-helices is well known[19]. The hinge region of caerin 1.1 assists this basic peptide with the efficient interaction of different types of bacterial membrane surfaces, particularly those containing an excess of anionic residues. Replacement of either one or both proline residues with alanine or glycine increases the overall helicity of caerin 1.1, but significantly reduces the antibacterial activity of this peptide. This indicates the importance of the hinge regions within caerin 1.1. The glandular secretions of *Litoria splendida* also contain endopeptidases that degrade caerin 1.1 after it has been on the skin for several minutes, forming the inactive peptides caerin 1.1.1 and caerin 1.1.2.

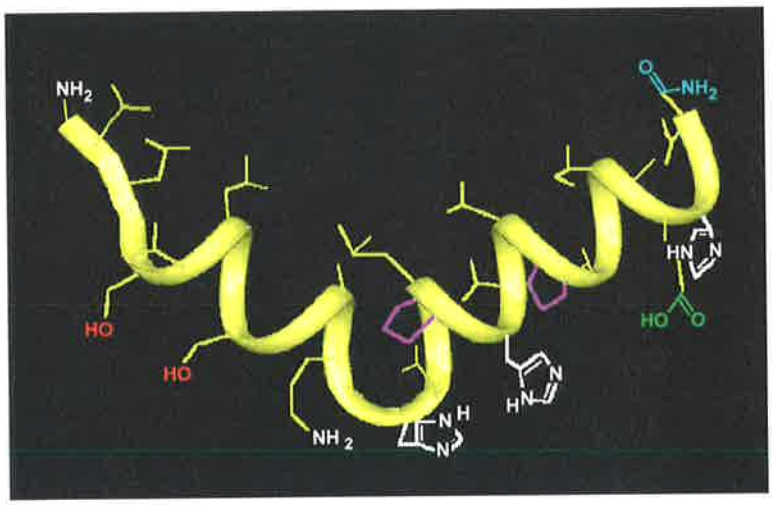


Figure 2.5. Three-dimensional structure of caerin 1.1.

Caerin 2.1 is active against *Pasteurella haemolytica* and caerin 3.1 is active against *Micrococcus luteus*, however, both peptides are not active against the other test organisms listed in Table 2.2. Caeridin 1.1 shows no antibacterial activity. It was of interest to determine whether any of the peptides, listed in Table 2.1, are present in the tadpoles of *Litoria splendida* prior to metamorphic climax.

2.1c. Development cycle of *Litoria splendida*

The amphibian development cycle of *Litoria splendida* has been divided into 46 stages of development (Figure 2.6)[20]. Metamorphic climax is the most spectacular stage. It is at this stage that the aquatic tadpole loses its tail and transforms into a terrestrial adult that has fully developed limbs[18]. Initial formation of granular glands also takes place during metamorphosis. The development cycle of *Litoria splendida* is complete within 13 weeks.

The aims of the work presented in this chapter are threefold:

(i) To monitor the tadpole (larval) development cycle of *Litoria splendida* for the production of peptides.

(ii) To investigate whether any peptides are present before metamorphosis in this particular species.

(iii) To investigate whether the peptides, if present, appear in a specific order.

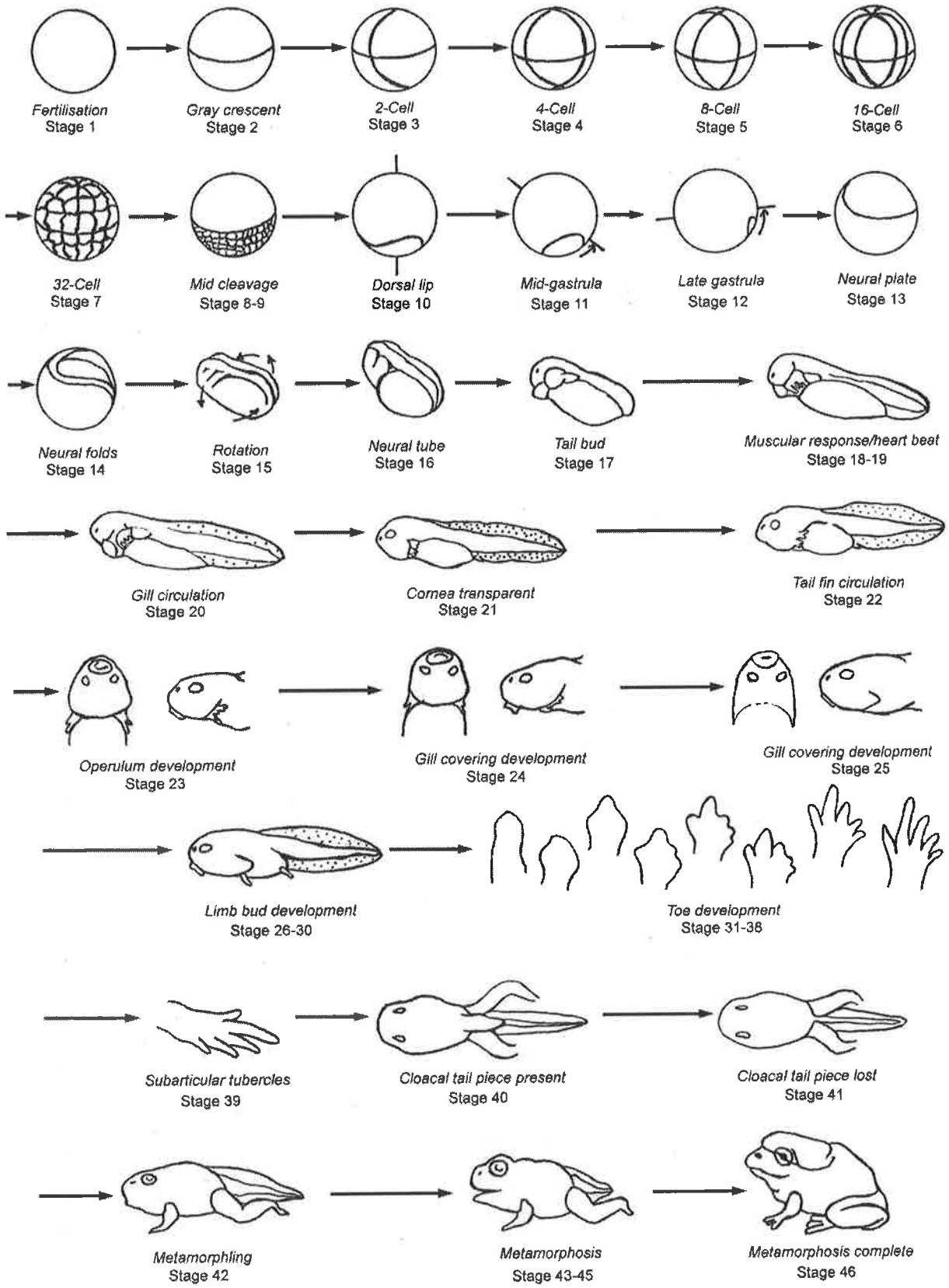


Figure 2.6. Simplified representation of the stages of development of *Litoria splendida* larvae.

2.2. Results and Discussion

2.2a. General

Specimens of *Litoria splendida* were obtained from the Kimberley region of Western Australia. Several specimens were collected in 1994, averaging 12 cm in length, and have been kept in captivity ever since. The skin secretion containing peptide material was obtained from the rostral and parotoid glands by the SES method. The amphibian was held while its skin was moistened with distilled water and mild electrical stimulation induced the release of the skin secretion. This process could be repeated without harm to the amphibian, on a monthly basis. On average about 70 mg of peptide material was obtained from the milking of each adult anuran.

The HPLC chromatogram (Figure 2.7), derived from 3 mg of crude peptide material, identified an extra peptide within the skin secretion of *Litoria splendida* to those identified by *Stone et al.*[14]. This is due to the fact that the HPLC separation of peptides using the VYDAC C_{18} column, as used in this research, is superior to that produced previously for the adult *Litoria splendida* using a Brownlee Aquapore RP-300 analytical column. This enhanced resolution has resulted in the isolation of a component not detected in the previous study. This component was identified as caerin 1.6 (structural details of this peptide are recorded in Table 2.4), an antibacterial peptide also present in the skin secretions of the related species *Litoria xanthomera* and *Litoria chloris*[21, 22].

The peptides shown in Figure 2.7 were identified by fast atom bombardment mass spectrometry (FAB MS). Excluding the peptide fraction representing that of caerin 1.6, the molecular weights of the remaining peptide fractions obtained indicated the peptides were identical to those discovered 8 years ago by *Stone et al.*[14]. On average, each milking produced 10 mg of caerulein, 0.15 mg of caerin 1.1.2, 0.4 mg of caerin 1.1.1, 1 mg of caeridin 1.1, 9 mg of caerin 3.1, 4 mg of caerin 2.1, 2 mg of caerin 1.6 and 18 mg of caerin 1.1.

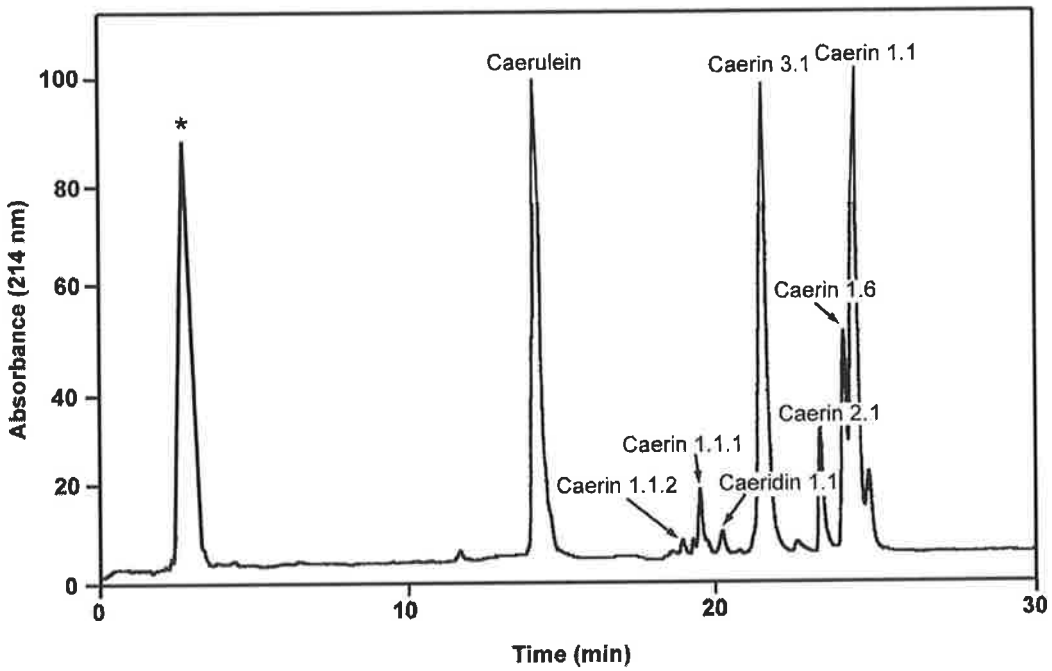


Figure 2.7. HPLC chromatogram from the crude skin secretions of *Litoria splendida*. (See Experimental section for details).

Anurans are seasonal breeders and reproduction depends heavily on temperature, day length, moisture and atmospheric pressure[23]. As fertilisation occurs in water, rainfall in tropical environments may serve as the final trigger of reproductive behaviour. Breeding can be very difficult when the anuran is in captivity. This problem was overcome by injecting two *Litoria splendida* anurans, which were chosen as the breeding pair, with a hormone known as luteinising hormone releasing hormone (LHRH)*. Within 24 hours of the hormone injection, approximately 9000 eggs spawned with the majority successfully fertilised. The fertilised eggs were sampled almost immediately. Sampling of the larval stages was then continued frequently usually twice a week over a period of 13 weeks. During the initial stages of development the material derived from the whole tadpole was analysed. This crude material continued to be monitored until the tadpoles were large enough for just the skins to be analysed.

* LHRH induces ovulation and prepares the eggs within the female for fertilisation. This hormone also sexually excites the male and in doing so initiates the breeding process.

2.2b. Structure determination

The structures of the peptides isolated were determined by a combination of FAB mass spectrometry, methylation and enzymic sequencing procedures. FAB MS was used to determine the molecular weight of each peptide. Each peptide was methylated and analysed by FAB MS to determine the C-terminal end group. The molecular weight of each resulting methyl ester was then determined by FAB mass spectrometry. Additional structural information was obtained by cleaving the peptides using a Lys-C enzyme digest. The chosen peptide fragments were then collisionally activated and sequenced by their resulting MIKE spectra.

2.2c. Isolation of peptides from larval *Litoria splendida*

With such a high frequency of sampling, many HPLC separations were performed, which produced a large quantity of analytical chromatograms (summarised in Table 2.3). The first significant result was obtained when the tadpoles were 10 days old*. At this stage the tadpole had just left its embryonic stage and was in the process of losing its gills. The HPLC chromatogram taken at this stage (Figure 2.8) indicated a peak with a retention time of 14.3 minutes, which matched that of caerulein, as found in the adult. Co-injection of the tadpole sample with a synthetic sample of caerulein confirmed the peak found at this particular stage as being caerulein. At this stage, about 5 ng of caerulein was estimated per tadpole (total weight of tadpole, 6 mg).

* Age is dated from the onset of fertilisation.

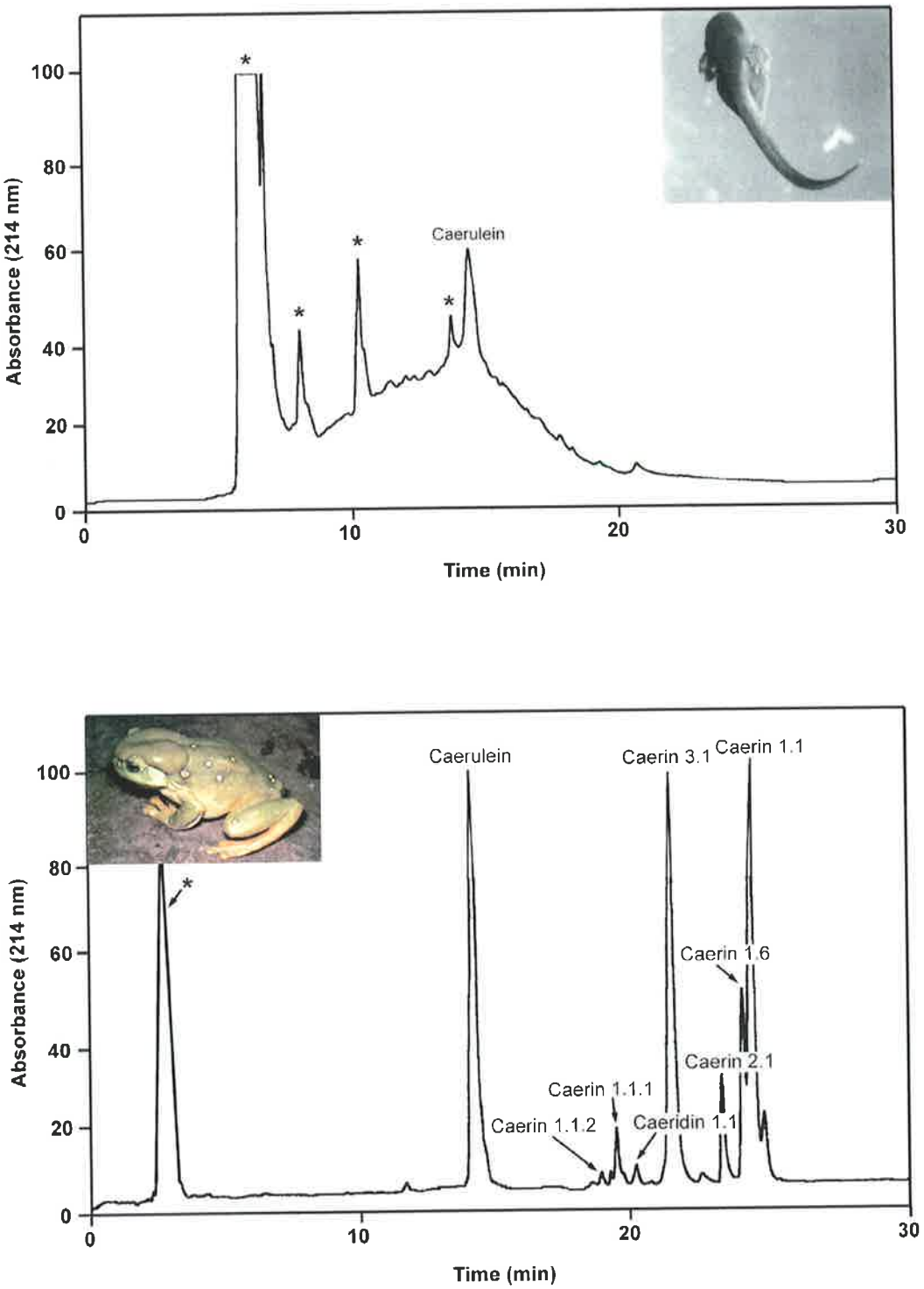


Figure 2.8. HPLC chromatogram derived from whole specimens of larval *Litoria splendida* (10 days old), in comparison with the adult *Litoria splendida* chromatogram. * Non peptide material.

The next significant observation was made when the tadpoles were 14 days old. At this stage, the gills of the tadpoles had been lost and their front limbs were beginning to develop. The HPLC chromatogram taken at this stage (Figure 2.9) indicated the appearance of 3 new peaks with retention times of 25.0, 23.8 and 22.5 minutes. These retention times match those of caerin 1.1, caerin 2.1 and caerin 3.1 respectively. Peptides [total weight of tadpole (average, 12 mg)]: approximately 30 ng (caerulein), 35 ng (caerin 3.1), 10 ng (caerin 2.1), 15 ng (caerin 1.6) and 40 ng (caerin 1.1) were isolated at this stage from six tadpoles.

The peptide showing a retention time of 25 minutes, which was suspected to be caerin 1.1, was purified by HPLC and analysed by FAB mass spectrometry. The molecular weight obtained was 2582 Da, which matched that of caerin 1.1. Methylation experiments together with FAB mass spectrometry indicated a primary amide C-terminal end group for this peptide. Further structural information was gained by specifically cleaving the peptide at lysine (Lys^{11}) using a Lys-C digest. This yielded two smaller peptide fragments that were detected by FAB mass spectrometry as having molecular weights of 1043 and 1558 Da.

The collisional activation MS/MS spectrum of the fragment ion MH^+ 1043 is shown in Figure 2.10. The mass spectrum shows considerable background noise, but was still the best obtainable considering the minute quantity of material used, and the type of mass spectrometer used for this measurement. Information gained from the B and Y+2 fragment ions established the structure of the enzyme digest fragment ion as being Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys (OH).

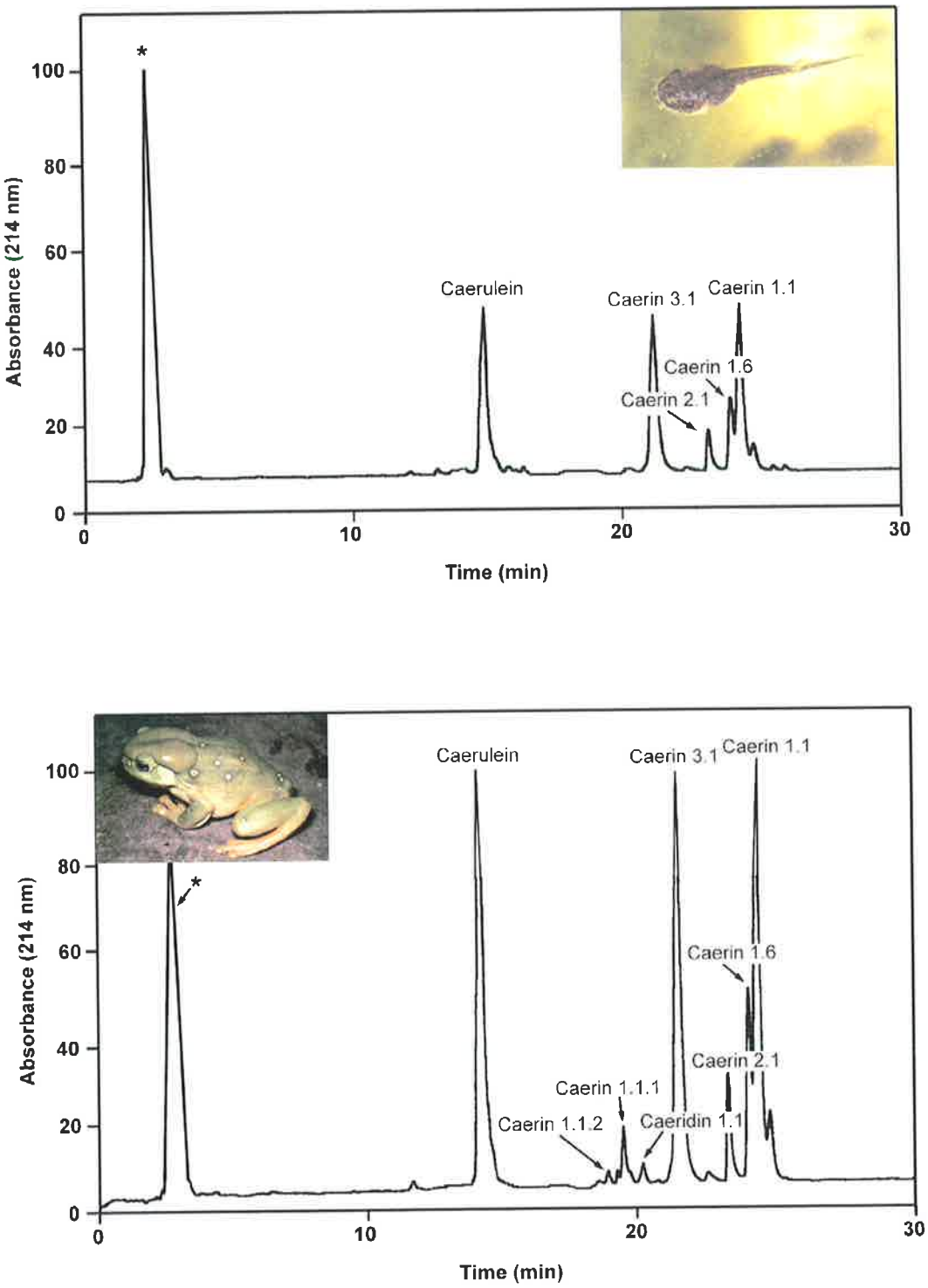


Figure 2.9. HPLC chromatogram derived from whole specimens of larval *Litoria splendida* (14 days old), in comparison with the adult *Litoria splendida* chromatogram. * Non peptide material.

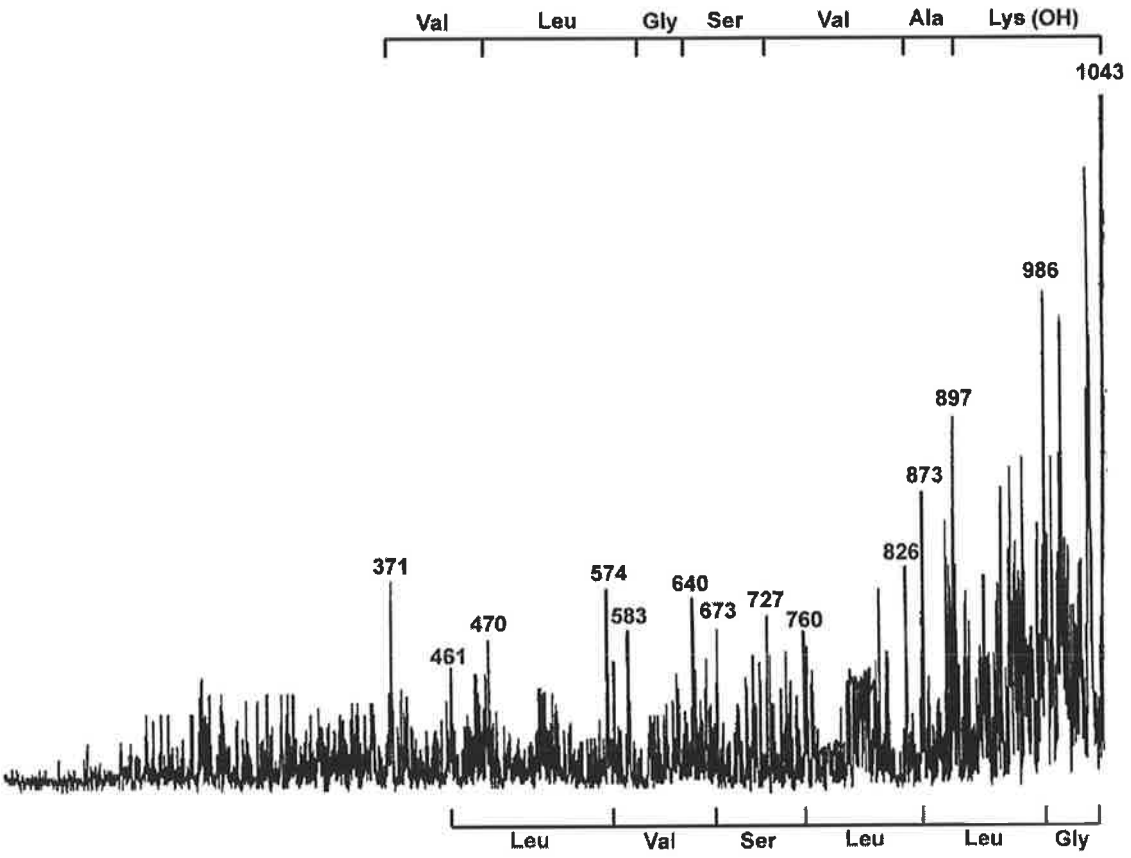


Figure 2.10. CA MS/MS of the peptide fragment ion MH^+ 1043. The sequence above the spectrum is determined by the B fragmentations, while underneath is the sequence determined by the Y+2 fragmentations.

The collisional activation MS/MS spectrum of the other fragmentation ion of MH^+ 1558 is shown in Figure 2.11. Information gained from the B and Y+2 cleavages determined His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2) as the structure of this fragment ion. This allowed full structural determination of the parent peptide, confirming it to be structurally identical to caerin 1.1 already isolated from the adult *Litoria splendida*. The structural determination methods used in this example were applied for all the peptides isolated from the larval extracts. Details for each peptide are summarised in Table 2.4.

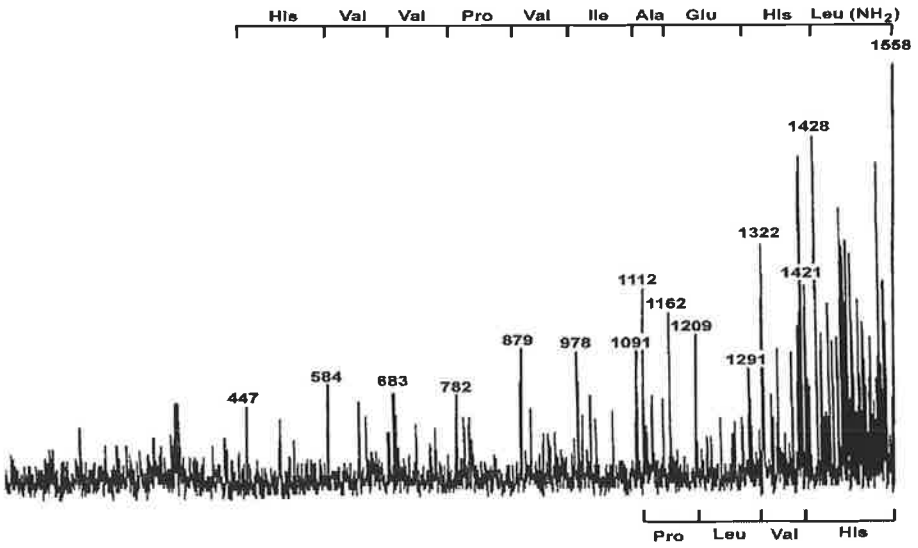


Figure 2.11. CA MS/MS of the peptide fragment ion MH^+ 1558. The sequence above the spectrum is determined by the B fragmentations, while underneath is the sequence determined by the Y+2 fragmentations.

At this stage of development (14 days after fertilisation), the tadpoles were large enough for the skins to be removed. The HPLC chromatogram derived from the skin samples is shown in Figure 2.12, together with the previous HPLC chromatogram derived from the whole tadpole at exactly the same stage. The two HPLC chromatograms appeared to be identical indicating that the peptide material represented by these peaks is present within the skin. From this stage onwards, just the skin samples from the tadpoles were analysed.

The final significant observation was made when the tadpoles were 84 days old. At this stage the tadpoles were going through metamorphosis: their tails had started to regress and their front and hind limbs were nearly fully developed. The HPLC chromatogram taken at this stage (Figure 2.13) indicated the appearance of 3 new peaks with retention times of 19.6, 19.0 and 20.4 minutes. These retention times match those of caerin 1.1.1, caerin 1.1.2 and caeridin 1.1, as found in the adult. Peptides [weight per metamorph skin (average, 12 mg)]: approximately 2 μg (caerulein), 60 ng (caerin 1.1.2), 80 ng (caerin 1.1.1), 70 ng (caeridin 1.1), 3 μg (caerin 3.1), 1 μg (caerin 2.1), 0.5 μg (caerin 1.6) and 3 μg (caerin 1.1) were isolated at this stage from six tadpoles. In comparison, the two HPLC chromatograms of the larval *Litoria splendida* (84 days old) and adult *Litoria splendida* were identical. Mass spectrometry analysis confirmed the peptides represented by the two corresponding chromatograms to be identical.

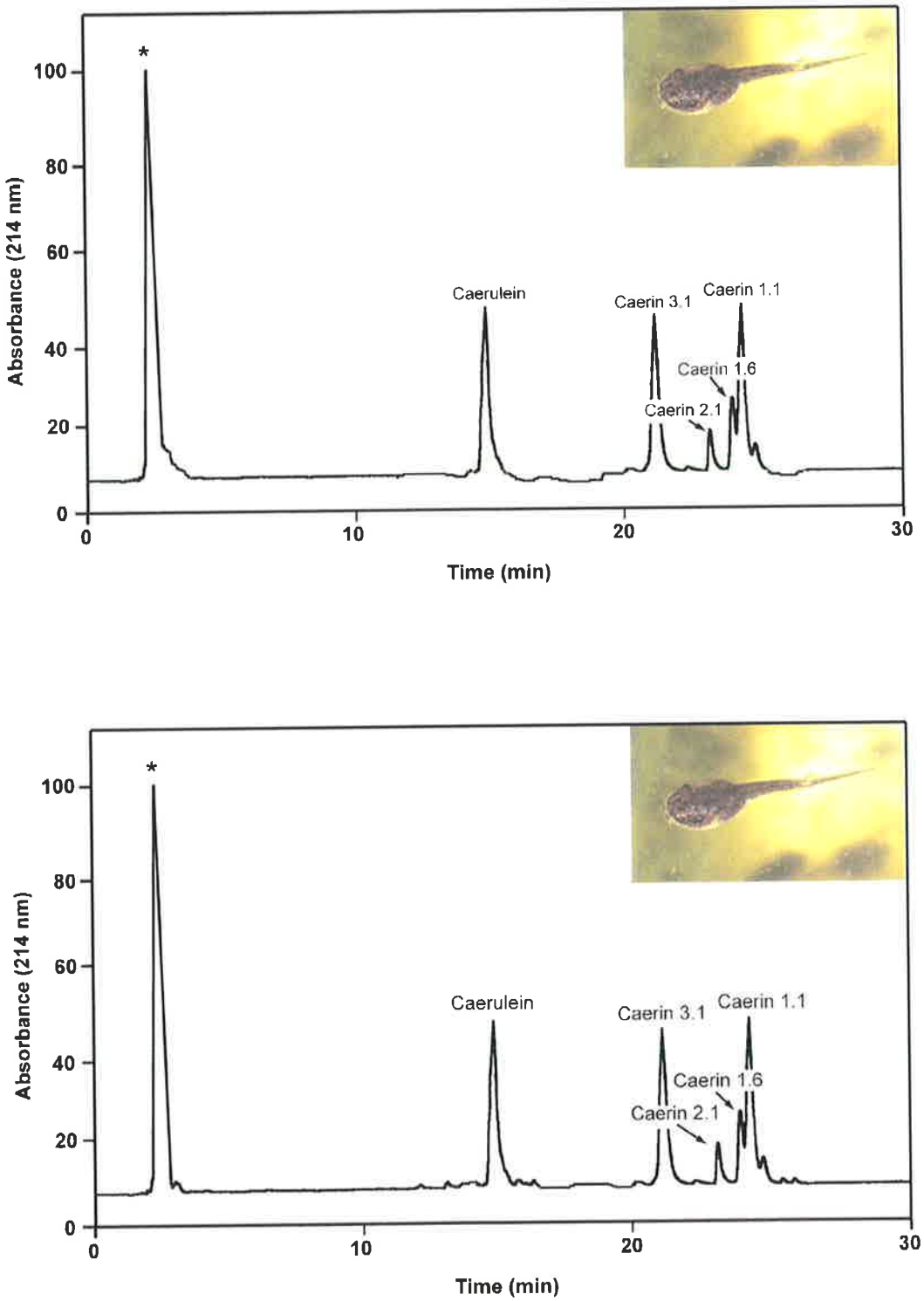


Figure 2.12. HPLC chromatogram derived from the skin samples of larval *Litoria splendida* (14 days old), in comparison with the HPLC chromatogram derived from whole specimens of larval *Litoria splendida* (14days old). * Non peptide material.

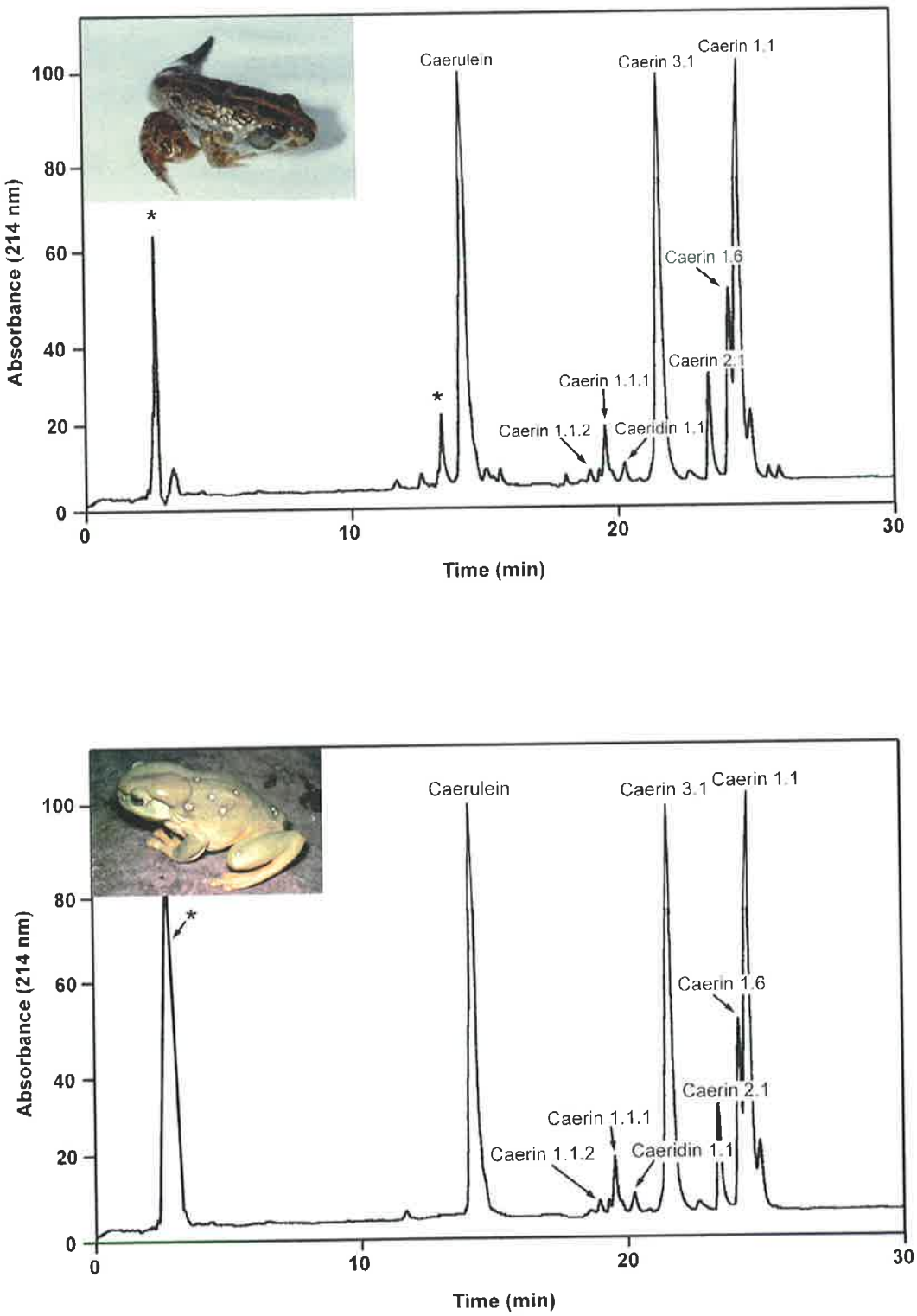


Figure 2.13. HPLC chromatogram derived from the skin samples of larval *Litoria splendida* (84 days old), in comparison with the adult *Litoria splendida* chromatogram. * Non peptide material.

Stage*	Maturity†	No.‡	Weight§ (mg)	Whole tadpole	Skin only	Peptides isolated during the various stages of development							
						C	c1.1	c2.1	c3.1	c1.6	c1.1.1	c1.1.2	cd1.1
0-9	0.5	12	7.2	✓									
10-13	2	12	7.6	✓									
14-17	5	6	18	✓									
17-18	8	6	23	✓									
21	10	6	35	✓		✓							
21-22	11	6	38	✓		✓							
22-24	12	6	47	✓		✓							
24-25	14	6	55	✓		✓	✓	✓	✓	✓			
24-25	14	6	8		✓	✓	✓	✓	✓	✓			
26	16	6	68	✓		✓	✓	✓	✓	✓			
26	16	6	10		✓	✓	✓	✓	✓	✓			
26	19	6	74	✓		✓	✓	✓	✓	✓			
26	19	6	11		✓	✓	✓	✓	✓	✓			
26-27	22	6	72	✓		✓	✓	✓	✓	✓			
26-27	22	6	10		✓	✓	✓	✓	✓	✓			
26-27	25	6	75	✓		✓	✓	✓	✓	✓			
26-27	25	6	11		✓	✓	✓	✓	✓	✓			
27	28	6	14		✓	✓	✓	✓	✓	✓			
27	31	6	20		✓	✓	✓	✓	✓	✓			
27-28	34	6	20		✓	✓	✓	✓	✓	✓			
27-28	38	3	21		✓	✓	✓	✓	✓	✓			
27-28	38	6	90	✓		✓	✓	✓	✓	✓			
27-28	42	6	25		✓	✓	✓	✓	✓	✓			
28	46	6	28		✓	✓	✓	✓	✓	✓			
28-29	49	6	28		✓	✓	✓	✓	✓	✓			
29-30	52	3	33		✓	✓	✓	✓	✓	✓			
29-30	52	6	140	✓		✓	✓	✓	✓	✓			
30	55	6	38		✓	✓	✓	✓	✓	✓			
31-32	58	6	40		✓	✓	✓	✓	✓	✓			
33-34	61	6	45		✓	✓	✓	✓	✓	✓			
35-36	63	6	46		✓	✓	✓	✓	✓	✓			
37-38	66	3	50		✓	✓	✓	✓	✓	✓			
37-38	69	6	170	✓		✓	✓	✓	✓	✓			
39-40	72	6	53		✓	✓	✓	✓	✓	✓			
40	75	6	58		✓	✓	✓	✓	✓	✓			
40-41	78	6	70		✓	✓	✓	✓	✓	✓			
41	81	6	72		✓	✓	✓	✓	✓	✓			
41	84	6	72		✓	✓	✓	✓	✓	✓	✓	✓	✓
42	84	3	80		✓	✓	✓	✓	✓	✓	✓	✓	✓
42	84	6	200	✓		✓	✓	✓	✓	✓	✓	✓	✓
42-43	87	6	86		✓	✓	✓	✓	✓	✓	✓	✓	✓
44-45	90	6	115		✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 2.3. Summary of the analytical HPLC chromatograms taken during the development cycle of *Litoria splendida*. * Stages of development, as represented in Table 2.1. † Maturity represented as days after fertilisation. ‡ Number of specimens used for analysis. § Total tissue weight of specimens used for analysis. C = caerulein, c1.1 = caerin 1.1, c2.1 = caerin 2.1, c3.1 = caerin 3.1, c1.6 = caerin 1.6, c1.1.1 = caerin 1.1.1, c1.1.2 = caerin 1.1.2 and cd1.1 = caeridin 1.1.

Caerin 1.1 [MH^+ 2582]

Methylation gives a methyl ester, MH^+ 2611 (one CO_2H and one $CONH_2$ group)
Lys-C digestion gives MH^+ 1043 and 1558

MH^+ 1043 MS/MS	'B ions'	*m/z* 1025, 897, 826, 727, 640, 583,470, 371 [Val, Leu Gly Ser Val Ala Lys (OH)]
	'Y+2 ions'	*m/z* 1043, 986, 873, 760, 673, 574, 461 [Gly Leu Leu Ser Val Leu]
	Sequence	Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys (OH)
MH^+ 1558 MS/MS	'B ions'	*m/z* 1541, 1428, 1291, 1162, 1091, 978, 879, 782, 683, 584, 447 [His Val Val Pro Val Ile Ala Glu His Leu (NH_2)
	'Y+2 ions'	*m/z* 1558, 1421, 1322, 1209, 1112 [His Val Leu Pro]
	Sequence	His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2)

Full sequence of caerin 1.1, as determined from mass spectrometric and automated Edman data is:

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 2.1 [MH^+ 2392]

Methylation gives a methyl ester, MH^+ 2435 (one CO_2H and two $CONH_2$ groups)
Lys-C digestion gives MH^+ 1823 and 587

MH^+ 1823 MS/MS	'B ions'	*m/z* 1805, 1677, 1577, 1478, 1364, 1293, 1180, 1067, 1010, 953, 840 [Leu Gly Gly Leu Leu Ala Asp Val Val Lys (OH)
	'Y+2 ions'	*m/z* 1823, 1766, 1653, 1553, 1466, 1379, 1266, 1209, 1053, 982, 869 [Gly Leu Val Ser Ser Ile Gly Arg Ala Leu]
	Sequence	Gly Leu Val Ser Ser Ile Gly Arg Ala Leu Gly Gly Leu Leu Ala Asp Val Val Lys (OH)

Table 2.4. MS data for peptides isolated from larval *Litoria splendida*.

Caerin 2.1 [MH^+ 2392] (Continued)

MH^+ 587 MS/MS	'B ions'	*m/z* 569, 498, 401, 273, 216 [Gly Gln Pro Ala (OH)
	'Y+2 ions'	*m/z* 587, 500, 372, 315 [Ser Lys Gly]
	Sequence	Ser Lys Gly Gln Pro Ala (OH)

Full sequence of caerin 2.1, as determined from mass spectrometric and automated Edman data is:

Gly Leu Val Ser Ser Ile Gly Arg Ala Leu Gly Gly Leu Leu Ala Asp Val Val Lys Ser Lys Gly Gln Pro Ala (OH)

Caerin 3.1 [MH^+ 2382]

Methylation gives a methyl ester, MH^+ 2425 (one CO_2H and two $CONH_2$ groups)
Lys-C digestion gives MH^+ 1529 and 871

MH^+ 1529 MS/MS	'B ions'	*m/z* 1511, 1383, 1284, 1227, 1098, 999, 886, 829, 742 [Ser Gly Ile Val Glu Gly Val Lys (NH_2)]
	'Y+2 ions'	*m/z* 1529, 1413, 1285, 1214, 1127, 998, 885, 786, 699 [Asp Lys Ala Ser Glu Leu Val Ser]
	Sequence	Asp Lys Ala Ser Glu Leu Val Ser Gly Ile Val Glu Gly Val Lys (NH_2)
MH^+ 871 MS/MS	'B ions'	*m/z* 853, 725, 612, 484, 356 [Gln Lys Ile Lys (OH)]
	'Y+2 ions'	*m/z* 871, 813, 700, 514, 386 [Gly Leu Trp Gln]
	Sequence	Gly Leu Trp Gln Lys Ile Lys (OH)

Full sequence of caerin 3.1, as determined from mass spectrometric and automated Edman data is:

Gly Leu Trp Gln Lys Ile Lys Asp Lys Ala Ser Glu Leu Val Ser Gly Ile Val Glu Gly Val Lys (NH_2)

Caerin 1.6 [MH^+ 2591]

Methylation gives a methyl ester, MH^+ 2620 (one CO_2H and one $CONH_2$ group)
Lys-C digestion gives MH^+ 1061 and 1549

MH^+ 1061 MS/MS	'B ions'	*m/z* 1043, 915, 844, 745, 674, 617, 504, 405, 318, 171 [Phe Ser Val Leu Gly Ala Val Ala Lys OH)]
	'Y+2 ions'	*m/z* 1061, 1004, 891, 744, 657, 558 445, 388, 317, 218 [Gly Leu Phe Ser Val Leu Gly Ala Val]
	Sequence	Gly Leu Phe Ser Val Leu Gly Ala Val Ala Lys (OH)

Table 2.4. *(Continued)*

Caerin 1.6 [MH^+ 2591] (Continued)

MH^+ 1549 MS/MS	'B ions'	*m/z* 1532, 1419, 1291, 1162, 1091, 978, 879, 782, 683, 584 [Val Val Pro Val Ile Ala Glu Lys Leu(NH_2)]
	'Y+2 ions'	*m/z* 1549, 1412, 1313, 1200, 1103, 966, 867, 768, 671, 572 [His Val Leu Pro His Val Val Pro Val]
	Sequence	His Val Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2)

Full sequence of caerin 1.6, as determined from mass spectrometric and automated Edman data 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)

Caerin 1.1.1 [MH^+ 2412]

Methylation gives a methyl ester, MH^+ 2441 (one CO_2H and one $CONH_2$ group)

Lys-C digestion gives MH^+ 1558 and 872

MH^+ 1558 MS/MS	'B ions'	*m/z* 1541, 1428, 1291, 1162, 1091, 978, 879, 782, 683, 584, 447 [His Val Val Pro Val Ile Ala Glu His Leu (NH_2)
	'Y+2 ions'	*m/z* 1558, 1421, 1322, 1209, 1112 [His Val Leu Pro His]
	Sequence	His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2)
MH^+ 872 MS/MS	'B ions'	*m/z* 854, 726, 655, 556, 469, 412 [Gly Ser Val Ala Lys (OH)]
	'Y+2 ions'	*m/z* 872, 758, 671, 572, 459, 402 [Leu Ser Val Leu Gly]
	Sequence	Leu Ser Val Leu Gly Ser Val Ala Lys (OH)

Full sequence of caerin 1.1.1, as determined from mass spectrometric and automated Edman data is:

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)

Table 2.4. *(Continued)*

Caerin 1.1.2 [MH^+ 2299]

Methylation gives a methyl ester, MH^+ 2328 (one CO_2H and one $CONH_2$ group)

Lys-C digestion gives MH^+ 1558 and 759

MH^+ 1558 MS/MS	'B ions'	*m/z* 1541, 1428, 1291, 1162, 1091, 978, 879, 782, 683, 584, 447 [His Val Val Pro Val Ile Ala Glu His Leu (NH_2)]
	'Y+2 ions'	*m/z* 1558, 1421, 1322, 1209, 1112 [His Val Leu Pro His]
	Sequence	His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu (NH_2)
MH^+ 759 MS/MS	'B ions'	*m/z* 741, 613, 542, 443, 356, 299, 186 [Val Ala Lys His Ala Lys (OH)]
	'Y+2 ions'	*m/z* 759, 672, 573, 460, 403, 316, 217 [Ser Val Leu Gly Ser Val]
	Sequence	Ser Val Leu Gly Ser Val Ala Lys His Ala Lys (OH)

Full sequence of caerin 1.1.2, as determined from mass spectrometric and automated Edman data is:

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

Caeridin 1.1 [MH^+ 1140]

Methylation gives a methyl ester, MH^+ 1169 (one CO_2H and one $CONH_2$ group)

MH^+ 1140 MS/MS	'B ions'	*m/z* 1122, 1009, 952, 839, 738, 681, 568, 455 [Leu Leu Gly Thr Leu Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 1140, 1083, 970, 857, 724, 685, 572 [Gly Leu Leu Asp Gly Leu]
	Sequence	Gly Leu Leu Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2)

Full sequence of caeridin 1.1, as determined from mass spectrometric and automated Edman data is:

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

Table 2.4. *(Continued)*

2.2d. Conclusions

The peptides found during the larval development cycle of *Litoria splendida* have been detected in the specific order summarised in Table 2.5.

