Amphibian Neuropeptides:
Isolation, Sequence Determination and Bioactivity

A Thesis submitted
for the
Degree of Doctor of Philosophy
in the
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by

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Chapter 4- Antimalarial Activity of Anuran Peptides

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Abstract

The skin extracts from amphibians have been investigated for over fifty years and have been found to contain numerous components with therapeutic and medicinal uses. Host-defence compounds are secreted onto the dorsal surface of the animal from specialised granular glands in response to a variety of stimuli, such as stress induced by a predator. Isolated peptides can exhibit either pharmacological properties or antibiotic activity.

Previous studies isolated a potent hypotensive neuropeptide, crinia angiotensin II, within skin secretions of the Australian frog *Crinia georgiana*. This prompted further investigations into the isolation and sequence determination of host-defence compounds from other species in this genus- *C. signifera*, *C. riparia* and *C. deserticola*. Fifteen novel peptides were identified. The major peptide components were potent disulfide containing neuropeptides of a type not observed in other Australian anurans that have been previously investigated. The remaining peptides demonstrate either antibiotic activity or inhibit the enzyme neuronal nitric oxide synthase.

The skin components from anurans of the *Litoria* genus have been extensively studied, with a number of peptides exhibiting both antibacterial and pharmacological activity. The skin secretion of *Litoria dentata* has been investigated, with five novel peptides identified. The neuropeptide tryptophyllin L 1.3 was previously isolated from the related frog *L. rubella*. Other components that are unique in structure have not yet been tested for biological activity.

The parasitic disease malaria is responsible for over one million deaths per year. The increase in resistance of current antimalarial compounds has led to the development of new treatments from various animal-derived peptide antimicrobials. A number of amphibian peptides and their derivatives were investigated as potential antiplasmodial agents against the malaria parasite *Plasmodium falciparum*. Results indicate that these compounds inhibit parasite growth with minimal haemolytic activity, making them promising tools for malaria research.

The defence chemistry of amphibian neuropeptides has been extensively studied and is important in understanding both the ecology and physiology of the vertebrate. Neuropeptides are classified into groups with similar structural characteristics. Biological activity occurs via interaction with a G protein-coupled receptor. The most studied of all amphibian neuropeptides is caerulein, which has a similar spectrum of activity to the mammalian peptide cholecystokinin. This includes smooth muscle contraction that occurs via interaction with cholecystokinin receptors.

The pharmacological activity of Australian anuran neuropeptides from various genera was investigated. Two biological assays were conducted- a smooth muscle contraction test and a lymphocyte proliferation assay. A range of neuropeptides contracted smooth muscle at nanomolar concentrations, while others only proliferated lymphocytes. Some peptides were inactive in both assays.

Young marsupials are born at an immature stage of development and rely on immune protection provided by the mother. Eugenin is a host-defence compound isolated from pouch secretions of the Tammar wallaby. The immunomodulator activates CCK_2 receptors, resulting in lymphocyte proliferation. Therefore, eugenin stimulates immune cells in the pouch providing vital immune protection for pouch young.

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.

I give consent for this copy of my thesis, when deposited in the University Library, to be available for loan and photocopying.

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Vita Marie Maselli Date

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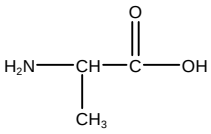
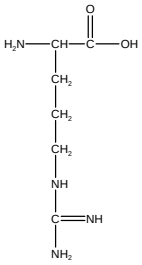
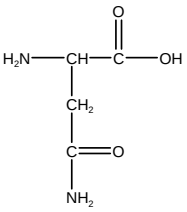
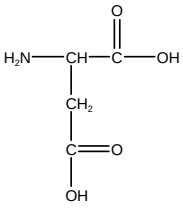
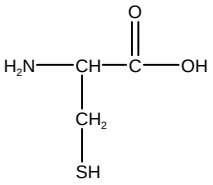
The 20 Common Amino Acids

Alanine Ala (A)	**Arginine** Arg (R)	**Asparagine** Asn (N)	**Aspartate** Asp (D)	**Cysteine** Cys (C)
				
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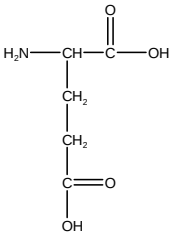	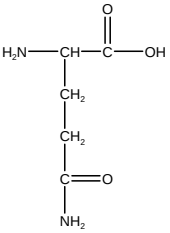	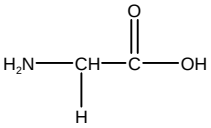	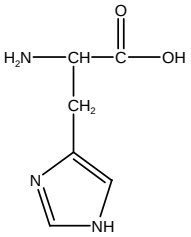	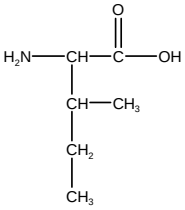
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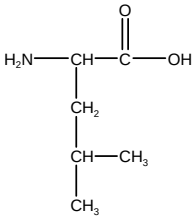	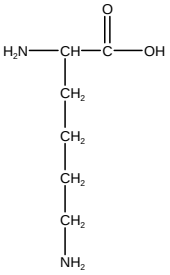	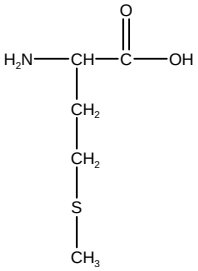	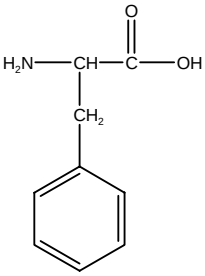	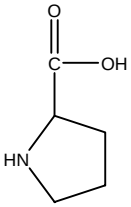
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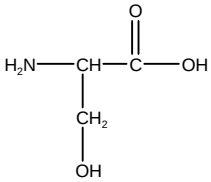
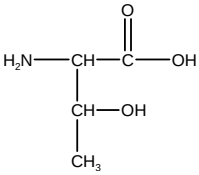
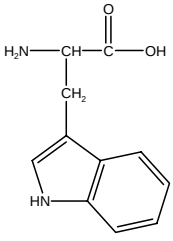
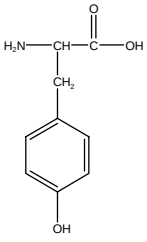
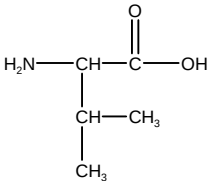
				

Chapter 1- Amphibians and their Peptides

1.1 Amphibian Peptides

1.1a Amphibians

For centuries, extracts from amphibians, particularly skin extracts, have been found to contain numerous active components with therapeutic and medicinal uses [1]. The isolation, identification and testing of new amphibian peptides has led to the treatment of various medical conditions including conjunctivitis, gastrointestinal infections and cancer.

Amphibian skin secretions have been investigated for over fifty years in an effort to identify their contents and clarify their biological functions [2]. Research in the 1960s found that the skin glands of amphibians contain rich chemical arsenals. Subsequent analysis has revealed a wide variety of biologically active peptides within the arsenals, including biogenic amines, steroidal compounds, alkaloids and peptides. Some of these peptides are homologous or identical to mammalian hormones or neurotransmitters, and may provide an insight into the activity of the unknown peptides and its receptor site. Previously, peptides were isolated from extracted frog skins with an organic solvent like methanol [3]. These were then recovered by batch absorption and resolved by ion-exchange chromatography or reversed-phase high performance liquid chromatography [3]. Currently, secretions containing peptides are collected from the surface of the skin and studied following stimulation of their release using non-invasive methods, such as mild electrical shock [4].

Amphibian populations have been declining since the 1960s [5]. Probable causes for this diminution currently being investigated are the introduction of predators, exposure to pesticides, exposure to ultra-violet blue light and pathogens including the chytrid fungus *Batrachochytrium dendrobatidis* [6]. Amphibian protection is primarily due to their ability to

generate a defence mechanism exhibiting biological activity involving a number of host-defence compounds found in the skin secretions [7].

The skin of amphibians is used as a buffer between the frog and its environment [8]. Importantly, it provides for gas and water exchange, defence, sensation and camouflage. Host-defence compounds within skin secretions originate from specialised glands within the dermal layers of the skin. The dermal layer of the skin contains a non-homogenous distribution of two distinct types of glands: the mucous glands and the granular glands [1]. These glands are primarily dispersed along the dorsal surface of the animal and communicate directly with the exterior surface by means of secretory ducts [1]. The mucous glands secrete a clear, watery fluid which moistens the skin and facilitates gas exchange, whilst the granular glands contain active peptides that are stored as inactive processed peptides. The central nervous system controls the action of the glands through the release of secretions by an adrenergic stimulator. Host-defence compounds are secreted within a gel from specialised granular glands onto the dorsal surface and into the gut of the amphibian in response to a variety of stimuli [9, 10], such as stress [11]. The structures of the glands are unique to amphibians and are strategically concentrated in areas most exposed to predatory attack [12], most commonly by birds and snakes [13]. Any stress induced by a predator will stimulate discharge of the secretions [12], which are generated by the frog's nervous system and are noxious to the nervous system of the predatory organism [13]. The peptides also provide antimicrobial defence and play a role in defending the animal from environmental threats like bacteria. Therefore, the peptides act on both macroscopic and microscopic predators [13].