Stage	Days from fertilisation	Morphology	Peptide/s detected
21	10	*Tadpole had just past the embryonic stage. Gills beginning to be lost.*	caerulein
24-25	14	*Gills had been lost. Front limbs beginning to be developed.*	caerulein caerin 1.1 caerin 2.1 caerin 3.1 caerin 1.6
42	84	*Tadpoles starting to go through metamorphosis. Tails regressing, and front and hind limbs nearly fully developed.*	caerulein caerin 1.1 caerin 2.1 caerin 3.1 caerin 1.6 caerin 1.1.1 caerin 1.1.2 caeridin 1.1

Table 2.5. Summary of results.

The metamorphic and adult forms of *Litoria splendida* store the procaerulein and procaerin peptides in their rostral and parotoid glands, and it is from these glands that the active caerulein and caerins are released when required. However, it has been shown that the neuropeptide caerulein is first detected 10 days into the development cycle of *Litoria splendida*, with the antibacterial peptides first detected at 14 days.

The fact that peptide material has been shown present before metamorphosis is interesting. It is interesting because: (i) the granular glands within the skin of *Litoria splendida* are only developed during metamorphosis and (ii) in the past, it has been assumed that amphibians need these storage glands in order for the peptides to be present within the skin. The mechanism/s by which these active peptides are being formed and liberated within the tadpole are currently uncertain.

Although these peptides were detected at the stages indicated, it may not necessarily mean that they are definitely absent prior to these stages. Peptide material may be present a lot earlier than detected. This assumption may be plausible, as the peptide material is present in only very small amounts during the early stages of development. Since the peptides are present in only minute quantities early in development, they may therefore be undetectable by the analytical methods used in this research*. In conclusion, peptide material has been detected before metamorphosis for this particular species. If there are no peptides produced during the beginning of the development cycle of *Litoria splendida*, the defence mechanisms that are protecting the tadpoles against microbial pathogens are yet to be determined.

* No pair of *Litoria splendida* has been induced to breed since these initial experiments. Consequently, further experiments using a recently obtained LCQ mass spectrometer have not been possible in the reinvestigation of this reproductive cycle between egg deposition and the 10th day of tadpole development.

2.3. Experimental

2.3a. Collection and preparation of adult *Litoria splendida* secretions

The skin secretions from *Litoria splendida* were provided courtesy of Associate Professor Mike Tyler, Department of Environmental Biology, University of Adelaide. The secretion was obtained by the SES method, using a C.F. Palmer student model electrical stimulator attached with a bipolar electrode of 21G platinum. *Litoria splendida* was held by the back legs, while its skin was moistened with distilled water, and rubbed gently with the electrode in a circular method using pulse duration of 2 milliseconds at 10V. The skin secretion was washed from the amphibian with distilled water and collected into a plastic container. An equal volume of methanol was added to the solution. The resulting solution was centrifuged, with the supernatant liquid removed and filtered through a Millipore 0.45 μm filter. The solution was then concentrated under vacuum.

2.3b. Collection and preparation of larval *Litoria splendida* samples

Larval *Litoria splendida* were provided courtesy of Associate Professor Mike Tyler, Department of Environmental Biology, University of Adelaide. Each member of the breeding pair of *Litoria splendida* was injected subcutaneously with luteinising hormone releasing hormone (10 μl in 0.1 ml of normal saline) into the dorsal lymph sac. The pair accomplished amplexus an hour from injection of the hormone, and commencing 6 hours from injection, the female deposited about 9000 eggs. The majority of the eggs were fertile. Developing tadpoles were kept in groups of about 100 within shallow, aerated, 5 litre tanks, with the temperature maintained at 28°C. Dechlorinated tap water in the tanks was changed daily.

Samples were collected almost immediately after fertilisation, with collection being continued at 3-day intervals until the end of the developing cycle. Initially 12 tadpoles were collected at a time. After 3 weeks from fertilisation, the sample size was decreased to 6 tadpoles from that point on.

The samples were worked up ready for HPLC separation in the following manner. The tadpoles were frozen in liquid nitrogen and allowed to defrost. Once thawed a pair of fine tipped forceps was used to remove the skins, except during the early stages of development when the tadpoles were ground up whole. To this material a solution of H_2O/MeOH (1:1) was added (25 ml). The solution was centrifuged, the supernatant liquid removed and filtered through a Millipore 0.45 μm filter. The resulting solution was then concentrated under vacuum.

2.3c. HPLC separation

HPLC separation was achieved for each sample using a VYDAC C_{18} Protein and Peptide (218TP54) reverse phase column equilibrated with 10% acetonitrile/ aqueous 0.1% TFA. Each lyophilised sample was dissolved in deionised water (25 μl) and injected onto the column. The elution profile increased from 10-70% acetonitrile over a period of 30 minutes using a flow rate of 1 ml/min.

2.3d. Methylation of peptides

Purified peptide (*ca* 5-10 μg) in deionised water (40 μl) was lyophilised in an Eppendorf tube. 'Acidified methanol'* was added, and the resulting mixture heated at 45°C for 30 minutes. The solvent was removed in a stream of nitrogen and the solid product analysed by FAB mass spectrometry.

* The 'acidified methanol' was prepared by adding methanol (858 μl) to a 10ml screw top test tube. This was cooled in dry ice for 5 minutes, acetyl chloride (142 μl) was added under nitrogen, the test tube sealed and cooled again in dry ice for 5 minutes. The solution was allowed to warm to 20°C over a period of 1 hour, flushing with nitrogen every 15min. The reagent was stored at -4°C for a maximum of 5 days.

2.3e. Enzyme digestion using Lys-C

Lyophilised peptide (*ca* 5μg) was added to an Eppendorf tube and dissolved in deionised water (5 μl). An aqueous ammonium hydrogen carbonate (0.1M, 1 μl, pH=8) buffer solution and endoprotease Lys-C (0.5 μl) was then added. The resulting solution was incubated at 45°C for 15 minutes and then analysed by mass spectrometry.

2.3f. Mass spectrometry analysis

The molecular weights of the individual peptides were determined by FAB mass spectrometry with a Vacuum Generators ZAB 2HF mass spectrometer, and collisionally activated mass analysed kinetic energy spectra (CA MIKES) of MH^+ ions were used to provide sequencing data. The mass spectrometer was equipped with an ion FAB gun using an argon beam with a source pressure of 10^{-6} Torr. The samples were mixed with glycerol and 1,4-dithiothritol/erythritol (DTT/DTE) matrices on the probe tip before inserting the probe into the FAB source. CA MS/MS were obtained by focussing the magnetic sector to transmit the ion of interest; the ions were collisionally activated in the second collision cell [containing argon at a pressure of $2x10^{-6}$ Torr (equivalent to single collision conditions)]. The electric sector was then scanned to produce CA MS/MS. All of the peptides described were analysed in the positive mode. FAB spectra for molecular weight determination, including the products from methylation experiments and enzyme digestion were obtained by scanning the magnet over a mass range from *m/z* 500-3000, using a mass resolution of 1500. CA MS/MS experiments were performed using a mass resolution of 300-500.

2.3g. Antibacterial testing

The Microbiology Department of the Institute of Medical and Veterinary Science (Adelaide, Australia) carried out antibacterial testing. The method used involved the measurement of inhibition zones (produced by the applied peptide) on a thin agarose plate containing the microorganisms under study. The method follows a standard testing procedure[24].

2.3h. Preparation of synthetic peptides

Peptides were synthesised commercially (by Chiron Mimotopes, Clayton, Victoria, Australia) using L-amino acids through the standard N-α-Fmoc method[25]. Each synthetic peptide was shown to be identical with the natural peptide by (i) electrospray mass spectrometry, (ii) automated Edman sequencing, and (iii) co-elution of the synthetic and natural peptides on HPLC.

2.3i. Automated Edman sequencing

Sequences that contained the isomeric residues Ile or Leu, or the isobaric residues Lys or Gln, were further analysed by automated Edman sequencing. Automated Edman sequencing was performed by the Department of Biochemistry, University of Adelaide, using an applied Biosystem 470A sequencer equipped with a 900A data analysis module.

2.4. References

[1] Moore, K., Bevins, C., Brasseur, M., Tomassini, N., Turner, K., Eck, H. and Zasloff, M., *J. Biol. Chem.*, **266**, 19851-19857 (1991).

[2] Reilly, D., Tomassini, N. and Zasloff, M., *Dev. Biol.*, **162**, 123-133 (1994).

[3] Moore, K., Bevins, C., Tomassini, N., Huttner, K., Sadler, K., Moreira, J., Reynolds, J. and Zasloff, M., *J. Histochem. Cytochem.*, **40**, 367-378 (1992).

[4] Bevins, C. and Zasloff, M., *Annu. Rev. Biochem.*, **59**, 395-399 (1990).

[5] Jacob, L. and Zasloff, M., in *Antimicrobial Peptides: Ciba Foundation Symposium 186*, 197-208 (Wiley and Sons, New York, NY, 1994).

[6] Resnick, N., Maloy, W., Gut, H. and Zasloff, M., *Cell*, **66**, 541-554 (1991).

[7] Williams, R., Starman, R., Taylor, K., Gable, K., Beeler, T., Zasloff, M. and Covell, D., *Biochemistry*, **29**, 4490-4496 (1990).

[8] Flucher, B., Lenglachner-Bachinger, C., Pohlhammer, K., Adam, H. and Mollay, C., *J. Cell Biol.*, **103**, 2299-2309 (1986).

[9] Seki, T., Kikuyama, S. and Yanaihara, N., *Cell Tissue Res.*, **258**, 483-489 (1989).

[10] Kreil, G., in *Antimicrobial Peptides: Ciba Foundation Symposium 186*, 72-92 (Wiley and Sons, New York, NY, 1994).

[11] Gibson, B.W., Poulter, L., Williams, D.H. and Maggio, J.E., *J. Biol. Chem.*, **261**, 5341-5349 (1986).

[12] Clark, D.P., Durell, S., Maloy, W.L. and Zasloff, M., *J. Biol. Chem.*, **14**, 10849-10855 (1994).

[13] Tyler, M.J., in *Encyclopaedia of Australian animals* (ed. Strahan, R.) 3-42 (Harper Collins, London, UK, 1992).

[14] Stone, D.J.M, Waugh, R.J., Bowie, J.H., Wallace, J.C. and Tyler, M.J., *J. Chem. Soc. Perkin. Trans I.*, **11**, 3173-3177 (1992).

[15] Anastasi, A., Erspamer, V. and Endean, R., *Arch. Biochem. Biophys.*, **125**, 57-68 (1968)

[16] Lazarus, L.H. and Attila, M., *Prog. Neurobiol.*, **41**, 473-507 (1993).

[17] Erspamer, V. and Melchiorri, P. *Pure Appl. Chem.*, **35**, 463-494 (1973).

[18] Wong, H., Bowie, J.H. and Carver, J.A., *Eur. J. Biochem.*, **247**, 545-557 (1997).

[19] Bazzo, R., Tappin, M.J., Pastore, A., Harvey, T.S., Carver, J.A. and Campbell, I.D., *Eur, J. Biochem.*, **173**, 139-146 (1988).

[20] Gosner, K., *Herpetologica*, **70**, 183-187 (1960).

[21] Steinborner, S.T., Waugh, R.J., Bowie, J.H., Wallace, J.C., Tyler, M.J. and Ramsay, S.L., *J. Peptide Sci.*, **3**, 181-185 (1997).

[22] Wabnitz, P.A., Steinborner, S.T., Currie, G.J., Bowie, J.H. and Tyler, M.J., *Rapid Commun. Mass Spectrom.*, **11**, 997-1001 (1997).

[23] Barker, J., Grigg, G. and Tyler, M.J., in *A field guide to Australian frogs* (Surrey Beatty, Norton, NSW, 1995).

[24] Jorgensen, J.H., Clealand, W.A., Craig, G., Doern, M., Ferraro, J., Finegold, C.M., Hansen, S.L., Jenkins, S.G., Novick, W.J., Pfaller, M.S., Preston, D.A., Reller, L.B. and Swanson, J.M., *National committee for clinical laboratory standards Doc. M7-A3*, **13**, 1-12 (1993).

[25] Maeji, N.J., Bray, R.M., Valerio, R.M. and Wang, W., *Peptide Res.*, **8**, 33-38 (1995).

CHAPTER 3. Three-Year Peptide Profiling of Male and Female *Litoria splendida*

3.1. Introduction

3.1a. General

During the course of studying *Litoria splendida*, it has been noticeable that the peptide profiles between specimens vary from time to time. The variation is only slight and not always obvious. Consequently, it seemed appropriate to further investigate this occurrence, and in particular, to determine whether there is any variation between the peptide profiles of the male and female adults of *Litoria splendida* at different times of the year. A peptide that is present in only one sex of an anuran has not yet been reported. The existence of such a peptide would be extremely interesting, as it could be a sex pheromone. With the exception of a suspected alarm pheromone in a particular species of tadpole, there has been no report or identification of a pheromone from an anuran[1]. Most pheromones found in terrestrial animals tend to be volatile in nature. However, although *Litoria splendida* is primarily a tree frog, it moves to an aquatic environment during the mating season. This is the likely time when a water-soluble sex pheromone would be most needed. Therefore, a peptide that is both non-volatile and water soluble, could act as a sex pheromone for this particular vertebrate. *Litoria splendida* was an ideal species for this research as it has been studied extensively[2, 3]. The HPLC peptide profile for this species is also ideal to monitor, as it is not overly complicated, allowing any subtle variations to be noticed more easily. Since any variations could possibly be occurring seasonally, it seemed reasonable to monitor the HPLC peptide profile monthly over a three-year period.

The aims of the research presented in this chapter are:

(i) To monitor the peptide profiles regularly within a group of adult male and female *Litoria splendida* over a three-year period.

(ii) To isolate and characterise any new peptides discovered as a result of this research.

3.2. Results and Discussion

3.2a. General

Skin secretions were obtained separately from both male and female adult groups of *Litoria splendida* once a month over a total period of thirty-seven months. Peptide profiling was achieved by analysing each crude secretion by HPLC. The results from each chromatogram were then tabulated with particular attention being focussed on any subtle changes between peptide profiles. The work-up procedure and HPLC analysis followed immediately after the secretion was collected from each specimen. This was due to the fact that most peptides derived from the skin secretions of adult specimens of *Litoria splendida* degrade within a period of 10-30 minutes. An endopeptidase is the likely cause of this degradation, as it has already been shown that the caerin 1 antibiotic peptides are degraded by an endopeptidase[4]. The magnitude of degradation, within the skin secretions of *Litoria splendida* adults, can be seen when comparing the HPLC chromatograms taken from a secretion that has been left untreated for 45 minutes (Figure 3.1) to one that has been worked-up and analysed immediately after collection from the skin (Figure 3.2).

From a total of seventy-six HPLC chromatogram traces, taken over a three-year period, some distinct variations within the peptide profiles were found. These variations can be divided into two main categories:

(i) The discovery of seasonal variations within the caerulein region of the peptide profile in both the males and females.

(ii) The discovery of two additional peptides within the male secretion that are not present in the secretion of the female.

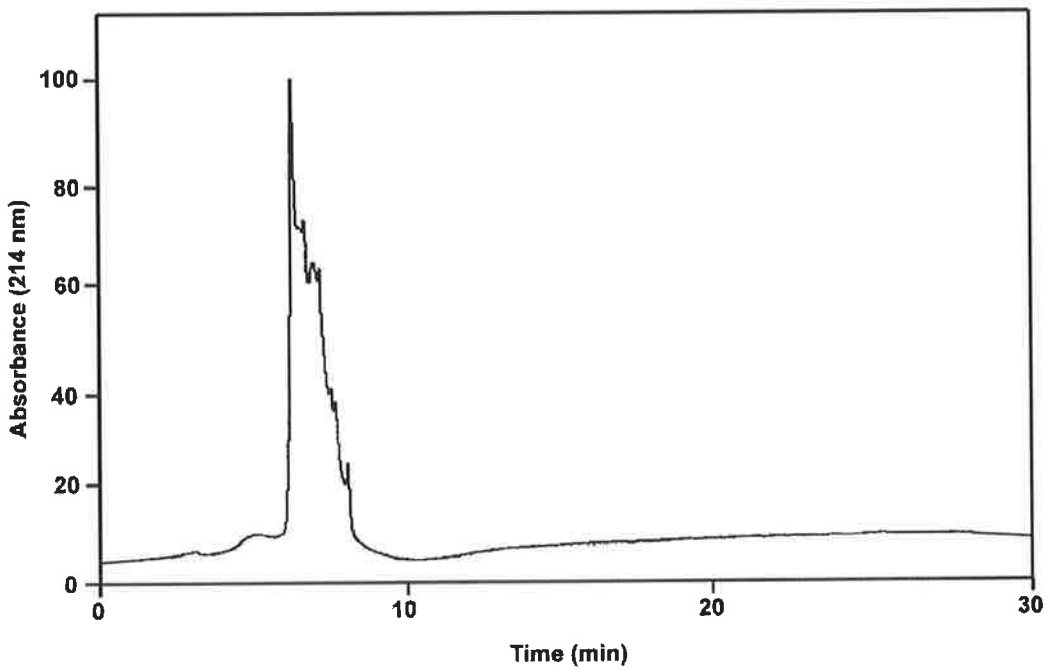


Figure 3.1. Chromatogram of the HPLC separation of a crude skin secretion analysed 45 minutes after being obtained from the skin glands of an adult *Litoria splendida* specimen. (See Experimental section for full details).

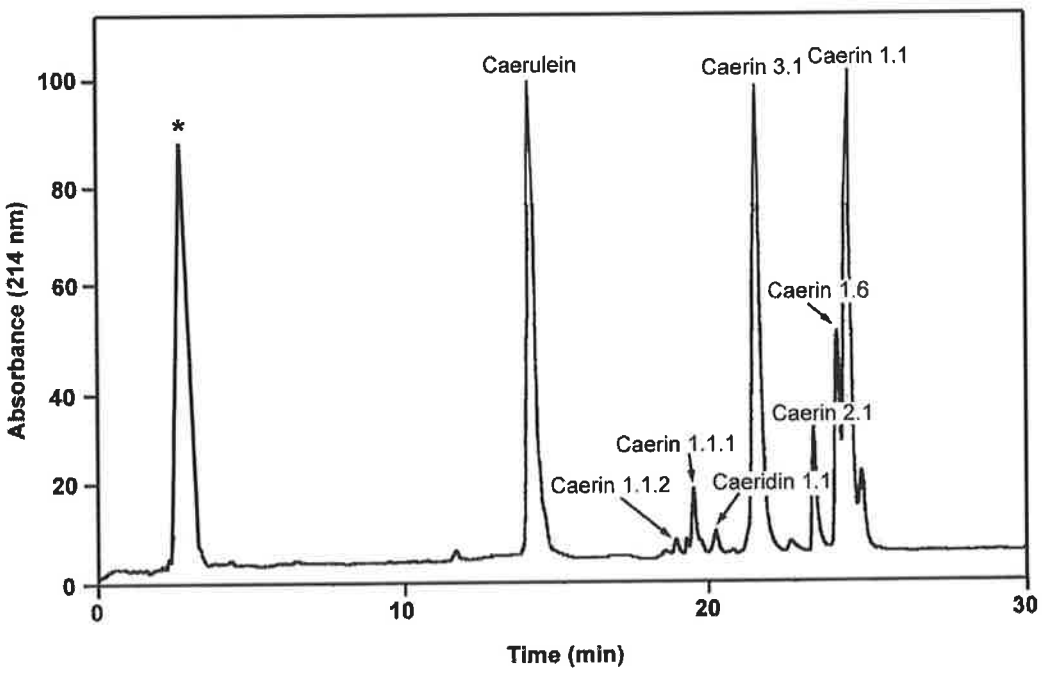


Figure 3.2. Chromatogram of the HPLC separation of a crude skin secretion analysed immediately after being obtained from the skin glands of an adult *Litoria splendida* specimen. (See Experimental section for full details).

3.2b. Seasonal variation of the caerulein region

Investigation of the peptide profiles from *Litoria splendida* male and female adults over the three-year period led to the observation of an intriguing pattern. On closer examination, the peptide with retention time of 16.7 minutes, which represented that of caerulein (Figure 3.3), appeared to fluctuate in abundance in a series of cycles. A new peptide, with a retention time of 13.4 minutes and not observed before within the peptide profile of *Litoria splendida*, also ~~showed to emerge~~ appeared at particular periods. The appearance of this new peptide was very specific as it emerged only during certain months of the year. The structure of the new peptide was identified by ESMS. Positive ion ESMS determined the molecular weight of the peptide to be *m/z* 1368. Negative ion CA MS/MS of the parent ion (Figure 3.4) showed a pronounced loss of 80 Da, indicating the loss of a sulfate (SO_3) group. A combination of methylation experiments and positive ion CA MS/MS of the parent ion (Figure 3.5) determined the structure of this peptide to be pGlu Gln Asp Tyr (SO_3) Thr Gly Trp Phe Asp Phe (NH_2). Full structural determination details are recorded in Table 3.1. The peptide was named Phe^8-caerulein, as it corresponds to the structure of caerulein, except that the Phe^8 replaces the Met^8 residue.

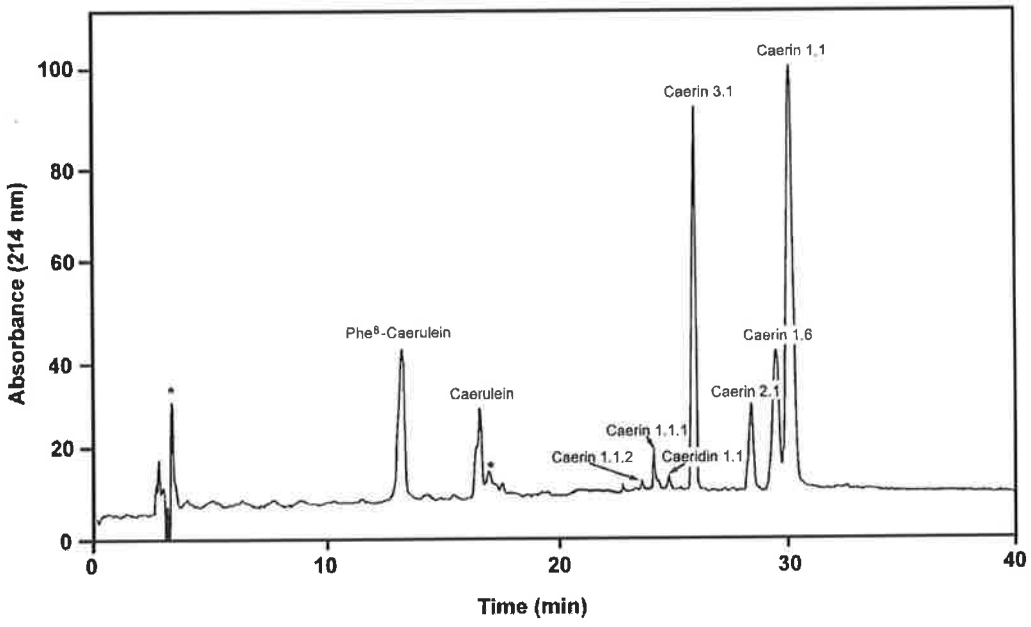


Figure 3.3. Chromatogram of the HPLC separation of crude skin secretions taken from *Litoria splendida*. Secretion taken during the month of July (winter season). (See Experimental section for full details).

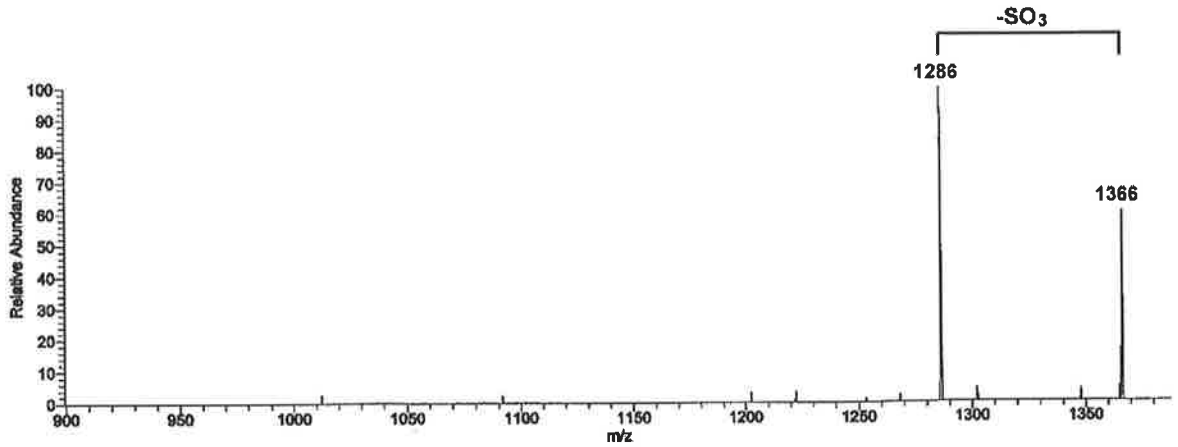


Figure 3.4. Negative ion CA MS/MS of $[M-H]^-$ (*m/z* 1366) ion of Phe[8]-caerulein. Note the loss of 80 Da. (See Experimental section for full details).

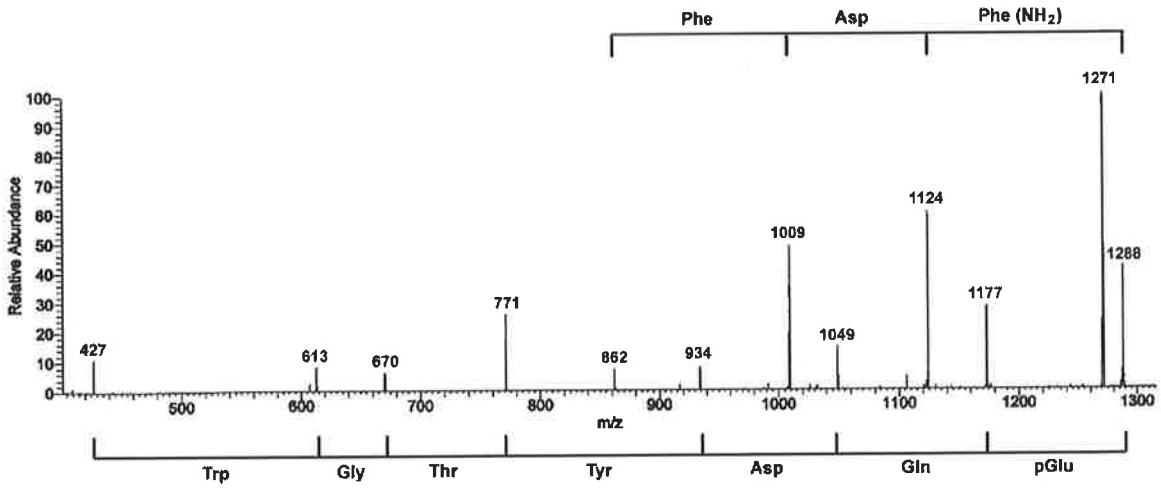


Figure 3.5. Positive CA MS/MS of the $[MH^+ -SO_3]$ ion of Phe[8]-caerulein (*m/z* 1288). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

The cyclic fluctuation of the abundance of both caerulein and Phe[8]-caerulein appeared to correspond together with the seasonal changes throughout the year. For example, over the entirety of the three-year period of this research, Phe[8]-caerulein was not produced throughout the majority of the year within the secretions of either the male and female adults of *Litoria splendida*. At the same time however, caerulein was present in considerable abundance. In contrast, Phe[8]-caerulein appeared during the winter periods (June-August) at which time the abundance of caerulein was reduced. This phenomenon is summarised in Figures 3.6 and 3.7.

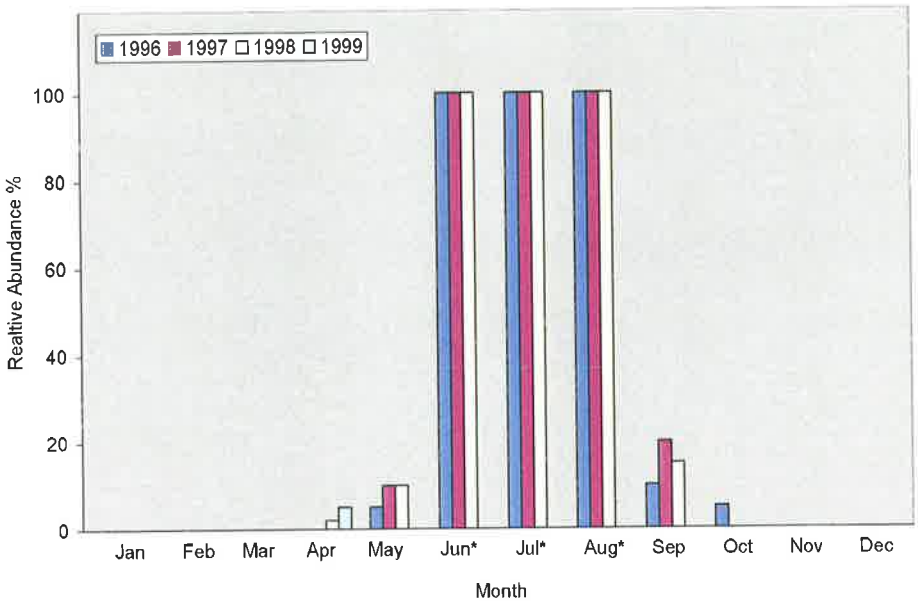


Figure 3.6. Relative abundance of Phe^{8}-caerulein over a three-year period within male and female *Litoria splendida* secretions. Legend shows the corresponding year in which the peptide was monitored. * Denotes the winter months.

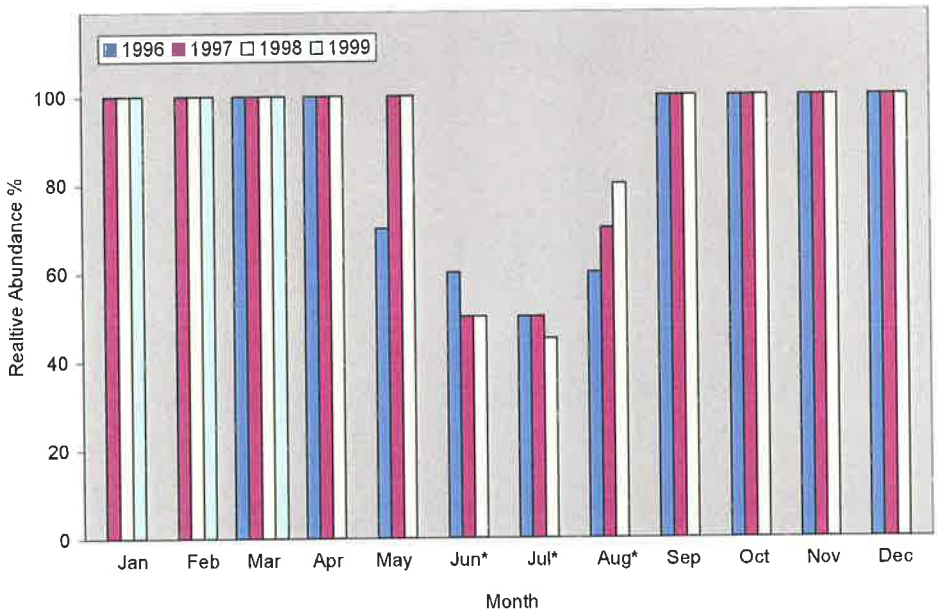


Figure 3.7. Relative abundance of caerulein over a three-year period within male and female *Litoria splendida* secretions. Legend shows the corresponding year in which the peptide was monitored.
* Denotes the winter months.

Caerulein [MH^+ 1352]

Methylation gives a methyl ester, MH^+ 1362 (one pGlu, one Tyr (SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1442 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

MH^+ 1352	'B ions'	*m/z* 1335, 1188, 1073, 942, 756
MS/MS		[Trp Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1352, 1241, 1113, 998, 755, 654, 597
	Sequence	[pGlu Gln Asp Tyr(SO_3) Thr Gly]

Negative ion CA MS/MS gives predominantly $[M-H]^-$ 1350 and 1270, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of Caerulein as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Thr Gly Trp Met Asp Phe (NH_2)

Phe^8-Caerulein [MH^+ 1368]

Methylation gives a methyl ester, MH^+ 1378 (one pGlu, one Tyr(SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1458 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

$[MH^+ - SO_3]$ 1288	'B ions'	*m/z* 1271, 1124, 1009, 862
MS/MS		[Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1288, 1177, 1049, 934, 771, 670, 613, 427
	Sequence	[pGlu Gln Asp Tyr Thr Gly Trp]

Negative ion CA MS/MS gives predominantly $[M-H]^-$ 1366 and 1286, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of Phe^8-Caerulein as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Thr Gly Trp Phe Asp Phe (NH_2)

Table 3.1. MS data for peptides isolated from the caerulein region of the *Litoria splendida* secretion.

3.2c. Additional peptides in the adult male *Litoria splendida* skin secretions

Seventy-six skin secretions from male and female adults of *Litoria splendida* were obtained monthly and analysed by HPLC and ESMS over a three-year period. Two minor components were found present only within the male secretion. These two minor components are always present in the male secretion but absent in the female secretion. This can be seen by comparing the partial HPLC profiles of the skin secretions of the adult male with that of the female (Figure 3.8). Structural analysis revealed that one of the two peptides was a known peptide. The peptide with retention time of 28.9 minutes is caerin 2.3, while the other peptide with a retention time of 30.4 minutes is a new peptide that was named caerin 1.10. Collision activation mass spectra (MS/MS) of the $[M+2H]^{2+}$ ion of caerin 2.3 (*m/z* 1183) is shown in Figure 3.9, and the CA MS/MS of the $[M+2H]^{2+}$ ion of caerin 1.10 (*m/z* 1288) is shown in Figure 3.10. Full structural determination details are summarised in Table 3.2.

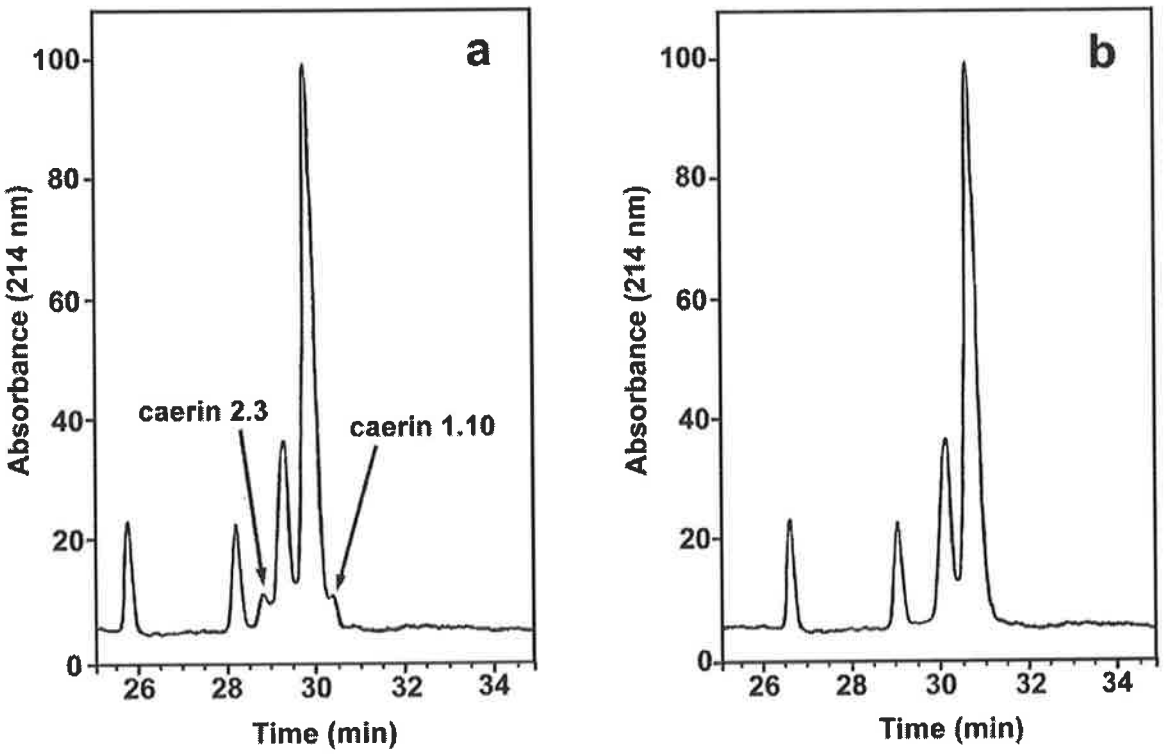


Figure 3.8. Partial HPLC chromatograms of the skin secretions of **a**, male and **b**, female *Litoria splendida*. (See Experimental section for full details).

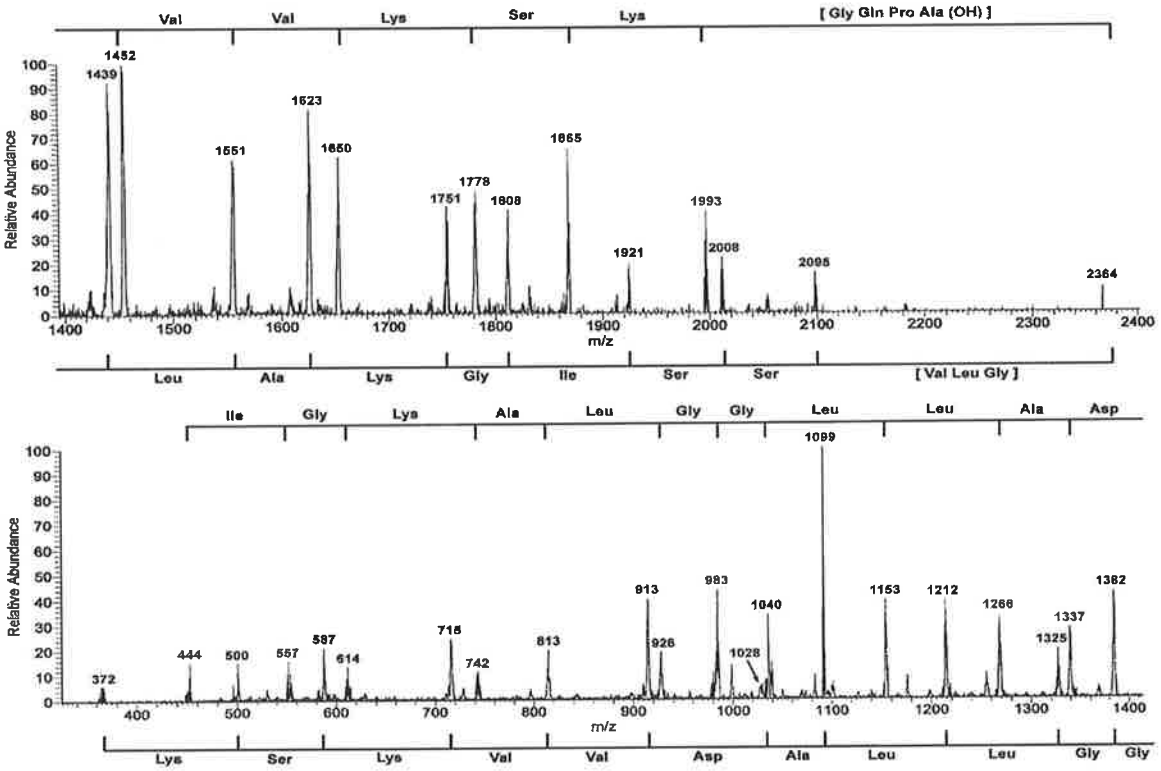


Figure 3.9. CA MS/MS of the $[M+2H]^{2+}$ ion of caerin 2.3 (*m/z* 1183). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

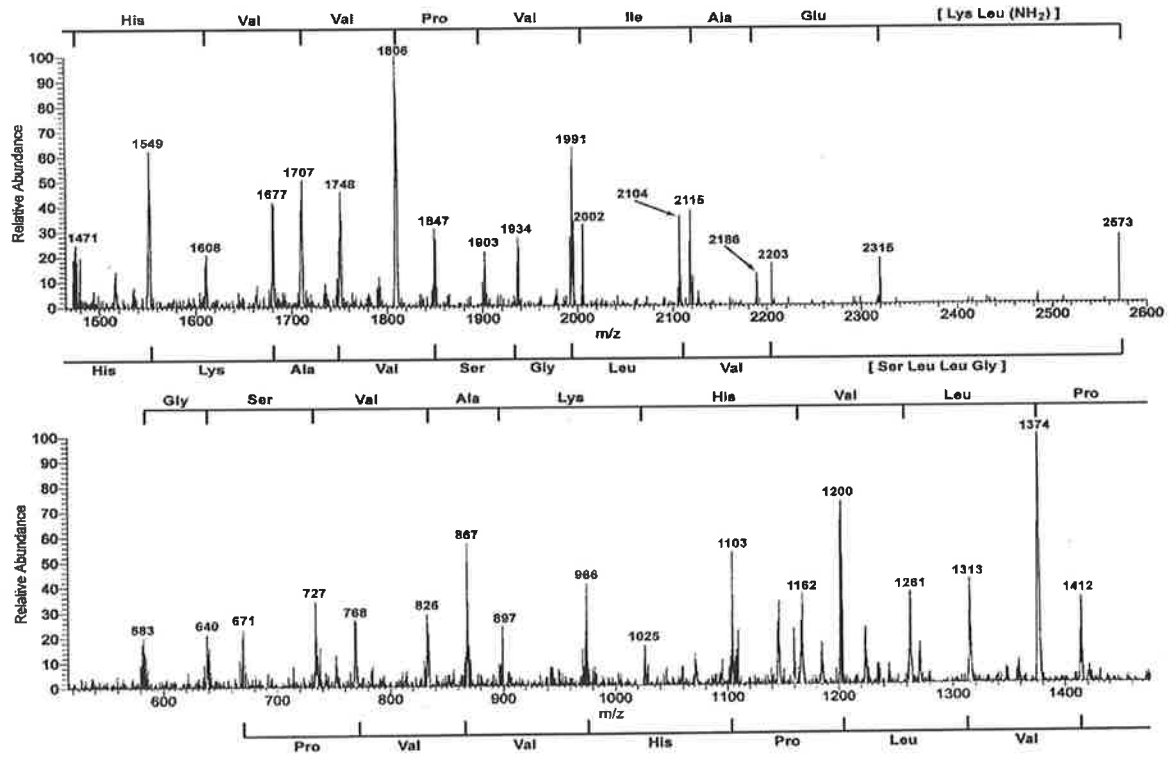


Figure 3.10. CA MS/MS of the $[M+2H]^{2+}$ ion of caerin 1.10 (*m/z* 1288). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

The abundance of caerin 2.3 fluctuates regularly throughout the three-year period. The concentration of caerin 2.3 peaked during the breeding season of *Litoria splendida* (January-March) and decreased tenfold during the period June to November, as summarised in Figure 3.11. Caerin 1.10 remained constant in abundance throughout the three-year period of this research.

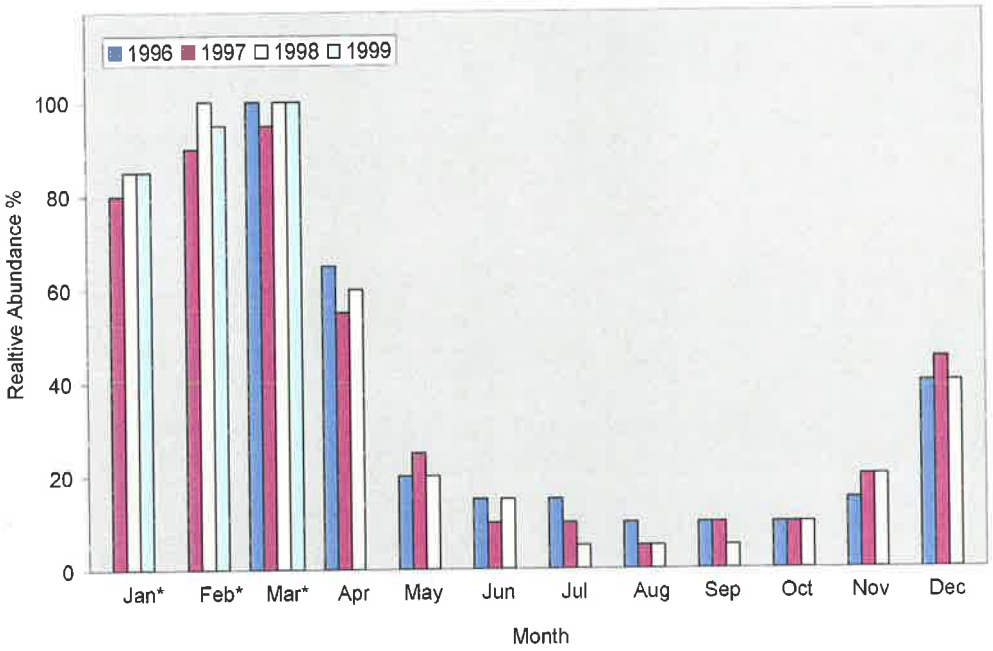


Figure 3.11. Relative abundance of caerin 2.3 over a three-year period within male *Litoria splendida* secretions. Legend shows the corresponding year in which the peptide was monitored. * Denotes the breeding period of *Litoria splendida*.

Caerin 2.3 [MH^+ 2364]

Prominent $[M+2H]^{2+}$ 1183 also present in spectra.

Methylation gives a methyl ester, MH^+ 2407 (two CO_2H and one $CONH_2$ group).

$[M+2H]^+$ 1183	'B ions'	*m/z* 1993, 1865, 1778, 1650, 1551,
MS/MS		1452, 1337, 1266, 1153, 1040, 983,
		926, 813, 742, 614, 557, 444
		[Ile Gly Lys Ala Leu Gly Gly Leu Leu Ala
		Asp Val Val Lys Ser Lys -(OH)]
	'Y+2 ions'	*m/z* 2095, 2008, 1921, 1808, 1751, 1623, 1552, 1439, 1382, 1325, 1212, 1099, 1028, 913, 814, 715, 587, 500, 372
		[Ser Ser Ile Gly Lys Ala Leu Gly Gly Leu
		Leu Ala Asp Val Val Lys Ser Lys]
	Sequence	Ser Ser Ile Gly Lys Ala Leu Gly Gly Leu
		Leu Ala Asp Val Val Lys Ser Lys –(OH)]

Lys-C digestion gives MH^+ 760 and 587.

MH^+ 760	'B ions'	*m/z* 742, 614, 557, 444, 357, 270
MS/MS		[Ser Ser Ile Gly Lys (OH)]
	'Y+2 ions'	*m/z* 760, 703, 590, 491, 404, 317, 204
		[Gly Leu Val Ser Ser Ile]
	Sequence	Gly Leu Val Ser Ser Ile Gly Lys (OH)
MH^+ 587	'B ions'	*m/z* 569, 498, 401, 273, 216
MS/MS		[Gly Gln Pro Ala (OH)]
	'Y+2 ions'	*m/z* 587, 500, 372, 315, 187
		[Ser Lys Gly Gln]
	Sequence	Ser Lys Gly Gln Pro Ala (OH)

Full sequence of Caerin 2.3 as determined from mass spectrometric and automated Edman data is:

Gly Leu Val Ser Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Ala Asp Val Val Lys Ser Lys Gly Gln Pro Ala (OH)

Table 3.2. MS data for the additional peptides isolated only from male *Litoria splendida* secretions.