1.1b The Role of Anuran Peptides

Although plants have traditionally been a major source of active compounds for pharmaceutical products, the quest for pharmaceuticals derived from natural origin extends beyond such material. Significantly, amphibian skin secretions produce a number of peptides with a variety of functions. It is this high level of biochemical diversity that makes amphibians suitable subjects for chemical prospecting [14].

Peptide activity can be classified as:

i) neuropeptides-including neurotransmitters and hormones,
ii) antibacterial, anticancer and antifungal agents,
iii) antimalarial agents,
iv) pheromones, and
v) miscellaneous agents.

A description of each category of peptide is summarised in section 1.2. Many of these peptides are present in significant quantities [13, 15] and preliminary research suggests they provide diverse physiological and defence functions. Moreover, amphibian research facilitates medical advancement through the detection of hormones and neuropeptides, which may contain antibacterial or anticancer properties [9]. Secretions have also been the subject of biophysical research, biochemical taxonomy and evolutionary relationships [14].

1.2 The Pharmacology of Peptides

Modern methods allow most peptides to be fully characterised and synthesised. Many peptides are known to be active chemical mediators, however specialised knowledge of the pharmacology of biologically active peptides and of their therapeutic utilisation remains relatively sparse [16]. This is due to their:

i) ability to hydrolyse in the stomach and be poorly absorbed when given orally,
ii) expensive manufacturing cost on a commercial basis,
iii) rapid degradation by plasma and tissue peptidases that result in a short biological half-life, and
iv) inability to penetrate the blood-brain barrier.

Presently, peptides isolated from amphibian skin are classified into two groups [13]:

i) pharmacologically active peptides- these exhibit activity like smooth muscle contraction and relaxation, and
ii) antibiotic peptides- these exhibit non-specific interactions upon the bacterial cell membrane [13].

More than thirty five species of Australian frog and toad have been studied over the past twenty years from the genera *Litoria, Uperoleia*, *Limnodynastes*, *Cyclorana* and *Crinia* [17]. The majority of frogs studied have a variety of peptides within the skin glands. Over one hundred and thirty peptides have been isolated and sequenced with a range of biological activities. Each species of frog generally includes a neuropeptide and a powerful broad-spectrum antibiotic. Peptides exhibit anticancer properties, pheromone activity, antimalarial activity and neurotransmitter functions. These are further discussed below.

1.2a Neuropeptides

Neuropeptides are chemical messengers that are released from sensory neurons and act on neuronal receptors. They generally produce intracellular effects that alter the synaptic activity of particular neurons by activating a second messenger system [18]. The isolation, structure determination and pharmacological activity of anuran peptides have been investigated.

Erspamer and his research group first pioneered the work on the host-defence chemistry of anuran neuropeptides in the 1960s. Anuran neuropeptides are classified as:

i) bombesins,
ii) caeruleins,
iii) tachykinins,
iv) bradykinins,
v) tryptophyllins, and
vi) dermorphins and deltorphins.

There are also a number of miscellaneous neuropeptides. These are further discussed in section 1.2h.

Neuropeptides play a pivotal role in the animal's defence system, as well as assisting with the regulation of dermal physiological action. Neuropeptide activity can include smooth muscle activity, analgaesic and antihistamine activity, and immunomodulatory functions.

The majority of frogs of the genus *Litoria* and toadlets of the *Uperoleia* genus contain at least one neuropeptide, which is frequently the major host-defence peptide in the glandular secretion. A potent neuropeptide found in almost all of the Australian *Litoria* species studied is caerulein **(1)** [17]. Caerulein is a post-translationally modified peptide of the C-terminal end group, has transformation of the first residue into pyroglutamate, and sulfation of the tyrosine residue. The occurrence of caerulein in dermal secretions is usually accompanied by a number of caerulein-like analogues, which are presumed to possess similar activity [19]. The seasonal

changes of caerulein may be involved in the thermoregulation of the animal during the breeding cycle [12].

Caerulein's structure is similar to cholecystokinin-8 **(2)**, a mammalian gastrin-like neuropeptide [20], and hence exhibits a similar spectrum of activity. Biological activity includes contraction of smooth muscle, enhancement of blood circulation, modification of satiety, sedation and thermoregulation. It is also an analgaesic several thousand times more potent than morphine and has been used clinically [20].

(1) Caerulein pEQDY(SO_3)TGWMDF-NH_2

(2) Cholecystokinin-8 DY(SO_3)MGWMDF-NH_2

Uperolein **(3)** was first isolated from toadlets of the genus *Uperoleia*. Uperolein exhibits potent vasodilator and hypertensive action [20].

(3) Uperolein pEPDPNAFYGLM-NH_2

A number of amphibian peptides have been found to possess neuronal nitric oxide synthase (nNOS) inhibition activity [21]. The enzyme nNOS is responsible for the production of the neurotransmitter nitric oxide. Nitric oxide (NO) behaves as a defensive cytotoxin against tumour cells and pathogens. The immune system uses the toxic properties of NO to kill or inhibit the growth of invading organisms. These peptides interfere with communication between Ca^{2+} CaM (calmodulin) which is responsible for the activation of NOS and nNOS.

Three general groups have been identified:

i) short basic peptides, possessing an amphipathic α-helical structure and terminating with an amide group - this includes lesueurin **(4)**, the aureins 1 and the citropins 1,

ii) frenatin 3 **(5)** type peptides, which are basic and terminate with a carboxylic acid group- this includes the caerin 2 peptides, and

iii) peptides with a helix-hinge-helix type structure- the caerin 1 family **(6)**.

(4) Lesueurin GLLDILKKVGKVA-NH_2

(5) Frenatin 3 GLMSVLGHAVGNVLGGLFKPKS-OH

(6) Caerin 1.8 GLFKVLGSVAKHLLPHVVPVIAEKL-NH_2

A group of opioid peptides in the dermorphin and deltorphin group possess potent analgaesic effects. This group is described in more detail in section 5.1g.

In addition, there are many amphibian neuropeptides yet to be classified into a particular group. These include the recently identified disulfide peptides isolated from the *Crinia* genus (see chapter 2 and section 5.5), which possess smooth muscle contractile activity and immunomodulatory properties [22, 23].

1.2b Hormonal Peptides

Hormones are chemicals that are stored in secretory vesicles and secreted into the blood stream to act on a distant tissue in response to a variety of demands [18]. Hormonal peptides can be dissolved within the plasma to readily flow through the blood stream due to its hydrophobicity and low lipid solubility. They fall into three main categories:

i) peptides and proteins,

ii) amines, and

iii) steroids.

The majority of hormones are of the peptide and protein category. Hormonal effects can be caused by the stimulation of various types of smooth muscle throughout the central nervous system to produce a change in blood pressure or stimulation of the urinary, reproductive and gastrointestinal tracts [11]. They are also secreted in organs like the kidneys, pancreas, heart and liver.

1.2c Antibacterial Peptides

Numerous antibacterial and antimicrobial peptides have been isolated and characterised. The dermal secretions of most Australian frog species contain at least one broad-spectrum antibiotic and a number of peptides with varied specificity to allow enhanced protection against a range of bacteria. In the mid 1980s, antibacterial peptides from anuran skin secretions invoked great interest, when a peptide from a particular anuran species was found to exhibit broad-spectrum antibacterial activity [24]. Peptide activity is mediated by disruption of the target cell membrane by disturbing the phospholipid bilayer situated on the membrane surface [25]. Peptides from vertebrates have three characteristic properties:

i) they are relatively small, containing between 10 - 46 amino acid residues,
ii) they are usually basic, having an overall positive charge due to basic residues like histidine, arginine and lysine, and
iii) they have the ability to form amphipathic helical structures.

The most active antibacterial peptides include caerin 1.1 **(7)**, maculatin 1.1 **(8)**, citropin 1.1 **(9)** and aurein 1.2 **(10)**.

(7)	Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2
(8)	Maculatin 1.1	GLFGVLAKVAAHVVPAIAEHF-NH_2
(9)	Citropin 1.1	GLFDVIKKVASVIGGL-NH_2
(10)	Aurein 1.2	GLFDIIKKIAESF-NH_2

1.2d Anticancer Agents

Amphibian peptides have also been tested against cancer cells *in vitro*. Generally, anticancer activity operates similarly to antibacterial activity. This is likely due to a similar mechanism of action at both bacterial and cancer cells [26].

1.2e Antifungal Peptides

The chytrid fungus (*Batrachochytrium dendrobatidis*) is currently adversely affecting anuran populations' worldwide. The antibiotic peptides of some frogs, like the temporins [27] and ranateurins **(11)** [28] from *Rana* species, are active against the chytrid fungus. Similarly, some antibiotic peptides from Australian anurans like the caerins 1 **(12)**, uperins and citropins 1 **(13)**, kill the chytrid fungus in the micromolar concentration range [29].

(11) Ranatuerin-2TRa GIMDSIKGAAKEIAGHLLDNLKCKITGC-OH

(12) Caerin 1.10 GLLSVLGSVAKHVLPHVVPVIAEKL-NH_2

(13) Citropin 1.3 GLFDIIKKVASVIGGL-NH_2

1.2f Antimalarial Peptides

Malaria is one of the most widespread diseases in the world and is promulgated by mosquitoes. The disease is caused by an intracellular protozoan, which spends most of its life cycle within the host red blood cell [30]. The control measures of malaria are becoming less effective as there is an increasing resistance of the parasite to classical malarial drugs.

Anurans breed in aquatic environments rife with mosquitoes. Some European ranid frogs are prone to infestation by malaria parasites, however, they are still able to reduce the effect of infection by the parasites [31]. A group of peptides named dermaseptins **(14)**, isolated from the *Phyllomedusa* family, have been widely studied as antiplasmodial agents [32]. Certain Australian amphibian peptides have been investigated for their ability to inhibit the growth of

the malaria parasite, *Plasmodium falciparum*. Their mode of action is also being investigated [33] (see chapter 4).

(14) Dermaseptin S4 ALWMTLLKKVLKAAAKAALNAVLVGANA-OH

1.2g Pheromones

The first anuran sex pheromone, splendipherin **(15)**, was isolated from *Litoria splendida* in 1997 [12]. Pheromones are substances that are released to cause a behavioural response in a conspecific, and are commonly involved in mating and courtship [12].

(15) Splendipherin GLVSSIGKALGGLLASVVKSKGQPA-OH

1.2h Miscellaneous Peptides

The activity of a number of peptides abundant in the glandular secretions of anuran species remains unknown. These include the caeridins **(16)**, dynastins, frenatins **(17)**, rubellidins **(18)**, electrins **(19)**, tryptophyllins and rotheins **(20)** [34-36]. These peptides exhibit neither neuropeptide nor antibacterial activity, and their role in the defence system of anurans is as yet undetermined.

(16) Caeridin 1 GLLDGLLGTGL-NH_2

(17) Frenatin 1 GLLDALSGILGL-NH_2

(18) Rubellidin 4.1 GLGDILGLLGL-NH_2

(19) Electrin 2.1 NEEEKVKWEPDVP-NH_2

(20) Rothein 2.1 AGGLDDLLEPVLNSADNLVHGL-NH_2

1.3 Peptide Biosynthesis

Anurans encode and synthesise all active peptides as part of larger parent peptides. There are three major components of the peptide: a signal peptide (pre), a spacer peptide (pro) and a bioactive peptide (pre-pro-peptide) (Figure 1.1) [37]. The signal peptide is removed by an endoprotease enzyme, leaving the inactive pro-peptide to be transported and stored in secretory granules within the glands of the skin. The spacer peptide is cleaved in the endoplasmic reticulum and Golgi complex to obtain the active peptide [18]. Upon stimulation, the active peptide is released onto the skin or gut [9]. The active peptide may be cytotoxic to the host, however, frogs possess proteolytic enzymes to guard them against damaging their own cells [10].

Peptides can also undergo post-translational modification, including C-terminal amidation, blocking of the N-terminus by conversion of glutamic acid to pyroglutamate, sulfation of tyrosine residues and isomerisation of L-amino acids to the D- form [38]. Peptide modifications, including degree of helicity, charge state and hydrophobicity have been found to be important for activity.

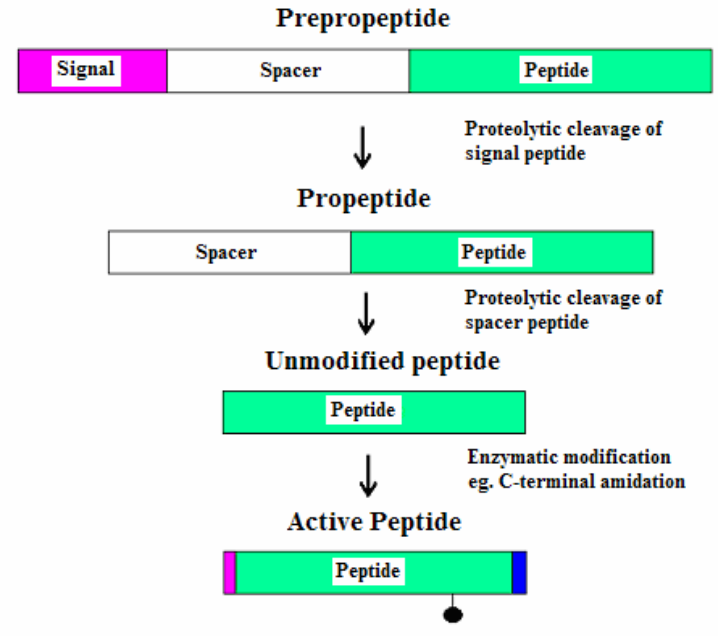


Figure 1.1. The biosynthetic pathway of peptides

1.4 Methodology

1.4a Collection of Frog Secretions

Frogs possess two types of glands on the skin surface:

i) the mucous glands- these produce a watery secretion for cooling and aiding in respiration, and
ii) the granular glands- these store the active components of the secretion [39].

The dorsal glands are best illustrated by *Litoria splendida*, which has large paratoid and rostral glands on the head. The sympathetic nervous system, which is involved with the 'fight-or-flight' response, controls both gland types.

Previously, peptides were extracted from the dried skin using an organic solvent [40]. This required the sacrifice of thousands of animals in order to obtain sufficient material for testing [3, 40]. However, evidence of diminishing frog populations [41] led to the current practice of collecting secretions through a non-invasive technique that leaves the animals unharmed.

The surface electrical stimulation (SES) method [4], developed in 1992, is used to effect the release of glandular secretions. The method is simplistic, efficient and harmless, with 'milking' being repeated on a monthly basis.

The SES method involves gently massaging a platinum electrode (that is attached to an electrical stimulator) over the glandular region of the frogs' dorsal surface to produce a mild electrical stimulation, while moistening the skin with distilled water (Figure 1.2). Skin secretions are discharged from the dorsal surface of the amphibian and the crude secretion is rinsed from the skin with distilled water and collected. Any active peptides that are cytotoxic to the amphibian can be deactivated 5-30 minutes after the secretion is released onto the skin by enzymes. To eliminate the risk of active peptides degrading if the enzymes are not removed

or deactivated, methanol is added to the aqueous extract (1:1) to both denature the protein and to reduce its solubility. The resulting crude secretion is filtered, concentrated and then separated and purified by high performance liquid chromatography (HPLC) (see section 1.4b below).

(a) **(b)**

Figure 1.2. (a) 'milking' *L. caerulea* for skin secretions using the SES method and **(b)** the crude material collected

1.4b Analysis by High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a powerful separation technique that allows for the exploration of biological systems and the quantitation of minute amounts of biologically active components. HPLC enables the purification and separation of crude secretions in order to obtain the constituent peptides that can range in molecular weight from one hundred to several thousand Da.

The separation of amphibian peptides utilises a reverse phase HPLC column. Crude secretions are injected into the HPLC machine and the peptides absorb onto the hydrophobic surface from the mobile phase. Peptides are displaced once sufficient concentration of organic solvent reaches the peptide [42].

The separation of peptides is dependent on differences in the amino acid sequence as this leads to differences in the hydrophobic face of the peptide. Acetonitrile is used as the organic modifier due to its ultraviolet transparency at low wavelengths, low viscosity resulting in low column backpressure and high column efficiency, and high volatility that permits the facile removal of solvent from fractions containing peptides. The aqueous solvent contains trifluoroacetic acid (TFA) as the acidic modifier. This adjusts the pH as well as solubilising the peptide. In addition, TFA assists with high-resolution separations and is volatile and easily removed. A wavelength between 214 - 215 nm detected amide bonds by measuring the $\pi \rightarrow \pi^*$ transition of the amide carbonyl group.

1.4c Mass Spectrometry

Mass spectrometry (MS) is a technique that allows the molecular weight of a molecule to be measured. Mass spectrometers perform three basic functions [43]:

i) to produce ions from vapourised compounds (gas-phase ionisation),
ii) to separate these ions according to their mass to charge (*m/z*) ratios, and
iii) to distinguish and record the resulting separated ions.

Fragmentation of the molecular ions occur due to excess vibrational energy from the ionisation process or by collisions with inert gas molecules [44]. The resulting fragment ions (or ‘daughter’ ions) provide information about the structure of the original molecular ‘parent’ ion, allowing the peptide sequence to be deduced from the fragmentation pattern.

The present research utilised the Q-TOF 2 hybrid quadrupole time of flight mass spectrometer to identify peptide primary structures. The methods employed by the Q-TOF 2 to carry out gas-phase ionisation, analysis and detection will be discussed below in section 1.4d.

1.4d Q-TOF 2 Hybrid Quadrupole Time of Flight Mass Spectrometer

The Q-TOF 2 mass spectrometer is an ideal instrument for analysing large biological molecules in minimal quantities. A schematic diagram of the principal components of the Q-TOF 2 mass spectrometer is shown in Figure 1.3.