Caerin 1.10 [MH^+ 2573]

Prominent $[M+2H]^{2+}$ 1288 also present in spectra.

Methylation gives a methyl ester, MH^+ 2602 (one CO_2H and one $CONH_2$ group).

$[M+2H]^+$ 1288 MS/MS	'B ions'	*m/z* 2315, 2186, 2115, 2002, 1903, 1806, 1707, 1608, 1471, 1374, 1261, 1162, 1025, 897, 826, 727, 640, 583 [Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu- (NH_2)]
	'Y+2 ions'	*m/z* 2203, 2104, 1991, 1934, 1847, 1748, 1677, 1549, 1412, 1313, 1200, 1103, 966, 867, 768, 671 [Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro]
	Sequence	Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu- (NH_2)

Lys-C digestion gives MH^+ 1043, 1437 and 1549.

MH^+ 1043 MS/MS	'B ions'	*m/z* 1025, 897, 826, 727, 640, 583,470 [Leu Gly Ser Val Ala Lys (OH)]
	'Y+2 ions'	*m/z* 1043, 986, 873, 760, 673, 574, 461 [Gly Leu Leu Ser Val Leu]
	Sequence	Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys (OH)
MH^+ 1549 MS/MS	'B ions'	*m/z* 1532, 1419, 1291, 1162, 1091, 978, 879, 782, 683, 584, 447 [His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2)]
	'Y+2 ions'	*m/z* 1549, 1412, 1313, 1200, 1103, 966, 867, 768, 671, 572 [His Val Leu Pro His Val Val Pro Val]
	Sequence	His Val Leu Pro His Val Val Pro Val Ile Ala Glu Lys Leu (NH_2)

Full sequence of Caerin 1.10 as determined from mass spectrometric and automated Edman data is:

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

Table 3.2. *(Continued)*

3.2d. Biological activity determination

The biological activity of caerulein as a powerful neuropeptide has been known for some time, and it has been applied medically as a muscle relaxant in various clinical procedures [5]. Phe[8]-caerulein is present in both male and female *Litoria splendida*. This is a new peptide not reported from any other biological system, as indicated by searches of various peptide and protein database libraries. Phe[8]-caerulein is likely to be a potent neuropeptide. Unfortunately, this peptide is very difficult to synthesise, since synthetic preparations lack the sulfate group. This appears to be lost following removal of the peptide from the resin support. Consequently, neuropeptide activity testing has not been possible for Phe[8]-caerulein to this time. The biological testing work is being continued by the group of Professor Vittorio Erspamer (La Sapienza, Rome) but to this time, they have also been unable to synthesise Phe[8]-caerulein.

The first peptide only found in the male secretion was caerin 2.3. This is a known peptide, already isolated from another Australian tree frog, *Litoria caerulea*, a species closely related to *Litoria splendida*. Caerin 2.3 was tested against a range of microorganisms using a MIC test, as shown in Table 3.3. The results were negative; caerin 2.3 is not an antibacterial agent.

The second peptide, found only in the male secretion, has been named caerin 1.10. This new peptide shows significant wide spectrum antibacterial activity (Table 3.3).

Peptide	Reference	Sequence	Antibacterial activity
caerin 2.3	C2.3	GLVSS I GKALGGLLADVVKSKGQPA (OH)	inactive
caerin 1.10	C1.10	GLLSVLGDVAKHVLPHVVPV I AEKL (NH_2)	active

	MIC (μg/ml)	
	C2.3	C1.10
Bacillus cereus		
Escherichia coli		
Leuconostoc lactis		6
Listeria innocua		50
Micrococcus luteus		25
Pasteurella multocida		100
Staphylococcus aureus		
Staphylococcus epidermidis		100
Streptococcus uberis		50

Table 3.3. Antibacterial activities of caerin 2.3 and caerin 1.10 from male *Litoria splendida*. Inactive in this context means the activity is > 100 μg/ml. Where there is no figure listed the MIC value is > 100 μg/ml.

3.2e. Conclusions

This research has revealed some interesting biological patterns within the skin secretions of adult *Litoria splendida*. The discovery of the new peptide Phe[8]-caerulein (which shows analogy with caerulein and is thought to be a neuropeptide) is of interest because of its seasonal fluctuation. *Litoria splendida*, in its natural habitat hibernates during the winter season. Consequently, the fact that this peptide appears during the winter months, and yet disappears from the skin secretion for the remainder of the year, may possibly imply that its role is directly related to temperature regulation. Previous research has implicated that certain neuropeptides can indirectly regulate the control of thermogenesis in mammals and amphibians [6]. Neuropeptides that influence the levels of galanin (GAL) and nitric oxide synthase (NOS) can also effect the temperature regulation of an anuran. For example, the results of an experimental protocol involving amphibians have shown a temperature regulated expression of GAL and NOS in the hypothalamus and preoptic areas of the CNS of the toad, suggesting that GAL and NOS may effect hibernation in these animals[6]. Since *Litoria splendida* has a hibernation period during the winter months, this formation of Phe[8]-caerulein during the winter period might possibly relate to its role in thermogenesis during the hibernation period of this animal.

The discovery of caerin 2.3 and caerin 1.10 within the skin secretions of only the males of *Litoria splendida* is intriguing, particularly the observation of the cycling pattern of caerin 2.3. The increase in concentration of caerin 2.3 occurs during the breeding cycle of this species. The concentration of caerin 2.3 is at a maximum during January to March (breeding period of *Litoria splendida*) and a minimum throughout the months of May to November. This suggests the possibility of a relationship between caerin 2.3 and the reproductive cycle of this animal. The role(s) of caerin 2.3 and caerin 1.10 need to be further investigated. This has been done using behavioural studies specifically targeted towards the possibility of pheromone activity, and is reported in Chapter 4.

3.3. Experimental

3.3a. Collection and preparation of male and female *Litoria splendida* secretions

The skin secretions from male and female *Litoria splendida* were provided courtesy of Associate Professor Mike Tyler, Department of Environmental Biology, University of Adelaide. The secretions were obtained by the SES method, as described in section 2.3a. The resulting secretion was washed off the skin with water (distilled 25 ml). Methanol (25 ml) was immediately added to the aqueous extract, the mixture centrifuged, and concentrated to approximately 1 ml. Samples were taken separately from both male and female adult groups of *Litoria splendida* once a month over a total period of 37 months.

3.3b. HPLC separation

HPLC separation was achieved for each sample using a VYDAC C_{18} Protein and Peptide (218TP54) reverse phase column equilibrated with 10% acetonitrile/ aqueous 0.1% TFA. Additional purification was achieved using an elution profile of 40-65% acetonitrile over a period of 60 minutes. Each sample was filtered through a Millipore 0.45 μm filter and injected onto the column. The conditions for the various HPLC chromatograms represented in this chapter are as follows:

Figure 3.1. The elution profile increased from 10-70% acetonitrile over a period of 30 minutes using a flow rate of 1 ml/min. The crude secretion was diluted in deionised water (500 μl), left untreated for a period of 45 minutes before being injected onto the column, and analysed.

Figure 3.2. The elution profile increased from 10-70% acetonitrile over a period of 30 minutes using a flow rate of 1 ml/min.

Figures 3.3 and 3.8. The elution profiles increased from 10-75% acetonitrile over a period of 40 minutes using a flow rate of 1 ml/min.

3.3c. Mass spectrometry analysis

Electrospray mass spectra were determined using a Finnigan LCQ ion trap mass spectrometer. The samples were dissolved in methanol/water (1:1) and infused into the electrospray source at a flow rate of 8 µl/min. Collision activation mass spectral data in positive mode were obtained using collision energy of 35%. In the negative mode, a collision energy of 45% was used. Electrospray conditions were as follows: source voltage 4.2 kV, source current 17 µA, capillary temperature 200°C, capillary voltage 3V and sheath gas flow 30 psi. Mass spectra were acquired with the automatic gain control on, a maximum ion time of 400 milliseconds, and using 3 microscans per scan, averaging over approximately 20 scans. Molecular weights of peptides were determined from both MH^+ or $[M+2H]^{2+}$ ions and $[M-H]^-$ ions as appropriate.

3.3d. Additional information

The remaining procedures are identical to those described elsewhere, as detailed in the table below.

Procedure	Section in which it is described
Methylation of peptides	Section 2.3d (page 74)
Enzyme digestion using Lys-C	Section 2.3e (page 75)
Antibacterial testing	Section 2.3g (page 76)
Preparation of synthetic peptides	Section 2.3h (page 76)
Automated Edman sequencing	Section 2.3i (page 76)

3.4. References

[1] Birch, M. C., in *Pheromones* (eds. Neuberger, A and Tatum, E. L) 1-14 (North-Holland Research Monographs, Frontiers of Biology, Amsterdam, 1974).

[2] Stone, D., Waugh, R., Bowie, J., Wallace, J. and Tyler, M., *J. Chem. Soc. Perkin Trans I.*, **11**, 3173-3176 (1992).

[3] Wabnitz, P. A., Walters, H., Tyler, M. J., Wallace, J. C. and Bowie, J. H., *J. Peptide Res.*, **52**, 477-481 (1998).

[4] Resnick, N.M., Maloy, W.L., Guy, H.R. and Zasloff, M., *Cell*, **66**, 541-554 (1991).

[5] Anastasi, A., Erspamer, V. and Endean, R., *Experientia*, **25**, 659-670 (1968).

[6] Gonzalez Nicolini, M.V., Orezzoli, A.A. and Villar, M.J., *Gen. Comp. Endo.*, **110**, 175-181 (1998).

CHAPTER 4. *Litoria splendida* behavioural studies

4.1. Introduction

4.1a. Pheromones

Information in regard to the outside world is crucial to every life form. Humans acquire this information primarily through sight and sound. Many creatures on the other hand rely on chemical signals as their predominant source of information concerning the world around them. These chemical signals may be messages that come from other organisms or events in the environment [1]. The ability to sense and respond to chemicals is vital for species as diverse as seaweeds, grapevine moths and house mice [2]. Even bacteria respond to certain chemicals, enabling them to move towards amino acids and away from toxic compounds [3].

One very important group of these chemical signals is the pheromones, which are the signals that members of a species use to communicate with one another [3]. The word pheromone was originally derived from the Greek words *pherin* (to transfer) and *hormon* (to excite) [4]. Pheromone research first began in the late 19th century on a particular species of silk moth [5]. It was shown that the female moths could attract the male of their species, sometimes over distances of several kilometres [6]. By the 1930s, researchers had determined that this attraction was due to minute quantities of volatile chemical compounds discharged by the female moth and detected by the male in his antennae [3]. The first pheromone was discovered and isolated by the German organic chemist, Aldolf Butenandt. His research, which began in the late 1930s, lasted 20 years and finally led to the identification of the sex pheromone of the female silkworm moth (*Bombyx mori*) [3, 7]. From half a million moths, Butenandt and his co-workers managed to obtain 6.4mg of the purified pheromone. The pheromone, which they named bombykol, was (10*E*, 12*Z*)-hexadececadien-1-ol (Figure 4.1).

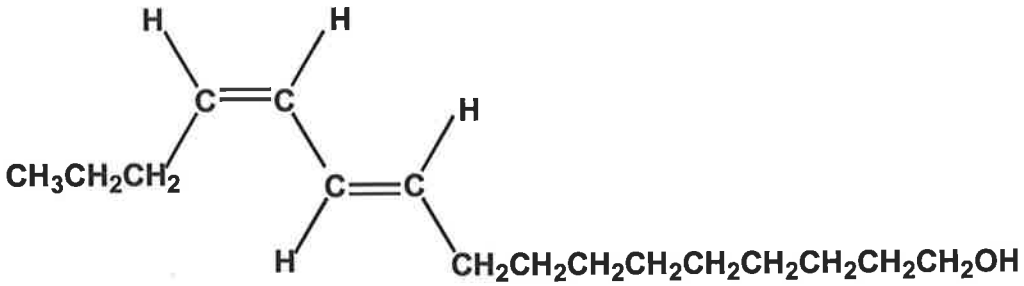


Figure 4.1. (10*E*, 12*Z*)-Hexadececadien-1-ol (bombykol).

The discovery of bombykol generated an interest by researchers worldwide in learning the chemical structures and functions of other pheromones, as this was an exciting new area of research that combined chemistry, biology and behavioural studies.

4.1b. Pheromone categories

Pheromones are generally active in minute quantities. They may evoke a behavioural response as a single compound or a complex mixture [4]. In either case, the complex of compounds or the single compound is a pheromone if it stimulates a behavioural reaction in the receiving organism(s) [8]. This behavioural response is dependent on the type of signal or message that the pheromone is carrying. Some different categories of pheromones are listed (Table 4.1), together with the kind of behavioural reactions that they can stimulate.

Pheromone Category	Some of the Related Behavioural Responses
Recognition Pheromones	*Recognize individual organisms of the same species.* *Recognize the physiological or social status of the species.* *Recognize the organism's own home, nest or home range.*
Aggregation Pheromones	*Aggregate others to a food source.* *Aggregate others to a suitable habitat that is to be colonized.* *Regulate the activities of some social insects.* *Important role in the parent-young relationships of vertebrates.*
Dispersion Pheromones	*Maintain optimal separation between individual organisms.* *Maintain separation between territorial social groups.* *Cause dispersion of threatening situations (Alarm Pheromones).* *Counteract the influence of aggregation stimuli when aggregation is inappropriate.*
Aggression Pheromones	*Induce aggression in the more dominant organisms when they perceive the odour of subordinates.*
Sex Pheromones	*Stimulate reactions in the opposite sex that either directly or indirectly enhance the likelihood that mating will occur, therefore promoting reproduction.*

Table 4.1. Some pheromone categories.

4.1c. Alarm pheromones

Alarm pheromones are used in communication systems that usually operate over very small distances and for short periods of time [6]. These kinds of pheromones are released by organisms that may be injured or that perceive a threatening situation, causing a dispersion of any nearby organisms of the same species [4, 6]. Alarm pheromones have been found present in a variety of vertebrate and invertebrate animals [6]. An example is the alarm pheromone of the common sea anemone, *Anthopleura elegantissima* [9]. If the anemone is wounded or threatened by a predator, it produces an alarm pheromone that warns neighbouring anemones of the same species of the danger. An anemone that receives this signal instantly protects itself. It shakes its tentacles, pulls them into its mouth cavity, and closes its mouth, all in less than three seconds. When the alarm signal is no longer present, the anemone opens and resumes its

normal activities within about two hours [3, 9]. The pheromone responsible for this message was named anthopleurine (Figure 4.2), and was the first ionic pheromone to be reported.

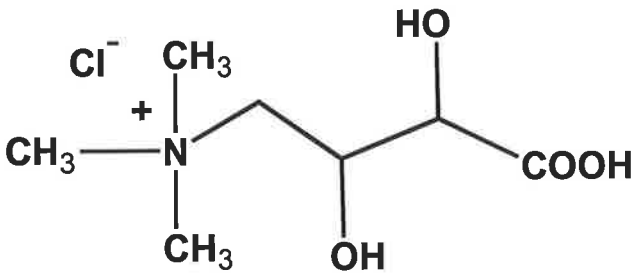


Figure 4.2. Anthopleurine (as the chloride).

4.1d. Sex pheromones

Sex pheromones are chemicals that are secreted by organisms of one sex and cause behavioural reactions in the opposite sex that facilitate mating [6]. Of all pheromone types, sex pheromones have been the most extensively studied. Organisms that use this type of pheromone may exhibit a variety of reactions to it. These reactions fall into two main categories:

(i) Reactions that lead to aggregation near the pheromone source.

(ii) Reactions that lead to close-range courtship or direct sexual behaviour.

For most organisms to date, there is a trend that the female releases the aggregation-inducing pheromone in order to attract the male [6]. Usually, the male approaches the female following an aerial odour trail (or the aquatic equivalent) to her vicinity, as previously shown in the example of the female silkworm moth (*Bombyx mori*). In a few animal species however, females have shown be attracted to pheromone releasing males[10, 11].

4.1e. The pheromone communication system

A typical communication system consists of three components:

(i) A mechanism for message emission.

(ii) A medium for message transmission.

(iii) A mechanism for message reception.

In reference to pheromone communication, the emitting mechanism usually involves a glandular organ associated with a specialized mechanism that transfers the chemical molecules into the surrounding medium [6]. The types and locations of pheromone glands found in insects and mammals vary considerably. Most of the pheromone glands found in insects and mammals are constituted of modified epidermal glands [12]. The gland may consist of a single layer of cells on the exterior of the organism, or may be complex and associated with internal reservoirs [6].

The medium used for transmission is either air or water, and is dependent on the lifestyle of the organism and the medium in which it lives. Pheromones are transmitted and dispersed throughout the water/air by diffusion and passive transport. Diffusion involves the random movement of molecules, and is important for the distribution of pheromones when the medium is not flowing. Diffusion rates in water are a lot slower than those in air, and are dependent on the rate of release of pheromone and the overall molecular concentration. Passive transport, however, is attributed to the movement or flow of the medium. Since air and water are rarely static the medium is usually flowing.

Pheromone reception is usually perceived by a process known as olfaction*. The exact mechanism for olfaction is quite complex, but it is generally understood that the receptors for olfaction in both vertebrates and invertebrates are quite similar in structure. They primarily consist of a series of specialized sensory neurons extending into either a cavity or sensory fibres [13]. A layer of liquid film surrounds these extended neurons [14]. In both vertebrates and invertebrates the pheromones must be absorbed into this liquid bathing the neurons, and then move to the receptor sites within the neurons by diffusion [15]. Pheromones may therefore be considered as molecular ligands that bind to receptors in the olfactory sensory neurons, giving rise to the sensory response [16].

* Olfaction refers to the sense of smell.

4.1f. Pheromones in amphibians

Compared to insects and mammals, very little is known about pheromones of non-mammalian vertebrates. Of these, the fish pheromones have been the most extensively studied. Even though there is little known about pheromones in amphibians and reptiles, the alarm reaction of some anuran tadpoles has been recognized [4], and one sex pheromone has been discovered in a particular ureodole species [11].

The alarm reaction of tadpoles of the toad *Bufo bufo* was first observed in the 1950s when crushed *Bufo bufo* tadpole was brought near a tadpole school of that same species. A strong alarm reaction resulted that was thought to be due to a pheromone. The tadpoles closest to the extract became disturbed and swam further away, while the neighbouring tadpoles avoided the region that was occupied by the crushed tadpole. The effect of the extract was shown to diminish within two minutes. The experiments also showed that the olfactory organ perceived the alarm substance, as tadpoles that had their olfactory nerves transected no longer responded. The mystery of the origin of this alarm pheromone remains, since there are no skin glands present in tadpoles prior to metamorphosis.

The sex pheromone, sodefrin, was isolated from the cloacal gland of the male red-bellied newt, *Cynops pyrrhogaster*. It demonstrated female attraction, which was rare and was shown to be species specific. Sodefrin, a decapeptide (Figure 4.3), was the first and only amphibian pheromone identified and the first and only peptide pheromone identified in a vertebrate to date.

Ser Ile Pro Ser Lys Asp Ala Leu Leu Lys (OH)

Figure 4.3. Sodefrin.

Anurans are amphibians, and therefore they could have both terrestrial (volatile) and aquatic (water-soluble) pheromones. Since anurans may breed in aquatic conditions, and deposit their eggs in water, it is possible that they will produce peptides as water-soluble pheromones. This proposal has been supported by the identification of sodefrin. Besides this example, no other peptide pheromone has ever been identified in a vertebrate, and to date no anuran pheromone has ever been identified.

The aims of the work presented in this chapter are:

(i) To conduct behavioural studies on *Litoria splendida* involving caerin 1.10.

(ii) To conduct behavioural studies on *Litoria splendida* involving caerin 2.3.

4.2. Results and Discussion

4.2a. Behavioural studies procedure

Adult specimens of *Litoria splendida* were used for the behavioural study experiments. These specimens were originally obtained from the Kimberley region of Western Australia in 1994 and have been held in captivity ever since. The experiment was conducted in a two-metre glass aquarium tank to which water was added producing a water depth of approximately 2cm. Two sterile gauze pads were placed at opposite ends of the tank as shown in Figure 4.4.

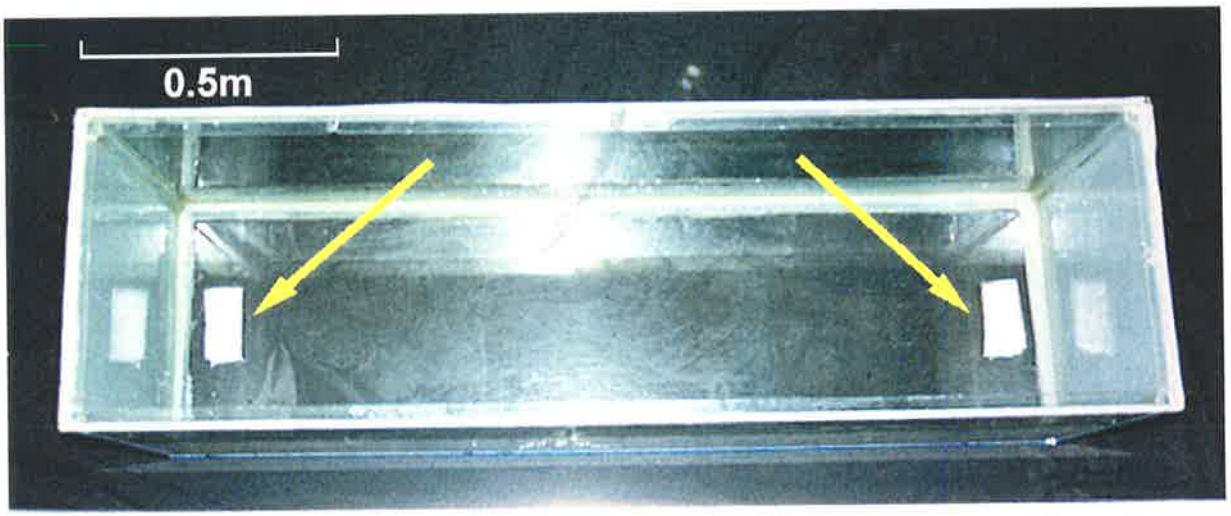


Figure 4.4. Aerial view of aquarium layout. Gauze pads indicated by the two yellow arrows.

Specimens of adult *Litoria splendida* were placed in the centre of the tank, where they were left for a period of 5 minutes. This allowed the amphibian to condition itself to the new surroundings. The aquarium was identical to that in which the animal lived. After this period of stabilisation, the peptide solution was introduced to only one of the two gauze pads, and at this point timing began. The movements of *Litoria splendida* were carefully recorded and timed for a period of 30 minutes. The amphibian was then removed from the tank and returned to its original environment. Tests were repeated in the same manner using the same series of samples and concentrations over a total period of 4 weeks to confirm reproducibility.

The samples used for this experiment involved the peptides caerin 1.10 and caerin 2.3, which are present only in the male *Litoria splendida*. Both the naturally derived peptides and synthesized ones were used. The synthetic peptides were all made with amino acids in their L-isomeric form, and were identical in primary structure to the natural peptides. The purity of the peptides was confirmed by high performance liquid chromatography and electrospray mass spectrometry prior to use. Concentrations of peptide within the tank ranged from 10^{-14} M to 10^{-6} M.

4.2b. Behavioural studies of caerin 1.10 with female and male *Litoria splendida*

Caerin 1.10 of varying concentrations was first tested on a series of adult female *Litoria splendida*. The results showed random movement on all tests and overall indicated no significant result.

Caerin 1.10 was also tested on a series of adult male *Litoria splendida* for comparison. The results showed some agitation from the males. Overall however, random movement was again noted. The experiments were repeated over a period of several weeks (see Table 4.2 and Table 4.3). It was determined that caerin 1.10 had no significant result in terms of behavioural response on either male or female adult specimens of *Litoria splendida*. This was true at all concentrations from 10^{-14} to 10^{-6} M of the test substance within the container.

Test Number	Caerin 1.10 Pad	Blank Pad
1	×	×
2	×	×
3	×	×
4	×	×

Table 4.2. Success rate of caerin 1.10 as an attractant on females of *Litoria splendida*. Experiment conducted in a series of tests, with the test specimens not being used more than once. ✓ Indicates test specimen successfully comes into contact with the pad containing the test substance. × Indicates test specimen does not have any contact with the pad containing the test substance.

Test Number	Caerin 1.10 Pad	Blank Pad
1	×	×
2	×	×
3	×	×
4	×	×

Table 4.3. Success rate of caerin 1.10 as an attractant on males of *Litoria splendida*. Experiment conducted in a series of tests, with the test specimens not being used more than once. ✓ Indicates test specimen successfully comes into contact with the pad containing the test substance. × Indicates test specimen does not have any contact with the pad containing the test substance.

4.2c. Behavioural studies of caerin 2.3 with female *Litoria splendida*

Synthetically derived caerin 2.3 of varying concentrations was tested on a number of adult females of *Litoria splendida*. Results of interest were first observed at 30ng of caerin 2.3, which produced a total concentration within the container of 10^{-10} M* and are described as follows. Within a period of about twenty seconds after the peptide solution containing caerin 2.3 was introduced onto the gauze pad, a few notable changes occurred. The addition of caerin 2.3 elicited a distinct change in posture and an increased degree of alertness in the anuran within the 20 seconds of the introduction of the peptide. Within a few minutes, the female of *Litoria splendida* approached the peptide saturated gauze pad, sat on it, and remained seated until removed. This occurred in a series of timed movements as represented in Figure 4.5.

* Concentration assuming sample completely dispersed in tank. Actual concentration of peptide in the vicinity of the anuran is likely to be much less than this.

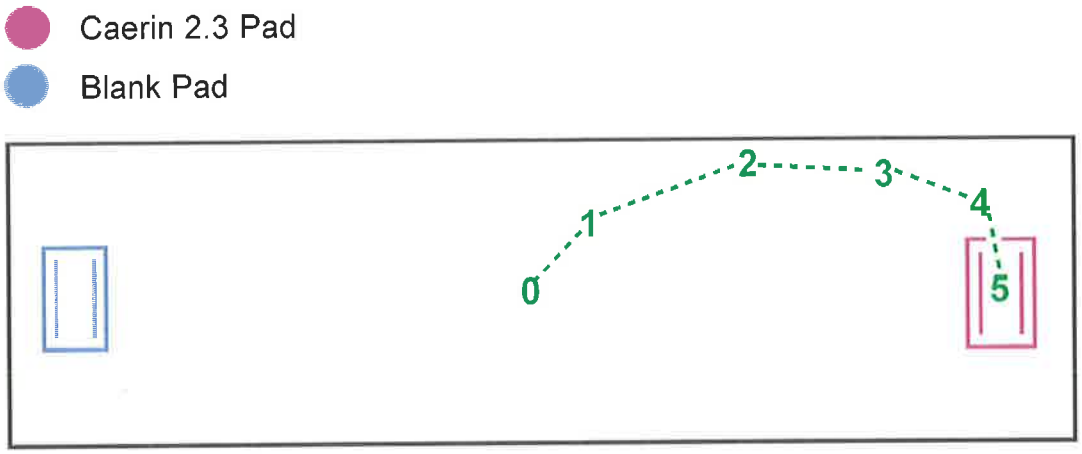


Figure 4.5. Schematic representation of the attracting effect of caerin 2.3 on an adult female *Litoria splendida*. Timed movements of the test specimen are numerically indicated: (0) initially on introduction of test substance; (1) 2 minutes; (2) 3 minutes; (3) 4.5 minutes; (4) 5 minutes; (5) 6.5 minutes.

This is further shown below in Figure 4.6.

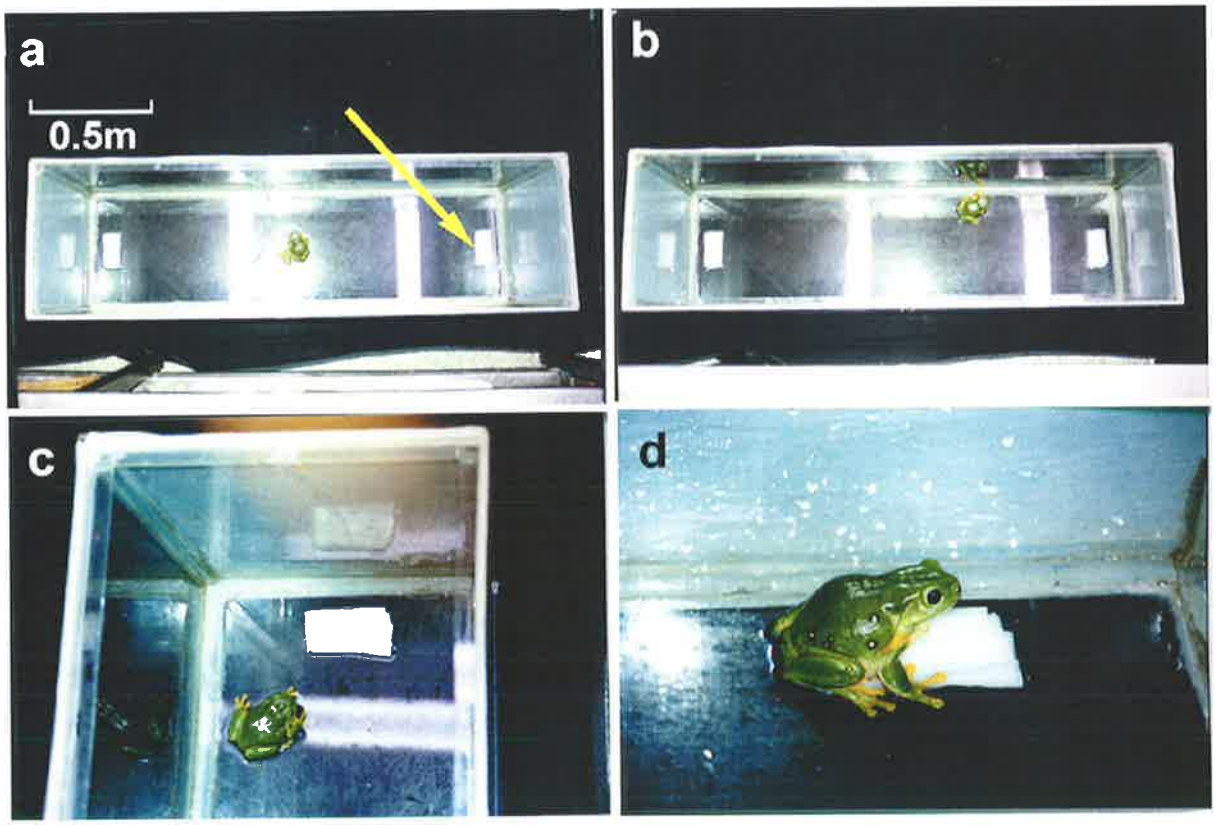


Figure 4.6. Aerial views of an aquarium with an adult female *Litoria splendida*, demonstrating the attracting effect to caerin 2.3: (a) immediately after caerin 2.3 has been introduced, the gauze pad containing caerin 2.3 is shown by the arrow; (b) 3 minutes after introduction of caerin 2.3, female is moving towards the peptide region; (c) 1.5 minutes after b; (d) 2 minutes after c, female is now directly on the gauze pad containing caerin 2.3.

The fact that this particular amphibian remained on the gauze pad for a considerable period was unusual as *Litoria splendida*, being a tree frog, has a natural tendency to climb up the sides of the aquarium.

After each test, the aquarium was emptied and thoroughly washed with methanol and distilled water (see chapter 4.3 for full experimental details). The experiment was performed on a number of occasions with 6 different females. The success rate was 100% and the average time from the introduction of the pheromone until the time the female sits on the peptide containing gauze pad was 7 minutes. The end of the aquarium in which the test peptide was introduced was also alternated. In another experiment, the peptide solution containing caerin 2.3 was introduced onto the gauze pad at the opposite end of the aquarium to the previous example. The female again moved from the centre of the aquarium towards the area containing the peptide. After exactly 9 minutes the female was directly on top of the gauze pad. The timed movements of this second example are represented in Figure 4.7.

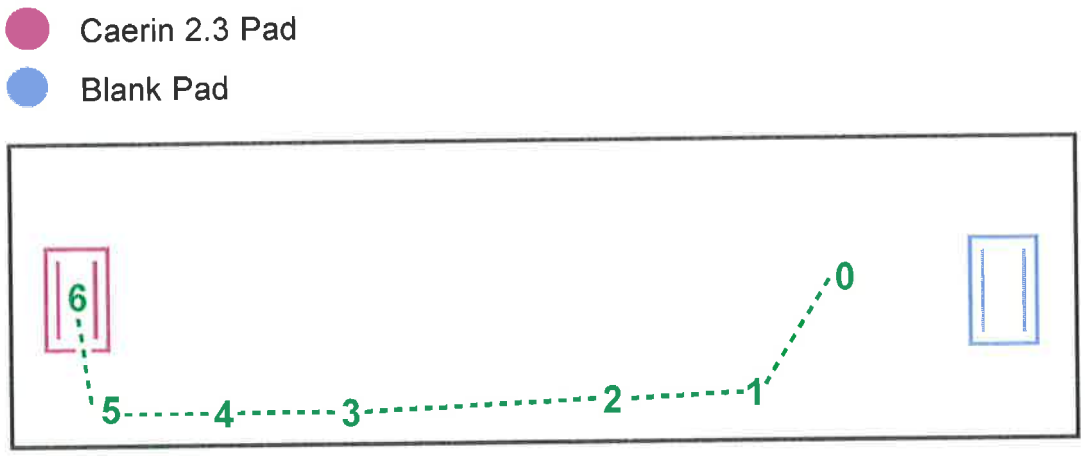


Figure 4.7. Schematic representation of the attracting effect of caerin 2.3 on an adult female *Litoria splendida*. Timed movements of the test specimen are numerically indicated: (0) initially on introduction of test substance; (1) 5 minutes; (2) 6 minutes; (3) 7.5 minutes; (4) 8 minutes; (5) 8.5 minutes; (6) 9 minutes.

The observation that the amphibian moved in the opposite direction compared to the previous example, but still towards the peptide region, ruled out the possibility that the amphibian was moving towards an external attractant like a light source or sound.

The results of these experiments are summarised in Table 4.4. The optimal concentration range of caerin 2.3 within the container was also determined to be somewhere between 10^{-11} and 10^{-8} M (results represented in Figure 4.8). The anurans did not respond to the pheromone when the concentrations of caerin 2.3 < 10^{-11} M. When the concentration of pheromone was > 10^{-7} M the females became confused and agitated, ultimately climbing up the walls of the tank.

Test Number	Caerin 2.3 Pad	Time Taken for Test Specimen to Reach Caerin 2.3 Pad (min)	Blank Pad
1	✓	6.5	×
2	✓	9	×
3	✓	7	×
4	✓	7.5	×
5	✓	8.5	×
6	✓	8	×

Table 4.4. Success rate of caerin 2.3 as an attractant on females of *Litoria splendida*. Experiment conducted in a series of tests, with the test specimens not being used more than once. ✓ Indicates test specimen successfully comes into contact with the pad containing the test substance. × Indicates test specimen does not have any contact with the pad containing the test substance.

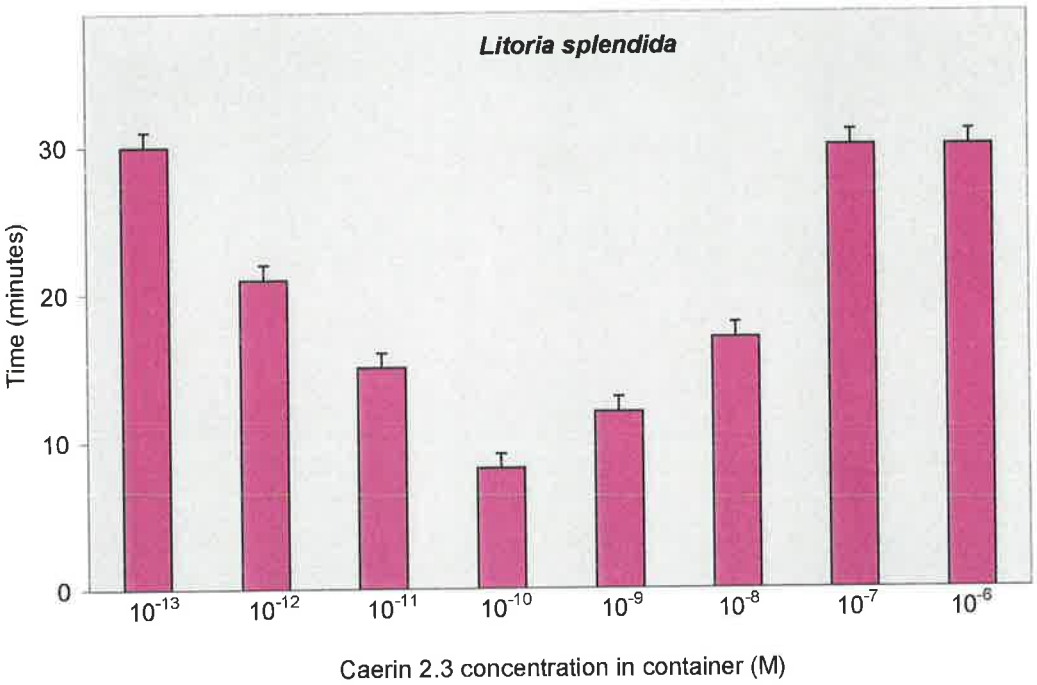


Figure 4.8. Determination of the minimum effective concentration of caerin 2.3 on females of *Litoria splendida*. Attracting effect represented as the amount of time taken for the test specimen to reach the gauze pad containing the test substance. The minimum effective concentration was determined as the minimum concentration of caerin 2.3 to induce attraction on the test specimen. Results represent mean values (± SE) of three tests.

Most biologically active peptides that have been isolated from Australian amphibians have a C-terminal $CONH_2$ end group[17]. Caerin 2.3 isolated from male *Litoria splendida* however, has a C-terminal CO_2H end group. A synthetically modified version of caerin 2.3 with a C-terminal $CONH_2$ end group was tested for pheromone activity. This was carried out in order to confirm the specificity of these behavioural results in relation to the peptide with a carboxyl terminal end. The synthetically modified caerin 2.3 was tested for pheromone activity on four specimens of female *Litoria splendida* (see Table 4.5).

Test Number	C-Terminal Modified Caerin 2.3 Pad	Blank Pad
1	×	×
2	×	×
3	×	×
4	×	×

Table 4.5. Success rate of C-terminal modified caerin 2.3 as an attractant on females of *Litoria splendida*. Experiment conducted in a series of tests, with the test specimens not being used more than once. ✓ Indicates test specimen successfully comes into contact with the pad containing the test substance. × Indicates test specimen does not have any contact with the pad containing the test substance.

The results indicated that there was no attraction of the females towards the synthetically modified caerin 2.3. This indicated that the attraction was specific to the naturally occurring peptide with the free acid terminal group.

4.2d. Behavioural studies of caerin 2.3 with male *Litoria splendida*

Caerin 2.3 was then tested on several adult male *Litoria splendida* for comparison. The peptide displayed no attractive effect on any male, and only random movement was noted (see Table 4.6). There was no evidence of aggressive behaviour towards the pheromone (for example, against a competing male).

Test Number	Caerin 2.3 Pad	Blank Pad
1	×	×
2	×	×
3	×	×
4	×	×

Table 4.6. Success rate of caerin 2.3 as an attractant on males of *Litoria splendida*. Experiment conducted in a series of tests, with the test specimens not being used more than once. ✓ Indicates test specimen successfully comes into contact with the pad containing the test substance. × Indicates test specimen does not have any contact with the pad containing the test substance.

4.2e. Behavioural studies of caerin 2.3 with female and male *Litoria caerulea*

As described in section 3.2d, the closely related species *Litoria caerulea* also contains caerin 2.3 within its skin secretion. Caerin 2.3 was tested on *Litoria caerulea* for pheromone activity. Caerin 2.3 of varying concentrations was tested on a series of adult female *Litoria caerulea*. The peptide showed no effect on the amphibian, and only random movements were noted (Table 4.7).

Test Number	Caerin 2.3 Pad	Blank Pad
1	×	×
2	×	×
3	×	×
4	×	×

Table 4.7. Success rate of caerin 2.3 as an attractant on females of *Litoria caerulea*. Experiment conducted in a series of tests, with the test specimens not being used more than once. ✓ Indicates test specimen successfully comes into contact with the pad containing the test substance. × Indicates test specimen does not have any contact with the pad containing the test substance.

Caerin 2.3 was also tested on a series of adult male *Litoria caerulea*. The results indicated no significant effect on the amphibian, and only random movements were noted (Table 4.8). Therefore, no behavioural response from either the male or female adults of the species *Litoria caerulea* was produced by the caerin 2.3 peptide.

Test Number	Caerin 2.3 Pad	Blank Pad
1	×	×
2	×	×
3	×	×
4	×	×

Table 4.8. Success rate of caerin 2.3 as an attractant on males of *Litoria caerulea*. Experiment conducted in a series of tests, with the test specimens not being used more than once. ✓ Indicates test specimen successfully comes into contact with the pad containing the test substance. × Indicates test specimen does not have any contact with the pad containing the test substance.

4.2f. Conclusions

The results of these behavioural studies indicated that caerin 2.3 is the aquatic sex aggregation pheromone of the male *Litoria splendida*. Caerin 2.3 has been renamed splendipherin, to indicate pheromone activity. The biological role of splendipherin is that it attracts the female *Litoria splendida* to the male in aquatic conditions. In the natural habitat, *Litoria splendida* is a terrestrial animal that spends most of its time high above land within the protection of trees. Only during the mating season does it move down into an aquatic environment to mate and deposit the eggs. *Litoria splendida* does not drink water through its mouth, but instead absorbs it through its permeable skin layer. Therefore, as these behavioural tests were conducted in only a shallow depth of water (2cm deep), the amphibian must be absorbing the pheromone through its skin. This process is rapid, as a behavioural response related to its change in posture and attentive stance is noted almost immediately after splendipherin is introduced into the tank. The speed of this recognition of the pheromone means that it cannot be due to the peptide diffusing through the solution. The amphiphilic peptide must be acting as a surfactant, with the hydrophilic zone of the helical peptide interacting with the surface of the water, and ultimately being absorbed through the skin of the anuran. When the amount of pheromone added to the pad is greater than 4000 nanograms, the female although still alert, is unable to find the source of the pheromone. Decreasing the amount to below 4 nanograms elicits no response from the female. The optimal concentration range of splendipherin within the container was determined to be between 10^{-11} and 10^{-8} M. Splendipherin evokes no

behavioural response in male *Litoria splendida* using identical pheromone tests. No behavioural response was noted when either the female or male of the closely related species *Litoria caerulea* was exposed to splendipherin. The aquatic sex pheromone splendipherin is therefore species specific.

It was also shown that only splendipherin containing a free acid carboxyl terminal end was active as an attractant, and not its modified version with an amide C-terminal end. This is interesting since most of the biologically active peptides isolated from Australian amphibians have C-terminal $CONH_2$ end group functionality[17]. Natural splendipherin and synthetic (all L amino acids) splendipherin had identical female-attracting activity. This confirmed that all the amino acids were L-isomers, which is usually true for all naturally occurring biologically active peptides[18].

Anurans are an understudied group in terms of their chemical communication[19]. Splendipherin is the first anuran pheromone to be isolated and identified. Splendipherin is also the second peptide pheromone to date identified in a vertebrate. Given the fact that *Litoria splendida* reproduces in an aquatic environment, a non-volatile but water-soluble peptide is a reasonable form to expect as a pheromone in this amphibian.

The reason why the antibiotic caerin 1.10 occurs only in the male skin of *Litoria splendida* remains a mystery. There is no evidence that it plays a sex-related role.

4.3. Experimental

4.3a. General

Adult male and female specimens of *Litoria splendida* and *Litoria caerulea* were used for all of the described behavioural tests. The specimens of *Litoria splendida* were originally from the Kimberley region of Western Australia, and have been held in captivity since 1994. Test specimens were selected from a total of eight males and seven female adults of *Litoria splendida*. Adult male and female specimens of *Litoria caerulea* were originally from the Northern Territory, and have been held in captivity since 1995. Test specimens were selected from fourteen males and twelve female adults of *Litoria caerulea*. Both species have been held in temperature and humidity regulated aquariums since captivity. The aquarium and the room in which the experiments were conducted were regulated at a constant temperature of 28°C and 65% humidity with ultraviolet lighting overhead, mimicking their natural environment. Caerin 1.10 and splendipherin (caerin 2.3) were derived from male *Litoria splendida* as described in chapter 3. Both peptides were synthesised (Chiron Mimotopes, Victoria see section 2.3h) in order to provide more material for the behavioural tests. The test substances were dissolved in deionised water. All experiments were repeated, comprising a series of at least six tests for each experiment. After each test was conducted, the aquarium was thoroughly washed with methanol and water and then finally rinsed with water. The aquarium was filled with fresh water and new gauze pads in readiness for the next test. No test female was used more than once. The optimal concentration was determined using varying concentrations of the test substance, ranging from 10^{-14} to 10^{-6} M (assuming dispersal into the whole volume of water in the tank).

4.3b. Experimental procedure

A glass aquarium, with dimensions 650mm x 2000mm x 750mm, was filled with 1600 ml of tap water. Two gamma sterilized cotton gauze pads (dimensions 100mm x 200mm) were folded in half and placed at opposite ends of the glass aquarium. The test specimen was then placed in the middle of the aquarium. Five minutes after the specimen was stabilised, the test

substance was introduced and absorbed onto one of the gauze pads within the aquarium, while the other gauze pad only contained tap water. At this point all movements of the test animal were recorded and timed. Thirty minutes after the test substance was introduced the experiment concluded.

4.4. References

[1] Berliner, D. L., Jennings-White, C. and Lavker, M., *J. Steroid Biochem. Molec. Biol.*, **39**, 671-678 (1991).

[2] Guerin, P., Arn, H. and Buser, H. R., in *Semiochemistry Flavours and Pheromones* (eds. Acree, T. and Soderlund, D.) 239-254 (Proceedings American Chemical Society Symposium, Washington, DC, 1983).

[3] Agosta, W. C., *J. Chem. Ed.*, **71**, 242-259 (1994).

[4] Birch, M. C., in *Pheromones* (eds. Neuberger, A. and Tatum, E. L.) 3-14 (North-Holland Research Monographs, Frontiers of Biology, Amsterdam, 1974).

[5] Singer, A. G., *J. Steroid Biochem. Molec. Biol.*, **39**, 627-632 (1991).

[6] Shorey, H. H., in *Animal Communication by Pheromones*, (Academic Press, New York, 1976), chap. 2-10.

[7] Butenandt, A. and Hecker, E. Z., in *Techniques in Pheromone Research* (eds. Hummel, H. E. and Miller, T. E.) 5-34 (Springer, New York, 1984).

[8] Johnston, R. E., in *Pheromones and Reproduction in Mammals* (ed. Vandenbergh, J. G.) 5-18 (Academic Press, New York, 1983).

[9] Howe, N. R. and Sheikh, Y. M., *Science*, **189**, 386-390 (1975).

[10] Greet, D. N., *Nature*, **204**, 96-99 (1964).

[11] Kikuyama E.A., *Science*, **267**, 1643-1649 (1995).

[12] Kauer, J. S., *J. Physiol.*, **243**, 695-671 (1974).

[13] Scott, J. W., *J. Steroid Biochem. Molec. Biol.*, **39**, 593-600 (1991).

[14] Evershed, R. P., in *CRC Handbook of Natural Pesticides Volume IV Pheromones Part A* (eds. Morgan, D. and Mandava, B.) 4-25 (CRC Press, Inc., Florida, 1988).

[15] Pace, U., Hanski, E., Salomon, Y. and Lancet, D., *Nature*, **316**, 255-232 (1985).

[16] Shepherd, G. M. and Firestein, S., *J. Steroid Biochem. Molec. Biol.*, **39**, 583-588 (1991).

[17] Bowie, J.H., Wegener, K.L., Chia, B.C.S., Wabnitz, P.A., Carver, J.A., Tyler, M.J. and Wallace, J.C., *Protein and Peptide Letters*, in press.

[18] Lazarus, L.H. and Attila, M., *Prog. Neurobiol.*, **41**, 473-507 (1993).