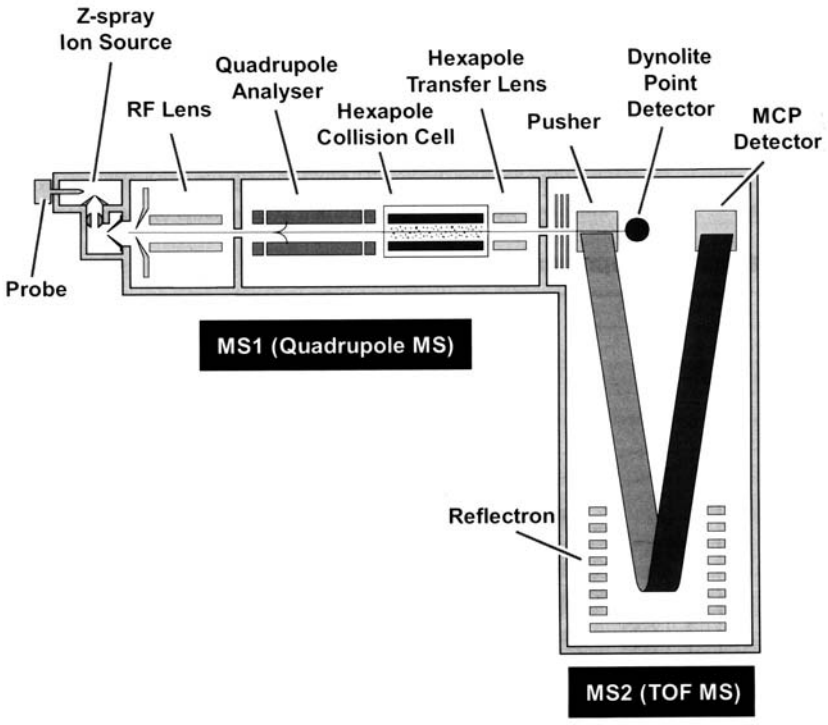


Figure 1.3. Schematic diagram of the Q-TOF 2 mass spectrometer

The Q-TOF 2 mass spectrometer uses a soft ionisation technique [45] that has the ability to record spectra at nanomolar concentrations and to analyse molecules with a mass range to 10,000 Da. These features are advantageous for analysing the small quantities of peptide isolated from anuran secretions.

The operational mode of the Q-TOF 2 involves electrospray ionisation (ESI) (Figure 1.4). ESI is a soft ionisation process that is capable of transforming parent ions in solution into ions with single or multiple charges in the gas phase. Gas-phase ions are produced in the Z-spray ion source [46]. The sample is introduced into a liquid chromatograph (~ 1-10 µL/min) through a heated capillary to which a high voltage electric field (~ 3-6 kV) is applied. Separation of the sample occurs as the chemical components move through the column. The capillary is maintained at the high voltage as the ESI needle ejects the sample solution into a fine mist of droplets, resulting in an electrically charged droplet surface. The electrical charge density at the surface of the droplets increases due to evaporation, until a critical point is attained where the forces due to electrostatic repulsion equal those due to surface tension. The resulting instability divides the droplets into an array of smaller daughter droplets. These smaller droplets also evaporate and 'explode' into smaller assemblies, until the droplets contain only the charged solute molecule [47]. Positive ions are formed by protonation to yield $(M + nH)^{n+}$ ions, and negative ions are formed by deprotonation to yield $(M - nH)^{n-}$ ions.

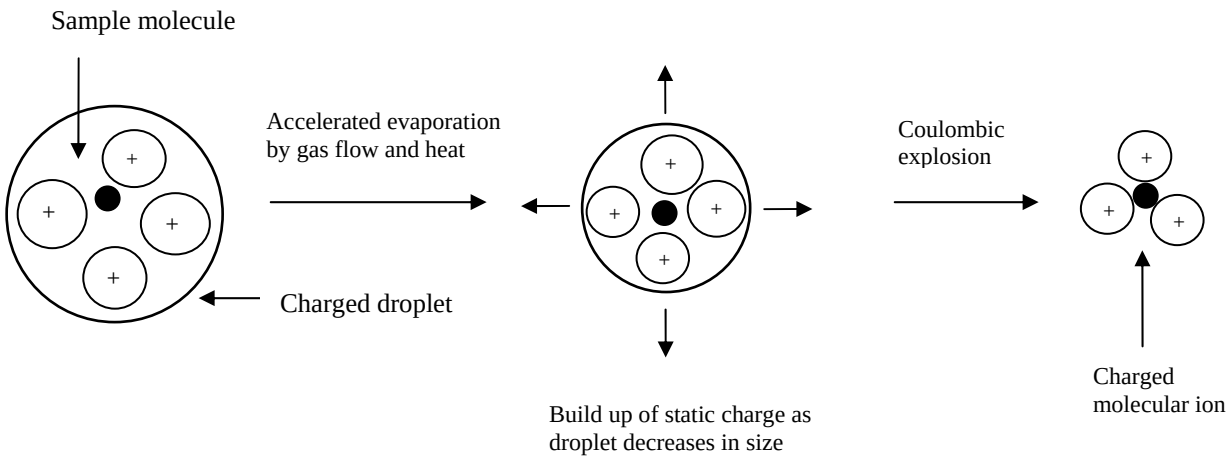


Figure 1.4. The mechanism of electrospray ionisation

The free ions are drawn through the sample cone aperture and into the ion block, before being transmitted into the analyser. The analyser consists of a quadrupole mass analyser and an orthogonal acceleration time of flight (TOF) mass spectrometer, which is separated by a hexapole collision cell. Positively charged ions or negatively charged ions are transmitted to the mass analyser depending on the polarity of potentials in the ionisation source. The mass to charge ratio (*m/z*) of produced ions determines the charge state of ions, as the mass difference between these peaks is inversely proportional to the charge. Ions can be separated according to their flight times, with ions of larger mass taking longer to reach the detector. Ions are fragmented in the hexapole collision cell, with the resulting daughter ions scanned by the TOF mass sector.

The Q-TOF 2 consists of numerous features that enable a peptide to be entirely sequenced. Introduction of a sample into the system produces parent ions by the ion source. A single mass spectrometry scan corresponds to a single stage of mass analysis where no collision induced fragmentation of the parent ion eventuates. The Q-TOF 2 has a microchannel plate detector that records a full spectrum every 50 µs. Collisional activated dissociation is utilised when fragmentation of a particular ion is required. The ion of interest is isolated within the trap and undergoes a series of high energy rapid collisions with argon atoms. The resulting daughter ions produce MS/MS data of the selected ion.

1.5 Peptide Sequencing

1.5a Positive and Negative Ion Mass Spectrometry

The primary structures of host-defence peptides isolated from anuran skin secretions are determined by the complementary use of mass spectrometry and automated Edman sequencing.

Primary peptide sequences can be elucidated using mass spectrometry in the positive and negative ion mode. The mass spectrometry method is advantageous as results can be obtained quickly and sequences can be determined from minute quantities of sample. Mass spectrometry is also able to identify post-translational modifications to the primary sequence that may be difficult or impossible to detect by automated Edman degradation [48], including N-terminal pyroglutamate residues. A limitation to mass spectrometry is the inability to distinguish between isomeric Ile and Leu (113 Da) and isobaric Lys and Gln (128 Da) amino acid residues (unless high-resolution capability is available) within the peptide sequence. Automated Edman sequencing is used to identify between isomers Ile and Leu. We use both Edman sequencing and enzyme digestion experiments (using Lys-C) to distinguish between isobaric Lys and Gln. Lys-C cleaves at the C-terminal end of Lys.

The sequencing of peptides using positive ion mass spectrometry has been used for some time and has been extensively reported [48]. Peptide structural information is provided by peptide fragmentation, which is initiated by the collision activation of the chosen peptide. In the positive mode, peptides undergo characteristic mass spectral fragmentations. We have predominantly used B and Y+2 cleavage ions to elucidate the primary structure of peptides [48] (Figure 1.5). These fragmentations occur as a result of cleavage between the carbon and nitrogen atoms of the peptide bond. Sequencing information from the C-terminal end of the peptide is supplied by simple B fragmentations, whilst Y+2 fragmentations provide sequencing information from the N-terminal [49].

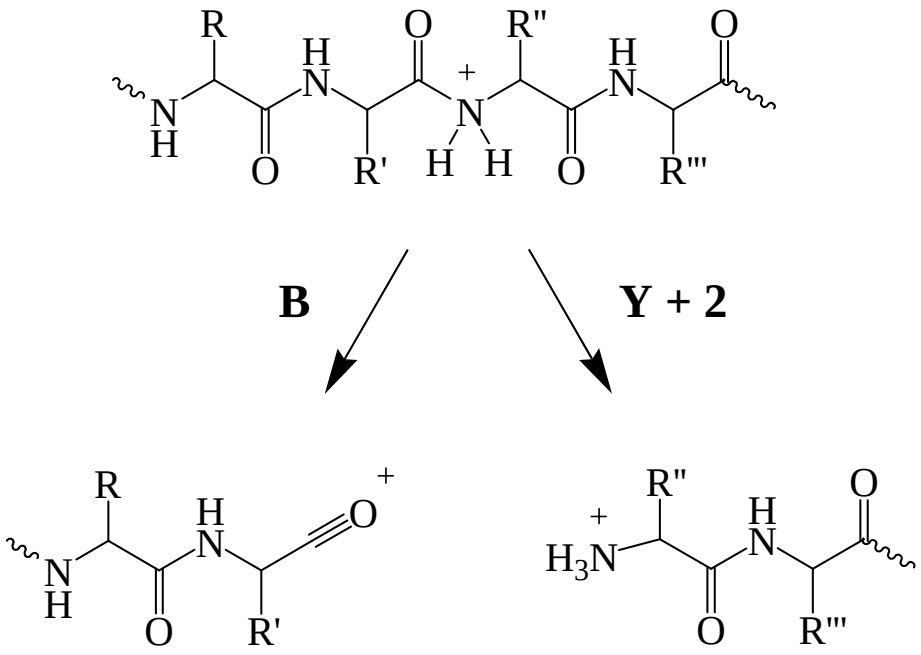


Figure 1.5. The B and Y+2 fragmentation ions formed in the positive ion mode

Negative ion mass spectrometry can also assist in the primary sequencing of peptides. The α and β cleavage processes provide analogous information to that provided by the B and Y+2 fragmentations in the positive mode. The α cleavage process provides sequence information from the N-terminal end of the peptide, while sequence information from the C-terminal end is provided by β cleavages (Figure 1.6) [50].

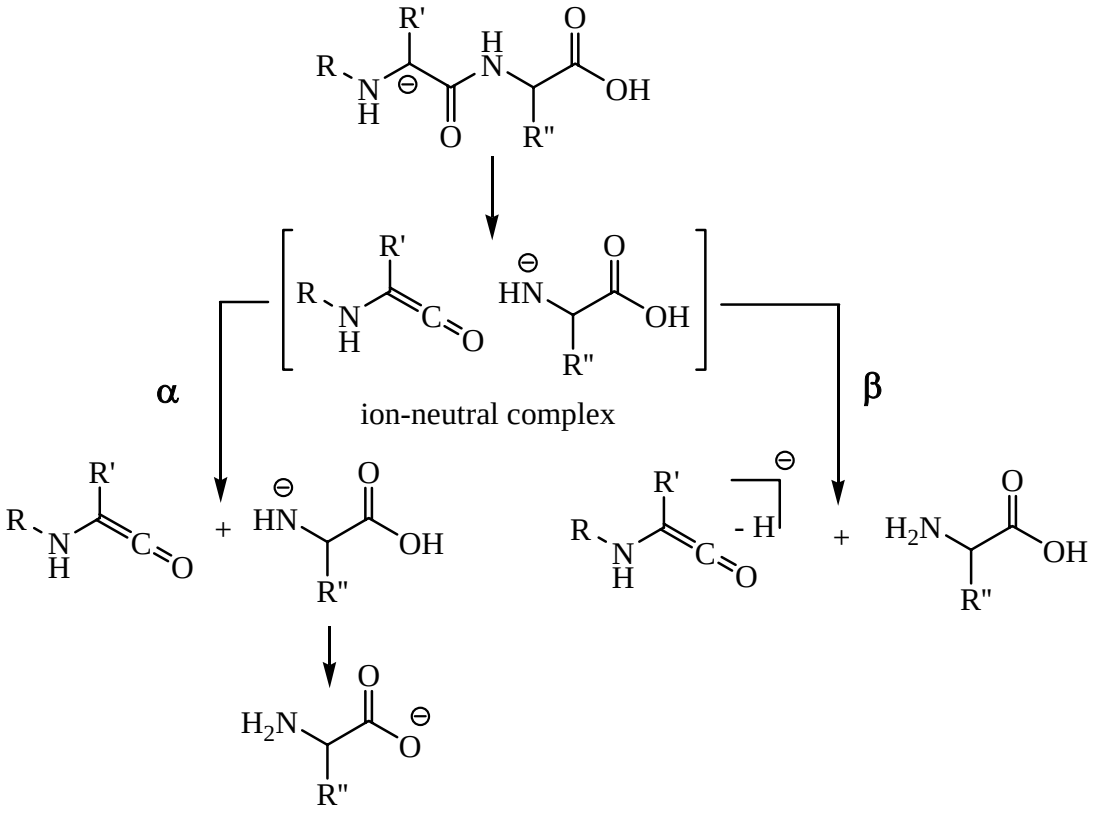


Figure 1.6. The α and β fragmentation ions formed in the negative ion mode

Several other cleavages have also been discovered that provide additional sequence information. These cleavages identify specific residues and/or the position of these residues in the peptide. These fragmentations involve backbone cleavages initiated from specific residues. The fragmentations result from cleavage of the bond between the NH and the αC, generating two possible ions depending on where the charge resides. They are δ (charge resides on the N-terminal fragment) and γ (charge resides on the C-terminal fragment) cleavage ions (Figure 1.7). Amino acid residues that undergo this type of fragmentation are Ser [50], Thr [50], Glu [50], Cys [51], Gln [50], Asp [50], Asn [50], and Phe [52].

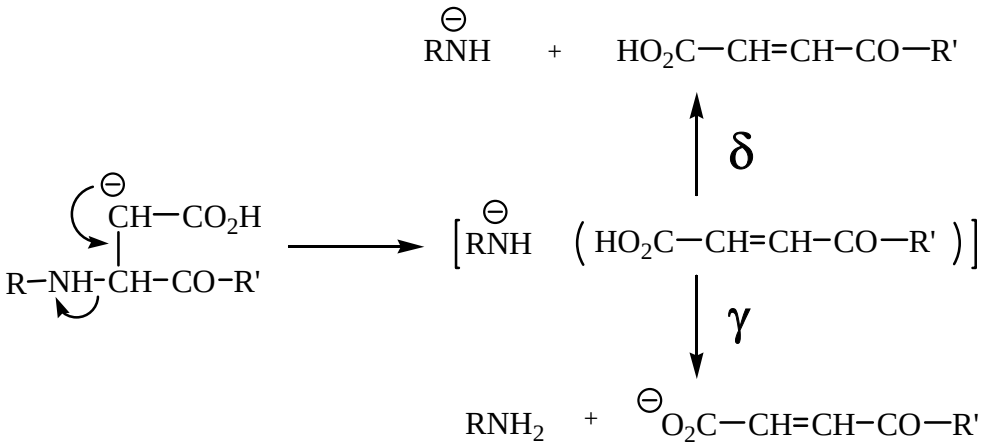


Figure 1.7. The mechanism for the formation of the δ and γ fragmentation ions formed from Asp

1.5b Automated Edman Sequencing

A standard procedure is used for all automated Edman sequencing using an applied Biosystem 470A proscribe sequencer equipped with a 900A data analysis module [53]. Determination of the primary peptide sequence occurs by the sequential removal of amino acids from the N-terminal end of the peptide. Each residue is identified by its HPLC retention time. Limitations to this technique [53] include peptides with blocked N-terminal ends that are unable to be processed, as the blocked group prevents coupling with phenyl isothiocyanate. Modified or uncommon peptides are also unable to be identified. Also, as the peptide is sequentially cleaved and its size decreases, it becomes more soluble and is likely to be washed from the solid support, losing the remaining portion of the peptide. Thus Edman sequencing is not always successful but can provide complementary information to mass spectrometric sequence data.

1.5c Enzyme Digestion

Enzyme digestion is used in conjunction with mass spectrometry to enable further sequencing information [54]. Enzyme digestion of peptides cleaves particular amide bonds to result in smaller fragments, which are subsequently analysed by mass spectrometry. Enzyme digestion using Lys-C endoprotease can be used to differentiate between lysine and glutamine residues. Cleavage occurs at the carboxyl side of the lysine residue (Figure 1.8).

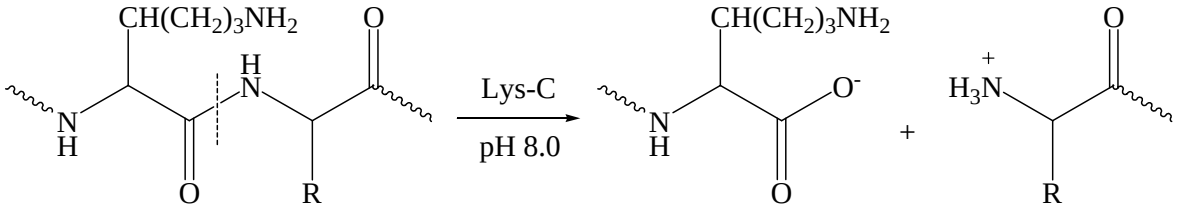


Figure 1.8. Enzyme digestion of peptides using Lys-C

1.5d Determination of the C-terminal End Group

Amphibian peptides possess either C-terminal CO_2H or $CONH_2$ end groups. Differentiation of C-terminal end groups is identified by methylation of peptides [55]. This is achieved by converting peptides to their methyl esters and analysing the resulting molecular weight by mass spectrometry. The mass of the MH^+ parent ion of the methyl ester minus the mass of the original MH^+ peptide allows the determination of the number of CO_2H and $CONH_2$ groups in the peptide. Upon methylation, an amide group will show an increase of 15 Da, whilst an acid group will display an increase of 14 Da (Figure 1.9).