[19] Houck, L.D., *Amer. Zool.*, **38**, 108-117 (1998).

CHAPTER 5. Peptides from *Litoria citropa*

5.1. Introduction

5.1a. *Litoria citropa*

Litoria citropa (Figure 5.1), also known as the 'Blue Mountains tree frog', has been described as one of the most beautiful tree frogs in Australia [1]. As an adult, it measures about 4-6.5 cm in length, and is found in the eastern regions of Victoria and New South Wales (Figure 5.2)[2]. This tree frog is quite unique as it has two separate types of skin glands that are distinct from each other, compared with most frogs that have only one type of skin gland. The two types of skin glands of *Litoria citropa* are:

(i) Dorsal granular glands, which are distributed evenly throughout the dorsal surface.

(ii) The submental gland, which is a relatively large gland located underneath the throat region.

Figure 5.1. *Litoria citropa*.

Figure 5.2. Map of Australia indicating the location of *Litoria citropa*. Distribution indicated by the black region.

To date, all other Australian tree frogs that have been studied for skin peptides have contained only one type of skin gland responsible for skin secretion. The extra submental gland of *Litoria citropa* is quite rare. Consequently, it is of interest to investigate the role of this gland, and to observe whether it secretes host defence peptides of the type produced from the dorsal glands of other amphibians.

The aims of the research presented in this chapter are:

(i) To isolate and determine the structure of the peptides of *Litoria citropa* secreted from both dorsal and submental glands.

(ii) To compare the peptide profiles of the two distinct glandular secretions.

(iii) To determine the antibacterial activity of any new peptides discovered, and to compare this spectrum of activity with those of the antibacterial peptides isolated from previously studied tree frogs of the genus *Litoria*.

5.2. Results and Discussion

5.2a. General

A single specimen of *Litoria citropa* was obtained in 1997. Two separate skin secretions were obtained using the surface electrical stimulation method [3]. The first one was taken from the dorsal glands and the other from the submental gland. On average, about 8mg of solid peptide material was acquired from the dorsal glands and 3mg from the submental gland after work-up. The peptides within each secretion were separated using HPLC. The HPLC chromatogram from the dorsal gland secretion (Figure 5.3) was similar to that obtained from the submental gland secretion (Figure 5.4), except for four peptides that were found only in the dorsal secretion.

In all, twenty-one peptides were isolated and characterised following HPLC separation. The first two peptide fractions from both secretions have been excluded from this chapter, and from the HPLC chromatograms, and will be discussed in more detail in the next chapter. Nineteen peptides, which are in the chromatogram regions of 30-60 min, are discussed in this chapter. The component peptides under consideration are labelled A-S in both HPLC chromatograms. The amino acid sequences of the nineteen peptides, which were isolated and characterised from the dorsal and submental secretions, are listed in Table 5.1. These amphibian peptides are new and various data bank searches have indicated that there are no reported analogues from any living system. The peptides were named citropins from the name *Litoria citropa*. Both the dorsal and submental glands produced fifteen of these citropin peptides, with the dorsal glands producing four additional citropin peptides.

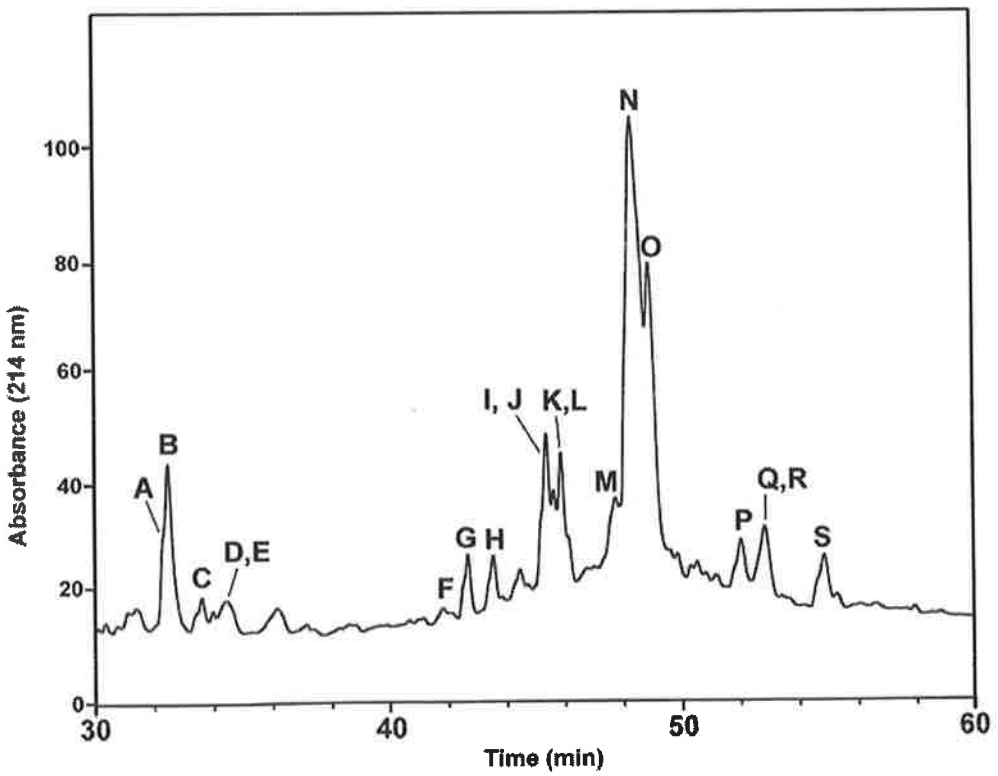


Figure 5.3. Partial chromatogram of the HPLC separation of crude skin secretions taken from the dorsal glands of *Litoria citropa*. (See Experimental section for full details).

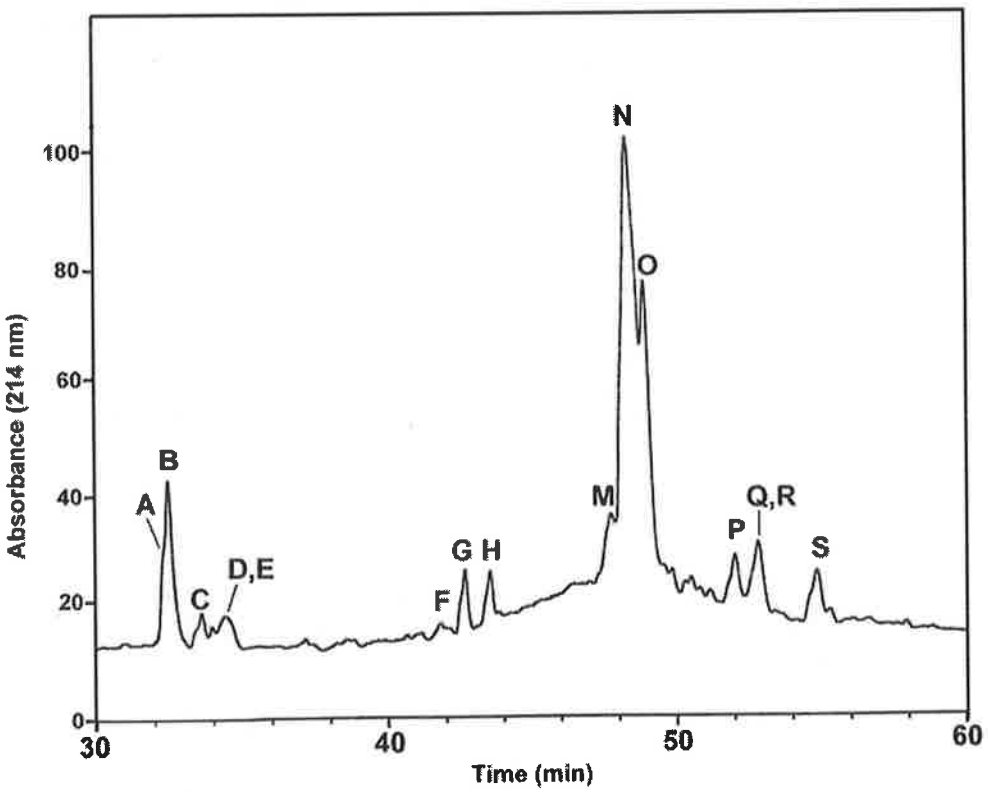


Figure 5.4. Partial chromatogram of the HPLC separation of crude skin secretions taken from the submental gland of *Litoria citropa*. (See Experimental section for full details).

Citropin	MW	Sequence	HPLC fraction	Gland
1.1	1614	GLFDVIKKVASVIGGL (NH_2)	N	Dor./ Sub.
1.1.1	1444	FDV I KKVASV I GGL (NH_2)	E	Dor./ Sub.
1.1.2	1297	DV I KKVASV I GGL (NH_2)	B	Dor./ Sub.
1.1.3	1813	GLFDV I KKVASV I GLASP (OH)	J	Dor.
1.1.4	1844	GLFDV I KKVASV I GLASQ (OH)	L	Dor.
1.2	1614	GLFD I I KKVASVVGGL (NH_2)	O	Dor./ Sub.
1.2.1	1444	FD I I KKVASVVGGL (NH_2)	D	Dor./ Sub.
1.2.2	1297	D I I KKVASVVGGL (NH_2)	A	Dor./ Sub.
1.2.3	1189	GLFD I I KKVAS (NH_2)	C	Dor./ Sub.
1.2.4	1813	GLFD I I KKVASVVGLASP (OH)	K	Dor.
1.2.5	1844	GLFD I I KKVASVVGLASQ (OH)	I	Dor.
1.3	1628	GLFD I I KKVASV I GGL (NH_2)	M	Dor./ Sub.
2.1	2160	GL I GS I GKALGGLLVDVLKPKL (OH)	S	Dor./ Sub.
2.1.1	2288	GL I GS I GKALGGLLVDVLKPKLQ (OH)	Q	Dor./ Sub.
2.1.2	2430	GL I GS I GKALGGLLVDVLKPKLQAA (OH)	R	Dor./ Sub.
2.1.3	2517	GL I GS I GKALGGLLVDVLKPKLQAAS (OH)	P	Dor./ Sub.
3.1	2456	DLFQV I KEKLKELTGGVIEG I Q (OH)	F	Dor./ Sub.
3.1.1	2513	DLFQV I KEKLKELTGGVIEG I QG (OH)	G	Dor./ Sub.
3.1.2	2612	DLFQV I KEKLKELTGGVIEG I QGV (OH)	H	Dor./ Sub.

Table 5.1. Peptides isolated from skin secretions taken from the dorsal and submental glands of *Litoria citropa*. Dor. indicates peptides isolated from the dorsal glands (on the back). Sub. indicates peptides isolated from the submental gland (underneath throat).

There are three groups of citropin peptides isolated and characterised from *Litoria citropa*. These are categorised as citropins 1, 2 and 3. Citropins 1.1, 1.1.2, 1.1.3, 1.2, 1.3, 2.1.1, 2.1.3 and 3.1 were synthesised. The native and synthetic peptides were shown to be identical using HPLC and mass spectrometry. One peptide from each group of citropin peptides will now be described including the MS structure determination of that peptide in detail. The structure determinations of all the other citropin peptides are summarised in Tables 5.2, 5.3 and 5.4.

5.2b. Citropin 1.1

All skin peptides derived from *Litoria citropa* were analysed by ESMS to determine their structures. Where applicable automated Edman sequencing was used to differentiate between Leu and Ile, and Lys-C digestion together with ESMS experiments were used to distinguish Lys from Gln. Methylation experiments together with ESMS identified all C-terminal end groups, and gave an indication of the number of the CO_2H and $CONH_2$ groups within the peptides.

Citropin 1.1 is one of the major fractions from the *Litoria citropa* skin secretion. Analysis of citropin 1.1 by ESMS indicated a very prominent $[M+2H]^{2+}$ peak at *m/z* 808 and a MH^+ peak at *m/z* 1614 within the spectrum. Methylation of citropin 1.1 yielded a methyl ester, MH^+ 1644. The difference in mass between citropin 1.1 and the methyl ester is 29Da, which indicates the presence of one CO_2H group and one $CONH_2$ group. Collision activation mass spectrum (MS/MS) of the parent ion of citropin 1.1 (*m/z* 1615) is shown in Figure 5.5.

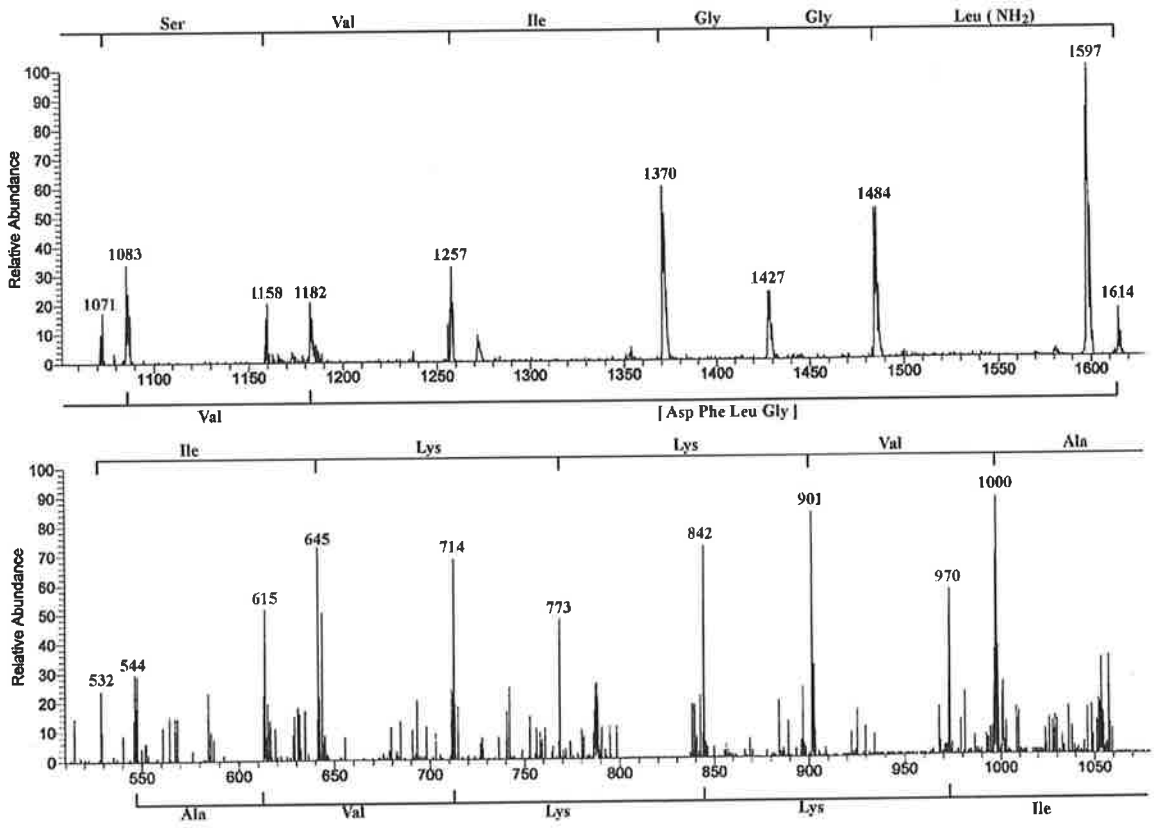


Figure 5.5. CA MS/MS of the MH^+ ion of citropin 1.1 (*m/z* 1614). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

The amino acid sequence determined from the CA MS/MS data is 432 –Val Ile Lys Lys Val Ala Ser Val Ile Gly Leu (NH_2). A number of residues (432 Da) at the N-terminal end of citropin 1.1 could not be identified from this spectrum. Enzyme digestion using the Lys-C /ESMS procedure was used to identify the unknown sequence. Lys-C digestion of citropin 1.1 gave four peptide fragments, *m/z* 919, 842, 791 and 714. These fragments confirmed the presence of the Lys^7 and Lys^8 residues. Sequence data was obtained for all four peptides; however, the spectrum of *m/z* 919 was all that was required to finalise the sequence of citropin 1.1. The collision induced spectrum (MS/MS) of *m/z* 919 (Figure 5.6) indicates the sequence of this fragment to be Gly Leu Phe Asp Val Ile Lys Lys (OH).

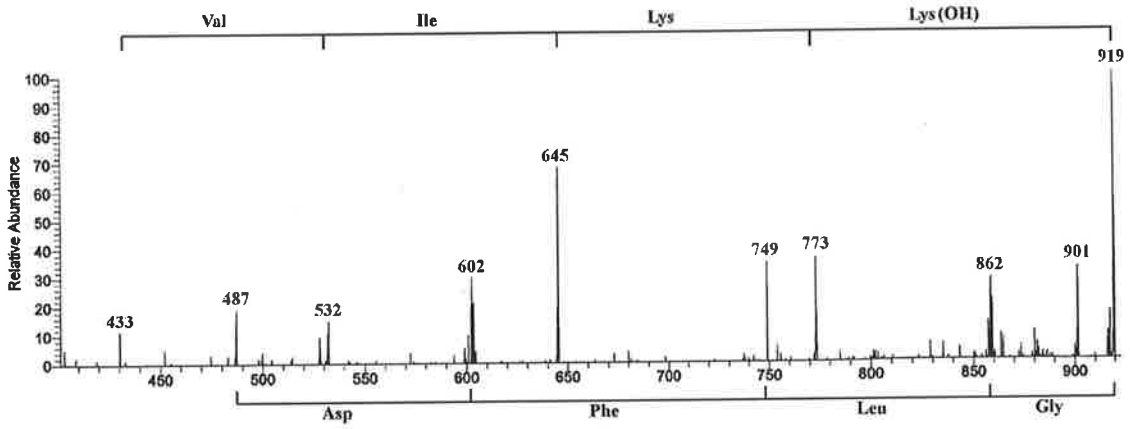


Figure 5.6. CA MS/MS of the Lys-C digest fragment ion of citropin1.1 (*m/z* 919). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

Combination of all the MS data gives the following sequence for citropin 1.1; Gly Leu Phe Asp Val Ile Lys Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2). This sequence, particularly Leu and Ile, was confirmed by the automated Edman procedure. Structural determination details of all the citropin 1 peptides are summarised in Table 5.2.

Citropin 1.1 [MH^+ 1614]

Prominent [M+2H] $^{2+}$ 808 also present in spectrum.
Methylation gives a methyl ester, MH^+ 1643 (one CO_2H and one $CONH_2$ group).

MH^+ 1614 MS/MS	'B ions'	*m/z* 1597, 1484, 1427, 1370, 1257, 1158, 1071, 1000, 901, 773, 645, 532 [Ile Lys Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 1614, – 1182, 1083, 970, 842, 714, 615, 544 [- Val Ile Lys Lys Val Ala]
	Sequence	Val Ile Lys Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2)

Lys-C digestion gives MH^+ 714, 791, 842 and 919

MH^+ 919 MS/MS	'B ions'	*m/z* 901, 773, 645, 532, 433 [Val Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 919, 862, 749, 602, 487 [Gly Leu Phe Asp]
	Sequence	Gly Leu Phe Asp Val Ile Lys Lys (OH)

Full sequence of Citropin 1.1 as determined from mass spectrometric and automated Edman data is:
Gly Leu Phe Asp Val Ile Lys Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2)

Citropin 1.1.1 [MH^+ 1444]

Prominent [M+2H] $^{2+}$ 723 also present in spectra.
Methylation gives a methyl ester, MH^+ 1473 (one CO_2H and one $CONH_2$ group).

MH^+ 1444 MS/MS	'B ions'	*m/z* 1427, 1314, 1257, 1200, 1087, 988, 901,830 [Ala Ser Val Ile Gly Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 1444, - 1182, - 970, 842, 714, 615, 544 [- Lys Lys Val Ala]
	Sequence	Lys Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2)

Lys-C digestion gives MH^+ 621, 714, 749 and 842

MH^+ 749 MS/MS	'B ions'	*m/z* 731, 603, 475, 362, 263 [Val Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 749, 602, 487, 388 [Phe Asp Val]
	Sequence	Phe Asp Val Ile Lys Lys (OH)

Full sequence of Citropin 1.1.1 as determined from mass spectrometric and automated Edman data is:
Phe Asp Val Ile Lys Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2)

Table 5.2. MS data for citropin 1 peptides isolated from *Litoria citropa*.

Citropin 1.1.2 [MH^+ 1297]

Prominent [M+2H] $^{2+}$ 650 also present in spectra.
Methylation gives a methyl ester, MH^+ 1326 (one CO_2H and one $CONH_2$ group).

MH^+ 1297 MS/MS	'B ions'	*m/z* 1280, 1167, 1110, 1053, 940, 841, 754, 683, 584 [Val Ala Ser Val Ile Gly Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 1297, 1182 [Asp]
	Sequence	Asp – Val Ala Ser Val Ile Gly Gly Leu (NH_2)

Lys-C digestion gives MH^+ 602, 714 and 842

MH^+ 602 MS/MS	'B ions'	*m/z* 584, 456, 328, 215 [Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 602, 487, 388, 275 [Asp Val Ile]
	Sequence	Asp Val Ile Lys Lys (OH)

Full sequence of Citropin 1.1.2 as determined from mass spectrometric and automated Edman data is:
Asp Val Ile Lys Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2)

Citropin 1.1.3 [MH^+ 1813]

Prominent [M+2H] $^{2+}$ 908 also present in spectra.
Methylation gives a methyl ester, MH^+ 1841 (two CO_2H groups).
Lys-C digestion gives MH^+ 791, 913, 919 and 1041

MH^+ 1041 MS/MS	'B ions'	*m/z* 1023, 926, 839, 768, 655, 598, 485 [Ile Gly Leu Ala Ser Pro (OH)]
	'Y+2 ions'	*m/z* 1041, 913, 814, 743, 656, 557, 444 [Lys Val Ala Ser Val Ile]
	Sequence	Lys Val Ala Ser Val Ile Gly Leu Ala Ser Pro (OH)
MH^+ 919 MS/MS	'B ions'	*m/z* 901, 773, 645, 532, 433 [Val Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 919, 862, 749, 602, 487 [Gly Leu Phe Asp]
	Sequence	Gly Leu Phe Asp Val Ile Lys Lys (OH)

Full sequence of Citropin 1.1.3 as determined from mass spectrometric and automated Edman data is:
Gly Leu Phe Asp Val Ile Lys Lys Val Ala Ser Val Ile Gly Leu Ala Ser Pro (OH)

Table 5.2. *(Continued)*

Citropin 1.1.4 [MH^+ 1844]

Prominent $[M+2H]^{2+}$ 923 also present in spectra.
Methylation gives a methyl ester, MH^+ 1887 (two CO_2H and one $CONH_2$ group).
Lys-C digestion gives MH^+ 791, 919, 944 and 1072

MH^+ 1072 MS/MS	'B ions'	*m/z* 1054, 926, 839, 768, 655, 598, 485 [Ile Gly Leu Ala Ser Gln (OH)]
	'Y+2 ions'	*m/z* 1072, 944, 845, 774, 687, 588, 475, 418 [Lys Val Ala Ser Val Ile Gly]
	Sequence	Lys Val Ala Ser Val Ile Gly Leu Ala Ser Gln (OH)
MH^+ 919 MS/MS	'B ions'	*m/z* 901, 773, 645, 532, 433 [Val Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 919, 862, 749, 602, 487 [Gly Leu Phe Asp]
	Sequence	Gly Leu Phe Asp Val Ile Lys Lys (OH)

Full sequence of Citropin 1.1.4 as determined from mass spectrometric and automated Edman data is:
Gly Leu Phe Asp Val Ile Lys Lys Val Ala Ser Val Ile Gly Leu Ala Ser Gln (OH)

Citropin 1.2 [MH^+ 1614]

Prominent $[M+2H]^{2+}$ 808 also present in spectra.
Methylation gives a methyl ester, MH^+ 1643 (one CO_2H and one $CONH_2$ group).

MH^+ 1614 MS/MS	'B ions'	*m/z* 1597, 1484, 1427, 1370, 1271, 1172, 1085, 1014, 915, 787, 659, 546 [Ile Lys Lys Val Ala Ser Val Val Gly Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 1614, – 1182, 1069, 956, 828 [- Ile Ile Lys]
	Sequence	Ile Ile Lys Lys Val Ala Ser Val Val Gly Gly Leu (NH_2)

Lys-C digestion gives MH^+ 700, 805, 828 and 933

MH^+ 933 MS/MS	'B ions'	*m/z* 915, 787, 659, 546, 433 [Ile Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 933, 876, 763, 616, 501 [Gly Leu Phe Asp]
	Sequence	Gly Leu Phe Asp Ile Ile Lys Lys (OH)

Full sequence of Citropin 1.2 as determined from mass spectrometric and automated Edman data is:
Gly Leu Phe Asp Ile Ile Lys Lys Val Ala Ser Val Val Gly Gly Leu (NH_2)

Table 5.2. *(Continued)*

Citropin 1.2.1 [MH^+ 1444]

Prominent [M+2H] $^{2+}$ 723 also present in spectra.
Methylation gives a methyl ester, MH^+ 1473 (one CO_2H and one $CONH_2$ group).

MH^+ 1444 MS/MS	'B ions'	*m/z* 1427, 1314, 1257, 1200, 1101,1002, 915, 844, 745, 617, 489 [Lys Lys Val Ala Ser Val Val Gly Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 1444, - 1182, - 956, 828, 700 [- Lys Lys]
	Sequence	Lys Lys Val Ala Ser Val Val Gly Gly Leu (NH_2)

Lys-C digestion gives MH^+ 635, 700, 763 and 828

MH^+ 763 MS/MS	'B ions'	*m/z* 745, 617, 489, 376, 263 [Ile Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 763, 616, 501 [Phe Asp]
	Sequence	Phe Asp Ile Ile Lys Lys (OH)

Full sequence of Citropin 1.2.1 as determined from mass spectrometric and automated Edman data is:
Phe Asp Ile Ile Lys Lys Val Ala Ser Val Val Gly Gly Leu (NH_2)

Citropin 1.2.2 [MH^+ 1297]

Prominent [M+2H] $^{2+}$ 650 also present in spectra.
Methylation gives a methyl ester, MH^+ 1326 (one CO_2H and one $CONH_2$ group).

MH^+ 1297 MS/MS	'b ions'	*m/z* 1280, 1167, 1110, 1053, 954, 855, 768, 697, 598 [Val Ala Ser Val Val Gly Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 1297, 1182, 1069 [Asp Ile]
	Sequence	Asp Ile – Val Ala Ser Val Val Gly Gly Leu (NH_2)

Lys-C digestion gives MH^+ 616, 700 and 828

MH^+ 616 MS/MS	'B ions'	*m/z* 598, 470, 342, 229 [Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 616, 501, 388, 275 [Asp Ile Ile]
	Sequence	Asp Ile Ile Lys Lys (OH)

Full sequence of Citropin 1.2.2 as determined from mass spectrometric and automated Edman data is:
Asp Ile Ile Lys Lys Val Ala Ser Val Val Gly Gly Leu (NH_2)

Table 5.2. *(Continued)*

Citropin 1.2.3 [MH^+ 1813]

Prominent [M+2H] $^{2+}$ 908 also present in spectra.
Methylation gives a methyl ester, MH^+ 1841 (two CO_2H groups).
Lys-C digestion gives MH^+ 805, 899, 933 and 1027

MH^+ 1027 MS/MS	'B ions'	*m/z* 1009, 912, 825, 754, 641, 584, 485 [Val Gly Leu Ala Ser Pro (OH)]
	'Y+2 ions'	*m/z* 1027, 899, 800, 729, 642, 543, 444 [Lys Val Ala Ser Val Val]
	Sequence	Lys Val Ala Ser Val Val Gly Leu Ala Ser Pro (OH)
[MH^+ 933 MS/MS	'B ions'	*m/z* 915, 787, 659, 546, 433 [Ile Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 933, 876, 763, 616, 501 [Gly Leu Phe Asp]
	Sequence	Gly Leu Phe Asp Ile Ile Lys Lys (OH)

Full sequence of Citropin 1.2.3 as determined from mass spectrometric and automated Edman data is:

Gly Leu Phe Asp Ile Ile Lys Lys Val Ala Ser Val Val Gly Leu Ala Ser Pro (OH)

Citropin 1.2.4 [MH^+ 1844]

Prominent [M+2H] $^{2+}$ 923 also present in spectra.
Methylation gives a methyl ester, MH^+ 1887 (two CO_2H and one $CONH_2$ group).
Lys-C digestion gives MH^+ 805, 930, 933 and 1058

MH^+ 1058 MS/MS	'B ions'	*m/z* 1040, 912, 825, 754, 641, 584, 485 [Val Gly Leu Ala Ser Gln (OH)]
	'Y+2 ions'	*m/z* 1058, 930, 831, 760, 673, 574 [Lys Val Ala Ser Val Val]
	Sequence	Lys Val Ala Ser Val Val Gly Leu Ala Ser Gln (OH)
MH^+ 933 MS/MS	'B ions'	*m/z* 915, 787, 659, 546, 433 [Ile Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 933, 876, 763, 616, 501 [Gly Leu Phe Asp]
	Sequence	Gly Leu Phe Asp Ile Ile Lys Lys (OH)

Full sequence of Citropin 1.2.4 as determined from mass spectrometric and automated Edman data is:

Gly Leu Phe Asp Ile Ile Lys Lys Val Ala Ser Val Val Gly Leu Ala Ser Gln (OH)

Table 5.2. *(Continued)*

Citropin 1.2.5 [MH^+ 1189]

Methylation gives a methyl ester, MH^+ 1218 (one CO_2H and one $CONH_2$ group).

MH^+ 1189	'B ions'	*m/z* 1172, 1085, 1014, 915, 787, 659
MS/MS		[Lys Lys Val Ala Ser (NH_2)]
	'Y+2 ions'	*m/z* 1189, 1132, 1019, 872, 757, 644, 531
		[Gly Leu Phe Asp Ile Ile]

Full sequence of Citropin 1.2.5 as determined from mass spectrometric and automated Edman data is:

Gly Leu Phe Asp Ile Ile Lys Lys Val Ala Ser (NH_2)

Citropin 1.3 [MH^+ 1628]

Prominent $[M+2H]^{2+}$ 815 also present in spectra.

Methylation gives a methyl ester, MH^+ 1657 (one CO_2H and one $CONH_2$ group).

Lys-C digestion gives MH^+ 714, 805, 842 and 933

MH^+ 933	'B ions'	*m/z* 915, 787, 659, 546, 433
MS/MS		[Ile Ile Lys Lys (OH)]
	'Y+2 ions'	*m/z* 933, 876, 763, 616, 501
		[Gly Leu Phe Asp]
	Sequence	Gly Leu Phe Asp Ile Ile Lys Lys (OH)
MH^+ 842	'B ions'	*m/z* 825, 712, 655, 598, 485, 386
MS/MS		[Val Ile Gly Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 842, 714, 615, 544, 457, 358
		[Lys Val Ala Ser Val]
	Sequence	Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2)

Full sequence of Citropin 1.3 as determined from mass spectrometric and automated Edman data is:

Gly Leu Phe Asp Ile Ile Lys Lys Val Ala Ser Val Ile Gly Gly Leu (NH_2)

Table 5.2. *(Continued)*

5.2c. Citropin 2.1

Citropin 2.1 is a minor component of the *Litoria citropa* skin secretion. Analysis of citropin 2.1 by ESMS indicated a very prominent $[M+2H]^{2+}$ peak at *m/z* 1081 and a MH^+ peak at *m/z* 2160 in the spectrum. Methylation of citropin 2.1 yielded a methyl ester, *m/z* 2188, indicating the presence of two CO_2H groups. The collision induced mass spectrum (MS/MS) of the $[M+2H]^{2+}$ ion (*m/z* 1081) is shown in Figure 5.7.

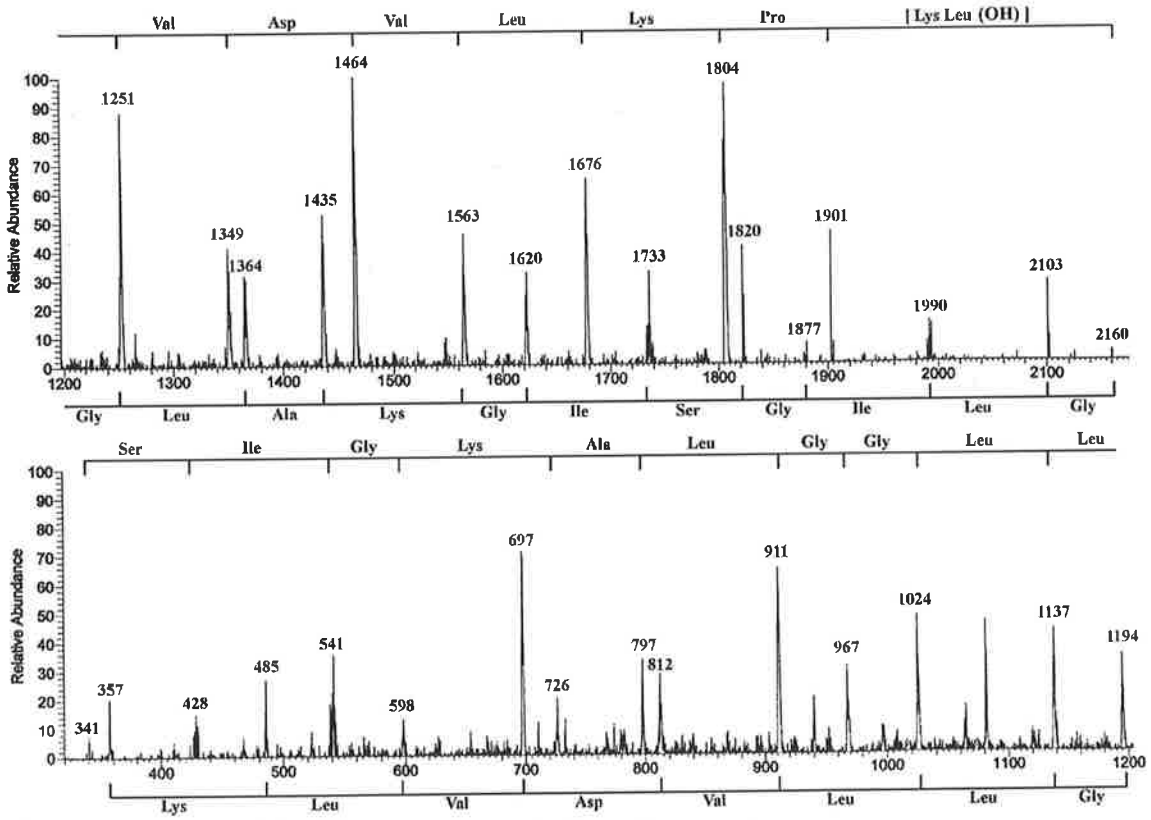


Figure 5.7. CA MS/MS of the $[M+2H]^{2+}$ ion of citropin 2.1 (*m/z* 1081). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

The amino acid sequence determined from this CA MS/MS spectrum is Gly Leu Ile Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro –259. The other residues (259 Da) at the C-terminal end of citropin 2.1 could not be identified from this spectrum. This segment was identified using the MS/MS/MS technique on the 'Y+2' fragment ion *m/z* 485. The collision induced mass spectrum (MS/MS/MS) of *m/z* 485 (Figure 5.8) indicates the sequence of this 'Y+2' fragment ion to be Lys Pro Lys Leu (OH).

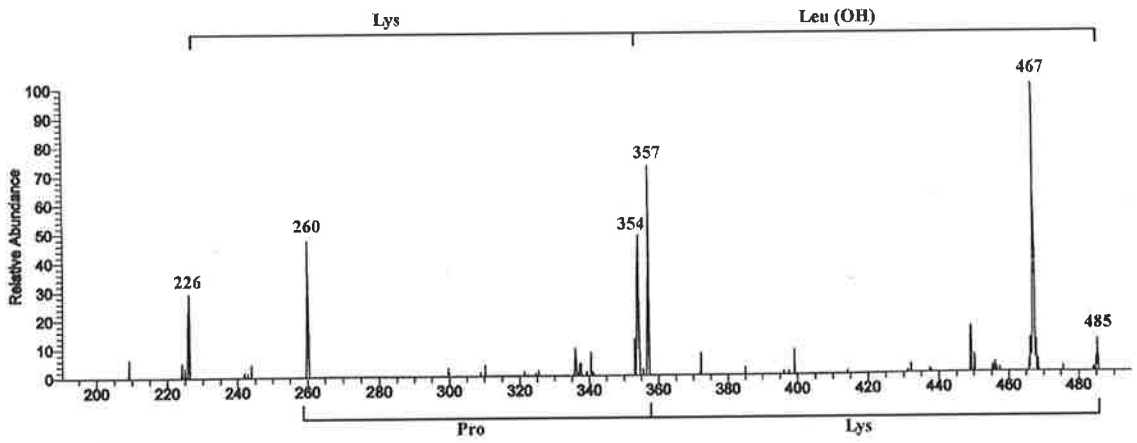


Figure 5.8. CA MS/MS/MS of the *m/z* 485 'Y+2' fragment ion of citropin 2.1. The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

The Lys-C digestion of citropin 2.1 gave four peptide fragments, *m/z* 2047, 1435, 1322 and 744. These fragments confirmed the presence of the Lys^{8}, Lys^{19} and Lys^{21} residues. Sequence data was obtained for all four peptides. Combination of all the MS data gave the following as the full sequence for citropin 2.1; Gly Leu Ile Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro Lys Leu (OH). The Leu and Ile residues were identified by automated Edman degradation, which also confirmed the full sequence. Structural determination details of all the citropin 2 peptides are summarised in Table 5.3.

Citropin 2.1 [MH^+ 2160]

Prominent [M+2H] $^{2+}$ 1081 also present in spectra.
Methylation gives a methyl ester, MH^+ 2188 (two CO_2H groups).

[M+2H] $^{2+}$ 1081 MS/MS	'B ions'	*m/z* 1901, 1804, 1676, 1563, 1464, 1349, 1250, 1137, 1024, 967, 910, 797, 726, 598, 541, 428, 341 [Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro - (OH)]
	'Y+2 ions'	*m/z* 2160, 2103, 1990, 1877, 1820, 1733, 1620, 1563, 1435, 1364, 1251, 1194, 1137, 1024, 911, 812, 697, 598, 485, 357 [Gly Leu Ile Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys]
	Sequence	Gly Leu Ile Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro (OH)
MH^+ 485 MS/MS/MS	'B ions'	*m/z* 467, 354, 226 [Lys Leu (OH)]
	'Y+2 ions'	*m/z* 485, 357, 260 [Lys Pro]
	Sequence	Lys Pro Lys Leu (OH)

Full sequence of Citropin 2.1 as determined from mass spectrometric and automated Edman data is:
Gly Leu Ile Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro Lys Leu (OH)

Citropin 2.1.1 [MH^+ 2288]

Prominent [M+2H] $^{2+}$ 1145 also present in spectra.
Methylation gives a methyl ester, MH^+ 2331 (two CO_2H and one $CONH_2$ group).

[M+2H] $^{2+}$ 1145 MS/MS	'B ions'	*m/z* 1464, 1349, 1250, 1137, 1024, 967, 910, 797, 726, 598 [Lys Ala Leu Gly Gly Leu Leu Val Asp - (OH)]
	'Y+2 ions'	*m/z* - 1861, 1748, 1691, 1563, 1492, 1379, 1322, 1265, 1152, 1039, 940, 825 [Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp]
	Sequence	Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp - (OH)

Table 5.3. MS data for citropin 2 peptides isolated from *Litoria citropa*.

Citropin 2.1.1 [MH^+ 2288] (Continued)

Lys-C digestion gives MH^+ 744, 1322, 1563 and 2048

MH^+ 1563 MS/MS	'B ions'	*m/z* 1545, 1417, 1304, 1176, 1079, 951, 838, 739, 624, 525 [Val Asp Val Leu Lys Pro Lys Leu Gln (OH)]
	'Y+2 ions'	*m/z* 1563, 1492, 1379, 1322, 1265, 1152, 1039, 940, 825, 726 [Ala Leu Gly Gly Leu Leu Val Asp Val]
	Sequence	Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro Lys Leu Gln (OH)]
MH^+ 744 MS/MS	'B ions'	*m/z* 726, 598, 541, 428, 341 [Ser Ile Gly Lys (OH)]
	'Y+2 ions'	*m/z* 744, 687, 574, 461, 404, 317, 204 [Gly Leu Ile Gly Ser Ile]
	Sequence	Gly Leu Ile Gly Ser Ile Gly Lys (OH)

Full sequence of Citropin 2.1.1 as determined from mass spectrometric and automated Edman data is:

Gly Leu Ile Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro Lys Leu Gln (OH)

Citropin 2.1.2 [MH^+ 2430]

Prominent $[M+2H]^{2+}$ 1216 also present in spectra.

Methylation gives a methyl ester, MH^+ 2473 (two CO_2H and one $CONH_2$ group).

$[M+2H]^{2+}$ 1216 MS/MS	'B ions'	*m/z* 1901, 1804, 1676, 1563, 1464,1349, 1250, 1137, 1024, 967, 910, 797 [Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro - (OH)]
	'Y+2 ions'	*m/z* 2090, 2003, 1890, 1833, 1705, 1634,1521, 1464, 1407, 1294, 1181, 1082, 967, 868, 755 [Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu]
	Sequence	Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro - (OH)
$[M+H]^+$ 868 MS/MS/MS	'B ions'	*m/z* 850, 779, 708, 580, 467, 339 [Lys Leu Gln Ala Ala (OH)]
	'Y+2 ions'	*m/z* 868, 755, 627, 530, 402, 289 [Leu Lys Pro Lys Leu]
	Sequence	Leu Lys Pro Lys Leu Gln Ala Ala (OH)

Table 5.3. *(Continued)*

Citropin 2.1.2 [MH^+ 2430] (Continued)

Lys-C digestion gives MH^+ 744, 1322, 1706 and 2048

MH^+ 744	'B ions'	*m/z* 726, 598, 541, 428, 341
MS/MS		[Ser Ile Gly Lys (OH)]
	'Y+2 ions'	*m/z* 744, 687, 574, 461, 404, 317, 204
		[Gly Leu Ile Gly Ser Ile]
	Sequence	Gly Leu Ile Gly Ser Ile Gly Lys (OH)

Full sequence of Citropin 2.1.2 as determined from mass spectrometric and automated Edman data is:

Gly Leu Ile Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro Lys Leu Gln Ala Ala (OH)

Citropin 2.1.3 [MH^+ 2517]

Prominent $[M+2H]^{2+}$ 1260 also present in spectra.

Methylation gives a methyl ester, MH^+ 2560 (two CO_2H and one $CONH_2$ group).

$[M+2H]^{2+}$ 1260	'B ions'	*m/z* 1901, 1804, 1676, 1563, 1464,1349,
MS/MS		1250, 1137, 1024, 967, 910, 797, 726,
		598, 541, 428, 341
		[Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu
		Val Asp Val Leu Lys Pro - (OH)]
	'Y+2 ions'	*m/z* 2234, 2177, 2090, 1977, 1920,
		1792, 1721, 1608, 1551, 1494, 1381,
		1268, 1169, 1054, 955, 842, 714, 617
		[Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu
		Leu Val Asp Val Leu Lys Pro]
	Sequence	Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu
		Leu Val Asp Val Leu Lys Pro - (OH)
$[M+H]^+$ 955	'B ions'	*m/z* 937, 850, 779, 708, 580, 467
MS/MS/MS		[Leu Gln Ala Ala Ser (OH)]
	'Y+2 ions'	*m/z* 955, 842, 714, 617, 489, 376
		[Leu Lys Pro Lys Leu]
	Sequence	Leu Lys Pro Lys Leu Gln Ala Ala
		Ser (OH)

Lys-C digestion gives MH^+ 744, 1322, 1793 and 2048

MH^+ 744	'B ions'	*m/z* 726, 598, 541, 428, 341
MS/MS		[Ser Ile Gly Lys (OH)]
	'Y+2 ions'	*m/z* 744, 687, 574, 461, 404, 317, 204
		[Gly Leu Ile Gly Ser Ile]
	Sequence	Gly Leu Ile Gly Ser Ile Gly Lys (OH)

Full sequence of Citropin 2.1.3 as determined from mass spectrometric and automated Edman data is:

Gly Leu Ile Gly Ser Ile Gly Lys Ala Leu Gly Gly Leu Leu Val Asp Val Leu Lys Pro Lys Leu Gln Ala Ala Ser (OH)

Table 5.3. *(Continued)*

5.2d. Citropin 3.1.2

Citropin 3.1.2 is another minor component of the skin secretion of *Litoria citropa*. Analysis of citropin 3.1.2 by ESMS shows a very prominent $[M+2H]^{2+}$ peak at *m/z* 1307 and a MH^+ peak at *m/z* 2612. Methylation of citropin 3.1.2 yielded a methyl ester, *m/z* 2712, indicating the presence of five CO_2H groups and two $CONH_2$ groups. Citropin 3.1.2 is the largest of the citropin peptides, with its structural determination being the most complex. The collision induced mass spectrum (MS/MS) of the $[M+2H]^{2+}$ ion (*m/z* 1307) is shown in Figure 5.9.

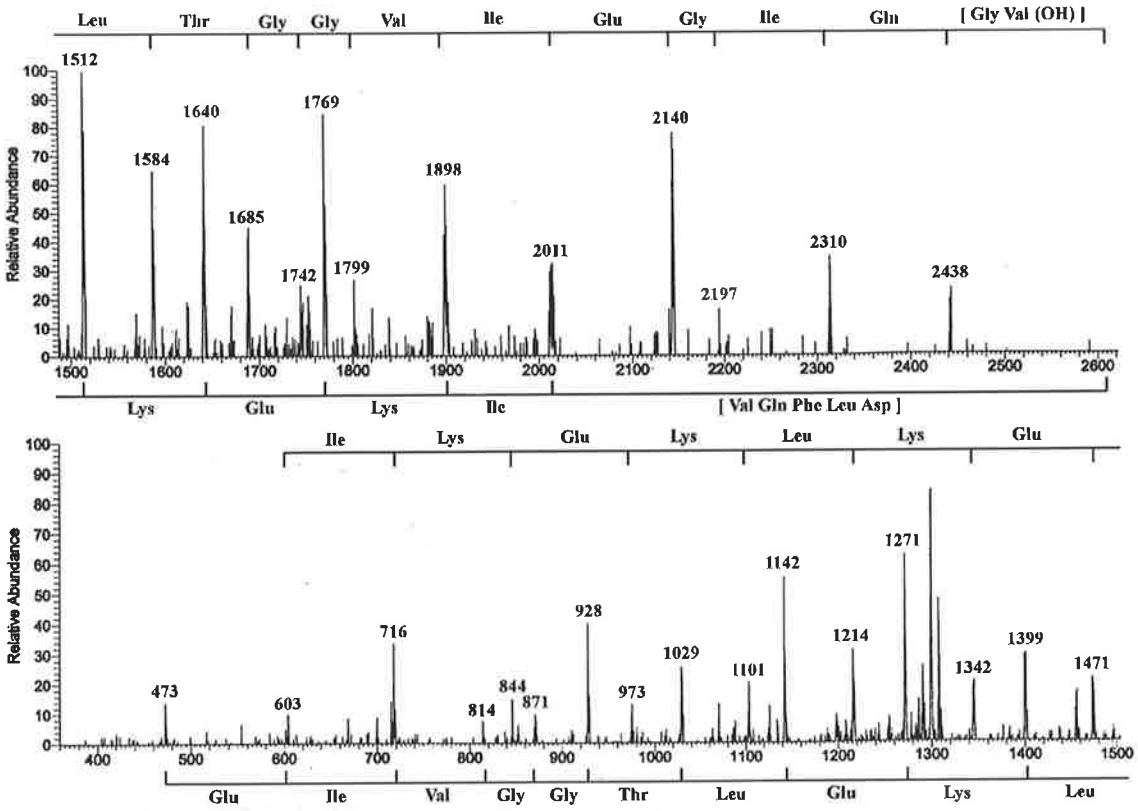


Figure 5.9. CA MS/MS of the $[M+2H]^{2+}$ ion of citropin 3.1.2 (*m/z* 1307). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

The amino acid sequence determined from this CA MS/MS spectrum is 601 – Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val Ile Glu Gly Ile Gln – 174, with the residues at the N-terminal end (601 Da) and at the C-terminal end (174 Da) not identified. The MS/MS/MS data for the 'Y+2' fragment ion *m/z* 928 identified the unknown residues at the C-terminal end. The collision induced mass spectrum (MS/MS/MS) of *m/z* 928 (Figure 5.10) gives the sequence Gly Gly Val Ile Glu Gly Ile Gln Gly Val (OH).

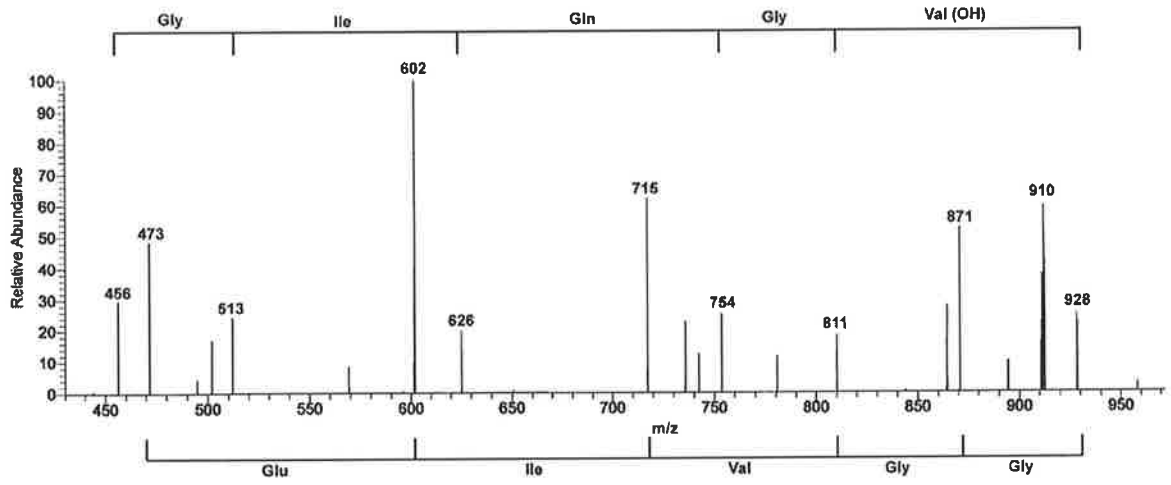


Figure 5.10. CA MS/MS/MS of the *m/z* 928 'Y+2' fragment ion of citropin 3.1.2. The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

The Lys-C digestion of citropin 3.1.2 gave five peptide fragments, *m/z* 1512, 1271, 1119, 862 and 517. These fragments confirmed the presence of Lys^{7}, Lys^{9} and Lys^{11} residues. The collision induced mass spectrum (MS/MS) of *m/z* 862 identifies the unknown residues at the N-terminal end. The collision induced mass spectrum (Table 5.4) of *m/z* 862 ion gives the sequence Asp Leu Phe Gln Val Ile Lys (OH). Combination of all the MS data, together with confirmatory Edman data, gives the full sequence for citropin 3.1.2 as Asp Leu Phe Gln Val Ile Lys Glu Lys Leu Lys Leu Lys Glu Leu Thr Gly Gly Val Ile Glu Gly Ile Gln Gly Val (OH). Structural determination details of all the citropin 3 peptides are summarised in Table 5.4.

Citropin 3.1 [MH^+ 2456]

Prominent $[M+2H]^{2+}$ 1229 also present in spectra.
Methylation gives a methyl ester, MH^+ 2556 (five CO_2H and two $CONH_2$ groups).

$[M+2H]^{2+}$ 1229 MS/MS	'B ions'	*m/z* 1898, 1799, 1742, 1685, 1584, 1471, 1342, 1214, 1101, 973, 844, 716, 603 [Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val - (OH)]
	'Y+2 ions'	*m/z* 1953, 1854, 1741, 1613, 1484, 1356, 1243, 1115, 986, 873, 772, 715, 658 [Val Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly]
	Sequence	Val Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val - (OH)
$[M+H]^+$ 658 MS/MS/MS	'B ions'	*m/z* 640, 512, 399, 342, 213 [Glu Gly Ile Gln (OH)]
	'Y+2 ions'	*m/z* 658, 559, 446, 317, 260 [Val Ile Glu Gly]
	Sequence	Val Ile Glu Gly Ile Gln (OH)

Lys-C digestion gives MH^+ 517, 862, 1115, 1119 and 1356

MH^+ 862 MS/MS	'B ions'	*m/z* 844, 716, 603, 504 [Val Ile Lys (OH)]
	'Y+2 ions'	*m/z* 862, 747, 634, 487, 359, 260 [Asp Leu Phe Gln Val]
	Sequence	Asp Leu Phe Gln Val Ile Lys (OH)

Full sequence of Citropin 3.1 as determined from mass spectrometric and automated Edman data is:
Asp Leu Phe Gln Val Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val Ile Glu Gly Ile Gln (OH)

Citropin 3.1.1 [MH^+ 2513]

Prominent $[M+2H]^{2+}$ 1258 also present in spectra.
Methylation gives a methyl ester, MH^+ 2613 (five CO_2H and two $CONH_2$ groups).

$[M+2H]^{2+}$ 1258 MS/MS	'B ions'	*m/z* 2140, 2011, 1898, 1799, 1742, 1685,1584, 1471, 1342, 1214, 1101, 973, 844, 716, 603, 504, 376 [Gln Val Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val Ile Glu - (OH)]
	'Y+2 ions'	*m/z* 2010, 1911, 1798, 1670, 1541, 1413, 1300, 1172, 1043, 930, 829, 772, 715, 616 [Val Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val Ile]
	Sequence	Gln Val Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val Ile Glu - (OH)

Table 5.4. MS data for citropin 3 peptides isolated from *Litoria citropa*.

Citropin 3.1.1 [MH^+ 2513] (Continued)

$[M+H]^+$ 715	'B ions'	*m/z* 697, 640, 512, 399, 342
MS/MS		[Gly Ile Gln Gly (OH)]
	'Y+2 ions'	*m/z* 715, 616, 503, 374, 317, 204
		[Val Ile Glu Gly Ile]
	Sequence	Val Ile Glu Gly Ile Gln Gly (OH)

Lys-C digestion gives MH^+ 517, 862, 1119, 1172 and 1413

MH^+ 862	'B ions'	*m/z* 844, 716, 603, 504, 376
MS/MS		[Gln Val Ile Lys (OH)]
	'Y+2 ions'	*m/z* 862, 747, 634, 487, 359, 260
		[Asp Leu Phe Gln Val]
	Sequence	Asp Leu Phe Gln Val Ile Lys (OH)

Full sequence of Citropin 3.1.1 as determined from mass spectrometric and automated Edman data is:

Asp Leu Phe Gln Val Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val Ile Glu Gly Ile Gln Gly (OH)

Citropin 3.1.2 [MH^+ 2612]

Prominent $[M+2H]^{2+}$ 1307 also present in spectra.

Methylation gives a methyl ester, MH^+ 2712 (five CO_2H and two $CONH_2$ groups).

$[M+2H]^{2+}$ 1307	'B ions'	*m/z* 2438, 2310, 2197, 2140,2011,1898
MS/MS		1799, 1742, 1685, 1584, 1471, 1342,
		1214, 1101, 973, 844, 716, 603
		[Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly
		Gly Val Ile Glu Gly Ile Gln - (OH)]
	'Y+2 ions'	*m/z* 2011, 1898, 1769, 1640, 1512,
		1399, 1271, 1142, 1029, 928, 871, 814,
		716, 603, 473
		[Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly
		Gly Val Ile Glu]
	Sequence	Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly
		Gly Val Ile Glu Gly Ile - (OH)
m/z 928	'B ions'	*m/z* 910, 811, 754, 626, 513, 456
MS/MS/MS		[Gly Ile Gln Gly Val (OH)]
	'Y+2 ions'	*m/z* 928, 871, 811, 715, 602, 473
		[Gly Gly Val Ile Glu]
	Sequence	Gly Gly Val Ile Glu Gly Ile Gln Gly
		Val (OH)

Lys-C digestion gives MH^+ 517, 862, 1119, 1271 and 1512

MH^+ 862	'B ions'	*m/z* 844, 716, 603, 504, 376
MS/MS		[Gln Val Ile Lys (OH)]
	'Y+2 ions'	*m/z* 862, 747, 634, 487, 359, 260
		[Asp Leu Phe Gln Val]
	Sequence	Asp Leu Phe Gln Val Ile Lys (OH)

Full sequence of Citropin 3.1.2 as determined from mass spectrometric and automated Edman data is:

Asp Leu Phe Gln Val Ile Lys Glu Lys Leu Lys Glu Leu Thr Gly Gly Val Ile Glu Gly Ile Gln Gly Val (OH)

Table 5.4. *(Continued)*

5.2e. Antibacterial activity determination

As described in chapter 1, most tree frogs of the genus *Litoria* have antibacterial peptides within their skin secretions. Wide-spectrum antibacterial peptides so far reported within this genus have generally been caerin or maculatin peptides. The specific examples of caerin 1.1 and maculatin 1.1 have been described in section 1.2c and 2.1b. The antibacterial activities of ten citropin peptides from *Litoria citropa* have been determined and are compared with those of caerin 1.1 and maculatin 1.1 in Table 5.5.

Peptide	Reference	Sequence	Antibacterial activity
Citropin 1.1	Cit1.1	GLFDVIKKVASVIGGL (NH_2)	active
Citropin 1.1.2	Cit1.1.2	DV I KKVASV I GGL (NH_2)	inactive
Citropin 1.1.3	Cit1.1.3	GLFDV I KKVASV I GLASP (OH)	active
Citropin 1.2	Cit1.2	GLFD I I KKVASVVGGL (NH_2)	active
Citropin 1.2.2	Cit1.2.2	D I I KKVASVVGGL (NH_2)	inactive
Citropin 1.2.3	Cit1.2.3	GLFD I I KKVAS (NH_2)	inactive
Citropin 1.3	Cit1.3	GLFD I I KKVASV I GGL (NH_2)	active
Citropin 2.1	Cit2.1	GL I GS I GKALGGLLVDVLKPKL (OH)	slightly active
Citropin 2.1.3	Cit2.1.3	GL I GS I GKALGGLLVDVLKPKLQAAS (OH)	slightly active
Citropin 3.1.2	Cit3.1.2	DLFQV I KEKLKELTGGVIEG I QGV (OH)	inactive
Caerin1.1	C1.1	GLLSVLGSVAKHVLPHVVPV I AEHL (NH_2)	active
Maculatin 1.1	M1.1	GLFGVLAKVAAHVVPA I AEHF (NH_2)	active

	MIC (μg/ml)							
	Cit1.1	Cit1.1.3	Cit1.2	Cit1.3	Cit2.1	Cit2.1.3	C1.1	M1.1
Bacillus cereus	50		25	25			50	25
Escherichia coli					50	100		
Leuconostoc lactis	6	50	3	6			1.5	3
Listeria innocua	25		100	25			25	50
Micrococcus luteus	12		12	12			12	50
Pasteurella multocida								50
Staphylococcus aureus	25		25	25			3	25
Staphylococcus epidermidis	12		25	25			12	12
Streptococcus uberis	25	100	12	12			12	12

Table 5.5. Antibacterial activities of some of the citropin peptides from *Litoria citropa*, in comparison with the antibacterial activities of caerin 1.1 and maculatin 1.1. Inactive in this context means the activity is > 100 μg/ml. Where there is no figure listed the MIC value is > 100 μg/ml.

Citropins 1.1, 1.2 and 1.3 showed potent antibacterial activity against the majority of test microorganisms. The antibacterial activity of these three citropin peptides is comparable to those of caerin 1.1 and maculatin 1.1. These three citropin peptides showed more pronounced wide-spectrum antibacterial activity against gram-positive than gram-negative microorganisms. None of the citropin 2 or citropin 3 peptides showed significant activity against the pathogens tested.

Citropins 1.1, 1.2 and 1.3 have only 16 amino acid residues. Amphibian peptides so far reported from the genus *Litoria* that have displayed antibacterial activity contain at least 20 amino acid residues. For example, caerin 1.1 and maculatin 1.1 consist of 25 and 21 amino acids respectively. This length prerequisite is important in order to comply with the 'barrel-stave' model of peptide antibacterial action, as described in section 1.2c. The citropin 1 peptides cannot span the entire width of the phospholipid bilayer of a bacterial cell wall. So the 'barrel-stave' model cannot explain their antibacterial activity.

5.2f. The 'carpet-like' mechanism

The 'carpet-like' model of antibacterial activity may explain the observed biological activity of the citropin 1 peptides [4]. This 'non-channel' forming mechanism is supported by the fact that relatively short peptides, consisting of only 12 –15 amino acids in length, have shown to display antibacterial activity [5]. This mechanism (Figure 5.11) is initially similar to that of the 'barrel-stave' mechanism, in that the peptides aggregate and bind in a parallel conformation to the negatively charged phospholipids of the bacterial cell membrane. The amphiphilic α-helical peptides then align themselves in such a manner that the positive charges of the basic amino acids interact with the negatively charged phospholipid head groups. The peptides then partially insert into the membrane by reorienting themselves so that their hydrophobic residues can interact with the hydrophobic core of the membrane, while the hydrophilic residues interact with both the polar head groups of the membrane and the aqueous solution.

Consequently, the lipid bi-layer expands and weakens in order to accommodate the bound peptides. When a threshold concentration is reached, the membrane is considerably weakened and disrupted, resulting in membrane penetration and ultimately the death of the cell[6]. In order for the citropin 1 peptides to be acting via the carpet mechanism, the peptides must be amphiphilic peptides with well-defined hydrophobic and hydrophilic zones.

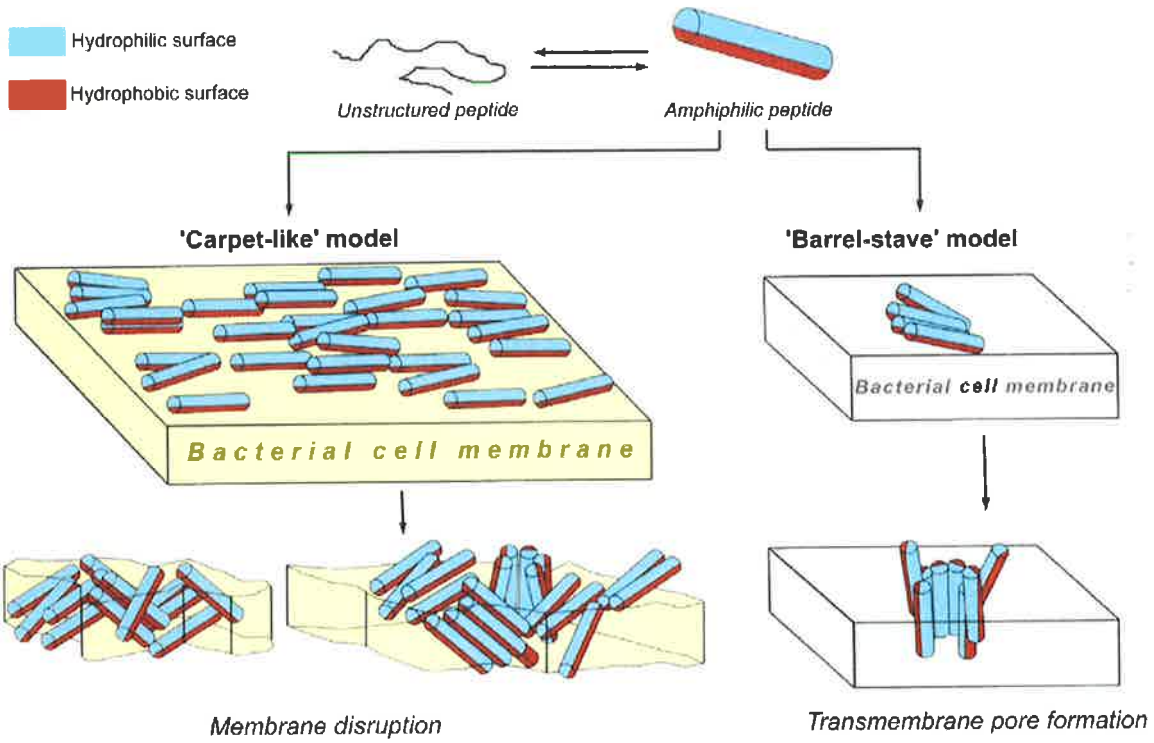


Figure 5.11. Illustration of the 'carpet-like' model, in comparison with the 'barrel-stave' model.

5.2g. Determination of structural conformation

The Edmundson helical wheel projections of the antibacterial citropins 1 show well defined hydrophobic and hydrophilic zones. For example, that of citropin 1.1 is shown in Figure 5.12. This suggests that citropins 1.1, 1.2 and 1.3 may be amphiphilic α-helical peptides. This has been confirmed by determining the tertiary structure of citropin 1.1 by nuclear magnetic resonance (NMR) experiments.

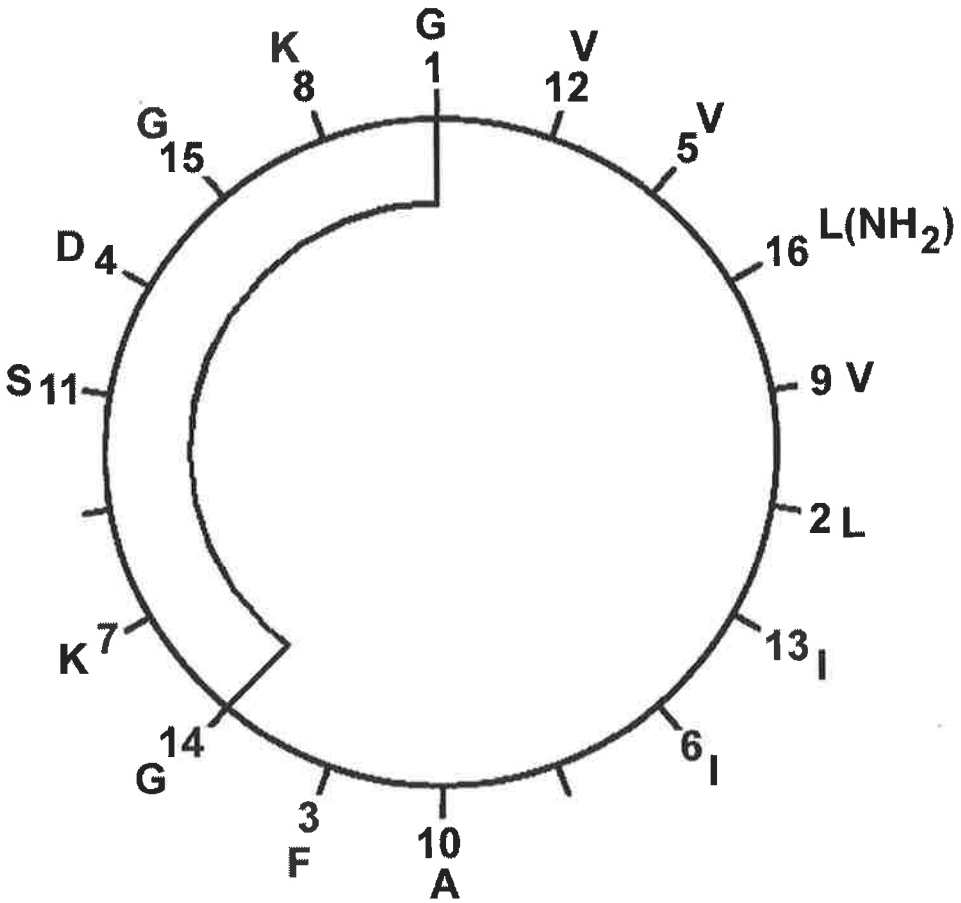


Figure 5.12. Edmundson projection of citropin 1.1. Note the well-defined hydrophobic and hydrophilic regions represented respectively by the right and left-hand sides of the figure.

The tertiary structure of citropin 1.1 has been determined using the solvent system of trifluoroacetic and water (1:1)[7]. This structure is shown in Figure 5.13, confirming the α-helical structure with well-defined hydrophobic and hydrophilic zones.

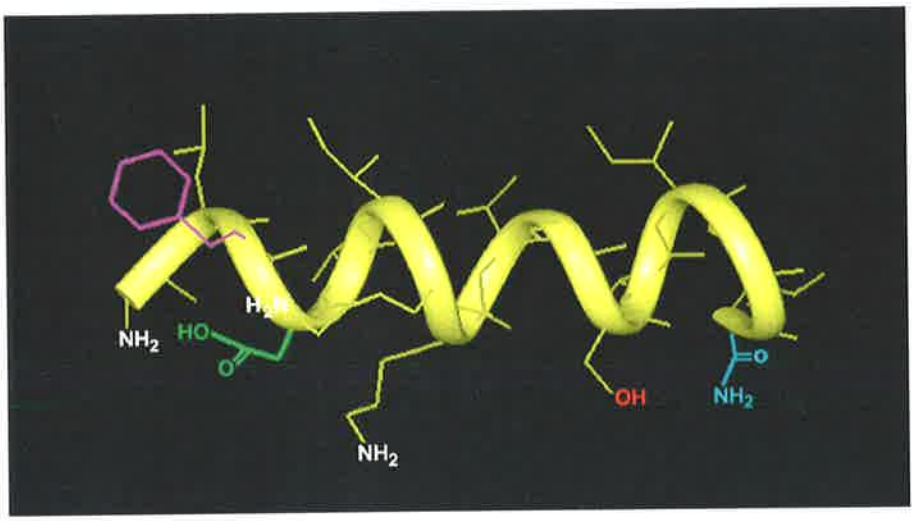


Figure 5.13. Three-dimensional structure of citropin 1.1.

The Edmundson projections of the citropins 2 and 3 do not show well-defined hydrophilic and hydrophobic zones. For example, the Edmundson projection of citropin 2.1.3 (Figure 5.14) indicates that hydrophobic and hydrophilic residues are scattered throughout an α-helix. The citropin 2 and 3 peptides are prevented from forming ideal α-helices as they contain central Gly and/or Pro residues. The presence of these residues destabilises the overall helical formation[8]. By way of illustration, citropin 2.1.3 contains Gly at positions 4, 7, 11 and 12 together with Pro at position 20. These observations may explain why the citropins 2 and 3 do not exhibit significant antibacterial activity, when compared to citropins 1.1, 1.2 and 1.3.

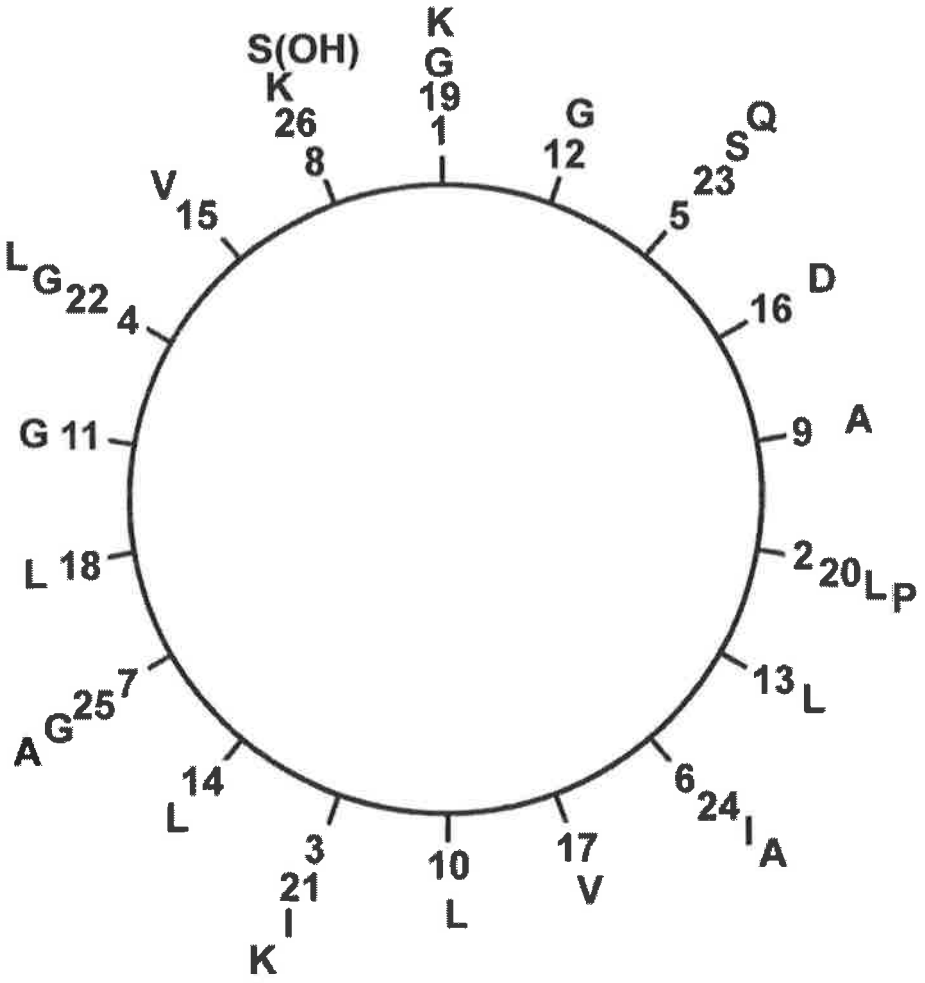


Figure 5.14. Edmundson projection of citropin 2.1.3.

5.2h. Synthetic modifications

Several synthetic modifications of citropin 1.1 have been prepared in order to investigate the significance of these changes on the overall antibacterial activity of citropin 1.1. The results are summarised in Table 5.6. Substituting Ala^4 for Asp^4 marginally increased antibacterial activity, while replacement of Lys^7 by Ala^7 significantly reduced the antibacterial activity of the peptide. The valine residue at position 12 appeared to be important for antibacterial activity as replacement of Val^{12} for Lys^{12} greatly reduced most of the activity against each microorganism except for *Leuconostoc lactis*, which remained essentially the same. Replacement of Gly^{14} for Lys^{14} increased the antibacterial activity against *Escherichia coli*.

Peptide	Reference	Sequence	Antibacterial activity
Citropin 1.1	Cit1.1	GLFDVIKKVASVIGGL (NH_2)	active
Citropin 1.1 mod 1	m1	GLF**E**VIKKVASVIGGL (NH_2)	active
Citropin 1.1 mod 2	m2	GLF**A**VIKKVASVIGGL (NH_2)	active
Citropin 1.1 mod 3	m3	GLFDVI**A**KVASVIGGL (NH_2)	slightly active
Citropin 1.1 mod 4	m4	GLFDVIKKVAS**K**IGGL (NH_2)	slightly active
Citropin 1.1 mod 5	m5	GLFDVIKKVASVI**K**GL (NH_2)	active
Citropin 1.1 mod 6	m6	GLFDVIKKVAS**K**I**K**GL (NH_2)	slightly active
Citropin 1.1 mod 7	m7	GLFDVIKKVASVI**KK**L (NH_2)	active

	MIC (μg/ml)							
	Cit1.1	m1	m2	m3	m4	m5	m6	m7
Bacillus cereus	50	50	25	100				
Escherichia coli		100	100			50		50
Leuconostoc lactis	6	6	3	25	12	3	6	3
Listeria innocua	25	50	25			25	100	50
Micrococcus luteus	12	50	12	100		25		50
Pasteurella multocida		100					100	100
Staphylococcus aureus	25	100	25	100		25		100
Staphylococcus epidermidis	12	50	12	100	100	25	100	12
Streptococcus uberis	25	50	25	100	100	25	100	50

Table 5.6. Antibacterial activities of citropin 1.1 and seven synthetically modified citropin peptides. Where there is no figure listed the MIC value is > 100 μg/ml.

5.2i. Conclusions

This research has investigated the citropin peptides from the skin secretions of *Litoria citropa*. Three distinct groups of citropin peptides have been discovered, namely, the citropins 1, 2 and 3. These peptides have no similarity with any other reported peptides from the *Litoria* genus [9]. Peptide material was obtained from both the dorsal and the submental glands. Both glands were shown to secrete the same 15 peptides, but in addition, the dorsal glands produced four peptides not contained in the submental gland.

Citropins 1.1, 1.2 and 1.3 are the major peptides produced by both the dorsal and submental glands: they are amphiphilic antibacterial peptides. The antibacterial potency of the citropin peptides is similar to that of caerin 1.1 and maculatin 1.1. The citropin 1 peptides are the simplest amphibian antibacterial peptides reported to date. Structurally, the citropins 1 contain the hydrophilic residues Asp^4, Lys^7, Lys^8 and Ser^{11}. They differ only in their nature of the hydrophobic residues at positions 5, 6, 12 and 13. Endoproteases that are secreted together

with the skin peptides are known to deactivate the major antibacterial peptides of other amphibians, usually within a period of 5–30 minutes [10]. For example, caerin 1.1 and maculatin 1.1 are secreted together with endoproteases that remove the first two amino acid residues from the N-terminal end of the peptide, deactivating the antibacterial activity [11, 12]. The same scenario holds for the citropins 1. For example, the citropins 1.1.1, 1.1.2, 1.2.1 and 1.2.2 are degradation products: they exhibit no antibacterial activity.

The citropin 2 and 3 peptides showed no significant antibacterial activity against the range of tested pathogens. The citropin 3 peptides are unusual in structure since their sequence commences with Asp. The only other reported amphibian peptides that commence with Asp are the hylambin and kassinin peptides, which both belong to the tachykinin family of neuropeptides: these show no structural relationship with the citropins 3 [13]. The anionic nature of the citropin 3 peptides suggests a possible function as spacer peptides for the inactive pro-citropin peptides, although this is just a proposal at this juncture and has not been proven. The biological roles of the citropin 2 are currently unknown.

Finally, the roles of the four extra citropin peptides produced by the dorsal glands are also unknown, particularly as to why they are only found within the dorsal gland secretion but absent in the submental gland secretion. The four peptides are structurally similar to citropin 1.1 and citropin 1.2, with the only difference being the presence of two additional residues in each case. There is a possibility that these peptides may function as neuropeptides or pheromones, but this has not been proven and the biological activity for these particular peptides remains to be determined.

5.3. Experimental

5.3a. Collection and preparation of *Litoria citropa* secretions

The skin secretions from *Litoria citropa* were provided courtesy of Associate Professor Mike Tyler, Department of Environmental Biology, University of Adelaide. The secretions were obtained by the SES method, as described in section 2.3a. The resulting secretion was washed off the skin with water (distilled 25 ml). Methanol (25 ml) was immediately added to the aqueous extract, the mixture centrifuged, and concentrated to approximately 1 ml. Samples were taken separately from both dorsal and submental glands of *Litoria citropa*. This procedure provides, on average, some 5 mg of solid material from the dorsal glands and 3 mg from the submental gland.

5.3b. HPLC separation

HPLC separation was achieved for each sample using a VYDAC C_{18} Protein and Peptide (218TP54) reverse phase column equilibrated with 10% acetonitrile/aqueous 0.1% TFA. Additional purification was achieved using an elution profile of 40-55% acetonitrile over a period of 60 minutes. Each sample was filtered through a Millipore 0.45 μm filter and injected onto the column. The conditions for the various HPLC chromatograms represented in this chapter are as follows:

Figure 5.3 and 5.4. The elution profiles increased from 10-75% acetonitrile over a period of 60 minutes using a flow rate of 1 ml/min.

5.3c. Mass spectrometry analysis

Electrospray mass spectra were determined using a Finnigan LCQ ion trap mass spectrometer. The samples were dissolved in methanol/water (1:1) and infused into the electrospray source at a flow rate of 8 μl/min. Collision activation mass spectral data were obtained using collision energy of 30-45 %. Electrospray conditions were as follows: source voltage 4.2 kV, source current 17 μA, capillary temperature 200°C, capillary voltage 3V and sheath gas flow

30 psi. Mass spectra were acquired with the automatic gain control on, a maximum ion time of 550 milliseconds, and using 5 microscans per scan, averaging over approximately 20 scans. Molecular weights of the peptides were determined from the MH^+ or $[M+2H]^{2+}$ ions.

5.3d. Additional information

The remaining procedures are identical to those described elsewhere, as detailed in the table below.

Procedure	Section in which it is described
Methylation of peptides	Section 2.3d (page 74)
Enzyme digestion using Lys-C	Section 2.3e (page 75)
Antibacterial testing	Section 2.3g (page 76)
Preparation of synthetic peptides	Section 2.3h (page 76)
Automated Edman sequencing	Section 2.3i (page 76)

5.4. References

[1] Barker, J., Grigg, G. and Tyler, M.J., in *A field guide to Australian frogs* (Surrey Beatty, Norton, NSW, 1995).

[2] Tyler, M.J., in *Encyclopaedia of Australian animals* (ed. Strahan, R.) 3-42 (Harper Collins, London, UK, 1992).

[3] Tyler, M. J., Stone, D. J. and Bowie, J. H., *J. Pharm. Toxicol. Methods,* **28**, 199-200 (1992).

[4] Shai, Y., *Trends. Biochem. Sci.*, **20**, 460-464 (1995).

[5] Andreu, D., Merrifield, R.B., Steiner, H. and Boman, H.G., *Biochemistry*, **24**, 1683-1688 (1985).

[6] Gazit, E., Boman, A., Boman, H.G. and Shai, Y., *Biochemistry*, **34**, 11479-11488 (1995).

[7] Wegener, K.L., Wabnitz, P.A., Carver, J.A., Bowie, J.H., Wallace, J.C. and Tyler, M.J., *Eur. J. Biochem.*, (1999), in press.

[8] Wong, H., Bowie, J.H. and Carver, J.A., *Eur. J. Biochem.*, **247**, 545-557 (1997).

[9] Bowie, J.H., Chia, B.S.C. and Tyler, M.J., *Pharm. News.*, **6**, 16-21 (1998).

[10] Resnick, N., Maloy, W., Crut, H. and Zasloff, M., *Cell*, **66**, 541-549 (1991).

[11] Steinborner, S.T., Waugh, R.J., Bowie, J.H., Tyler, M.J. and Wallace, J.C., *J. Peptide Sci.*, **3**, 181-185 (1997).

[12] Rozek, T., Waugh, R.J., Steinborner, S.T., Bowie, J.H., Wallace, J.C. and Tyler, M.J., *J. Peptide Sci.*, **4**, 111-115 (1998).

[13] Lazarus, L. H. and Attila, M., *Prog. Neurobiol.*, **41**, 473-507 (1993).

CHAPTER 6. Caerulein-like peptides from *Litoria citropa*

6.1. Introduction

6.1a. General

Caerulein, a decapeptide, was first isolated in the late 1960s from the skin extracts of the Australian tree frog *Litoria caerulea* [1, 2]. Since then, a relatively small number of peptides with structures analogous to that of caerulein have been discovered from various amphibian species around the world [3, 4]. Consequently, a new family of peptides evolved, known as the caeruleins. The caerulein family of peptides is characterised by a post-translated tyrosine sulfate residue and exhibits a spectrum of biological activities similar to the mammalian intestinal peptide hormones gastrin and cholecystokinin (CCK) [5]. The structural similarities existing between caerulein, gastrin II and CCK can be seen below in Table 6.1. Caerulein peptides that are de-sulfated are reported to lose their neurological activity, indicating the importance of the sulfate group in terms of the neuropeptide activity within this family of peptides [5].

Peptide	Sequence
Caerulein	pGlu Gln Asp Tyr(SO_3) Thr Gly Trp Met Asp Phe (NH_2)
C-terminal octapeptide of CCK	- Asp Tyr(SO_3) Thr Gly Trp Met Asp Phe (NH_2)
C-terminal hexapeptide of gastrin II	- Tyr(SO_3) Gly Trp Met Asp Phe (NH_2)

Table 6.1. Structural comparison between caerulein, gastrin II and CCK.

Caeruleins are biologically active within living systems. Specifically, they modify the biological activities *in vivo* of sedation, anti-nociception * and thermoregulation [6]. Caerulein is also an analgaesic as is CCK. Caerulein is several thousand times more potent than morphine

* Anti-nociception is the mechanism whereby harmful stimuli are inhibited from being transmitted to the central nervous system.

as an analgaesic, but has no affinity for the opioid receptors within the CNS [5].

Caerulein itself has been subjected to extensive pharmacological investigation [6]. It has been shown to display an extremely powerful action on the muscular wall of the gall bladder. In terms of concentration, caerulein was sixteen times more potent than CCK and one hundred and seventy times more potent than gastrin II in contracting the gall bladder [6]. Caerulein has been used clinically on humans in the treatment of cholecystography and cholangiography*. Caerulein also stimulates the smooth muscle of the gastrointestinal tract, and has been used in the clinical treatment of patients suffering from post-operative paralysis of the abdomen [5, 6]. For clinical purposes, caerulein is administered either intravenously or by intramuscular injection. Interestingly, it has been demonstrated that even the C-terminal dipeptide of caerulein, Asp Phe (NH_2), displays pharmacological effects on intestinal smooth muscle preparations [6].

To date, the HPLC peptide profiles of both the dorsal and submental secretions of *Litoria citropa* display a group of unidentified fractions close to that containing caerulein. These unknown fractions were suspected to be caerulein-like neuropeptides.

Therefore, the aims of the research presented in this chapter are:

(i) To separate and characterise the new fractions discovered within the caerulein region of the skin secretion peptide profile of *Litoria citropa*.

(ii) To investigate the biological activities of any new peptides found.

* Cholecystography and cholangiography are surgical procedures by which radiological images of the gall bladder and the bile duct are obtained.

6.2. Results and Discussion

6.2a. General

Initially, the first two fractions of the *Litoria citropa* skin peptide profile, revealed in both HPLC peptide profiles of dorsal and submental skin secretions, appeared to consist of just two individual peptide fractions. These two fractions, which are labelled **A** and **B,** are shown in Figure 6.1. Subsequent HPLC separation revealed that a number of peptides were incorporated within each of the two larger fractions.

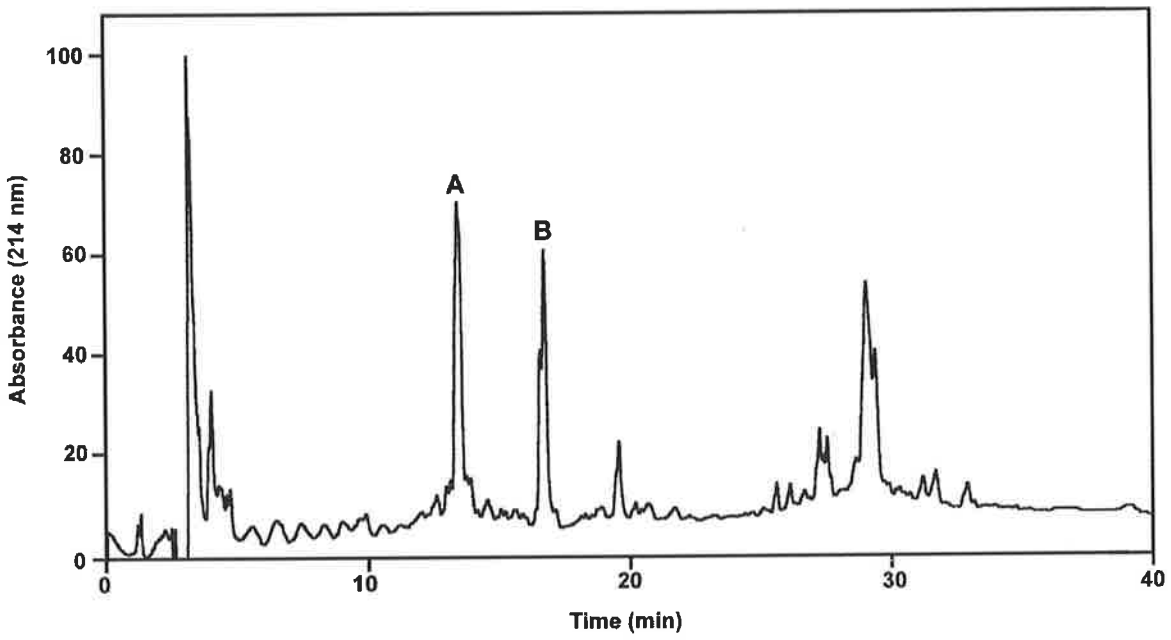


Figure 6.1. HPLC chromatogram from the crude secretions of *Litoria citropa*, indicating the fractions A and B. HPLC chromatogram taken from the dorsal secretion. However, fractions A and B are also present in the chromatogram taken from the submental secretion. (See Experimental section for details).

6.2b. HPLC analysis of fraction A

Additional separation and purification of fraction **A** exposed a total of eight peptide fractions within what was originally thought to be just one fraction. The resulting HPLC chromatogram derived from fraction **A** (Figure 6.2) indicated the presence of four major fractions (**b, c, f** and **g**) and four minor fractions (**a, d, e** and **h**). The relative abundances and corresponding retention times of these fractions are summarised in Table 6.2. Each fraction was then subjected to further purification by HPLC. The fractions were then ready for structural analysis.

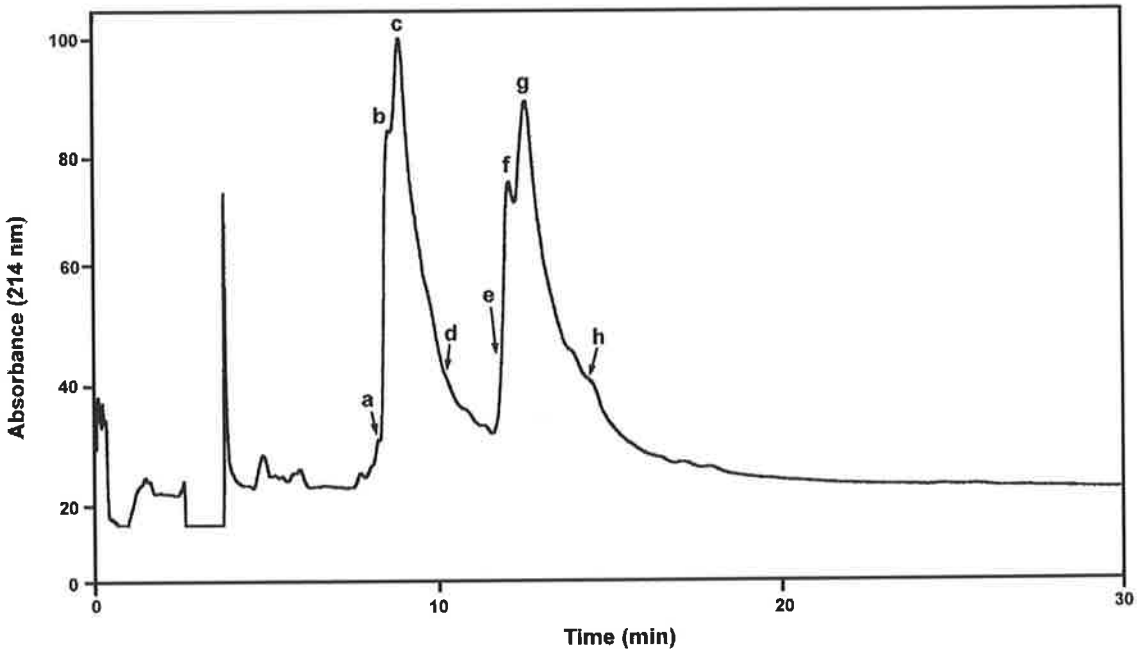


Figure 6.2. HPLC separation of fraction A. (See Experimental section for details).

HPLC fraction	Retention time (minutes)	Relative abundance (percent)
a	8.50	40
b	8.72	75
c	9.77	100
d	10.45	40
e	12.30	40
f	13.10	70
g	13.91	85
h	14.95	30

Table 6.2. Relative abundances and retention times of fraction A, as summarised from the chromatogram represented in Figure 6.2.

6.2c. HPLC analysis of fraction B

Additional separation and purification of fraction **B** also resulted in the separation of eight peptides. The resulting HPLC chromatogram derived from fraction **B** (Figure 6.3) indicated the presence of four major fractions (**j, k, n** and **o**) and four minor fractions (**i, l, m** and **p**). The relative abundances and corresponding retention times of these fractions are summarised in Table 6.3. Further separation and purification by HPLC was again performed on each fraction, to yield eight pure peptides.

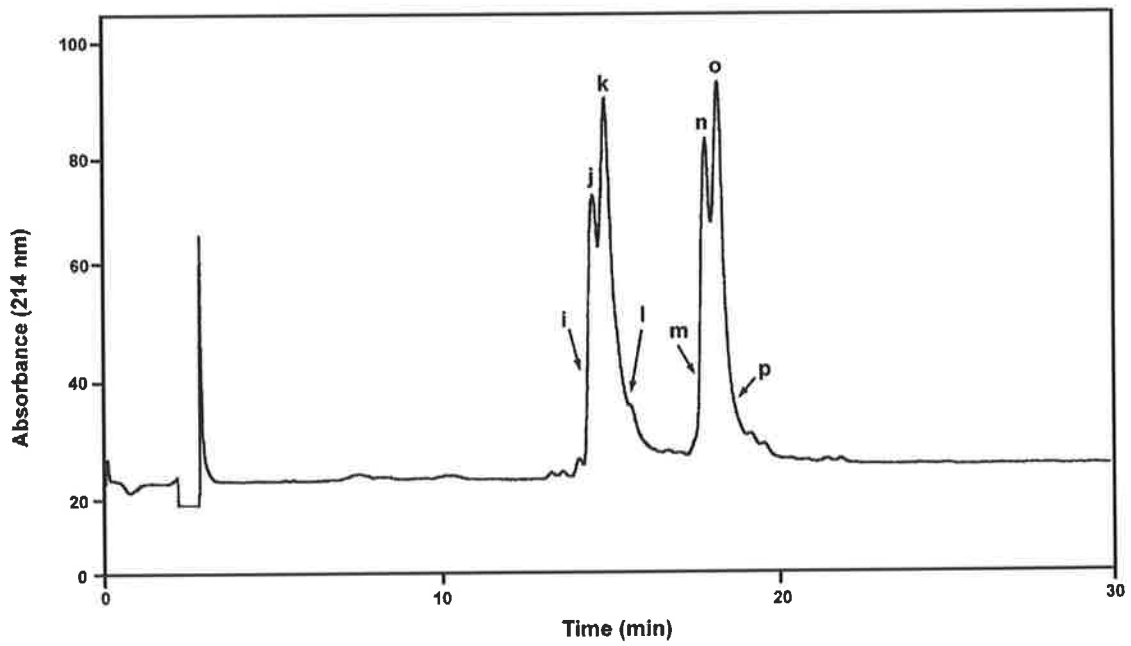


Figure 6.3. HPLC separation of fraction B. (See Experimental section for details).

HPLC fraction	Retention time (minutes)	Relative abundance (percent)
i	15.73	50
j	15.95	70
k	16.10	95
l	16.80	45
m	18.05	40
n	18.45	85
o	18.60	100
p	19.00	45

Table 6.3. Relative abundances and retention times of fraction B, as summarised from the chromatogram represented in Figure 6.3.

6.2d. Structural analysis of peptides derived from fraction A

Structural determination of all peptide fractions was carried out by ESMS experiments. The molecular weights were determined by ESMS in the positive mode. Methylation experiments together with ESMS identified all C-terminal end groups, and gave an indication of the number of the CO_2H and $CONH_2$ groups within the peptides. Lys-C digestion experiments together with ESMS differentiated between Lys and Gln residues. Collision activation mass spectra (MS/MS) of the parent ions, and of the daughter ions (MS/MS/MS), determined the full sequence of all peptides presented in this chapter.

The structural analysis results of the peptides **a-h**, as derived from fraction **A** of the *Litoria citropa* skin secretions, are recorded in Table 6.4. Details of the structural determination of these peptides are listed in Table 6.5.

HPLC fraction	MW	Sequence
a	1425	pGlu Gln Asp Tyr(SO_3) Gly Thr Gly Trp Phe Asp Phe (NH_2)
b	1390	pGlu Gln Asp Tyr(SO_3) Thr Gly Ala His Phe Asp Phe (NH_2)
c	1368	pGlu Gln Asp Tyr(SO_3) Thr Gly Trp Phe Asp Phe (NH_2)
d	1406	pGlu Gln Asp Tyr(SO_3) Thr Gly Ser His Phe Asp Phe (NH_2)
e	1345	pGlu Gln Asp Tyr Gly Thr Gly Trp Phe Asp Phe (NH_2)
f	1310	pGlu Gln Asp Tyr Thr Gly Ala His Phe Asp Phe (NH_2)
g	1288	pGlu Gln Asp Tyr Thr Gly Trp Phe Asp Phe (NH_2)
h	1326	pGlu Gln Asp Tyr Thr Gly Ser His Phe Asp Phe (NH_2)

Table 6.4. Sequences of peptides isolated and characterised from fraction A.