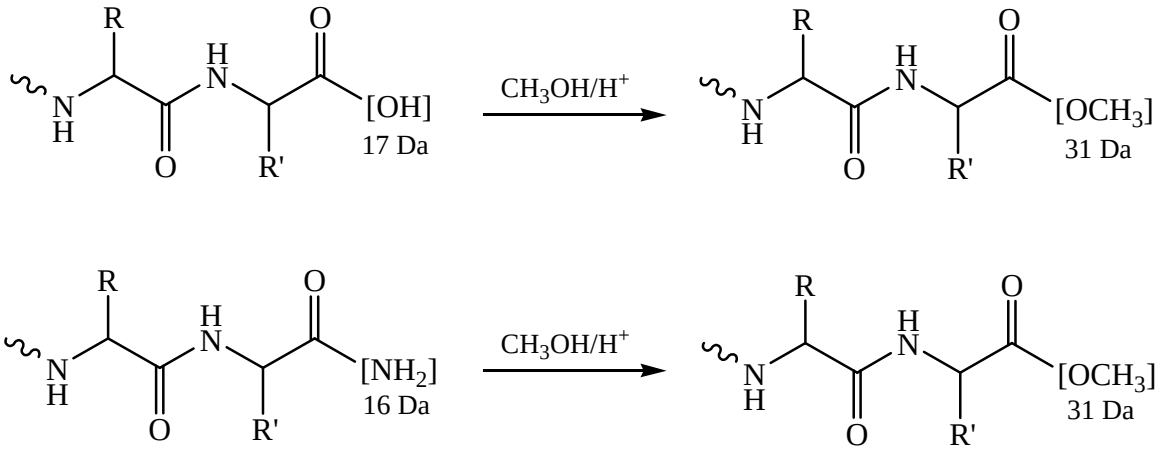


Figure 1.9. Methylation of peptides to determine the C-terminal end group

1.6 Bioactivity Testing

Isolated peptides were subjected to a number of biological tests. Peptides were synthesised to confirm the sequence of a natural peptide and to enable sufficient material for testing. Peptides were synthesised commercially by Genscript (U.S.A.) using L-amino acids through a standard N-α-Fmoc procedure [56]. HPLC and mass spectrometry confirmed all synthetic peptides to be identical to native peptides.

Antibacterial testing was carried out by the Microbiology Department at the Institute of Medical and Veterinary Science (IMVS), Adelaide, using the minimum inhibitory concentration (MIC) assay [57]. Synthetic peptides were measured against a number of microorganisms, with activities recorded as MIC values. These represent the minimum concentration of the test peptide required to completely inhibit the growth of a particular microorganism. If no inhibition is detected at concentrations less than 100 µg/mL, the peptide is deemed 'inactive' against this organism.

Peptides were screened for anticancer activity by the National Cancer Institute (Washington, D.C., U.S.A) by assessing the chemosensitivity of a number of human tumour cell lines to a synthetic peptide [58].

Neuronal nitric oxide synthase inhibition testing was conducted by the Australian Institute of Marine Science (Queensland, Australia). Inhibition was measured by monitoring the concentration of peptide that inhibited the reaction of [^{3}H]-arginine to [^{3}H]-citrulline by 50% [21].

Neuropeptide activity testing was conducted in the Department of Experimental and Clinical Pharmacology, the University of Adelaide, under the guidance of Dr. I. F. Musgrave. Pharmacological activity was determined by monitoring concentration-dose dependent curves using a smooth muscle contraction assay and a lymphocyte proliferation assay.

Chapter 2- Studies of Skin Secretions from the *Crinia* Genus

2.1 Introduction

2.1a General

The *Crinia* genus, belonging to the family Myobatrachidae, consists of fourteen species of amphibian that are distributed throughout Australia and New Guinea. Common traits displayed by this polymorphic genus include body size, a variety of dorsal patterns, long and unwebbed fingers and toes, smooth or granular skin and there may be a tympanum present [8].

We were initially interested in studying skin components from this genus as previous studies isolated a potent hypotensive neuropeptide, crinia angiotensin II **(1)** within skin secretions of the Australian frog *Crinia georgiana* [59]. This peptide has a structural relationship to human angiotensin **(2)**.

(1) Crinia angiotensin II APGDRIYVHPF-OH

(2) Human angiotensin DRVYIHPF-OH

Recently, we reported the isolation and sequence determination of the signiferin peptides from the skin secretion of the Common Froglet, *Crinia signifera* (Figure 2.1) [22], with sequences listed in Table 2.1. *C. signifera* is widely distributed throughout Australia (Figure 2.2). The major peptide, signiferin 1, is a potent disulfide containing neuropeptide of a type not observed in other Australian anurans that have been previously investigated [60]. The remaining signiferin peptides demonstrate either antibiotic activity or inhibit the formation of the ubiquitous chemical messenger NO from neuronal nitric oxide synthase (nNOS) (see Table 2.1).

Figure 2.1. *Crinia signifera*

Figure 2.2. The distribution of *Crinia signifera*

Table 2.1. Name, sequence and activity of major peptides identified from *Crinia signifera*

Signiferin	Sequence	MW	Activity
1	RLCIPYIIPC-OH	1187	a
2.1	IIGHLIKTALGMLGL-NH_2	1547	b,c
2.2	IIGHLIKTALGFLGL-NH_2	1563	b
3.1	GIAEFLNYIKSKA-NH_2	1451	d
4.1	GFADIFGKVANLIKS-NH_2	1577	e
4.2	GFADLFGKAVDFIKS-NH_2	1613	e
4.3	GFADLFGKAVDFIKSRV-NH_2	1868	f

(a) neuropeptide (smooth muscle active at 10^{-9} M); (b) wide-spectrum antibiotic; (c) anticancer agent; (d) nNOS inhibitor (IC_{50}, 81 µg/mL); (e) not tested, but likely to be nNOS inhibitors; (f) nNOS inhibitor (IC_{50}, 40 µg/mL)

This chapter further examines the peptide profile of this genus, with the isolation and sequence determination of peptides from skin secretions of *Crinia riparia* and *Crinia deserticola*. Peptide profiling assists with identifying different species of anuran, identifying different populations of the same species and investigating evolutionary trends [8].

Crinia riparia, commonly known as the Littlejohn and Martin Streambank Froglet (Figure 2.3), is South Australia's only endemic frog. Its distribution is uniquely located to the southern end of the Flinders Ranges, South Australia (Figure 2.4). Skin patterns vary greatly in colour, including black, brown or grey. Anurans live close to fast flowing streams and are found beneath boulders and stones on the edge of rock-strewn creeks [8]. Males range between 16.0 - 22.0 mm in length and females are between 19.0 - 25.0 mm in length. *Crinia riparia* can often be mistaken for *C. signifera* at the border of the southern end of the Flinders Ranges [8].

Figure 2.3. *Crinia riparia*

Figure 2.4. The distribution of *Crinia riparia*

Crinia deserticola, the Desert Froglet (Figure 2.5), exhibits less variability than other species. Individuals are pale grey in colour with complex triangular and rectangular markings along the middle of the back. Their distribution covers a broad geographic range in arid and tropical areas (Figure 2.6). *C. deserticola* assemble close to static temporary or permanent water bodies, sheltering under leaves, corrugated iron, timber and similar materials [8]. Males range in size from 13 - 18 mm and females from 15 - 20 mm in length. *C. deserticola* may be confused with other small frogs like *Uperoleia capitulata* and *U. rugosa*, but can be distinguished by their call, which resembles the chirruping sound of a sparrow [8].

Figure 2.5. *Crinia deserticola*

Figure 2.6. The distribution of *Crinia deserticola*

2.1b Cyclic Peptides

Cyclic peptides are found within a variety of species, including anurans from the European and Indian frogs of the *Rana* genus. They exhibit a range of properties including both pharmacological and antibacterial activity (Table 2.2).

Table 2.2. A selection of cyclic peptides and their activities

Peptide	Sequence	Location	Activity
Urotensin II [61]	ETPDCFWKYCV-OH	Vascular and cardiac tissue	Most potent mammalian vasoconstrictor
Endothelin I [16, 61]	CSCSSLMDKECVYPC-OH	Vascular endothelial cells	Endothelium vasoconstrictor
Brevinin I [62]	FLPVLAGIAAKVVPALFCKITKKC-OH	*Rana brevipoda* skin secretion	Antimicrobial
Tigerinin I [63]	FCTMIPIPRCY-OH	*Rana tigerina* skin secretion	Antimicrobial

Only recently have a unique group of Cys containing peptides been discovered among Australian amphibia from the *Crinia* genus [22, 23]. The cyclic peptides are often the major glandular component in the skin. Their biological and chemical properties are intriguing and their role in amphibian skin was initially unknown. At least one cyclic peptide exists in each *Crinia* species. Each peptide has a unique structure containing a disulfide bridge between two cysteine amino acid residues.