The results identified fraction **c** as Phe[8]-caerulein, identical to that isolated from *Litoria splendida* (see chapter 3). The remaining seven peptides are unique and have not been reported in any other living system. The two peptide groups, **a-d** and **e-f**, are related by the presence or absence of the sulfate group on the tyrosine residue. We are awaiting advice from our collaborator Vittorio Erspamer before we formally name these peptides.

Fraction a [MH^+ 1425]

Methylation gives a methyl ester, MH^+ 1435 (one pGlu, one Tyr (SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1515 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

MH^+ 1425 MS/MS	'B ions'	*m/z* 1408, 1261, 1146, 999, 813, 756 [Gly Trp Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1425, 1314, 1186, 1071, 828, 771, 670, 613, 427
	Sequence	[pGlu Gln Asp Tyr(SO_3) Gly Thr Gly Trp]

Negative ion CA MS/MS gives predominantly *m/z* 1423 and 1343, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of fraction a as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Gly Thr Gly Trp Phe Asp Phe (NH_2)

Fraction b [MH^+ 1390]

Methylation gives a methyl ester, MH^+ 1400 (one pGlu, one Tyr (SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1480 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

MH^+ 1390 MS/MS	'B ions'	*m/z* 1373, 1226, 1111, 964, 827, 756, 699 [Gly Ala His Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1390, 1279, 1151, 1036, 793, 692, 635, 564, 427
	Sequence	[pGlu Gln Asp Tyr(SO_3) Thr Gly Ala His]

Negative ion CA MS/MS gives predominantly *m/z* 1388 and 1308, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of fraction b as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Thr Gly Ala His Phe Asp Phe (NH_2)

Fraction c (Phe^8-Caerulein) [MH^+ 1368]

Methylation gives a methyl ester, MH^+ 1378 (one pGlu, one Tyr(SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1458 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

[MH^+ -SO_3] 1288 MS/MS	'B ions'	*m/z* 1271, 1124, 1009, 862 [Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1288, 1177, 1049, 934, 771, 670, 613, 427
	Sequence	[pGlu Gln Asp Tyr Thr Gly Trp]

Negative ion CA MS/MS gives predominantly *m/z* 1366 and 1286, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of fraction c as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Thr Gly Trp Phe Asp Phe (NH_2)

Table 6.5. MS data for peptides isolated from fraction A.

Fraction d [MH^+ 1406]

Methylation gives a methyl ester, MH^+ 1416 (one pGlu, one Tyr (SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1496 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

MH^+ 1406 MS/MS	'B ions'	*m/z* 1389, 1242, 1127, 980, 843, 756, 699 [Gly Ser His Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1406, 1295, 1167, 1052, 809, 708, 651, 564
	Sequence	[pGlu Gln Asp Tyr(SO_3) Thr Gly Ser]

Negative ion CA MS/MS gives predominantly *m/z* 1404 and 1324, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of fraction d as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Thr Gly Ser His Phe Asp Phe (NH_2)

Fraction e [MH^+ 1345]

Methylation gives a methyl ester, MH^+ 1435 (one pGlu, two CO_2H and two $CONH_2$ groups).

MH^+ 1345 MS/MS	'B ions'	*m/z* 1328, 1181, 1066, 919, 733, 676, 575, 518 [Gly Thr Gly Trp Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1345, 1234, 1106, 991, 828, 771, 670
	Sequence	[pGlu Gln Asp Tyr Gly Thr]

Full sequence of fraction e as determined from mass spectrometric data is:

pGlu Gln Asp Tyr Gly Thr Gly Trp Phe Asp Phe (NH_2)

Fraction f [MH^+ 1310]

Methylation gives a methyl ester, MH^+ 1400 (one pGlu, two CO_2H and two $CONH_2$ groups).

MH^+ 1310 MS/MS	'B ions'	*m/z* 1293, 1146, 1031, 884, 747, 676, 619, 518 [Thr Gly Ala His Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1310, 1199, 1071, 956, 793, 692
	Sequence	[pGlu Gln Asp Tyr Thr]

Full sequence of fraction f as determined from mass spectrometric data is:

pGlu Gln Asp Tyr Thr Gly Ala His Phe Asp Phe (NH_2)

Table 6.5. *(Continued)*

Fraction g [MH^+ 1288]

Methylation gives a methyl ester, MH^+ 1378 (one pGlu, two CO_2H and two $CONH_2$ groups).

MH^+ 1288	'B ions'	*m/z* 1271, 1124, 1009, 862,
MS/MS		[Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1288, 1177, 1049, 934, 771, 670, 613, 427
	Sequence	[pGlu Gln Asp Tyr Thr Gly Trp]

Full sequence of fraction g as determined from mass spectrometric data is:

pGlu Gln Asp Tyr Thr Gly Trp Phe Asp Phe (NH_2)

Fraction h [MH^+ 1326]

Methylation gives a methyl ester, MH^+ 1416 (one pGlu, two CO_2H and two $CONH_2$ groups).

MH^+ 1326	'B ions'	*m/z* 1309, 1162, 1047, 900, 763, 676
MS/MS		[Ser His Phe Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1326, 1215, 1087, 972, 809, 708, 651, 564
	Sequence	[pGlu Gln Asp Tyr Thr Gly Ser]

Full sequence of fraction h as determined from mass spectrometric data is:

pGlu Gln Asp Tyr Thr Gly Ser His Phe Asp Phe (NH_2)

Table 6.5. *(Continued)*

6.2e. Structural analysis of peptides derived from fraction B

The structural analysis results of the peptides **i-p**, as derived from fraction **B** of the *Litoria citropa* skin secretions, are recorded in Table 6.6. Details of the structural determination of these peptides are listed in Table 6.7. Three examples of the CA MS/MS spectra that were obtained from this section are shown in Figures 6.4, 6.5 and 6.6.

HPLC fraction	MW	Sequence
i	1409	pGlu Gln Asp Tyr(SO_3) Gly Thr Gly Trp Met Asp Phe (NH_2)
j	1374	pGlu Gln Asp Tyr(SO_3) Thr Gly Ala His Met Asp Phe (NH_2)
k	1352	pGlu Gln Asp Tyr(SO_3) Thr Gly Trp Met Asp Phe (NH_2)
l	1390	pGlu Gln Asp Tyr(SO_3) Thr Gly Ser His Met Asp Phe (NH_2)
m	1329	pGlu Gln Asp Tyr Gly Thr Gly Trp Met Asp Phe (NH_2)
n	1295	pGlu Gln Asp Tyr Thr Gly Ala His Met Asp Phe (NH_2)
o	1272	pGlu Gln Asp Tyr Thr Gly Trp Met Asp Phe (NH_2)
p	1310	pGlu Gln Asp Tyr Thr Gly Ser His Met Asp Phe (NH_2)

Table 6.6. Sequences of peptides isolated and characterised from fraction B.

These results identified fraction **k** as caerulein. The other seven peptides have not been reported previously. As with the peptides derived from fraction **A**, the two peptide groups (**i-l** and **m-p**) differ in structure by the presence or absence of the sulfate group on the tyrosine residue. Three of the desulphated peptides were synthesised commercially by Chiron Mimotopes. They are identical with the isolated peptides, as evidenced by their ESMS and HPLC characteristics. These peptides have not been formally named.

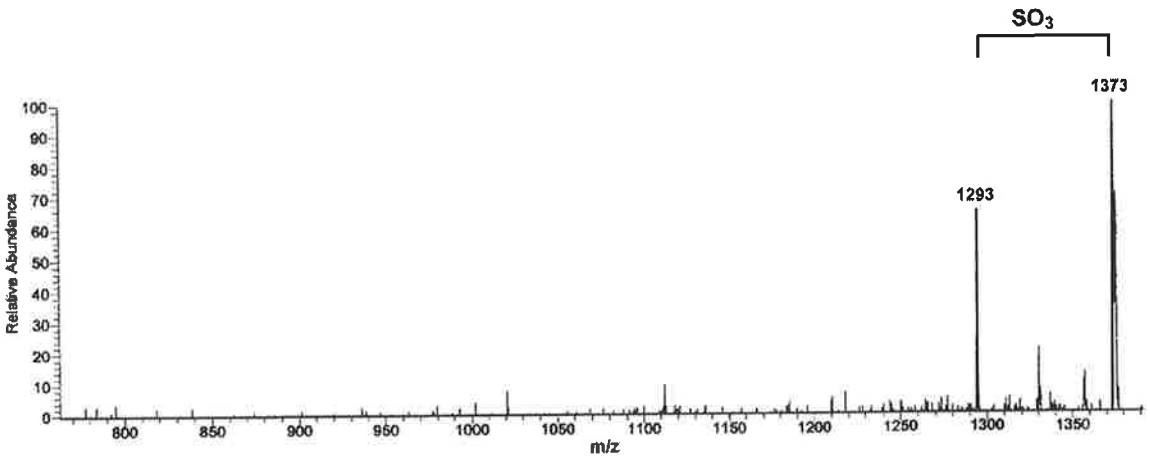


Figure 6.4. Negative ion CA MS/MS of $[M-H]^-$ (*m/z* 1373) ion of fraction j. Note the loss of 80 Da. (See Experimental section for full details).

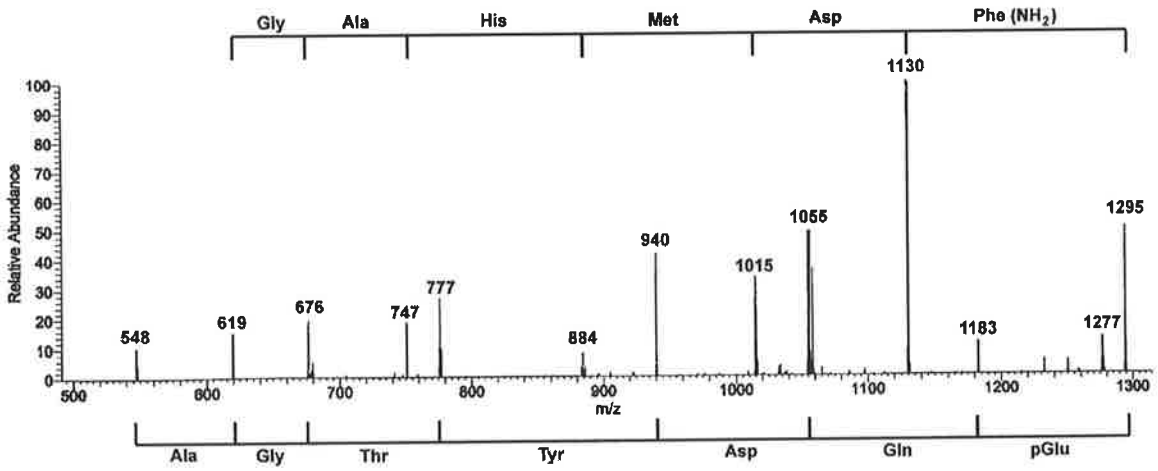


Figure 6.5. Positive CA MS/MS of the $[MH^+ -SO_3]$ ion of fraction j (*m/z* 1295). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

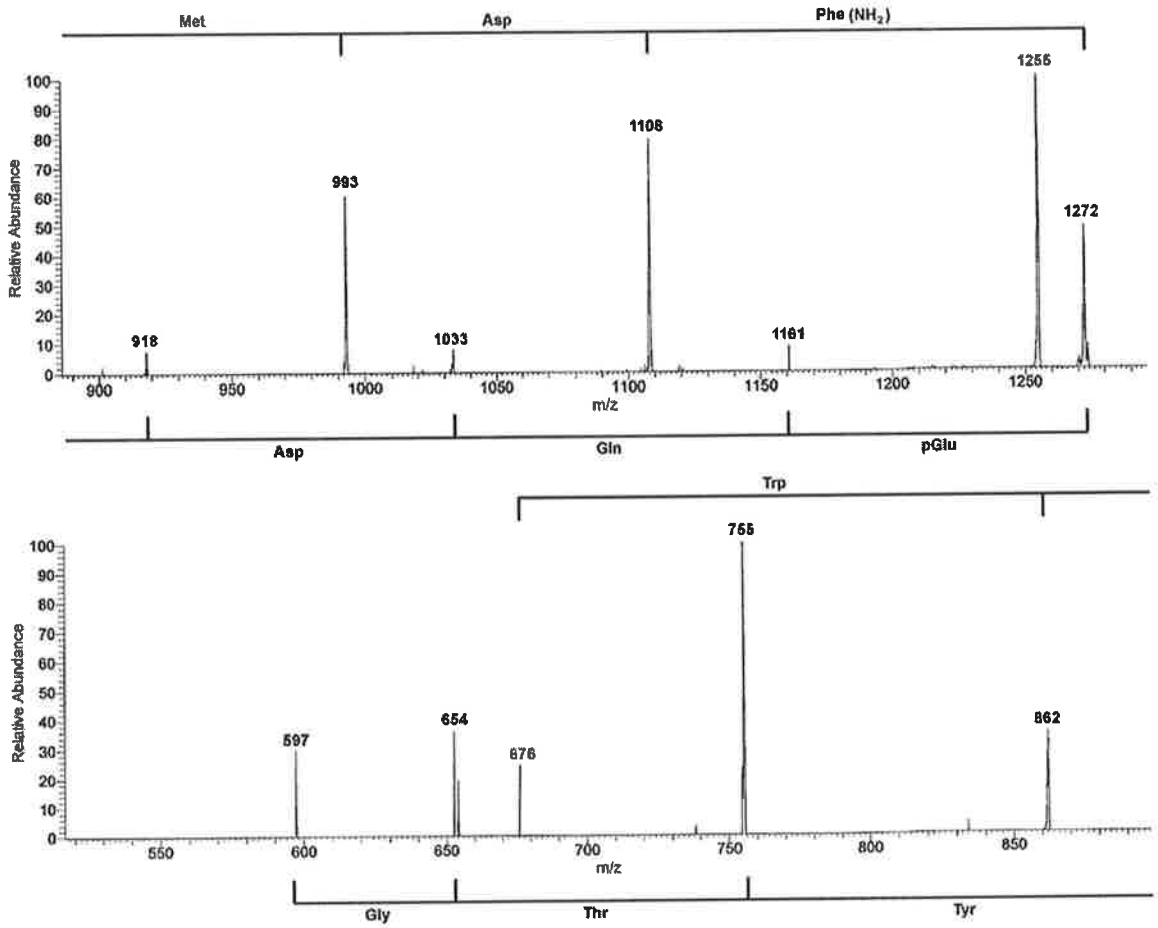


Figure 6.6. Positive CA MS/MS of the [MH^+ -SO_3] ion of caerulein (*m/z* 1272). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

Fraction i [MH^+ 1409]

Methylation gives a methyl ester, MH^+ 1419 (one pGlu, one Tyr(SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1499 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

MH^+ 1409 MS/MS	'B ions'	*m/z* 1392, 1245, 1130, 999, 813, 756, 655 [Thr Gly Trp Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1409, 1298, 1170, 1055, 812, 755 [pGlu Gln Asp Tyr(SO_3) Gly]

Negative ion CA MS/MS gives predominantly *m/z* 1407 and 1327, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of fraction i as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Gly Thr Gly Trp Met Asp Phe (NH_2)

Fraction j [MH^+ 1374]

Methylation gives a methyl ester, MH^+ 1384 (one pGlu, one Tyr(SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1464 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

MH^+ 1374 MS/MS	'B ions'	*m/z* 1357, 1210, 1095, 964, 827, 756 [Ala His Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1374, 1263, 1135, 1020, 777, 676, 619, 548 [pGlu Gln Asp Tyr(SO_3) Thr Gly Ala]

Negative ion CA MS/MS gives predominantly *m/z* 1373 and 1293, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of fraction j as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Thr Gly Ala His Met Asp Phe (NH_2)

Fraction k (Caerulein) [MH^+ 1352]

Methylation gives a methyl ester, MH^+ 1362 (one pGlu, one Tyr(SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1442 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

MH^+ 1352 MS/MS	'B ions'	*m/z* 1335, 1188, 1073, 942, 756 [Trp Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1352, 1241, 1113, 998, 755, 654, 597 [pGlu Gln Asp Tyr(SO_3) Thr Gly]

Negative ion CA MS/MS gives predominantly *m/z* 1350 and 1270, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of fraction k as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Thr Gly Trp Met Asp Phe (NH_2)

Table 6.7. MS data for peptides isolated from fraction B.

Fraction l [MH^+ 1390]

Methylation gives a methyl ester, MH^+ 1400 (one pGlu, one Tyr(SO_3), two CO_2H and two $CONH_2$ groups). NB: Should observe MH^+ 1480 after methylation. However, the SO_3 functionality is cleaved from the tyrosine in acidic conditions.

MH^+ 1390 MS/MS	'B ions'	*m/z* 1373, 1226, 1111, 980 [Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1390, 1279, 1151, 1036, 793, 692, 635 [pGlu Gln Asp Tyr(SO_3) Thr Gly]
	Sequence	pGlu Gln Asp Tyr(SO_3) Thr Gly – Met Asp Phe (NH_2)
m/z 1151 MS/MS/MS	'B ions'	*m/z* 1134, 987, 872, 741, 604, 517 [Ser His Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1151, 1036, 793, 692, 635, 548 [Asp Tyr(SO_3) Thr Gly Ser]
	Sequence	Asp Tyr(SO_3) Thr Gly Ser His Met Asp Phe (NH_2)

Negative ion CA MS/MS gives predominantly *m/z* 1388 and 1308, indicating a pronounced loss of 80 Da (SO_3).

Full sequence of fraction l as determined from mass spectrometric data is:

pGlu Gln Asp Tyr(SO_3) Thr Gly Ser His Met Asp Phe (NH_2)

Fraction m [MH^+ 1329]

Methylation gives a methyl ester, MH^+ 1419 (one pGlu, two CO_2H and two $CONH_2$ groups).

MH^+ 1329 MS/MS	'B ions'	*m/z* 1312, 1165, 1050, 919, 733, 676 [Gly Trp Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1329, 1218, 1090, 975, 812 [pGlu Gln Asp Tyr]
	Sequence	pGlu Gln Asp Tyr - Gly Trp Met Asp Phe (NH_2)
m/z 1090 MS/MS/MS	'B ions'	*m/z* 1073, 926, 811, 680 [Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1090, 975, 812, 755, 654, 597, 411 [Asp Tyr Gly Thr Gly Trp]
	Sequence	Asp Tyr Gly Thr Gly Trp Met Asp Phe (NH_2)

Full sequence of fraction m as determined from mass spectrometric data is:

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

Table 6.7. *(Continued)*

Fraction n [MH^+ 1295]

Methylation gives a methyl ester, MH^+ 1385 (one pGlu, two CO_2H and two $CONH_2$ groups).

MH^+ 1295 MS/MS	'B ions'	*m/z* 1277, 1130, 1015, 884, 747, 676, 619 [Gly Ala His Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1295, 1183, 1055, 940, 777, 676, 619, 548 [pGlu Gln Asp Tyr Thr Gly Ala]

Full sequence of fraction n as determined from mass spectrometric data is:
pGlu Gln Asp Tyr Thr Gly Ala His Met Asp Phe (NH_2)

Fraction o [MH^+ 1272]

Methylation gives a methyl ester, MH^+ 1362 (one pGlu, two CO_2H and two $CONH_2$ groups).

MH^+ 1272 MS/MS	'B ions'	*m/z* 1255, 1108, 993, 862, 676, 619, 518 [Thr Gly Trp Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1272, 1161, 1033, 918, 755, 654, 597 [pGlu Gln Asp Tyr Thr Gly]

Full sequence of fraction o as determined from mass spectrometric data is:
pGlu Gln Asp Tyr Thr Gly Trp Met Asp Phe (NH_2)

Fraction p [MH^+ 1310]

Methylation gives a methyl ester, MH^+ 1400 (one pGlu, two CO_2H and two $CONH_2$ groups).

MH^+ 1310 MS/MS	'B ions'	*m/z* 1293, 1146, 1031, 900 [Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1310, 1199, 1071, 956, 793, 692, 635 [pGlu Gln Asp Tyr Thr Gly]
	Sequence	pGlu Gln Asp Tyr Thr Gly – Met Asp Phe (NH_2)
m/z 1071 MS/MS/MS	'B ions'	*m/z* 1054, 907, 792, 661, 524, 437 [Ser His Met Asp Phe (NH_2)]
	'Y+2 ions'	*m/z* 1071, 956, 793, 692, 635, 548 [Asp Tyr Thr Gly Ser]
	Sequence	Asp Tyr Thr Gly Ser His Met Asp Phe (NH_2)

Full sequence of fraction p as determined from mass spectrometric data is:
pGlu Gln Asp Tyr Thr Gly Ser His Met Asp Phe (NH_2)

Table 6.7. *(Continued)*

6.2f. Conclusions

This research has uncovered sixteen caerulein-like peptides, of which fourteen are new and not reported from any other known living system. All of these peptides, which were isolated from the skin peptide profile of *Litoria citropa*, closely resembled the structure of the neuropeptide caerulein. The peptides isolated can be listed in two categories: (i) caerulein-like peptides containing a post-translated tyrosine sulfate residue, and (ii) caerulein-like peptides with a tyrosine residue minus the sulfate group. As mentioned earlier, caerulein-like peptides that lack a sulfate group within the tyrosine residue have been reported to exhibit no neuropeptide activity. Since the sulfated caerulein-like peptides from *Litoria citropa* all closely represent caerulein, gastrin and CCK in structure, they are likely to exhibit a similar spectrum of neurological activity. An enzyme that cleaves the sulfate group, consequently deactivating the neuropeptide, may be a protective and deactivating mechanism used by the anuran. This may explain why caerulein-like de-sulfated peptides are abundant within the secretions of this amphibian*.

Secretions used for this research were obtained from *Litoria citropa* during the winter month of July. As discovered and presented in chapter three, a pattern was observed within the *Litoria splendida* secretions whereby Phe^{8}-caerulein appeared to cycle seasonally throughout the year. The abundance of Phe^{8}–caerulein within *Litoria splendida* was at its greatest during the winter periods and markedly decreased outside of this period. Unfortunately, this pattern

* There is no evidence to suggest that these peptides have been degraded on work up or by HPLC separation. Neither caerulein nor Phe^{8}-caerulein is affected by these procedures.

could not be established for *Litoria citropa*, as the sole specimen died during the period of this research and a new specimen was unavailable.

All the caerulein-like peptides containing a post-translated tyrosine sulfate residue have proved difficult to synthesise commercially, as the sulfate group cleaves when the peptide is removed from the support. Currently, the synthesis and biological activity testing of the caerulein-like peptides containing post-translated tyrosine sulfate residues is being undertaken by the research group of Professor Vittorio Erspamer (La Sapienza, Rome). The biological activities of these new caerulein-like peptides are therefore presently unknown, but it is suspected that their neuropeptide activity will be similar to that of caerulein.

6.3. Experimental

6.3a. Collection and preparation of *Litoria citropa* secretions

The skin secretions from adult *Litoria citropa* were provided courtesy of Associate Professor Mike Tyler, Department of Environmental Biology, University of Adelaide. The secretions were obtained by the SES method, as described in section 2.3a. The resulting secretion was washed with water (distilled 25 ml). Methanol (25 ml) was immediately added to the aqueous extract, the mixture centrifuged, and concentrated to approximately 1 ml. Secretions were taken and combined from both dorsal and submental glands.

6.3b. HPLC separation

HPLC separation was achieved for each sample using a VYDAC C_{18} Protein and Peptide (218TP54) reverse phase column equilibrated with 10% acetonitrile/ aqueous 0.1% TFA. Additional purification was achieved using an elution profile of 20-45% acetonitrile over a period of 60 minutes. Each sample was filtered through a Millipore 0.45 μm filter and injected onto the column. The conditions for the various HPLC chromatograms represented in this chapter are as follows:

Figure 6.1. The elution profile increased from 10-75% acetonitrile over a period of 40 minutes using a flow rate of 1 ml/min.

Figure 6.2. The elution profile increased from 47-56% acetonitrile over a period of 60 minutes using a flow rate of 1 ml/min.

Figure 6.3. The elution profiles increased from 40-60% acetonitrile over a period of 60 minutes using a flow rate of 1 ml/min.

6.3c. Mass spectrometry analysis

Electrospray mass spectra were determined using a Finnigan LCQ ion trap mass spectrometer. The samples were dissolved in methanol/water (1:1) and infused into the electrospray source at a flow rate of 12 µl/min. Collision activation mass spectral data in the positive mode were obtained using collision energy of 30%. In the negative mode, collision energy of 35% was used. Electrospray conditions were as follows: source voltage 4.2 kV, source current 18 µA, capillary temperature 200°C, capillary voltage 3V and sheath gas flow 30 psi. Mass spectra were acquired with the automatic gain control on, a maximum ion time of 700 milliseconds, and using 4 microscans per scan, averaging over approximately 25 scans. Molecular weights of the peptides were determined from either MH^+ or $[M-H]^-$ ions as appropriate.

6.3d. Additional information

The remaining procedures are identical to those described elsewhere, as detailed in the table below.

Procedure	Section in which it is described
Methylation of peptides	Section 2.3d (page 74)
Preparation of synthetic peptides	Section 2.3h (page 76)

6.4. References

[1] Anastasi, A., Erspamer, V. and Endean, R., *Experientia*, **23**, 699-700 (1967).

[2] Anastasi, A., Erspamer, V. and Endean, R., *Arch. Biochem. Biophys.*, **125**, 57-68 (1968).

[3] Montecucchi, P.C., Falconieri Erspamer, G. and Visser, J., *Experientia*, **33**, 1138-1139 (1977).

[4] Erspamer, V., Falconieri Erspamer, G., Mazzanti, G. and Endean, R., *Comp. Biochem. Physiol.*, **77**, 99-108 (1984).

[5] Lazarus, L.H. and Attila, M., *Prog. Neurobiol.*, **47**, 473-507 (1993).

[6] Erspamer, V. and Melchiorri, P., *Pure Appl. Chem.*, **35**, 463-494 (1973).

CHAPTER 7. Neuronal Nitric Oxide Synthase Inhibition Activity and Preliminary Anticancer Activity of the Citropin 1 Peptides

7.1. Introduction

7.1a. Nitric oxide

The discovery that mammalian cells generate nitric oxide (NO), a gas previously considered to be merely an atmospheric pollutant, has provided important insight concerning many vital biological processes. In mammalian systems, NO is a major messenger molecule in the cardiovascular, immune and nervous systems[1]. The functions of NO throughout the human body are very diverse. In the brain and peripheral nervous systems, NO displays the properties of a neurotransmitter and it has been implicated in neurotoxicity associated with stroke and various neurodegenerative diseases[2]. Within the central nervous system, NO has also been considered as an important neurotransmitter involved with the phenomenon of memory[3]. Endogenous NO also regulates mammalian blood vessels as a vasodilator, and is involved with the endothelial derived relaxing factor (EDRF), which regulates blood pressure[4]. Nitric oxide also displays several important functions within the immune system. For example, in macrophages* NO mediates tumouricidal and bactericidal actions by promoting cell-mediated immune responses. Nitric oxide acts as a double-edged sword, beneficial as a messenger or modulator and for immunological self-defense, but at the same time, potentially toxic as illustrated in Table 7.1.

Nitric oxide is not stored in vesicles, but is synthesised on demand[5]. The actions of NO are localised and are usually mediated by diffusion to intracellular targets rather than by binding to specific plasma membrane receptors[6]. Nitric oxide has a very short half-life and consequently breaks down within a few seconds[8].

* A macrophage is a mononuclear cell that has the ability to devour foreign material.

Tissue	Messenger	Toxin
Blood vessels	EDRF, antithrombotic, ischemic protection, antiatherosclerotic, inhibition of smooth muscle migration and proliferation, antiadhesive	Septic shock, inflammation, reperfusion injury, microvascular leakage, atherosclerosis
Heart	Coronary perfusion, negative inotropic, ischemia	Myocardial "stunning", septic shock, reperfusion
Lungs	Ventilation promotion, bronchiociliar motility, mucus secretion, immune defense	Immune complex-induced alveolitis, silo fillers disease, asthma
Kidneys	Tubuloglomerular feedback, glomerular perfusion, rennin secretion	Acute kidney failure, glomerulonephritis
CNS	Synaptogenesis, synaptic plasticity, memory formation, cerebral blood flow and ischemia, neuroendocrine secretion, visual transduction, olfaction	Neurotoxic, proconvulsive, migraine, hyperalgaesia, reperfusion
Pancreas	Endocrine/exocrine secretion	β cell destruction
Stomach	Blood flow, peristalsis, exocrine secretion, mucosal protection, antibacterial	Mutagenesis, mucosal damage
Immune system	Antibacterial, antitumour agent	Antiallograft, graft versus host disease, inflammation, septic shock, tissue damage

Table 7.1. NO as a double-edged sword: messenger and toxin.

As NO cannot be stored in vesicles like many other chemical messengers, its release is regulated by the activity of the enzyme that manufactures it, nitric oxide synthase (NOS)[7].

7.1b. Nitric oxide synthase

Nitric oxide, one of the smallest (30 Da) and simplest biosynthetic products, is made by nitric oxide synthase enzymes that are among the largest (*ca* 300 kDa) and most complicated yet identified. NOS enzymes are dimeric in their natural state: their monomers themselves are two enzymes fused together[8]. The complexity of NOS enzymes involves several tightly bound redox cofactors. These are organised into discrete domains and are associated with a particular activity[9]. The NOS enzymes share significant homology with another enzyme known as

NADPH* cytochrome P-450 reductase. The enzymes contain a cytochrome P-450-type heme moiety, which is an essential feature of the synthesis of NO[10]. Three isotopic NOS enzymes are known; viz. neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The three enzymes share the same basic structure and catalytic mechanism. Nitric oxide is formed at the P-450 heme-containing, H_4biopterin-dependent oxygenase active site in the N-terminal half of each NOS [11]. The NOS enzymes catalyse the oxidation of L-arginine to form L-citruline and NO[12]. The C-terminal reductase domain binds flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) and passes reducing equivalents from NADPH to the heme region. This activity requires the binding of a regulatory protein known as calmodulin (CaM)[10]. All three enzymes are homodimeric, with the interface between subunits being formed by the oxygenase domain[5].

The major differences between the enzymes relate to their tissue distribution and regulation. Binding of CaM to nNOS and eNOS is calcium dependent, whereas iNOS binds CaM independently of calcium[13]. Each enzyme has a unique N-terminal sequence that appears not to be strictly necessary for catalytic activity. In the cases of eNOS and nNOS, there is evidence that the N-terminal domains function in the intracellular localisation of the enzymes[9].

Nitric oxide synthases are unique among eukaryotic enzymes in being dimeric, CaM-dependent (or CaM-containing) cytochrome P-450-like hemoproteins that combine reductase and oxygenase catalytic domains in one monomer, contain both FAD and FMN, and carry out a 5-electron oxidation of L-arginine with the aid of the electron carrier (and reductant) tetrahydrobiopterin (H_4B)[13].

7.1c. Inducible nitric oxide synthase

More than a century ago, it was shown that resistance to cancer could be enhanced in a nonspecific manner by bacterial products, and that this phenomenon was linked to macrophage interaction [14]. Recent evidence now suggests that this nonspecific immunity is associated with the induction of NOS[15]. Almost every eukaryotic cell is able to express calcium independent NOS (iNOS) during cell-mediated immune responses, which in itself is

* NADPH is the reduced form of nicotinamide adenine dinucleotide phosphate.

always nonspecific[7]. As NO is effective against various microbes (bacteria, parasites and viruses), tumour cells and alloantigens, it may be viewed as an immune modulator[16]. An illustration of the cell-mediated immune response and cytotoxic mechanism of iNOS within a macrophage is shown in Figure 7.1.

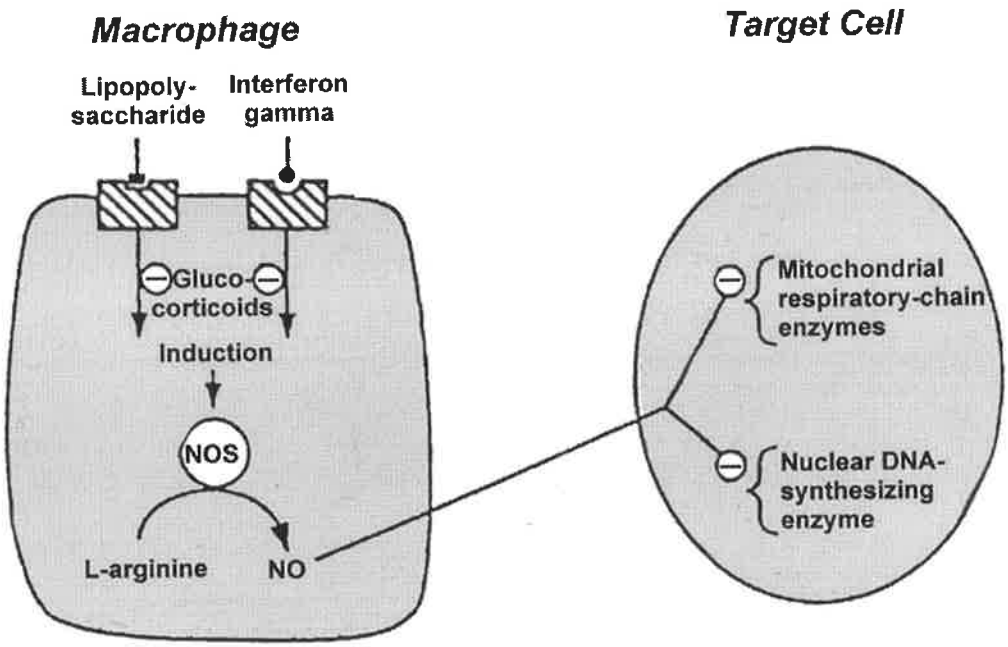


Figure 7.1. Mechanism of iNOS action, leading to cytostasis and cytotoxicity. The activation of the macrophage by lipopolysaccharide and/or interferon gamma results in the induction of the calcium-independent NOS. This induction, which is inhibited by glucocorticoids, results in the production of NO. The NO then diffuses to target cells such as tumour cells, bacteria, fungi and viruses. There the NO combines with iron-sulfur centres of the key enzymes responsible for the target cells respiratory cycle and pathway for DNA synthesis, causing inhibition. This then leads to cell death of the target cell. Minus signs indicate inhibition.

7.1d. Endothelial nitric oxide synthase

The endothelial nitric-oxide synthase (eNOS) is a crucial determinant of vascular homeostasis[15]. Endothelium-derived NO is a potent vasodilator that antagonises smooth muscle contraction, antagonises all stages of platelet activation and inhibits platelet adhesion[13]. Calcium dependent CaM binding regulates the eNOS. An increase in intracellular levels of calcium by specific extracellular signals leads to the activation of eNOS, which in turn produces increased levels of NO. The mechanism of action of eNOS within a vascular endothelial cell is illustrated in Figure 7.2.

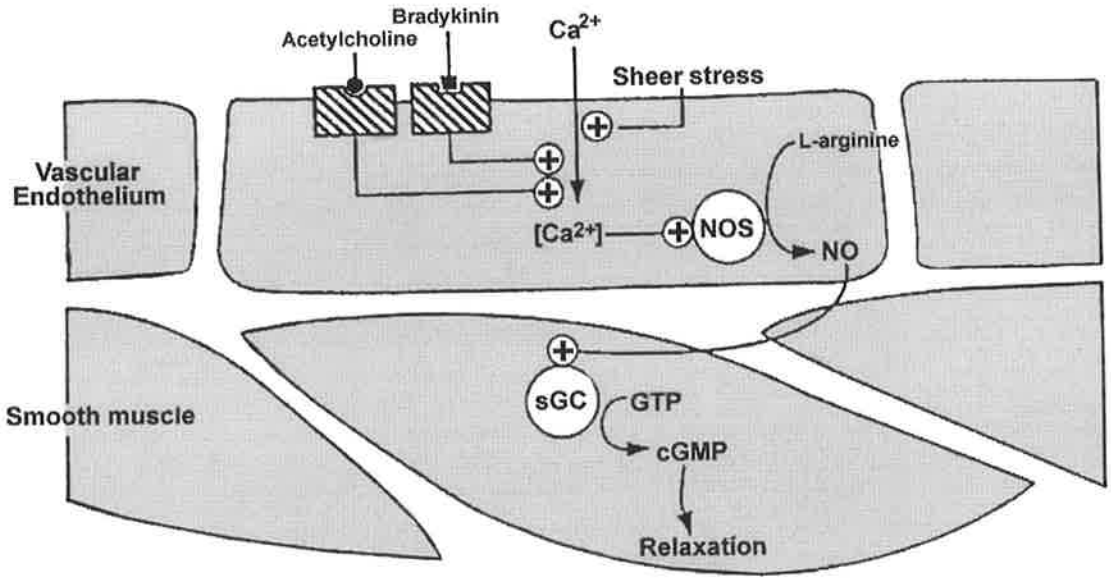


Figure 7.2. Mechanism of eNOS action, leading to vascular relaxation. Sheer stress or receptor activation of the vascular endothelium by bradykinin or acetylcholine results in the influx of calcium. This increase consequently activates eNOS, which then produces NO from L-arginine. Diffusion of NO to nearby smooth-muscle cells stimulates soluble guanylate cyclase (sGC), resulting in the increased synthesis of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). This increase of cGMP in the smooth muscle cells leads to relaxation. Plus signs indicate stimulation.

7.1e. Neuronal nitric oxide synthase

Of all three enzymes, the nNOS enzyme is the most relevant to the research presented within this chapter. Within the nervous system, NO functions as a neurotransmitter and as a hormone[6]. Its neuronal functions include peripheral neurotransmission in contractile and secretory tissues, synaptogenesis, sensory input processing, synaptic plasticity and memory formation. Excessive nNOS stimulation leads to an overproduction of NO that gives rise to a variety of pathophysiological conditions that include migraine, epilepsy, cerebral ischemia (that can lead to stroke), general neurotoxicity and other neurodegenerative diseases[17]. The mechanism of action of nNOS within a neuron is illustrated in Figure 7.3.

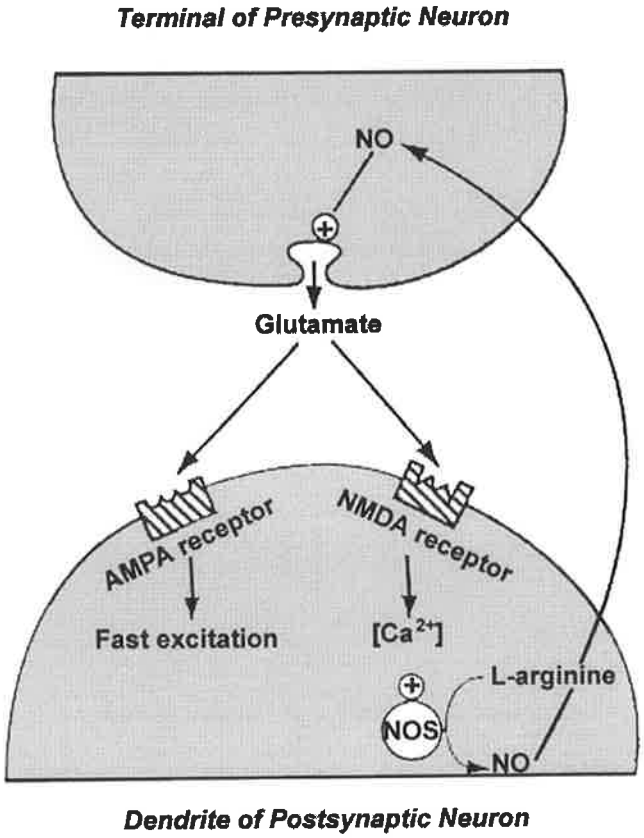


Figure 7.3. Mechanism of nNOS action, leading to increased neuronal activity. Glutamate released from the presynaptic nerve terminal activates different types of receptors on the dendrites of the postsynaptic neuron. Under normal conditions the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors mediate most of the effects of the glutamate. During high–frequency synaptic transmission, however, the N-methyl-D-aspartate (NMDA) receptors are activated. This then results in an increase in intracellular calcium levels, which then stimulates the nNOS. The NO that is consequently produced diffuses back to the presynaptic neuron, where it enhances the release of glutamate. This increased level of glutamate release leads to a greater activation of postsynaptic glutamate receptors, thereby increasing the effectiveness and neuronal activity of that particular synapse. Plus signs indicate stimulation.

7.1f. Calmodulin

Calmodulin regulates and plays a central role in the activity of all the NOS enzymes[18]. This 17-kDa protein serves as a calcium sensor in most eukaryotic cells, as it contains calcium-binding sites within its overall structure. The binding sites of CaM each consist of a 29-residue helix-loop-helix motif called the EF-hand that is responsible for high affinity calcium binding[19]. Calmodulin has four EF-hand domains, arranged as two pairs separated by an eight-turn central helix (Figure 7.4). The binding of calcium to the four binding sites in CaM induces conformational changes that convert it from an inactive to active form[16]. Activated CaM then binds to many enzymes (including the NOS enzymes) and other proteins in the cell

and modifies their activities. In regards to the NOS enzymes, the crucial CaM interaction functions as a molecular switch that allows electron transport from the C-terminal reductase domain of the NOS to its heme containing N-terminal domain that then leads to efficient catalysis[18].

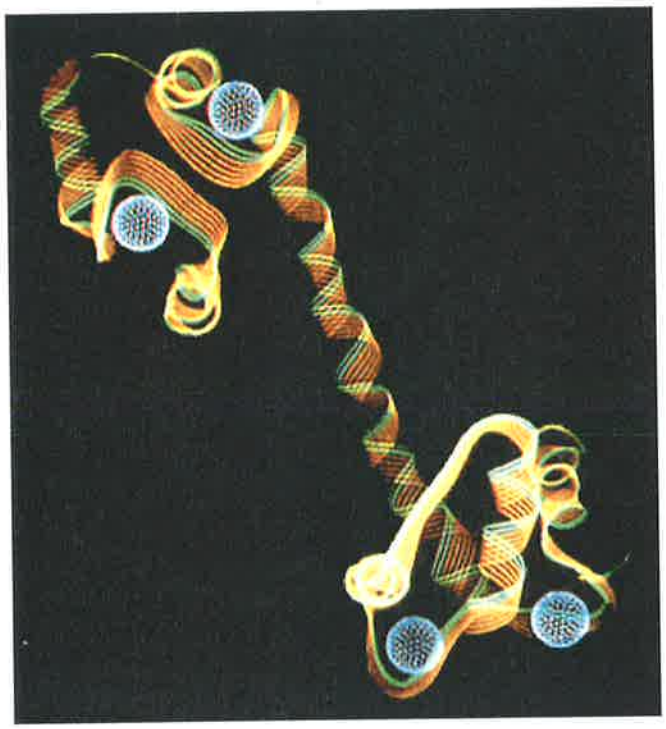
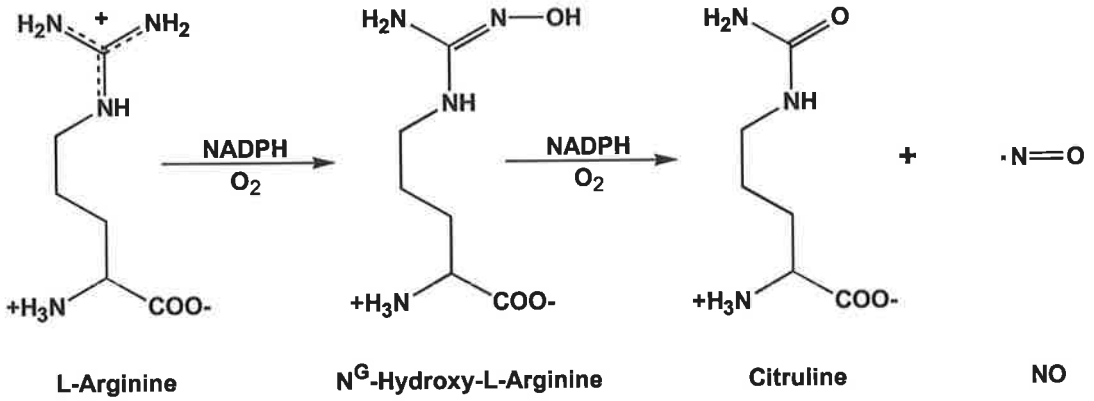


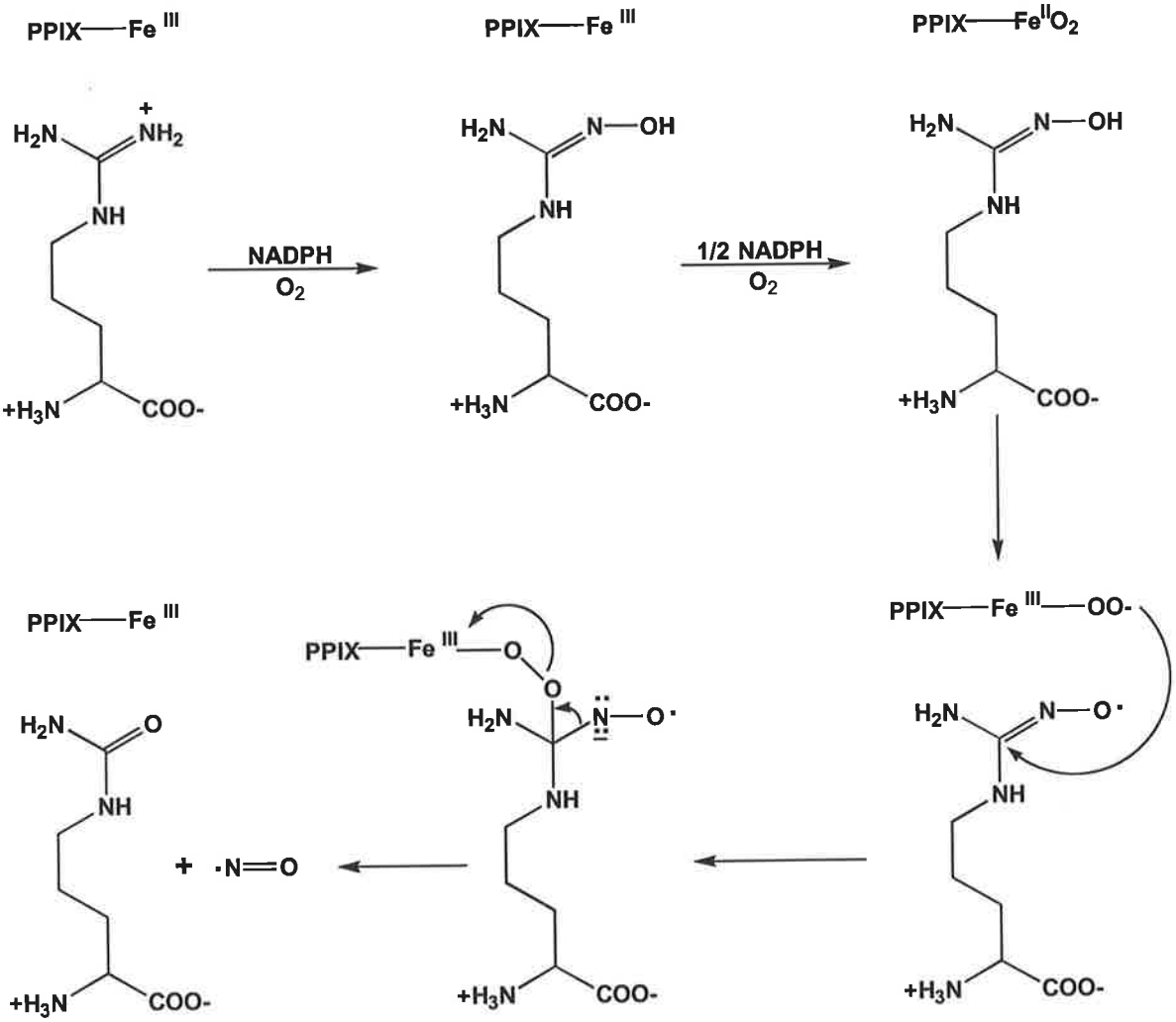
Figure 7.4. Structure of calmodulin. The four bound calcium ions are shown in blue.

7.1g. Nitric oxide synthase mechanism

The general reaction catalysed by the NOS enzymes is illustrated in Scheme 7.1. The enzymes specifically utilise L-arginine as the substrate, with the final products of the reaction being NO and citruline. As shown, the reaction requires molecular oxygen and reducing equivalents in the form of NADPH. The NOS reaction primarily involves an initial hydroxylation of L-arginine to generate N^G-hydroxy-L-arginine (NHA). This step involves the heme moiety of NOS. Although the exact chemistry involved in the conversion of NHA to NO and citruline is not entirely known, speculation based on enzymatic and chemical precedent has lead to the proposal that the heme is directly involved in the oxidation of NHA[20]. This proposed mechanism is outlined in Scheme 7.2 and involves a heme ferric peroxide (Fe^{III}-OO^-) nucleophile as a key reaction intermediate[21].



Scheme 7.1. The reaction catalysed by NOS.



Scheme 7.2. Mechanistic speculation on the formation of NO and citruline from L-arginine.

All NOS isoforms require NADPH as a source of the reducing equivalents needed for the production of citruline and NO[22]. The reductase domain of the NOS is within the C-terminus of the protein, while the heme-binding region is located in the N-terminus[23]. The C-terminal reductase domain binds the oxidising agents flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are crucial for the catalytic mechanism[11]. The reducing equivalents from the NADPH are passed to the heme. This activity requires the binding of CaM. NO is formed at the heme containing region and H_4biopterin-dependent oxygenase active site in the N-terminal half of the enzyme. Tetrahydro-L-biopterin (H_4B) is an important reducing agent that is also involved with the stabilisation of the NOS enzyme[23]. These processes are shown in Figure 7.5.

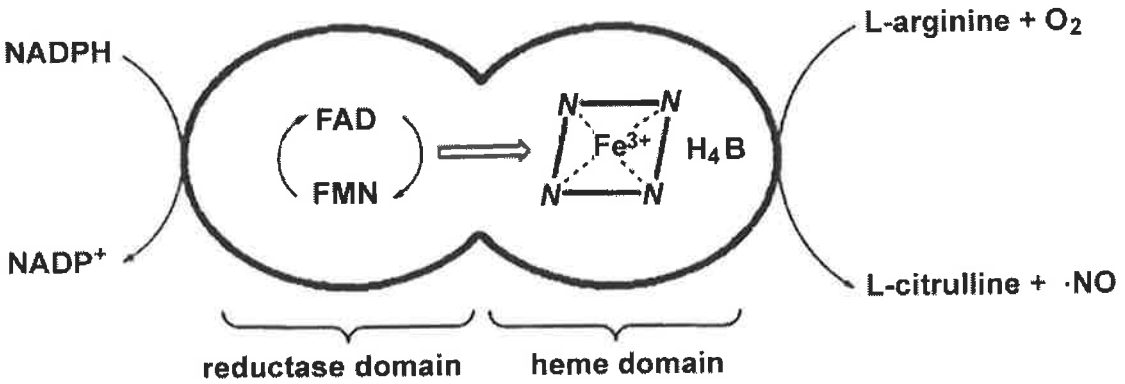


Figure 7.5. Schematic view of NOS indicating the interactions of the flavin reductase and oxidative heme domains.

7.1h. Binding domains of nNOS

Neuronal nitric oxide synthase has distinct recognition sites for FAD, FMN, NADPH and a CaM binding site[9]. These nNOS recognition sites are schematically represented in Figure 7.6. The CaM binding domain of nNOS has been discovered through recent assays and identified as the amino acid sequence Lys^{725}-Lys^{754} [12]. Various peptides have been synthesised and tested as mimics of the nNOS CaM binding domain with the intention of direct binding to CaM [12]. The consequence of this binding ultimately leads to an inhibition of nNOS activity. Therefore, the peptides are competing with the nNOS in binding to the CaM. A particular 23-residue

peptide, that mimicked the Lys^{732}-Lys^{754} sequence of nNOS, showed strong inhibition activity with an IC_{50}* of about 2 μg/ml. This peptide showed to successfully bind to the CaM, forming a complex with the CaM that then inhibited the activity of the nNOS. In contrast, two additional peptides, peptide 16 (Lys^{739}-Lys^{754}) and peptide 12 (Lys^{743}-Lys^{754}) that were shortened versions of peptide 23, showed no inhibition of the nNOS. These results indicated that Lys^{732}LysLeu in the nNOS sequence are critical residues for CaM binding. The sequence of the CaM binding domain, together with the sequences of peptides 23, 16 and 12, are indicated in Scheme 7.3.

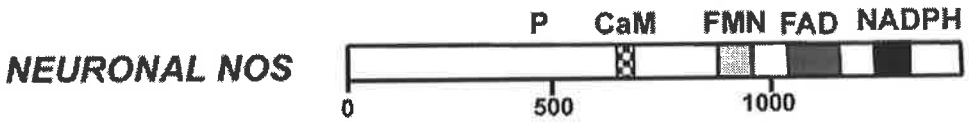


Figure 7.6. Schematic representation of nNOS recognition sites.

H-{-K R R A I G F K K L A E A V K F S A K L M G Q A M A K R V K-}-CO_2H ***nNOS***
725 754

K K L A E A V K F S A K L M G Q A M A K R V K ***P23***
732 754

K F S A K L M G Q A M A K R V K ***P16***
739 754

K L M G Q A M A K R V K ***P12***
743 754

Scheme 7.3. Sequence of the CaM binding domain of nNOS, together with the sequences of peptide 23 (P23), peptide 16 (P16) and peptide 12 (P12).

* IC_{50} is the concentration value that inhibits the activity of 50% of the test population.

It has also been shown that peptides that are of a hydrophobic/basic composition with the ability to form an amphiphilic helix are an important determinant for successful interaction with the CaM protein. These observations have also suggested that CaM binding to model peptides follow a general mechanism, driven primarily by strong hydrophobic interactions that may be relatively independent of the peptides exact primary sequence[18].

7.1i. Medical implications

As an excessive or irregular production of NO can contribute to many unwanted effects, regulation of NO biosynthesis has attracted a considerable amount of intensive study. Nitric oxide synthase inhibition is likely to be therapeutically important in certain clinical situations such as treating cerebral ischemia[23]. Cerebral ischemia* causes depolarisation of neurons due to excitotoxicity [14]. This depolarisation decreases the overall membrane potential and consequently increases the influx of calcium into the postsynaptic neuron. The increase of calcium concentration increases the calcium/CaM dependent cycle and leads to an over-activation of the nNOS. This in turn increases the levels of glutamate released from the presynaptic neuron and increases the activity of the negative feedback cycle involved with NO production [14]. Overproduction of NO is believed to cause the onset of cerebral ischemia and can eventually lead to cerebral infarction†. Therefore, a substance that can inhibit nNOS activity to a varying degree may have strong implications for people that are suffering from cerebral ischemia and may eventually prevent the onset of stroke.

The aim of the work presented in this chapter is to investigate the effect of some of the citropin peptides presented in chapter 6 on nNOS activity.

* Cerebral ischemia is a condition that involves the decrease of blood supplies to the brain and eventually can lead to cerebral infarction.
† Cerebral infarction is a medical term for stroke.

7.2. Results and Discussion

7.2a. nNOS and calcium channel activity testing

NOS and calcium channel activity tests were conducted courtesy of the Australian Institute of Marine Science (Queensland, Australia). The three peptides initially chosen as the test peptides for NOS biological activity testing consisted of citropin 1.1, citropin 1.2 and a synthetic modification of citropin 1.1, named citropin 1.1 M1. The sequences and the antibacterial activities of these peptides are listed in Table 7.2.

Peptide	Reference	Sequence	Antibacterial activity
citropin 1.1	Cit1.1	GLFDVIKKVASVIGGL (NH_2)	active
citropin 1.2	Cit1.2	GLFD I I KKVASVVGGL (NH_2)	active
citropin 1.1 M1	CitM1	GLFDVIKKVASVIKGL (NH_2)	active

	MIC (μg/ml)		
	Cit1.1	Cit1.2	CitM1
Bacillus cereus	50	25	
Escherichia coli			50
Leuconostoc lactis	6	3	3
Listeria innocua	25	100	25
Micrococcus luteus	12	12	25
Pasteurella multocida			
Staphylococcus aureus	25	25	25
Staphylococcus epidermidis	12	25	25
Streptococcus uberis	25	12	25

Table 7.2. Sequence and corresponding antibacterial activities of citropin 1.1, citropin 1.2 and citropin 1.1 M1. Where there is no figure listed the MIC value is > 100μg/ml.

The peptides were placed in solution (at a concentration of 2mg/ml in distilled water) and tested for activity using rat cerebellum. The results indicated that all three peptides were active against NOS and inhibited the conversion of [^{3}H] arginine to [^{3}H] citrulline by the nNOS assay. The IC_{50} values that were subsequently determined showed that citropin 1.1 and citropin 1.2 had similar activities, with IC_{50}s of 17.6±3.5 μg/ml and 16±2.8 μg/ml respectively. However, citropin 1.1 M1 was approximately 3 times more potent than both

citropin 1.1 and citropin 1.2, having an IC_{50} of 5.2±1.5 μg/ml. The results were reproducible and the competition curve of all three peptides is represented in Figure 7.7.

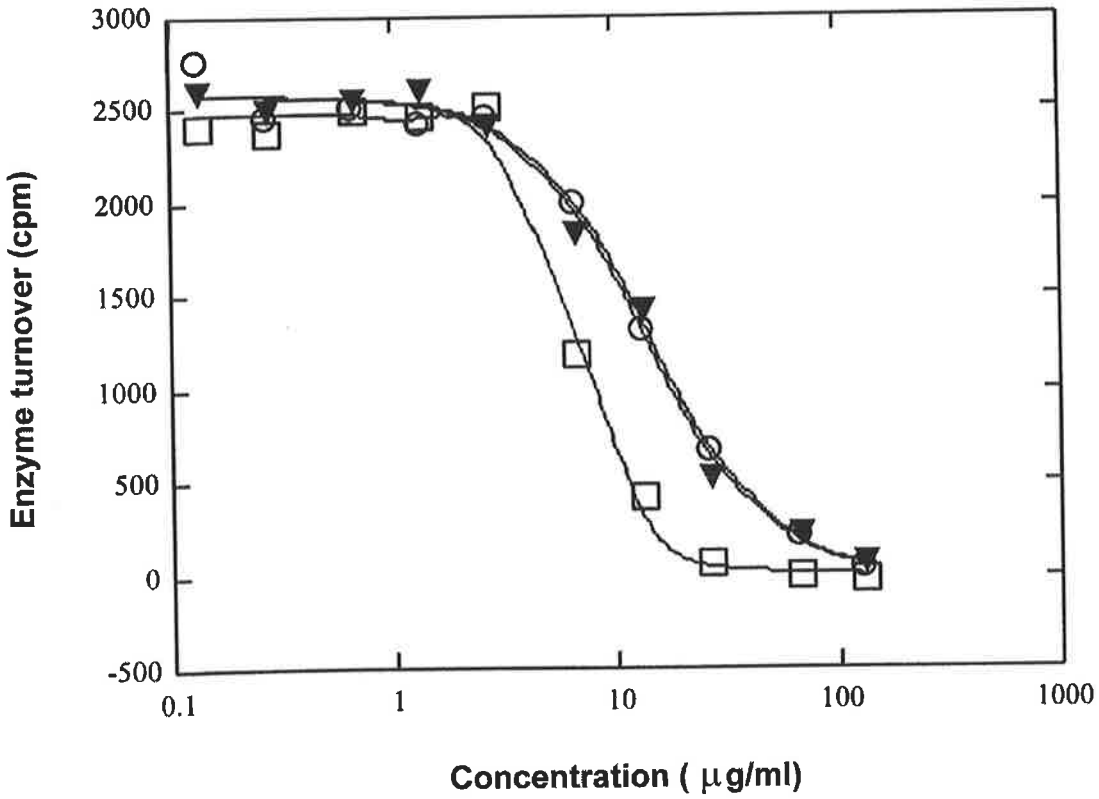


Figure 7.7. Competition curve analysis on peptides citropin 1.1 (O), citropin 1.2 (▼) and citropin 1.1 M1 (□). Data is representative of two separate experiments performed in duplicate.

All three peptides have shown inhibition activity on nNOS. As discussed previously, the Lys^{732} Lys Leu residues in the nNOS sequence are critical for CaM binding. All three citropin peptides have a Lys Lys Ile motif in their sequences, as shown in Scheme 7.4.

H-{-K R R A I G F K K L A E A V K F S A K L M G Q A M A K R V K-}-CO_2H ***nNOS***
725 754

H_2N-L G G I V S A V K K I V D F L G-H ***Cit1.1***

H_2N-L GG V V S A V K K I I D F L G-H ***Cit1.2***

H_2N-L G K I V S A V K K I V D F L G-H ***CitM1***

Scheme 7.4. Sequence of the CaM binding domain of nNOS, together with the sequences of citropin 1.1 (Cit1.1), citropin 1.2 (Cit1.2) and citropin 1.1 M1 (CitM1). The critical residues for CaM binding are underlined.

It is interesting that the synthetically modified version of citropin 1.1 (citropin 1.1 M1) is three times more potent than citropin 1.1. The only difference between the two peptides is that citropin 1.1 M1 has had the Gly^{14} residue replaced with Lys^{14}. The isoelectric point of glycine is 6, compared with the isoelectric point of lysine being 9.7. Since a greater value of the isoelectric point indicates a greater level of basicity, the lysine residue is considerably more basic than the glycine residue. Lysine is also more hydrophobic than glycine. The Edmundson wheel projection of citropin 1.1 is shown in comparison with the projection of citropin 1.1 M1 in Figure 7.8. The replacement of Gly^{14} with Lys^{14} increases the overall hydrophobicity of the amphiphilic peptide and produces a more distinct hydrophobic zone.

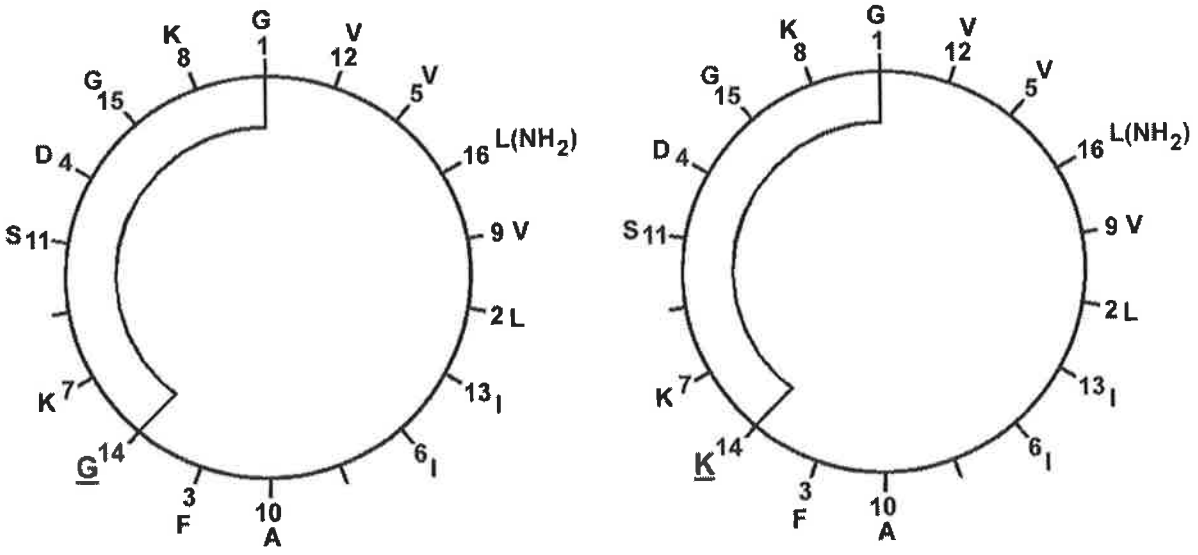


Figure 7.8. Edmundson projection of citropin 1.1 in comparison with the projection of citropin 1.1 M1. Note the well-defined hydrophobic and hydrophilic regions represented respectively by the right and left-hand sides of the figure. The residues that have been interchanged are underlined.

In addition, a further biological test for the three peptides was conducted. The experiment involved was the N-type calcium (Ca^{2+}) channel assay, which determines whether the peptides are blocking the Ca^{2+} channels. As Ca^{2+} is essential for the function of the NOS enzymes, this test was necessary to confirm that the peptides were inhibiting the production of citrulline by inhibiting the action of the nNOS enzyme activity, and not by the blocking of Ca^{2+} channels. The peptides had no significant effect on the Ca^{2+} channel assay. The results are summarised in Table 7.3.

Peptide	Ca2+ Channel Activity
Cit1.1	122.6±1.9 μg/ml
Cit1.2	111.5±3.5 μg/ml
CitM1	82.1±5.1 μg/ml

Table 7.3. Activity results from Ca^{2+} bioassay. Experiments were performed in duplicate and data presented as percent of control (distilled water) ±SE.

7.2b. Anticancer activity

Preliminary anticancer activity results of the citropin 1 peptides and some additional synthetically modified citropin peptides have been obtained from tests conducted by the National Cancer Institute (Washington DC, USA). The National Cancer Institute (NCI) has used an *in vitro* model, consisting of 3 human tumour cell lines as the primary anticancer screen, for preliminary investigation. The 3-cell line panel, of human tumour cells, consisted of MCF7 (Breast), NCI-H460 (Lung) and SF-268 (CNS). This 3-cell line, one-dose assay has been in use by the Developmental Therapeutics Program (DTP) for several years and has proven to be an effective pre-screen. The results for these peptides are listed in Table 7.4.

Peptide	Sequence	Growth Percentages (Lung) NCI-H460	(Breast) MCF7	(CNS) SF-268	Activity	Concentration of Activity (M)
Citropin 1.1	GLFDVIKKVASVIGGL(NH_2)	-90	-57	-92	Active	$1x10^{-4}$
Citropin 1.2.5	GLFDIIKKVAS(NH_2)	108	104	110	Inactive	-
Citropin 1.1 M1	GLFDVIKKVASVIKGL(NH_2)	96	97	75	Inactive	-
Citropin 1.1 M2	GLFEVIKKVASVIGGL(NH_2)	79	101	75	Inactive	-
Citropin 1.1 M3	GLFAVIKKVASVIGGL(NH_2)	-38	17	-37	Active	$1x10^{-6}$
Citropin 1.1 M4	GLFDVIAKVASVIGGL(NH_2)	57	109	79	Inactive	-
Citropin 1.1 M5	GLFDVIKKVASKIKGL(NH_2)	90	104	76	Inactive	-
Citropin 1.1 M6	GLFDVIKKVASVIKKL(NH_2)	-86	-78	-84	Active	$1x10^{-4}$

Table 7.4. Preliminary anticancer assay results. Results for each test agent are reported as the percentage of growth of the treated cells when compared to the untreated control cells. Compounds that reduce the growth of any one of the cell lines to 32% or less (negative numbers indicated cell kill) are passed on for evaluation in a full panel of 60 cell lines. (See Experimental section for further details).

7.2c. Conclusions

The three citropin 1 peptides have shown considerable activity with tests involving the inhibition of nNOS. In particular, citropin 1.1 M1 is three times more potent in inhibition activity than citropin 1.1 and citropin 1.2. Citropin 1.1 M1 is more hydrophobic and basic than the other two peptides, which may explain its greater potency.

Inhibition of nNOS by the three citropin 1 peptides may be due to these peptides mimicking the nNOS-binding site and competitively binding with CaM. This prevents CaM from effectively binding with nNOS. Consequently, NO production is decreased. The citropin peptides may be potentially useful in the medical treatment of patients susceptible to stroke, but a delivery mechanism must be found to enable the peptides to cross the blood brain barrier. A number of additional synthetic modifications of the citropin 1 peptides have been synthesised and are currently being tested for nNOS inhibition activity. These peptides include those that are listed in Table 7.4 and the all D-isomer of citropin 1.1.

Some of the peptides listed in Table 7.4 have shown activity in preliminary anticancer testing. These potential chemotherapeutic agents are currently undergoing a more detailed evaluation on a full panel of 60 tumour cell lines.

7.3. Experimental

7.3a. nNOS enzymic assay and N-type channel assay

Neuronal nitric oxide synthase enzymic assays and N-type calcium channel assay investigations were carried out by standard methods[24] by Mr. Jason R. Doyle and Dr. Lyndon E. Llewellyn (Australian Institute of Marine Science, Queensland).

7.3b. Anticancer assay

The 3-cell line panel used for this assay consisted of the human tumour cells MCF7 (Breast), NCI-H460 (Lung) and SF-268 (CNS). The National Cancer Institute (Washington DC, USA), using a standard protocol, carried out anticancer testing[25]. Results for each test agent were reported as the percentage of growth of the treated cells when compared to the untreated control cells. Compounds that reduced the growth of any one of the cell lines to 32% or less (negative numbers indicated cell kill) have been passed on for evaluation in a full panel of 60 cell lines.

7.3c. Peptide synthesis

The procedures used for the synthesis of the synthetic modifications are identical to those outlined in section 2.3h (page 76).

7.4. References

[1] Volke, V., Soosaar, A., Koks, S., Bourin, M., Mannisto, P.T. and Vasar, E., *Neuropeptides*, **30**, 323-326 (1996).

[2] Lowenstein, C. J., Glatt, C.S., Bredt, D.S. and Snyder, S.H., *Proc. Natl. Acad. Sci. USA*, **89**, 6711-6715 (1992).

[3] Garthwaite, J., Charles, S.L. and Chess-Williams, R., *Nature*, **336**, 385-387 (1988).

[4] Knowles, R.G., Palacious, M., Palmer, R. and Moncada, S., *Proc. Natl. Acad. Sci. USA*, **86**, 5159-5162 (1989).

[5] Garcia-Cardena, G., Martasek, P., Masters, B.S., Skidd, P.M., Couet, J. and Sessa, W.C., *J. Biol. Chem.*, **272**, 25437-25440 (1997).

[6] Schmidt, H.W. and Ulrich, W., *Cell*, **78**, 919-925 (1994).

[7] Jaffrey, S.R. and Snyder, S.H., *Science*, **274**, 774-777 (1996).

[8] Nathan, C. and Xie, Q.W., *Cell*, **78**, 915-918 (1994).

[9] Marletta, M.A., *Cell*, **78**, 927-930 (1994).

[10] Vorherr, T., Knopfel, L., Hofmann, F., Mollner, S., Pfeuffer, T. and Carafoli, E., *Biochemistry*, **32**, 6081-6088 (1993).

[11] Hemmens, B., Woschitz, S., Pitters, E. Klosch, B., Volker, C., Schmidt, K. and Mayer, B., *FEBS Lett.*, **430**, 397-400 (1998).

[12] Watanabe, Y., Hu, Y. and Hidaka, H., *FEBS Lett.*, **403**, 75-78 (1997).

[13] Nathan, C. and Xie, Q.W., *J. Biol. Chem.*, **269**, 13725-13728 (1994).

[14] Monacada, S. and Higgs, A., *N. Engl. J. Med.*, **329**, 2002-2012 (1993).

[15] Michel, J.B., Feron, O., Sacks, D. and Michel, T., *J. Biol. Chem.*, **272**, 15583-15586 (1997).

[16] Hibbs, J.B., Taintor, R.R. and Vavrin, Z., *Science*, **175**, 473-476 (1987).

[17] Bredt, D.S. and Snyder, S.H., *Proc. Natl. Acad. Sci. USA*, **86**, 9030-9033 (1989).

[18] Su, Z., Blazing, M.A., Fan, D. and George, S.E., *J. Biol. Chem.*, **270**, 29117-29122 (1995).

[19] Strynadka, N.C. and James, M.N., *Annu. Rev. Biochem.*, **58**, 951-998 (1989).

[20] Abu-Soud, H.M. and Stuehr, D.J., *Proc. Natl. Acad. Sci. USA*, **90**, 10769-10772 (1993).

[21] Bredt, D.S. and Snyder, S.H., *Proc. Natl. Acad. Sci. USA*, **87**, 682-685 (1990).

[22] Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D. and Wishnok, J.S., *Biochemistry*, **27**, 8706-8711 (1988).

[23] Marletta, M.A., *J. Biol. Chem.*, **268**, 12231-12234 (1993).

[24] Ichida, S., Wada, T., Sekiguchi, M., Kishino, H., Okazaki, Y. and Akimoto, T., *Neurochem. Res.*, **18**, 1137-1144 (1993).

[25] Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paul, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Cambell, H. and Mayo, J., *J. Natl. Cancer Inst.*, **83**, 757-766 (1991).

CHAPTER 8. Peptides from *Litoria electrica*

8.1. Introduction

8.1a. *Litoria electrica*

Litoria electrica (Figure 8.1) is a relatively small Australian tree frog[1], measuring about 3.5cm in length. This tree frog is also commonly known as the 'buzzing tree' frog, as the call it produces has been described as similar to that of the sound of a high voltage arc[1]. *Litoria electrica* is located in the northwestern corner of Queensland, south of the Gulf of Carpentaria. Secretions containing peptide material can be obtained from the glands distributed evenly over the whole dorsal surface.

Figure 8.1. *Litoria electrica*.

8.1b. Comparison of *Litoria electrica* with *Litoria rubella*

Litoria electrica is closely related to another Australian frog species known as *Litoria rubella* (Figure 8.2). J.E. Gray named *Litoria rubella* (commonly known as the red tree frog) in 1842 from early specimens collected at Port Essington in the Northern Territory[2]. It has since been widely reported throughout central and northern Australia, and is quite remarkable considering the extremes of climate in which it prospers[1, 2]. *Litoria rubella* is only a small amphibian, measuring about 2.5cm in length. Its skin secretion can also be obtained from the glands distributed over the whole dorsal surface.

Figure 8.2. *Litoria rubella*.

Specimens of *Litoria rubella* have been previously investigated from fifteen locations throughout Australia. Seventeen peptides have been identified from the various skin secretions [3,4,5]. *Litoria rubella* is unusual in that it does not produce antibacterial peptides of the type characteristic of the genus *Litoria*. Instead, several other peptides are produced, the most abundant of which are members of the tryptophyllin group. There are three major constituents of the tryptophyllin family (Table 8.1) observed in various populations of *Litoria rubella*. Tryptophyllin peptides show no significant activity as antibacterial agents, but exhibit minor smooth muscle activity at μM concentrations, which the Erspamer group considers as insignificant. It has been proposed that the tryptophyllins may play a role as neurotransmitters, since they are functionally similar to the human brain endomorphins*, which display a high affinity for neuronal γ receptors [6].

Name	Sequence	MH^+
Tryptophyllin L 1.2	Phe Pro Trp Leu (NH_2)	561
Tryptophyllin L 1.3	pGlu Phe Pro Trp Leu (NH_2)	672
Tryptophyllin L 3.1	Phe Pro Trp Pro (NH_2)	545

Table 8.1. The major tryptophyllins from *Litoria rubella*.

* e.g. Tyr Pro Trp Phe (NH_2) and Tyr Pro Trp Gly (NH_2)

A comparison of the skin peptide profiles of *Litoria rubella*, collected from different locations, suggested that a number of discrete populations existed throughout Australia. Three discrete populations of *Litoria rubella* surround the geographical location of *Litoria electrica*. These three populations of *Litoria rubella* are shown in Figure 8.3, which include the Adelaide River and Davenport Ranges populations together with the population along the eastern coastline of Queensland.

Figure 8.3. Location of *Litoria electrica* (1) and three neighbouring populations of *Litoria rubella*: (2) Adelaide River, (3) Davenport Ranges and (4) Queensland coastline.

The aims of the research presented in this chapter are twofold:

(i) To isolate and determine the structures of the skin peptides of *Litoria electrica*.

(ii) To compare the structures of these peptides with those already identified in the three populations of *Litoria rubella*, as shown in Figure 8.3

8.2. Results and Discussion

8.2a. General

Two specimens of *Litoria electrica* were obtained near Georgetown in Queensland. They are 3cm in length, and have been held in captivity since 1998. The skin secretion was taken from the dorsal glands using the surface electrical stimulation method [7]. On average, each milking gave about 3mg of peptide material after work-up. The peptides within the secretion were separated using HPLC. The HPLC chromatogram (Figure 8.4), derived from 0.1mg of crude peptide material, indicated two major peptide fractions and several minor fractions within the skin secretion. The component peptides have been labelled A-K in Figure 8.4. Fractions E and G yielded approximately 200 μg and 100 μg of solid material respectively with all other fractions yielding between 10 μg and 20 μg.

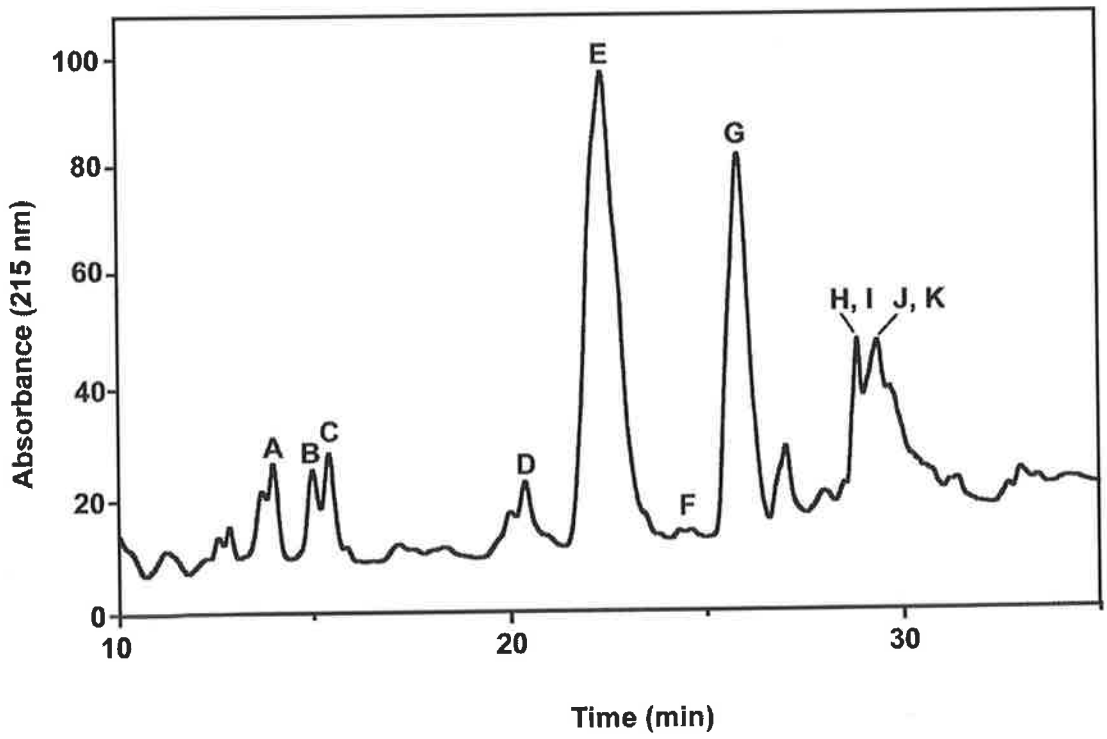


Figure 8.4. HPLC chromatogram from the crude skin secretions of *Litoria electrica*. (See Experimental section for full details)

8.2b. Structure determination

The structures of the peptides isolated were determined by a combination of ESMS, methylation and enzymic sequencing procedures. The molecular weight of each peptide was determined by ESMS. The peptides were then converted into methyl esters with acidified methanol. This procedure determined the number of CO_2H and $CONH_2$ groups in each peptide. Further information was obtained by cleaving some of the peptides with a Lys-C enzyme digest. The resulting fragments were then collisionally activated and sequenced using the MS/MS procedure.

Each peptide was sequenced using MS/MS data from either the MH^+ or $[M+2H]^{2+}$ ions. This procedure usually provided enough information to determine the full sequence. However, isobaric Gln and Lys were differentiated either by Lys-C digestion/ electrospray MS/MS or automated Edman sequencing, and isomeric Leu and Ile were differentiated by automated Edman sequencing. The amino acid sequences of the peptides isolated from the skin secretion of *Litoria electrica* are listed in Table 8.2. Details of the structural determination of each peptide are recorded in Table 8.3. An example of MS/MS data obtained from one of the major peptide fractions, electrin 1, is shown in Figure 8.5.

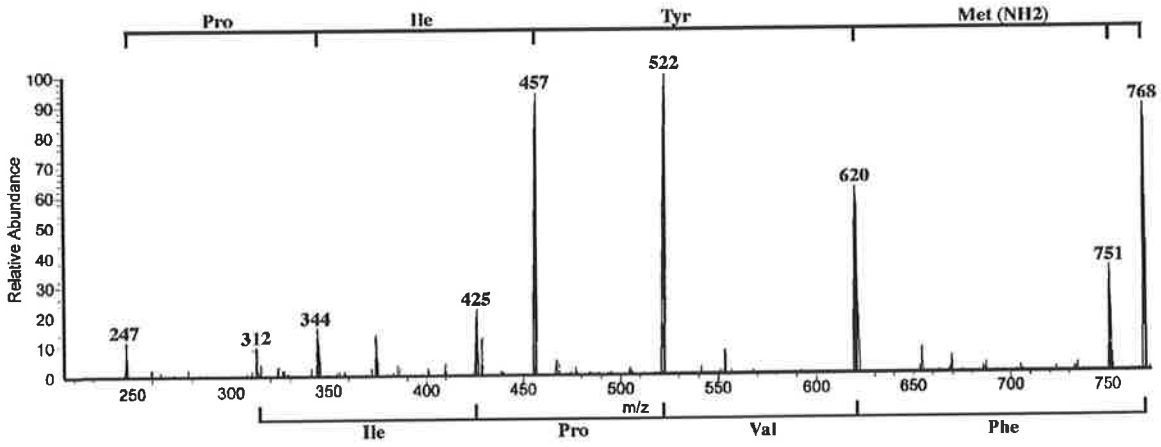


Figure 8.5. CA MS/MS of the MH^+ ion 768 (electrin 1). The sequence above the spectrum is determined by the 'B' fragmentations, while underneath is the sequence determined by the 'Y+2' fragmentations. (See Experimental section for full details).

Peptide	MW	Sequence	HPLC fraction
Tryptophyllin L 3.1	545	Phe Pro Trp Pro (NH_2)	E
Tryptophyllin L 1.2	561	Phe Pro Trp Leu (NH_2)	F
Rubellidin 3.2	642	Val Glu Phe Phe Thr (OH)	D
Rubellidin 4.2	883	Ala Gly Leu Leu Asp Ile Leu Gly Leu (NH_2)	J
Rubellidin 4.3	884	Ala Gly Leu Leu Asp Ile Leu Gly Leu (OH)	K
Electrin 1	768	Phe Val Pro Ile Tyr Met (NH_2)	G
Electrin 2.1	1744	Asn Glu Glu Glu Lys Val Lys Trp Glu Pro Asp Val Pro (NH_2)	H
Electrin 2.2	1743	Asn Glu Glu Glu Lys Val Lys Trp Gln Pro Asp Val Pro (NH_2)	I
Electrin 3	629	Phe Val His Pro Met (OH)	A
Electrin 4	615	Phe Ile Thr Val His (NH_2)	B
Electrin 5	833	Ile Tyr Glu Pro Glu Ile Ala (NH_2)	C

Table 8.2. Peptides isolated from *Litoria electrica* skin secretions.

Some of the peptides isolated and characterized were found to be unique to *Litoria electrica*, having no analogy to any other peptides isolated from amphibians to date. These new peptides were named electrins indicating their origin from *Litoria electrica*. The remaining peptides isolated are identical with, or show some similarity to peptides already isolated from *Litoria rubella*. For example, the major peptide found in the skin secretion of *Litoria electrica*, tryptophyllin L 3.1, is also a constituent of the skin secretion of *Litoria rubella*. The minor components, tryptophyllin L 1.2 and rubellidin 4.2, are also found in the skin secretion of *Litoria rubella*. Rubellidin 3.2 and rubellidin 4.3 are newly discovered peptides from *Litoria electrica*: these show structural resemblance to the rubellidin family of peptides.

Electrin 2.1 is very unusual in structure for a peptide derived from the skin secretion of an amphibian. It consists of thirteen anionic residues and eight hydrophilic residues. Some anionic peptides function as spacer peptides (see section 1.1e for a description of the biosynthesis of active peptides)[8]. Spacer peptides usually show no biological activity.

Tryptophyllin L 3.1 [MH^+ 545]

Methylation gives a methyl ester, MH^+ 560 (one $CONH_2$ group).

MH^+ 545	'B ions'	*m/z* 528, 431, 245
MS/MS		[Pro Trp Pro (NH_2)]
	'Y+2 ions'	*m/z* 545, 398, 301
		[Phe Pro]

Sequence of tryptophyllin L3.1 as determined from mass spectrometric data is:

Phe Pro Trp Pro (NH_2)

Tryptophyllin L 1.2 [MH^+ 561]

Methylation gives a methyl ester, MH^+ 576 (one $CONH_2$ group).

MH^+ 561	'B ions'	*m/z* 544, 431, 245
MS/MS		[Pro Trp Leu (NH_2)]
	'Y+2 ions'	*m/z* 561, 414, 317
		[Phe Pro]

Sequence of tryptophyllin L 1.2 as determined from mass spectrometric and automated Edman data is:

Phe Pro Trp Leu (NH_2)

Rubellidin 3.2 [MH^+ 642]

Methylation gives a methyl ester, MH^+ 670 (two CO_2H groups).

MH^+ 642	'B ions'	*m/z* 624, 523, 376, 229
MS/MS		[Phe Phe Thr (OH)]
	'Y+2 ions'	*m/z* 642, 543, 414, 267
		[Val Glu Phe]

Sequence of rubellidin 3.2 as determined from mass spectrometric data is:

Val Glu Phe Phe Thr (OH)

Rubellidin 4.2 [MH^+ 883]

Methylation gives a methyl ester, MH^+ 912 (one CO_2H and one $CONH_2$ group).

MH^+ 883	'B ions'	*m/z* 866, 753, 696, 583, 470, 355
MS/MS		[Asp Ile Leu Gly Leu (NH_2)]
	'Y+2 ions'	*m/z* 883, 812, 755, 642, 529, 414
		[Ala Gly Leu Leu Asp]

Sequence of rubellidin 4.2 as determined from mass spectrometric and automated Edman data is:

Ala Gly Leu Leu Asp Ile Leu Gly Leu (NH_2)

Table 8.3. MS data for peptides isolated from *Litoria electrica*.

Rubellidin 4.3 [MH^+ 884]

Methylation gives a methyl ester, MH^+ 912 (two CO_2H groups).

MH^+ 884	'B ions'	*m/z* 868, 753, 696, 583, 470, 355
MS/MS		[Asp Ile Leu Gly Leu (OH)]
	'Y+2 ions'	*m/z* 884, 813, 756, 643, 530, 415
		[Ala Gly Leu Leu Asp]

Sequence of rubellidin 4.3 as determined from mass spectrometric and automated Edman data is:

Ala Gly Leu Leu Asp Ile Leu Gly Leu (OH)

Electrin 1 [MH^+ 768]

Methylation gives a methyl ester, MH^+ 783 (one $CONH_2$ group).

MH^+ 768	'B ions'	*m/z* 751, 620, 457, 344, 247
MS/MS		[Pro Ile Tyr Met (NH_2)]
	'Y+2 ions'	*m/z* 768, 621, 522, 425, 312
		[Phe Val Pro Ile]

Sequence of electrin 1 as determined from mass spectrometric and automated Edman data is:

Phe Val Pro Ile Tyr Met (NH_2)

Electrin 2.1 [MH^+ 1744]

Prominent $[M+2H]^{2+}$ 873 also present in spectra.

Methylation gives a methyl ester, MH^+ 1844 (five CO_2H groups and two $CONH_2$ groups).

Lys-C digestion gives MH^+ 648, 875 and 888, confirming Lys^5 and Lys^7.

$[M+2H]^{2+}$ 873	'B ions'	*m/z* 1727, 1630, 1531, 1416, 1319,
MS/MS		1172, 1043, 857, 729, 630, 502, 373
		[Glu Lys Val Lys Trp Glu Phe Pro Asp Val Pro (NH_2)]
	'Y+2 ions'	*m/z* 1744, 1630, 1501, 1372, 1243, 1115, 1016, 888, 702, 573, 426, 329
		[Asn Glu Glu Glu Lys Val Lys Trp Glu Phe Pro]

Sequence of electrin 2.1 as determined from mass spectrometric data is:

Asn Glu Glu Glu Lys Val Lys Trp Glu Phe Pro Asp Val Pro (NH_2)

Table 8.3. *(Continued)*

Electrin 2.2 [MH^+ 1743]

Prominent $[M+2H]^{2+}$ 872 also present in spectra.
Methylation gives a methyl ester, MH^+ 1844 (four CO_2H groups and three $CONH_2$ groups).
Lys-C digestion gives MH^+ 648, 875 and 887 confirming Lys^5 and Lys^7.

$[M+2H]^{2+}$ 872 MS/MS	'B ions'	*m/z* 1726, 1629, 1530, 1415, 1318, 1171, 1043, 857, 729, 630, 502, 373 [Glu Lys Val Lys Trp Gln Phe Pro Asp Val Pro (NH_2)]
	'Y+2 ions'	*m/z* 1743, 1629, 1500, 1371, 1242, 1114, 1015, 887, 701, 573, 426, 329 [Asn Glu Glu Glu Lys Val Lys Trp Gln Phe Pro]

Sequence of electrin 2.2 as determined from mass spectrometric data is:
Asn Glu Glu Glu Lys Val Lys Trp Gln Phe Pro Asp Val Pro (NH_2)

Electrin 3 [MH^+ 629]

Methylation gives a methyl ester, MH^+ 643 (one CO_2H group).

MH^+ 629 MS/MS	'B ions'	*m/z* 612, 481, 384, 247 [His Pro Met (OH)]
	'Y+2 ions'	*m/z* 629, 483, 384, 247 [Phe Val His]

Sequence of electrin 3 as determined from mass spectrometric data is:
Phe Val His Pro Met (OH)

Electrin 4 [MH^+ 615]

Methylation gives a methyl ester, MH^+ 630 (one $CONH_2$ group).

MH^+ 615 MS/MS	'B ions'	*m/z* 598, 461, 362, 261 [Thr Val His (NH_2)]
	'Y+2 ions'	*m/z* 615, 468, 355, 254 [Phe Ile Thr]

Sequence of electrin 4 as determined from mass spectrometric and automated Edman data is:
Phe Ile Thr Val His (NH_2)

Electrin 5 [MH^+ 833]

Methylation gives a methyl ester, MH^+ 876 (two CO_2H group and one $CONH_2$ group).

MH^+ 833 MS/MS	'B ions'	*m/z* 816, 745, 632, 503, 406, 277 [Glu Pro Glu Ile Ala (NH_2)]
	'Y+2 ions'	*m/z* 833, 720, 557, 428, 331 [Ile Tyr Glu Pro]

Sequence of electrin 5 as determined from mass spectrometric and automated Edman data is:
Ile Tyr Glu Pro Glu Ile Ala (NH_2)

Table 8.3. *(Continued)*

8.2c. Biological activity determination

Tryptophyllin L 3.1, electrin 1 and electrin 2.1 were synthesised to enable testing for biological activity. The native and synthetic peptides were identical, as confirmed by HPLC and mass spectrometric analysis. The synthetic peptides showed no antibacterial activity at MIC values below 100 μg/ml over the range of pathogens tested. The synthetic peptides also showed no activity in the standard assays carried out by the Australian Institute of Marine Science (AIMS). These assays included activity determination specifically related to N-type Ca^{2+} channels, L-type Ca^{2+} channels, neuronal nitric oxide synthase, nitric oxide scavenging, serotonin receptor activity, DNA interaction and protein tyrosine phosphatase 1C activity.

Most Australian tree frogs contain biologically active peptides within their skin secretions. For example, the majority of species of the genus *Litoria* produce at least one neuropeptide* together with a wide spectrum antibiotic peptide†. However, neither *Litoria electrica* nor *Litoria rubella* contain caerulein type neuropeptides or antibacterial peptides in their skin secretions. The biological roles of the peptides from *Litoria rubella* and *Litoria electrica* remain a mystery.

8.2d. A comparison of the peptide profiles of *Litoria electrica* and *Litoria rubella*

The peptide components from *Litoria electrica*, along with those of the following three populations of *Litoria rubella*: (i) Darwin/Adelaide River, (ii) Davenport Ranges and (iii) Mount Carbine (from the Queensland coastline population) are listed in Table 8.4. The peptide profile of *Litoria electrica* showed some similarity to that of the *Litoria rubella* populations found to the south and to the east of the *Litoria electrica* location. There is no correlation between the skin peptides of *Litoria electrica* and those of the population of *Litoria rubella* centred on Darwin and the Adelaide River.

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* e.g. caerulein: pGluGlnTyr(SO_3H)TheGlyTrpMetAspPhe(NH_2)
† e.g. caerin1.1: GlyLeuLeuSerValLeuGlySerValAlaLysHisValLeuProHisValValProValIleAlaAsp HisLeu(NH_2)

Peptides *L.rubella* & *L.electrica* (from all locations)		***L. rubella*** (Darwin/Adelaide River)	***L. rubella*** (Davenport Ranges)	***L. rubella*** (Mt. Carbine)	***L. electrica***
Tryptophyllins					
1.1	PWL(NH_2)				
1.2	FPWL(NH_2)		✔	✔	✔
1.3	pEFPWL(NH_2)	✔			
1.4	FPFPWL(NH_2)			✔	
2.1	IPWL(NH_2)				
3.1	FPWP(NH_2)			✔	✔
3.2	FPWP(OH)		✔	✔	
3.3	pEFPWP(NH_2)				
4.1	LPWY(NH_2)				
4.2	FLPWY(NH_2)				
5.1	pEIPWYHR(NH_2)	✔			
Rubellidins					
1.1	VDFFA(OH)				
2.1	IEFFA(OH)	✔			
3.1	IEFFT(NH_2)				
3.2	VEFFT(OH)				✔
4.1	GLGDILGLLGL(NH_2)		✔		
4.2	AGGLLDILGL(NH_2)		✔		✔
4.3	AGGLLDILGL(OH)				✔
Electrins					
1	FVPIYM(NH_2)				✔
2.1	NEEEKVKWEPDVP(NH_2)				✔
2.2	NEEEKVKWQPDVP(NH_2)				✔
3	FVHPM(OH)				✔
4	FITVH(NH_2)				✔
5	IYEPEIA(NH_2)				✔

Table 8.4. Comparison of *Litoria rubella* peptide profiles [5] with that of *Litoria electrica*.

8.2e. Conclusions

A new class of peptide from the skin secretion of *Litoria electrica* has been named as the electrins. The electrins are only found in *Litoria electrica*, but other peptides isolated from this amphibian are identical or similar to skin peptides found in some populations of *Litoria rubella*. These observations support the concept that *Litoria electrica* and *Litoria rubella* are distinct species, which evolved from a common ancestor. This is in agreement with the morphological relationship between the two species, as derived from anatomical and other biological data [9, 10].

8.3 Experimental

8.3a. Collection and preparation of *Litoria electrica* secretions

The skin secretions from *Litoria electrica* were provided courtesy of Associate Professor Mike Tyler, Department of Environmental Biology, University of Adelaide. The secretions were obtained by the SES method, as described in section 2.3a. The resulting secretion was washed off the skin with water (distilled 25 ml). Methanol (25 ml) was immediately added to the aqueous extract, the mixture centrifuged, and concentrated to approximately 1 ml.

8.3b. HPLC separation

HPLC separation was achieved for each sample using a VYDAC C_{18} Protein and Peptide (218TP54) reverse phase column equilibrated with 10% acetonitrile/aqueous 0.1% TFA. Additional purification was achieved using an elution profile of 40-65% acetonitrile over a period of 60 minutes. Each sample was filtered through a Millipore 0.45 μm filter and injected onto the column. The conditions for the HPLC chromatogram represented in Figure 8.4 are as follows. The elution profile increased from 10-75% acetonitrile over a period of 40 minutes using a flow rate of 1 ml/min.