The peptides, named signiferin 1 (Table 2.1) and the riparins 1 (Table 2.3), are potent neuropeptides that contract smooth muscle tissue and/or proliferate lymphocytes via CCK_2 receptors. The biological activities are discussed in more detail in section 5.5.

2.2 Host-Defence Compounds from *Crinia riparia*

2.2a Results

2.2.1a Isolation and Sequence Determination of Active Components

Specimens of *Crinia riparia* were collected in field trips to the Flinders Ranges, South Australia. Skin secretions were acquired from the dorsal glands of eight amphibians by the surface electrical stimulation method [4]. Eight major peptides were identified within skin secretions, as shown in the HPLC chromatogram (fractions labelled A - H) (Figure 2.7). Material eluted prior to 16 minutes has not been investigated. Mass spectrometry and automated Edman sequencing identified the amino acid sequences (Table 2.3), which have been named riparins. The riparins are classed into four structural groups, with the structure determination of peptides described later in this section. A selection of peptides were synthesised and found to be identical to the native peptide by HPLC and mass spectrometry. Biological tests were conducted on synthetic peptides.

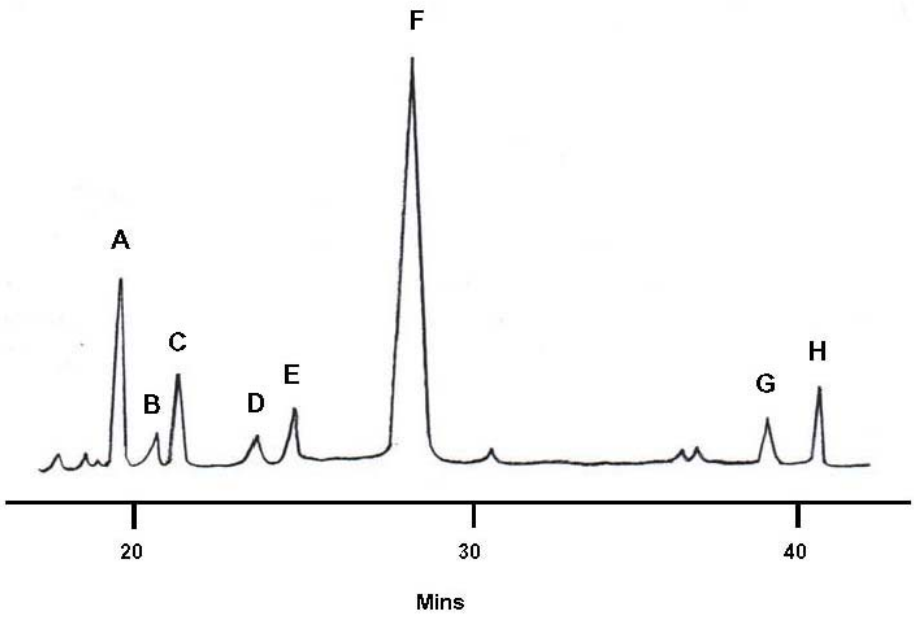


Figure 2.7. HPLC chromatogram of the glandular secretion from *Crinia riparia*

All peptides are unique to *C. riparia*, except signiferin 3.1, which is also produced by *C. signifera*.

Table 2.3. Name, sequence and activity of major peptides identified from *Crinia riparia*

Name	Sequence	MW	Activity	HPLC fraction
Riparin 1.1	RLCIPVIFPC-OH	1157	a	G
Riparin 1.2	FLPPCAYKGTC-OH	1196	a	C
Riparin 1.3	FPLPCAYKGTYC-OH	1359	b	D
Riparin 1.4	FFLPPCAYKGTC-OH	1343	b	F
Riparin 1.5	FFLPPCAHKGTC-OH	1317	b	E
Riparin 2.1	IIEKLVNTALGLLSGL-NH_2	1651	c	H
Signiferin 3.1	GIAEFLNYIKSKA-NH_2	1451	d	A
Riparin 5.1	IVSYPDDAGEHAHKMG-NH_2	1724	e	B

(a) immunomodulator; (b) not tested, but likely to be an immunomodulator; (c) narrow-spectrum antibiotic (active against *Leuconostoc lactis* at MIC 25 µM); (d) nNOS inhibitor (IC_{50}, 81 µg/mL); (e) no activity shown in our bioactivity screening programme

Peptide primary structures were principally determined using a combination of electrospray ionisation mass spectrometry (ESMS), methylation and enzymatic digest. C-terminal end groups were determined by calculating the difference in molecular weight of the parent ion and the methyl ester using mass spectrometry. Enzymatic digest using Lys-C differentiated between lysine and glutamine to identify the position of lysine residues. Automated Edman sequencing differentiated between leucine and isoleucine and was employed to confirm the primary peptide sequence.

2.2.2a The Riparin 1 Neuropeptides

The majority of the glandular contents of *C. riparia* consist of the riparin 1 group of peptides (riparin 1.1 - riparin 1.5). Analysis by positive ion mass spectrometry did not result in significant sequence information; for example, it does not immediately identify the presence of a disulfide bond. This problem was resolved utilising certain characteristic fragmentation patterns in the negative ion mode [64].

Analysis of riparin 1.1 by positive ion mass spectrometry shows a MH^+ peak at *m/z* 1158. The MS/MS of the MH^+ parent ion is shown in Figure 2.8. The B and Y+2 fragmentations were minimal, suggesting that riparin 1.1 is a cyclic peptide [65]. The data identifies the first two residues, Arg and Leu, corresponding to peaks *m/z* 157 and 270. The remaining peaks in this spectrum are formed by B cleavages within the disulfide unit, together with subsequent fragmentation of these ions.

RLCIPVIFPC-OH

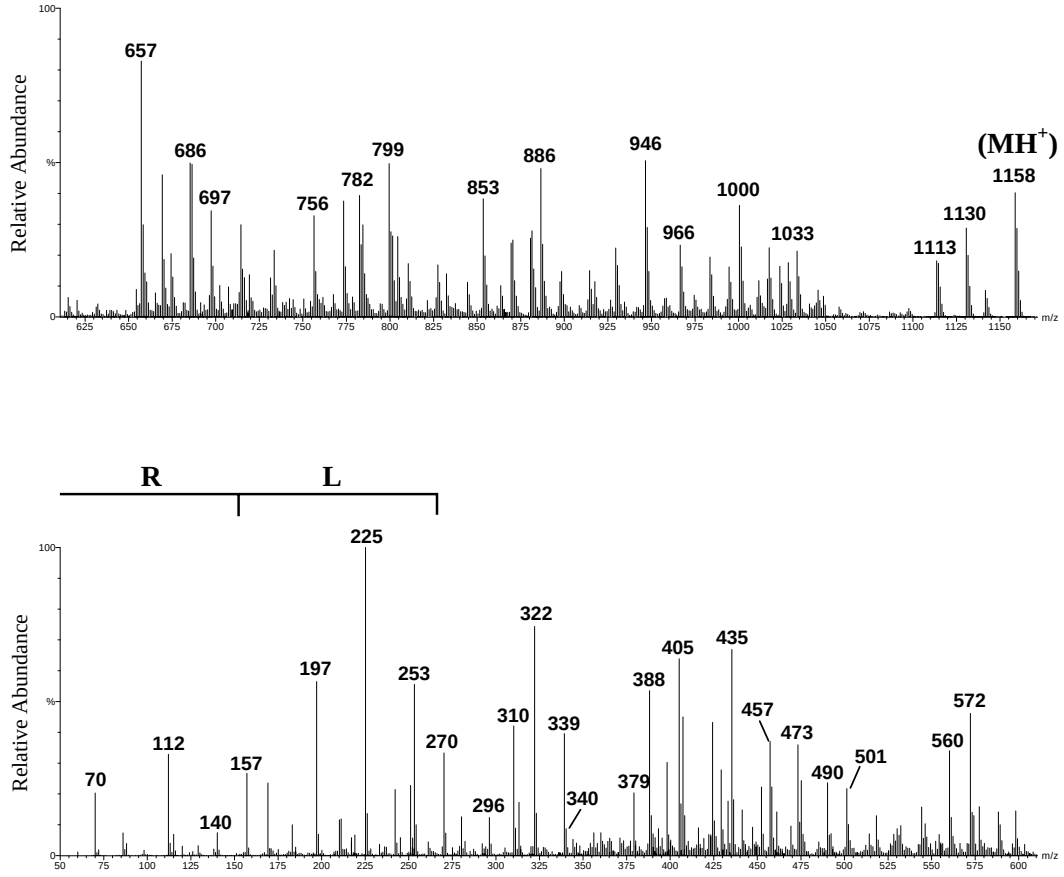


Figure 2.8. Mass spectrum (MS/MS) of the MH^+ parent ion of riparin 1.1

Negative ion mass spectrometry provides greater insight into the sequence of riparin 1.1. The MS/MS of the $(M-H)^-$ parent ion is as follows: [*m/z* (loss) relative abundance]; *m/z* 1156 $[(M-H)^-]$ 3; 1138 (H_2O) 2; 1090 (H_2S_2) 10; 1078 (CH_2S_2) 12; 1046 [(H_2S_2 + CO_2)] 100%. Figure 2.9 displays the MS/MS/MS data of the base peak of the spectrum, *m/z* 1046. This spectrum is also complex, however, identification of a disulfide link is achieved and it provides information on the position of Cys residues, with one at the C-terminal end [64].