8.3c. Mass spectrometry analysis

Electrospray mass spectra were determined using a Finnigan LCQ ion trap mass spectrometer. The samples were dissolved in methanol/water (1:1) and infused into the electrospray source at a flow rate of 8 μl/min. Collision activation mass spectral data were obtained using collision energy of 35-50%. Electrospray conditions were as follows: source voltage 4.2 kV, source current 17 μA, capillary temperature 200°C, capillary voltage 3V and sheath gas flow 30 psi. Mass spectra were acquired with the automatic gain control on, a maximum ion time of 200-400 milliseconds, and using 3 microscans per scan, averaging over approximately 20 scans. Molecular weights of peptides were determined from the MH^+ or $[M+2H]^{2+}$ ions.

8.3d. Additional information

The remaining procedures are identical to those described elsewhere, as detailed in the table below.

Procedure	Section in which it is described
Methylation of peptides	Section 2.3d (page 74)
Enzyme digestion using Lys-C	Section 2.3e (page 75)
Antibacterial testing	Section 2.3g (page 76)
Preparation of synthetic peptides	Section 2.3h (page 76)
Automated Edman sequencing	Section 2.3i (page 76)

8.4. References

[1] Barker, J., Grigg, G. and Tyler, M.J., in *A field guide to Australian frogs* (Surrey Beatty, Norton, NSW, 1995).

[2] Tyler, M.J., in *Encyclopaedia of Australian animals* (ed. Strahan, R.) 3-42 (Harper Collins, London, UK, 1992).

[3] Steinborner, S.T., Gao, C., Raftery, M.J., Bowie, J.H., Wallace, J.C. and Tyler, M.J., *Aust. J. Chem.*, **47**, 2099-2102 (1994).

[4] Steinborner, S.T., Wabnitz, P.A., Bowie, J.H. and Tyler, M.J., *Rapid Commun. Mass Spectrom.*, **10**, 92-94 (1996).

[5] Steinborner, S.T., Wabnitz, P.A., Waugh, R.J., Bowie, J.H., Gao, C., Tyler, M.J. and Wallace, J.C., *Aust. J. Chem.*, **49**, 955-958 (1996).

[6] Zadina, J.E., Hackler, L., Ge, L.J. and Kastin, A.J., *Nature*, **386**, 499-503 (1997).

[7] Tyler, M. J., Stone, D. J. and Bowie, J. H., *J. Pharm. Toxicol. Methods,* **28**, 199-200 (1992).

[8] Jacob, L. and Zasloff, M., in *Antimicrobial Peptides: Ciba Foundation Symposium 186*, 197-208 (Wiley and Sons, New York, NY, 1994).

[9] Ingram, G., and Corben, C., *Mem. Qld. Mus.*, **28**, 475-477 (1990).

[10] Tyler, M.J. and Davies, M., *Aust. J. Zool.*, **63**, 3-7 (1978).

CHAPTER 9. Summary and Conclusions

Peptides were discovered and isolated early in the development of tadpoles of the tree frog *Litoria splendida*, including antibacterial peptides and a neuropeptide/hormone. This is interesting, as tadpoles are resistant to pathogenic microorganisms and fungal growth at all stages of development and it was not exactly certain how this phenomenon was occurring. Past studies have indicated that antibacterial peptides were present during and after metamorphosis, but there was no evidence of their presence prior to metamorphosis. This research has reported the finding of peptides in tadpoles of *Litoria splendida* well before the onset of metamorphosis, which ultimately may be involved in protecting this amphibian against bacteria. The neuropeptide/hormone caerulein was detected 10 days after egg deposition, and the antibacterial peptides caerin 1.1, caerin 1.6 and caerin 3.1 first appeared at 14 days. The concentration of peptides increased with the onset of metamorphosis at 84 days, when the peptide profile is the same as that of the adult.

Examination of the skin secretions of adults of *Litoria splendida* over a period of three years, revealed interesting biological patterns. The seasonal fluctuations of a new peptide, Phe^8-caerulein, were noted. Phe^8-caerulein (which showed analogy with caerulein and is thought to be a neuropeptide) appeared only during the winter months over the entirety of the three-year study period. This suggested that Phe^8-caerulein might possibly be involved in the thermogenesis of this anuran during its hibernation period.

Comparisons between the skin secretions of adult males and females of *Litoria splendida* over the three-year study period found two minor component peptides. The two peptides, caerin 2.3 and caerin 1.10, were found to be present only within the male secretion. Caerin 1.10 showed antibacterial activity. Caerin 2.3 cycled in abundance throughout the three-year period, with the concentration peaking during the breeding season of *Litoria splendida* (January to March). The concentration of caerin 2.3 then markedly decreased during the period of June to November.

Behavioural studies of caerin 2.3 on adult females of *Litoria splendida* indicated pheromone activity. This aquatic sex aggregation pheromone of the adult male *Litoria splendida* was renamed as splendipherin. Splendipherin, which showed to be species specific, is the first anuran pheromone to be isolated and characterised. Splendipherin is also the second peptide pheromone to be identified in a vertebrate. Given the fact that *Litoria splendida* reproduces in an aquatic environment, a non-volatile but water-soluble peptide was a reasonable form to expect as a pheromone in this amphibian.

Investigation of the skin secretions of *Litoria citropa* initially led to the discovery of three distinct groups of citropin peptides, namely, the citropins 1, 2, and 3. These peptides showed to have no similarity with any other reported peptide from the *Litoria* genus. Nineteen citropin peptides were present in the dorsal secretion, while only fifteen of these peptides were also present in the secretion from the submental gland. The two major peptides, citropin 1.1, citropin 1.2 and a minor peptide, citropin 1.3 are wide-spectrum antibacterial peptides. The amphibian also showed to have an endopeptidase that deactivated the antibacterial peptides by removal of the N-terminal residues. The most abundant degradation products resulted from a loss of three residues from the N-terminal end. The additional four peptides produced by the dorsal glands were structurally related to the antibacterial citropin 1 peptides, except they contained three extra residues at their C-terminus. These additional peptides showed minimal antibacterial activity.

Both secretions, from the dorsal and submental glands of *Litoria citropa*, also contained a series of previously unreported peptides that were structurally related to the neuropeptide caerulein. In all, sixteen caerulein-like peptides were isolated and characterised. These peptides included eight peptides that contained a post-translated tyrosine residue, and eight peptides that contained a tyrosine residue minus the sulfate group. The sulfated caerulein-like peptides isolated from *Litoria citropa* are thought to be neuropeptides with similar biological actions as that of gastrin, CCK and caerulein.

Citropin 1.1, citropin 1.2 and a synthetic modification of citropin 1.1 all showed potent activity as inhibitors of nNOS, with citropin 1.M1 having a potency three times greater than that of citropins 1.1 and 1.2. Inhibition was thought to be due to the mimicking of the nNOS-binding site by these peptides, consequently producing competitive binding of the peptides with CaM and resulting in a decrease of NO production. Citropins 1.1, 1.1 M3 and 1.1 M6 showed anticancer activity on tumour cells responsible for lung, CNS and breast cancer. These peptides are now currently undergoing further evaluation on a full panel of 60 tumour cell lines by the National Cancer Institute (Washington DC, USA). The medical implications of these active citropin peptides may eventually be useful in the treatment of patients susceptible to stroke or those suffering with cancer.

Skin secretions of *Litoria electrica* have revealed eleven peptides, with the two most abundant being tryptophyllin L 3.1 and electrin 1. These two peptides showed neither significant antibacterial activity nor smooth muscle activity. Tryptophyllin L 3.1 is thought to be a neurotransmitter, and is similar in structure to the human endomorphins that act on the γ-receptor. Both *Litoria electrica* and *Litoria rubella* produce the tryptophyllin peptides, and are considered to have come from the same ancestor.

The results of this thesis have led to the isolation and characterisation of a number of new peptides from the skin secretions of three species of Australian amphibians. A considerable number of these novel peptides have shown significant biological activity. The biological implications of peptides such as these may eventually compel them into potentially useful therapeutic agents in the treatment of various medical conditions.

Publications

Journals Related to Thesis

Wabnitz, P.A., Bowie, J.H., Tyler, M.J. Wallace, J.C. and Smith, B.P., Splendipherin: The female-attracting sex pheromone of the male tree frog *Litoria splendida*., *Nature*, (1999), in press.

Wabnitz, P.A., Walters, H., Tyler, M. J., Wallace, J.C. and Bowie, J.H., First record of host defence peptides in tadpoles. The magnificent tree frog *Litoria splendida*., *J. Peptide Res*., **52**, 477-481 (1998).

Wabnitz, P.A., Bowie, J.H, Wallace, J.C. and Tyler, M.J., Peptides from the skin glands of the Australian buzzing tree frog *Litoria electrica*. Comparison with the skin peptides of the red tree frog *Litoria rubella*., *Aust. J. Chem*., (1999), in press.

Wabnitz, P.A., Bowie, J.H, Wallace, J.C. and Tyler, M.J., The citropin peptides from the skin glands of the Australian Blue Mountains tree frog *Litoria citropa*. Sequence determination using electrospray mass spectrometry., *Rapid Commun. Mass Spectrom*., (1999), in press.

Wegener, K.L., Wabnitz, P.A., Carver, J.A., Bowie, J.H., Wallace, J.C. and Tyler, M.J., Host defence peptides from the skin glands of the Australian Blue Mountains tree frog *Litoria citropa*. The antibacterial citropin 1 peptides. The solution structure of citropin 1.1., *Eur. J. Biochem*., (1999), in press.

Bowie, J.H., Wegener, K.L., Chia, B.C.S., Wabnitz, P.A., Carver, J.A., Tyler, M.J., and Wallace, J.C., Host defence antibacterial peptides from skin secretions of Australian amphibians. The relationship between structure and activity., *Protein and Peptide Letters*, (1999), in press.

Additional Journals

Wabnitz, P.A., Waugh, R.J., Eckersley, M.A., Dua, S., Blumenthal, T. and Bowie, J.H., The negative ion mass spectra of deprotonated 2,5-diketopiperazines., *Int. J. Mass Spectrom. Ion Proc*., **154**, 193-201 (1996).

Wabnitz, P.A., Steinborner, S.T., Currie, G.J., Bowie, J.H. and Tyler, M.J., New caerin 1 antibiotic peptides from the skin of the Australian tree frog *Litoria chloris*. Part 2. Sequence determination using electrospray mass spectrometry., *Rapid Commun. Mass Spectrom*., **12**, 53-56 (1998).

Steinborner, S.T., Wabnitz, P.A., Bowie, J.B. and Tyler, M.J., The application of mass spectrometry in the study of evolutionary trends in amphibians., *Rapid Commun. Mass Spectrom*., **10**, 92-95 (1996).

Steinborner, S.T., Wabnitz, P.A., Waugh, R.J., Bowie, J.B., Gao, C., Tyler, M.J. and Wallace, J.C., The structures of new peptides from the Australian red tree frog *Litoria rubella*. The skin peptide profile as a probe for the study of evolutionary trends of amphibians., *Aust. J. Chem*., **45**, 2099-2107 (1996).

Wabnitz, P.A., Walters, H., Tyler, M.J., Wallace, J.C., and Bowie, J.H., (1998) First record of host defence peptides in tadpoles. The magnificent tree frog *Litoria splendida.*
Journal of Peptide Research, v. 52 (6), pp. 477-481.

Wabnitz, P.A., Waugh, R.J., Eckersley, M.A., Dua, S., Blumenthal, T., and Bowie, J.H., (1996) The negative ion mass spectra of deprotonated 2,5-diketopiperazines. *International Journal of Mass Spectrometry and Ion Processes, v. 154 (3), pp. 193-201, July 1996*

Wabnitz, P.A., Steinborner, S.T., Currie, G.J., Bowie, J.H., and Tyler, M.J., (1998) New caerin 1 antibiotic peptides from the skin secretion of the Australian tree frog *Litoria chloris*. Part 2. Sequence determination using electrospray mass spectrometry. *Rapid Communications in Mass Spectrometry, v. 12 (2), pp. 53-56, January 1998*

HOST DEFENCE ANTIBACTERIAL PEPTIDES FROM SKIN SECRETIONS OF AUSTRALIAN AMPHIBIANS. THE RELATIONSHIP BETWEEN STRUCTURE AND ACTIVITY.

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Peptides have been isolated and characterised from the secretions of skin glands of twenty five species of Australian amphibian. Many peptides are host defence agents, showing, for example, neuropeptide and/or antibacterial activity. This review describes the relationship between activity and structure of the antibacterial peptides, particularly the caerin and uperin groups of peptide from the genera *Litoria* and *Uperoleia* .

1. Introduction

Amphibians have rich chemical arsenals in their skin glands. These form an integral part of their defence system, and also assist with the regulation of dermal physiological action [1,2]. In response to a variety of stimuli, host defence compounds are secreted, within a gel, from these specialised glands onto the dorsal surface and into the gut of the amphibian. Many of these compounds are bio-active peptides, including, e.g. the bombinin, brevinin and magainin families of antimicrobial peptides [2-4]. The magainin peptides also show some anticancer activity [5]. Some of the antibacterial peptides have attracted pharmaceutical interest [6].

During the last decade we have isolated and identified some 150 peptides from the secretions of skin glands of 25 Australian amphibians of the genera *Litoria*, *Uperoleia* and *Limnodynastes*. The peptides are separated by HPLC and sequenced using classical techniques, viz mass spectrometry and automated Edman sequencing [see e.g. 7,8]. Most amphibian skin secretions contain less than 10 major peptides. The record for largest number of peptides produced is, to date, held jointly by the Green Tree Frog *Litoria caerulea* [7,8] (Figure 1) and the Floodplain Toadlet *Uperoleia inundata* [9], each of which contain more than 40 peptides in their granular gland secretions. The Magnificent Tree Frog, *Litoria splendida* [8,10] (Figure 2), exudes up to 200 mg of active peptides from parotoid and rostral glands, while most other amphibians excrete less than 10 mg of active peptides onto their skins. We obtain glandular secretions by

electrical stimulation of the skin : animals are not sacrificed using this technique, and the process may be repeated at monthly intervals [11].

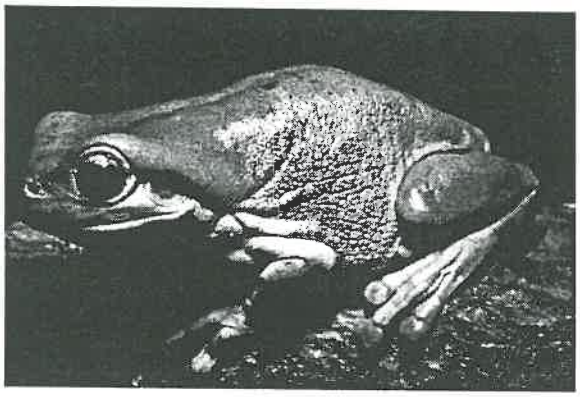

Figure 1. *L.caerulea*

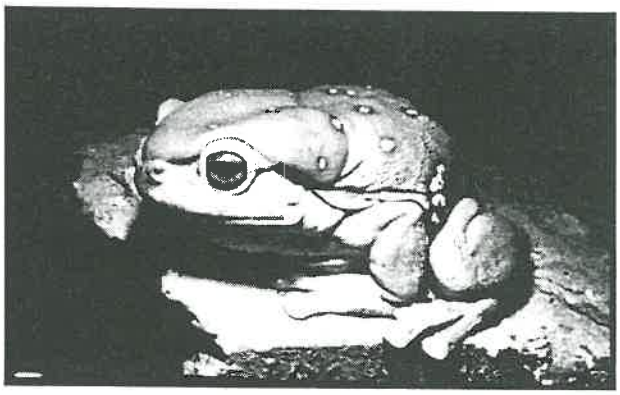

Figure 2. *L.splendida*.. Note the parotoid gland on the head extending rostrally.

The majority of the tree frogs of the genus *Litoria* and toadlets of the *Uperoleia* genus contain at least one neuropeptide of the caerulein [pEQDY(SO_3)TGWMDF-NH_2] and uperolein [pEPDPNAFYGLM-NH_2] groups respectively. These neuropeptides often constitute the major component(s) of the glandular secretion. Caerulein exhibits a spectrum of activity similar to that of the mammalian intestinal peptide gastrin, i.e. it modifies satiety, sedation and thermoregulation, is an analgaesic several thousand times more potent than morphine, and has been used clinically [1,2]. The uperolein peptides are members of the tachykinin group of neuropeptides, exhibiting potent vasodilator action, together with enhancement of capillary permeability [1,2]. Most species of the genera *Litoria* and *Uperoleia* also contain a number of wide-spectrum and narrow-spectrum antibacterial peptides in their glandular secretions. The structures of these peptides together with their activities constitute the subject matter of this review. We have shown that other peptide components of amphibian glandular secretions exhibit other bioactivities, including, for example, antiviral, anticancer, neuromodulator, and sex pheromone activity.

2 *Antibacterial Peptides*

Antibacterial peptides are encoded and synthesised by the amphibian as part of larger peptides generally containing three components, viz. signal peptide-spacer peptide-active peptide (pre-pro-peptide). Following synthesis of this large peptide in the cell, an endoprotease removes the signal peptide, and the residual pro-peptide, which is generally not bacterially active, is transported to and stored in the gland. Following either attack by a predator, or some other stimulus, a second endoprotease removes the spacer peptide and the active peptide is delivered from the appropriate gland onto the skin or into the gut [12]. In the case of wide-spectrum antibacterial peptides, a third endoprotease degrades and deactivates the peptide after it has been on the skin for some period (usually 5 - 30 minutes depending on the species) [13,14].

Tadpoles are largely immune to infection even though they live in aquatic media which abound with microbial pathogens [15]. Previously, it has been accepted that tadpoles do not produce host defence peptides, but that such peptides are first formed when the skin glands develop at metamorphic climax , i.e. when the tail has regressed and the limbs have developed. This has been confirmed experimentally for both

Xenopus laevis [16] and *Rana catesbeiana* [17]. This raises the question as to the nature of the immune systems of the tadpoles. In marked contrast, tadpoles of *Litoria splendida* produce the whole range of host defence peptides within 14 days of egg deposition. The concentration of peptides is small until the onset of metamorphosis, when the host defence peptide profile and concentration is essentially the same as that of the adult [18].

Antibacterial amphibian peptides are membrane active species, often having no close mammalian analogues. They are generally amphipathic α helices, containing 15 - 30 amino acid residues, that interact with the lipid bilayer, breaching the bacterial membrane by forming ion channels, ultimately leading to cell death. A number of proposals have been advanced to rationalise such penetration of the membrane by the peptide. The simplest of these involve initial aggregation of the peptides on the surface of the membrane followed by either (i), penetration through the lipid bilayer (the 'barrel stave' mechanism) (the peptide must have a minimum of 20 residues to effect full penetration of the membrane), or (ii) formation of pores on the membrane surface which ultimately breach the lipid bilayer (the 'carpet' mechanism) [e.g. 19-25].

TABLE 1
The Caerin and Related Peptides from *Litoria* Species

Name		Sequence	Species
Caerin	1.1***	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	a,b,c,d
	1.2***	GLLGVLGSVAKHVLPHVVPVIAEHL-NH_2	a
	1.3***	GLLSVLGSVAQHVLPHVVPVIAEHL-NH_2	a
	1.4***	GLLSSLSSVAKHVLPHVVPVIAEHL-NH_2	a
	1.5***	GLLSVLGSVVKHVIPHVVPVIAEHL-NH_2	a
	1.6***	GLFSVLGAVAKHVLPHVVPVIAEKL-NH_2	e,f
	1.7***	GLFKVLGSVAKHLLPHVAPVIAEKL-NH_2	e,f
	1.8***	GLFKVLGSVAKHLLPHVVPVIAEKL-NH_2	f
	1.9***	GLFGVLGSI AKHVLPHVVPVIAEKL-NH_2	f
	1.10***	GLLSVLGDVAKHVLPHVVPVIAEKL-NH_2	b
Maculatin	1.1***	GLFGVLAKVAAHVVPAIAEHF-NH_2	g
Caerin	2.1*	GLVSSIG**R**ALGGLLA**D**VV**K**S**K**GQPA-OH	b
	2.2*	GLVSSIG**R**ALGGLLA**D**VV**K**S**K**EQPA-OH	a
	2.3	GLVSSIG**K**ALGGLLA**D**VV**K**S**K**GQPA-OH	a,b
	2.4	GLVSSIG**K**ALGGLLA**D**VV**K**T**K**EQPA-OH	a
	2.5**	GLVASIG**R**ALGGLLA**D**VV**K**S**K**EQPA-OH	c
Citropin	2.1*	GLIGS IG**K**ALGGLLV**D**VL**K**P**K**L-OH	h
Frenatin	3*	GLMSVLG**H**AVGNVLGGLF**K**P**K**S-OH	i
Caerin	3.1**	GLWQKIKDKASELVSGIVEGVK-NH_2	b,c
	3.2*	GLWEKIKEKASELVSGIVEGVK-NH_2	a
	3.3*	GLWEKIKEKANELVSGIVEGVK-NH_2	a
	3.4*	GLWEKIREKANELVSGIVEGVK-NH_2	a
Caerin	4.1**	GLWQKIKSAAGDLASGIVEGIKS-NH_2	a
	4.2**	GLWQKIKSAAGDLASGIVEAIKS-NH_2	a
	4.3**	GLWQKIKQAAGDLASGIVEGIKS-NH_2	a

*** Wide spectrum antibacterial activity
** Major antibacterial activity against a particular organism
* Minor antibacterial activity.
a = *Litoria caerulea* [7,8]; b = *Litoria splendida* [8,10]; c = *Litoria gilleni* [26]; d = *Litoria ewingi* [27];
e = *Litoria xanthomera* [14]; f = *Litoria chloris* [28]; g = *Litoria genimaculata* [29]; h = *Litoria citropa* [30]; i = *Litoria infrafrenata* [31].

2.1 *The Caerin 1 Wide-Spectrum Antibacterial Peptides and Maculatin 1.1.*

Most of the tree frogs of the genus *Litoria* have a cocktail of antibacterial peptides including wide - and narrow spectrum components. Most common are the caerin 1 wide-spectrum antibacterial peptides (Table 1) which have already been reported from seven species (Table 1) and are present in several other tree frogs that we are currently examining. All the caerin 1 peptides have very similar structures, and are active mainly against gram positive organisms. Caerin 1.1 and caerin 1.4 are typical and their activities against a number of pathogens are shown in Table 2. Most of our detailed work has been done with caerin 1.1 [32]. A nuclear magnetic resonance (NMR) study has shown the solution structure of caerin 1.1 to be that shown in Figure 3; i.e. two helical regions separated by a central more flexible hinge region (initiated by Pro^{15}). Figure 3 indicates the amphipathic nature of caerin 1.1: the hydrophilic and hydrophobic zones are clearly defined. Like most other amphibian antibacterial peptides [cf. 1,2], caerin 1.1 is a membrane active peptide. This is confirmed by the activity of the natural L-caerin 1.1 being the same as that of synthetic D-caerin 1.1 [32]. The hinge of caerin 1.1 assists this basic peptide to interact efficiently with different types of membrane surfaces, particularly those containing an excess of anionic residues. We have carried out a number of synthetic modifications of caerin 1.1 in order to examine key features which affect the activity. The most significant observations are :- (i) replacement of either or both of the Pro residues with Ala or Gly increases the helicity but significantly reduces the activity, (ii) removal of residues from either the C- or N-terminal ends of the molecule destroys the activity [the amphibian itself deactivates a caerin 1 antibacterial peptide (after it has achieved its primary function) by releasing an endoprotease which removes the first two residues from the N-terminal end of the peptide], and (iii) replacing Lys^{10} with Ala^{10} reduces the activity.

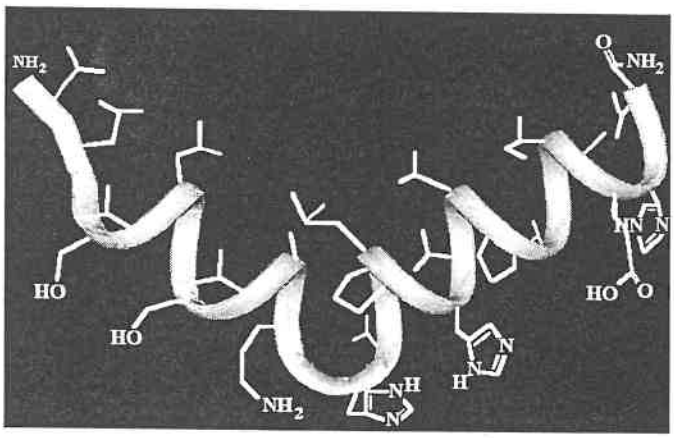

Figure 3. Solution structure of caerin 1.1.

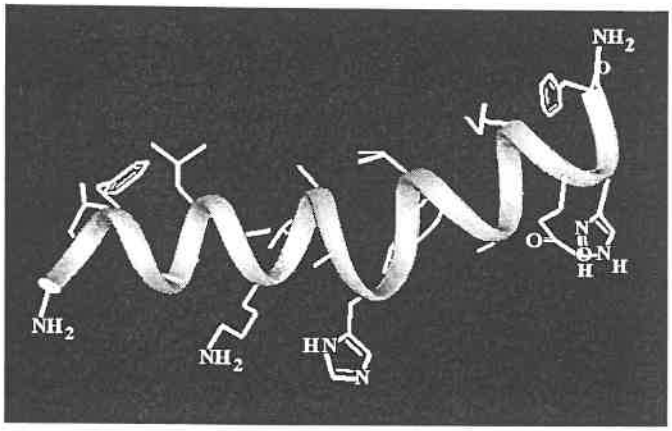

Figure 4. Solution structure of maculatin 1.1.

Our work with the caerin 1 antibacterial peptides had convinced us that it is necessary for such a peptide to have all 25 residues to achieve the appropriate activity and that any tampering with the central hinge should reduce the bioactivity. We were therefore surprised to find that the major antibacterial peptide from the tree frog *Litoria genimaculata* showed activity similar to that of a caerin 1 molecule, but has a structure in which four residues (including the first Pro) of a caerin 1 are missing. We called this molecule maculatin 1.1, and the sequence is shown in Table 1. The 3D structure of maculatin 1.1 (Figure 4) has been determined by NMR studies, both in solution and in the presence of an artificial phospholipid [33]. Maculatin 1.1 is essentially an α helix distorted at Pro^{15}. Replacement of Pro^{15} with Ala^{15} achieves an

almost perfect α helix, but the antibacterial activity of this synthetic modification is reduced in comparison with that of maculatin 1.1. Like caerin 1.1, maculatin 1.1 is a membrane active peptide, since the antibacterial activities of the natural L-maculatin 1.1 and synthetic all D-maculatin 1.1 are the same within experimental error [33]. The activities of caerin 1.1 and maculatin 1.1 are shown in Table 2.

TABLE 2

Most Active Antibacterial Peptides from Australian Frogs of the genera *Litoria* and *Uperoleia*

Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2
Caerin 1.4	GLLSSLS SVAKHVLPHVVPVIAEHL-NH_2
Maculatin 1.1	GLFGVLAKVAAHVVPAIAEHF-NH_2
Uperin 3.5	GVGDLIRKAVSV I KN IV-NH_2
Uperin 3.6	GVIDAAKKVVNVLKNLF-NH_2
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2
Citropin 1.2	GLFDIIKKVASVVGGL-NH_2
Caerin 2.5	GLVASIGRALGGLLADVVKSKEQPA-OH
Caerin 3.1	GLWQKIKDKASELVSGIVEGVK-NH_2
Caerin 4.1	GLWQKIKSAAGDLASGIVEGIKS-NH_2
Caerin 4.2	GLWQKIKSAAGDLASGIVEAIKS-NH_2

Organism	**MIC (μg/ml)[a,b]**										
	C1.1	C1.4	M1.1	U3.5	U3.6	Cit1.1	Cit 1.2	C2.5	C3.1	C4.1	C4.2
Bacillus cereus	50	50	25	25	25	25	25				
Escherichia coli		50								25	50
Leuconostoc lactis	1.5	12	3	3	3	3	6				
Listeria innocua	25	100	100	50	50	100	25				
Micrococcus luteus	12	0.4	12	25	50	25	12	<0.4	<0.4	12	6
Pasteurella haemolytica	25	-	-	12	25	-	-			<0.4	<0.4
Pasteurella multocida	25	25	50	100							
Staphylococcus aureus	3	100	6	50	25	25	25				
Staphylococcus epidermidis	12	25	12	12	12	25	25				
Streptococcus faecalis	25	25	25	-	-	-	-				
Streptococcus uberis	12	100	3	12	12	12	12				

(a) MIC is the minimum inhibitory concentration required to kill the organism.
(b) A dash means not tested. If there is no number indicated, the MIC value is > 100 μg/ml.

2.2 The Caerin Narrow Spectrum Antimicrobial Peptides

Some of the caerin 2, 3 and 4 peptides (Table 1) are not wide-spectrum antibacterial agents, but they do show activity against one or several pathogens. Caerins 2.5 and 3.1 show pronounced activity against *Micrococcus luteus*, while caerins 4.1 and 4.2 are very active against *Pasteurella* infections (Table 2). The caerins 4.1. and 4.2 also show activity against the gram negative organism *E. coli* : very few amphibian peptides (that we have studied) are active against this organism. The solution structure of caerin 4.1 has been determined by NMR methods: it is a perfect amphipathic α helix with well defined hydrophilic and hydrophobic zones [34].

The caerin 2 peptides are arguably the most unusual of all the caerin peptides. They are the only caerin molecules to have C-terminal CO_2H groups (the presence of a post translated C-terminal $CONH_2$ group is a good guide to possible bio-activity of amphibian peptides), and apart from caerin 2.5 they show either minimal or no antibacterial activity (Table 2). They all have similar structures: for example see the three basic and one acidic residues shown in bold in Table 1. We have not carried out NMR studies with any of these peptides, but their conventional Edmundson projections shows that if the molecules are α helices, their hydrophobic and hydrophilic residues do not occupy discrete and well defined zones. The caerin 2 peptides have structural similarity to similar peptides from other species of the genus *Litoria*: , for example citropin 2.1 (from *L.citropa*) and frenatin 3 (from *L. infrafrenata*) (see Table 1). These peptides must have some role in the amphibian integument, and we have recently found that caerin 2.3 (Table 1), which is found as a trace component in the glandular secretion of the male *L. splendida* (but not in that of the female), is the aquatic male sex pheromone of that species [35]. We are currently testing the other peptides to see if they are neuromodulators or neurotransmitters.

TABLE 3
The Citropin type Peptides from the genus *Litoria* and the Uperin Peptides from the Genus *Uperoleia*

Name		Sequence	Species
Aurein	1.1*	GLFDILKKLAQSL-NH_2	a
Aurein	3.1***	GLFDIVKKLAGHIAGST-NH_2	a
Citropin	1.1***	GLFDV IKKVASVIGGL-NH_2	b
Citropin	1.2***	GLFD I IKKVASVVGGL-NH_2	b
Citropin	1.3***	GLFD I IKKVASVIGGL-NH_2	b
Maculatin	2.1*	GFVDFLKKVAGTIANVVT-NH_2	c
Uperin	2.1**	GIVDFAKKVVGGIRNALGI-OH	d
	2.2	GFVDLAKKVVGGIRNALGI-OH	d
	2.3	GFFDLAKKVVGGIRNALGI-OH	d
	2.4*	GILDFAKTVVGGIRNALGI-OH	d
	2.5	GIVDFAKGVLGKIKNVLGI-OH	d
	2.6	GILDI AKKLVGGIRNVLGI-OH	e
	2.7	GII DI AKKLVGGIRNVLGI-OH	e
	2.8*	GILDVAKTLVGKLRNVLGI-OH	e
	3.1*	GVLDAFRKIATVVKNVV-NH_2	d
	3.2*	GVLDAFRKIATVVKNLV-NH_2	d
	3.3*	GVLDAFKKIATVVKNLV-NH_2	d
	3.4*	GVGDLIRKAVAAIKNIV-NH_2	e
	3.5***	GVGDLIRKAVSVIKNIV-NH_2	e
	3.6***	GVIDAAKKVVNVLKNLF-NH_2	e

*** Wide spectrum antibacterial peptide. ** Major antibacterial activity against a particular organism.
* Minor actibacterial activity.
a = *Litoria aurea* [36]; b = *Litoria citropa* [30]; c = *Litoria genimaculata* [29]; d = *Uperoleia inundata* [9]; e = *Uperoleia mjobergii* [37].

3.3 The Uperin Peptides from the genus Uperoleia *and Structurally Related Peptides from the* Litoria *genus.*

Toadlets of the genus *Uperoleia*, like the tree frogs of the genus *Litoria*, contain both a potent neuropeptide vasodilator and an array of antibacterial peptides. We have named the antibacterial peptides uperins: sequences of some of them are listed in Table 3. The sequences of the uperins are entirely different to those of the caerin 1 peptides, yet some, like uperins 3.5 and 3.6, show similar activities to those of the caerins 1 (see Table 2). It is of particular interest that some species of the genus *Litoria*, only

distantly related to the tree frogs of the *Litoria caerulea* group, have major wide-spectrum antibacterial peptides similar in structure to the uperin peptides. Amongst these are the aureins (from *Litoria aurea*), the citropins 1 (from *Litoria citropa*), and maculatin 2.1 (from *Litoria genimaculata*) (see Table 3).

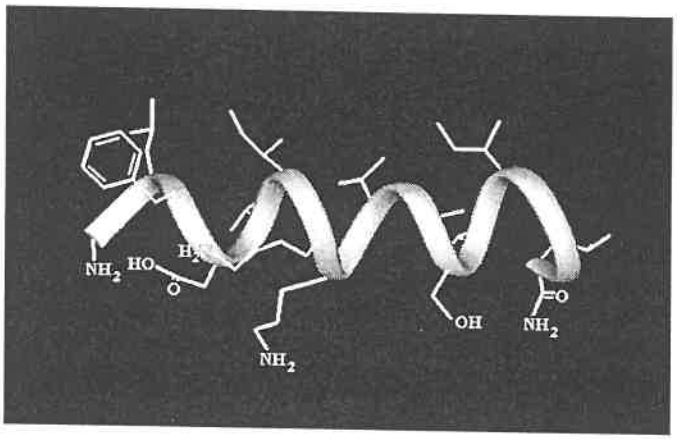

Figure 5. Solution structure of citropin 1.1.

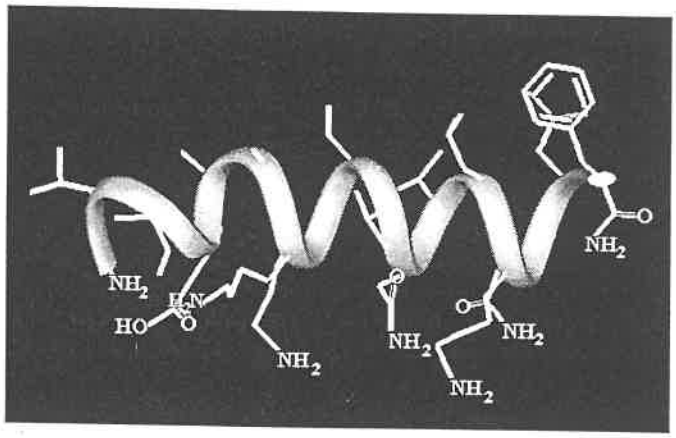

Figure 6. Solution structure of uperin 3.6.

The solution structures of citropin 1.1 [30] and uperin 3.6 [38] have been determined by NMR methods and are shown in Figures 5 and 6. These are amphipathic α helices, their structures are the simplest of all wide-spectrum amphibian antibacterial peptides so far reported, and searches of peptide and protein data banks have uncovered neither mammalian nor other analogues. The aurein 3 and citropin 1 antibacterial peptides are degraded and deactivated on the skin by endoproteases which remove the first two or three amino acid residues respectively: to date no similar behaviour has been detected for the uperin 3 peptides. Synthetic modifications of the citropins 1 have shown that the two basic residues at positions 7 and 8 (either Lys or Arg) are necessary for activity. Many of the uperins have an additional basic residue at position 15: this basic residue is not essential for activity. To date, we have not determined the minimum length of the peptide for appropriate activity, but is of interest that aurein 1.1 (with 13 residues) shows only minimal activity. The reason why these simple α helices show similar antibacterial activities to those of the caerin 1 peptides is not known. They are certainly more basic than the caerins 1: perhaps this makes them better able to bind with anionic sites on the surfaces of bacterial membranes.

3. *Some Amphibians which do not Produce Antibacterial Peptides*

We have described above how the majority of the tree frogs of the genus *Litoria* and toadlets of the *Uperoleia* genera contain at least one potent neuropeptide together with a cocktail of antibacterial peptides as part of their chemical arsenal in the glandular skin secretion. There are, surprisingly, exceptions to this scenario both in particular species of the *Litoria* genus, and in all of the frogs that we have studied from the *Limnodynastes* genus.

The Red Tree Frog *Litoria rubella* (Figure 7) is widespread throughout central and northern Australia and has evolved into a number of specific populations within this area [39]. It is a remarkable frog which can adapt to a range of climates from desert conditions to those of wet rain forests. There is a related frog called *Litoria electrica* but this is found only in northern Australia in a specific region just below the Gulf of Carpentaria [40]. Both of these frogs produce abundant glandular secretions on the

skin, but these secretions contain neither neuropeptides like caerulein nor any antibacterial peptides. This is most unusual, and it raises the question as to how these animals protect themselves from predators, both small and large? The granular glands produce 5 to 10 mg of a mixture of small peptides of the tryptophyllin family (see Table 4).

TABLE 4
The Tryptophyllin L Peptides From *Litoria rubella* and *Litoria electrica*

Name	Sequence	Species
1.1	PWL-NH_2	a
1.2	FPWL-NH_2	a,b
1.3	pEFPWL-NH_2	a
1.4	FPFPWL-NH_2	a
2.1	I PWL-NH_2	a
3.1	FPWP-NH_2	a,b
3.2	FPWP-OH	a
3.3	pEFPWF-NH_2	a
4.1	LPWY-NH_2	a
4.2	FLPWY-NH_2	a
5.1	pEIPWFHR-NH_2	a

a = *Litoria rubella* [39]; *Litoria electrica* [40].

Although these two frogs are unique amongst Australian frogs, tryptophyllin peptides were, in fact, first discovered in the South American hylid frog *Phyllomedusa rohdei* by Erspamer *et al* [41-43]. The tryptophyllins are quite similar in structure to the human brain endomorphins (e.g YPWF-NH_2 and YPWG-NH_2) which have a very high affinity for the γ-receptor [44]). So perhaps the tryptophyllins are neurotransmitters or neuromodulators. Erspamer has found that his and some of our tryptophyllins show either minimal or no smooth muscle activity, and we have shown (through the courtesy of the Australian Institute for Marine Science) that neither tryptophyllin L 1.2 nor 3.1 are active against neuronal nitric oxide synthase [45]. However one of Erspamer's tryptophyllins (FPPWM-NH_2) can induce sedation and behavioural sleep in birds, and is also immunoreactive to a set of cells in the rat adenohypophysis [46]. The role of the tryptophyllin peptides in amphibian skin is thus still to be determined.

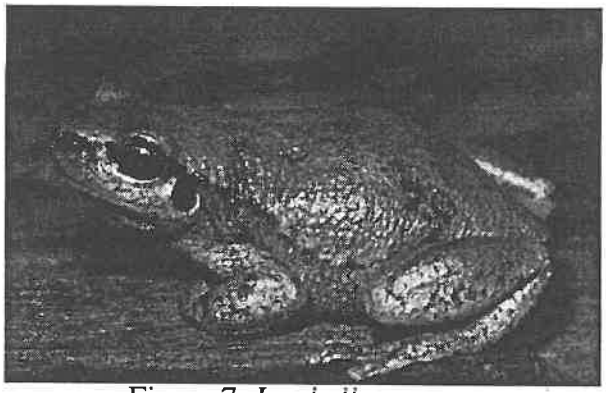

Figure 7. *L.rubella*

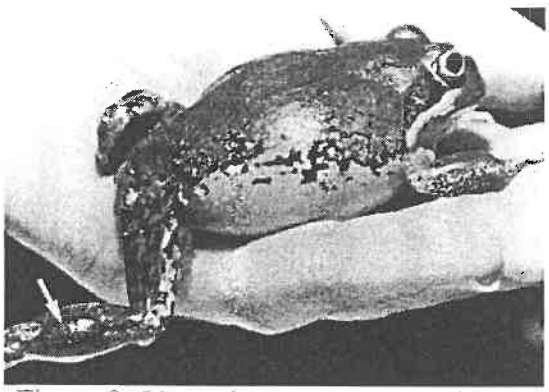

Figure 8. *Limnodynastes terraereginae.* Tibial gland arrowed.

TABLE 5.
Caeridin Type Peptides from Frogs of the Genera *Litoria* and *Limnodynastes*.

Name	Sequence	Species
Dynastin 1	GLVSNLGI-OH	a
Dynastin 2	GLLSSLGLNL-OH	b
Dynastin 3	GLVPNLLNNLGL-OH	c
Dynastin 4	GLVSNLGI-OH	d
Dynastin 5	GLISNLGI-OH	d
Dynastin 6	GAVSGLLTNL-OH	d
Dynastin 7	GAVSGLLTNLGL-OH	d
Fletcherin	AGPVSKLVSGIGL-OH	e
Caeridin 1.1	GLLαDGLLGTGL-NH_2	f,g,h,i,j
Caeridin 1.2	GLLβDGLLGTGL-NH_2	h
Caeridin 1.4	GLLαDGLLGGLGL-NH_2	i,j
Caeridin 1.5	GLLβDGLLGGLGL-NH_2	j
Caeridin 2	GLLDVVGNLLGGLGL-NH_2	g,h
Caeridin 3	GLFDAIGNLLGGLGL-NH_2	g,h
Caeridin 4	GLLDVVGNVLHSGL-NH_2	g
Caeridin 5	GLLGMVGSLLGGLGL-NH_2	g
Caeridin 6	GLLGFVGSLLGGLGI-NH_2	g
Frenatin 1	GLLDALSGI LGL-NH_2	k
Frenatin 2	GLLGTGNLLNGLGL-NH_2	k
Rubellidin 4.1	GLGDILGLLGL-NH_2	l
Rubellidin 4.2	AGLLDILGL-NH_2	l

a = *Limnodynastes interioris* [47]; b = *Limnodynastes dumerilii* [47]; c = *Limnodynastes terraereginae* [47]; d = *Limnodynastes salmini* [48]; e = *Limnodynastes fletcheri* [48].
f = *Litoria splendida* [8,10]; g = *Litoria caerulea* [7,8]; h = *Litoria gilleni* [26]; i = *Litoria xanthomera* [14]; j = *Litoria chloris* [28]; k = *Litoria infrafrenata* [31]; l = *Litoria rubella* [39].

Marsh frogs of the genus *Limnodynastes* produce copious secretions from their dorsal granular glands, and in some cases from tibial glands on their legs (see Figure 8 for *Limnodynastes terraereginae*). Only minute quantities of peptides are found in these secretions. We have named these peptides dynastins, and their sequences are listed in Table 5. The dynastins are all small anionic peptides, they contain C-terminal CO_2H residues, and they exhibit no bio-activity in any of the test regimes that we now use. It is clear that this genus of frog has a very different defence mechanism to that of others that we have studied, and we do not know what that is. It may be that the dynastins are aquatic pheromones of some type: we have not as yet carried out behavioural tests to confirm (or refute) this suggestion.

Finally, all of the tree frogs of the genus *Litoria* produce both caeridin and caerin peptides in their glandular secretions. The caeridin peptides show some structural resemblance to the dynastin peptides, but the caeridin type peptides are all post translationally modified (C-terminal $CONH_2$) (see Table 5). The caeridin peptides have shown no activity in any of our test regimes. We have not yet tested for pheromonal activity, but such activity perhaps seems unlikely when the sequences of the caeruleins are compared with that of the aquatic male sex pheromone of *Litoria splendida* (caerin 2.3 - see Table 1). Perhaps the caeridins are spacer peptides, but if that is so, why do they have C-terminal amide functionality? The

determination of their role(s) will have to wait for that part of the appropriate gene of *L. splendida* to be sequenced.

4. Conclusions

Amphibians evolved from fresh water fishes some 300 million years ago and their chemistry has been evolving since that time. We have data that show that the structures of peptides in the glandular secretions of some species have changed marginally within the last 5000 years, but whether this is a result of climatic change, change of predator, or some other variable is not known (this is beyond the scope of this review, but see [7, 39]). Amphibians have some of the most diverse yet simple host defence peptides to be reported in the animal kingdom, from neuropeptides with a wide range of activities to antibiotic peptides active against either a whole range of pathogens or against an individual pathogen. The range of 3D structures for the peptides, from simple α helices to α helices with a central hinge is quite spectacular, taking into consideration that most of these peptides have much the same antibacterial activities. The research that we carry out in this area is purely fundamental: we are enthralled by the biological chemistry of these fascinating and primative creatures. Yet the impressive range of bio-activities of these peptides indicates that consideration of certain of them for clinical and/or agricultural use needs to be explored.

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REFERENCES

1 Lazarus, L.H. and Attila, M. (**1993**) *Prog.Neurobiol, 41*, 473-507.
2 Erspamer, V (**1994**), in *Amphibian Biology, The Integument, 1*, 245-258.
3 Bevins, C.L. and Zasloff, M. (**1990**) *Annu.Rev.Biochem. 59*, 395-414.
4 Barra, D. and Simmaco, M (**1995**) *Trends Biotech. 13*, 205-209.
5 Soballe. P.W., Maloy, W.L., Myrga, M.L., Jacob, L.S. and Herlyn, M. (**1995**) *Int. J. Cancer 60*, 280-284.
6 Jacob, L. and Zasloff, M. (**1994**) *Ciba Found.Symposium 186*, 197-223.
7 Stone, D.J.M., Waugh, R.J., Bowie J.H., Wallace, J.C. and Tyler, M.J. (**1993**) *J.Chem.Research, S*, 138; *M*, 910-936.
8 Stone, D.J.M., Bowie, J.H., Tyler, M.J. and Wallace, J.C. (**1992**) *J.Chem.Soc.Chem. Commun.* 1224-1225.
9 Bradford, A.M., Raftery, M.J., Bowie, J.H., Tyler, M.J., Wallace, J.C., Adams.G.W. and Severini , C. (**1996**) *Aust. J. Chem. 49*, 475-484.
10 Stone, D.J.M., Waugh, R.J., Bowie, J.H., Wallace, J.C. and Tyler, M.J. (**1992**) *J. Chem. Soc. Perkin Trans 1*, 3173-3179.
11 Tyler, M.J., Stone, D.J.M. and Bowie.J.H. (**1992**) *J.Pharmacol.Toxicol.Methods, 28* 199-200.
12 Ganz, T. (**1994**) *Ciba Foundation Symposium 186*, 62-76.
13 Resnick, N., Maloy, W., Crut, H. and Zasloff, M. (**1991**) *Cell 66*, 541-549.
14 Steinborner, S.T., Waugh, R.J., Bowie, J.H., Wallace, J.C., Tyler, M.J. and Ramsay, S.L. (**1997**) *J.Peptide Science 3*, 181-185.
15 Kriel, G. (**1994**) *Ciba Foundation Symposium 186* 72-92.
16 Chirgwin, T.M., Przybyla, A.E., McDonald, R.J. and Rutter, W.J. (**1979**) *Biochemistry,*

Steinborner, S.T., Wabnitz, P.A., Bowie, J.H., and Tyler, M.J., (1996) The application of mass spectrometry to the study of evolutionary trends in amphibians.
Rapid Communications in Mass Spectrometry, v. 10 (1), pp. 92-95, 1996

Steinborner, S.T., Wabnitz, P.A., Waugh, R.J., Bowie, J.H., Gao, C.W., Tyler, M.J., and Wallace, J.C., (1996) The structures of new peptides from the Australian Red Tree Frog '*Litoria rubella*'. The skin peptide profile as a probe for the study of evolutionary trends of amphibians.
Australian Journal of Chemistry, v. 49 (9), pp. 955-963.