Sequence information is provided by the presence of peaks corresponding to eight α and five β backbone cleavages (for review see [50]). α cleavages are shown schematically above the spectrum, while the β cleavages are displayed below the spectrum.

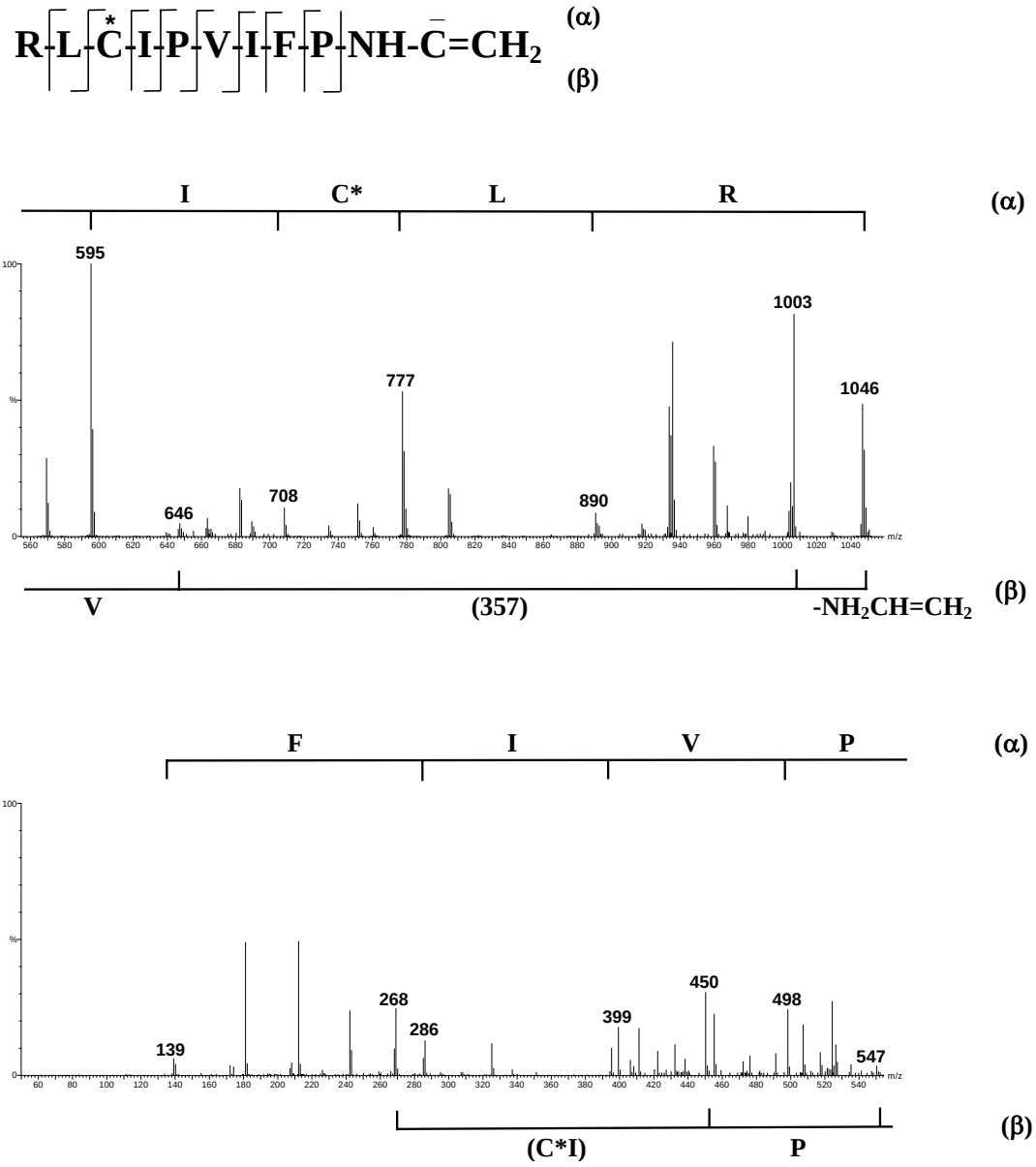


Figure 2.9. Mass spectrum (MS/MS/MS) of the $[(M-H)^- - (H_2S_2 + CO_2)]^-$ fragment anion of riparin 1.1. C* is $NHC(=CH_2)CO$

A combination of positive and negative ion mass spectrometry, together with Edman sequencing, gives the full sequence of riparin 1.1 as Arg Leu Cys Ile Pro Val Ile Phe Pro Cys-OH.

The remaining riparin 1 peptides contain either four or five residues outside the disulfide moiety at the N-terminal end of the peptide.

The positive ion mass spectrum of riparin 1.3 shows a MH^+ peak at *m/z* 1360 (Figure 2.10). Only residues outside the disulfide moiety are identified, but no sequence data within the disulfide ring are provided. From the data obtained from B and Y+2 cleavages, the sequence FPLP on the N-terminal side of the disulfide may be identified.

FPLPCAYKGTYC-OH

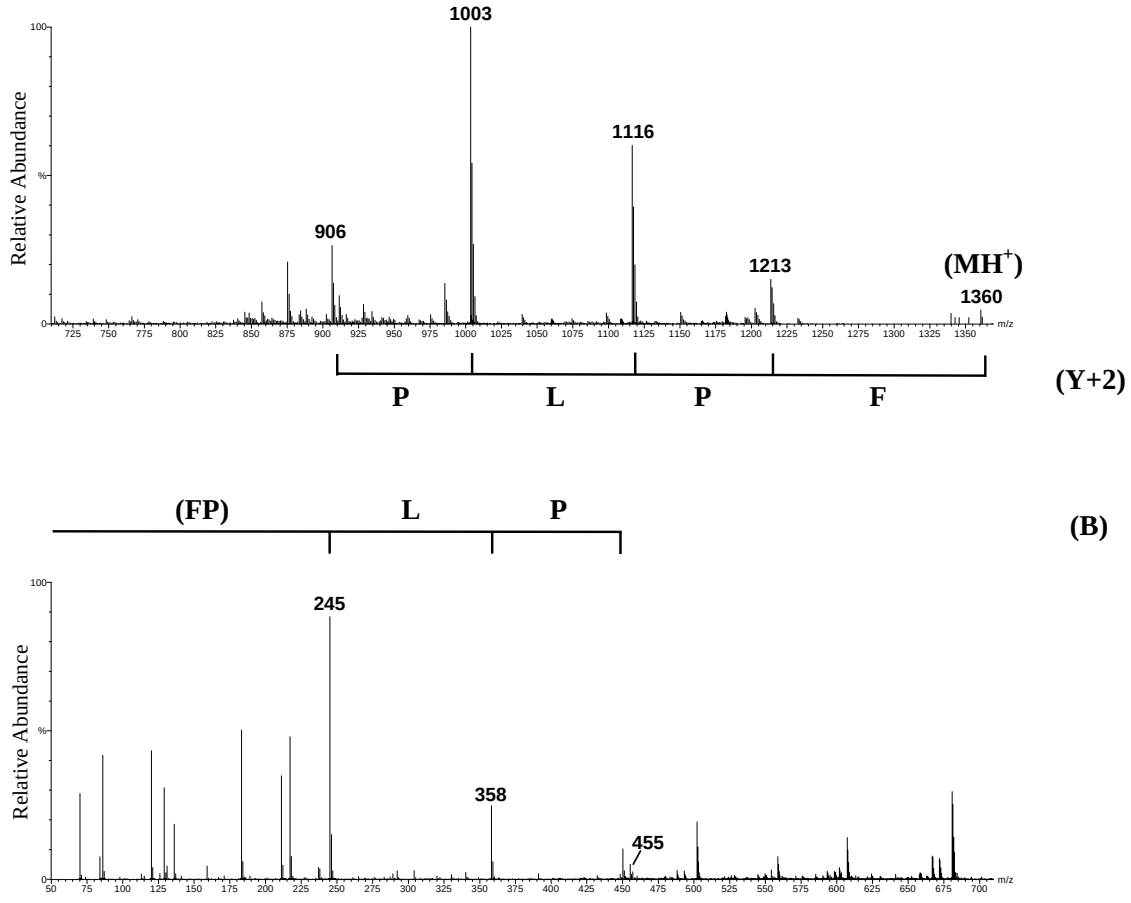


Figure 2.10. Mass spectrum (MS/MS) of the MH^+ parent ion of riparin 1.3

The mass spectrum of the $(M-H)^-$ parent ion is as follows: [mass (loss) relative abundance]; *m/z* 1358 [$(M-H)^-$] 100; 1292 (H_2S_2) 48; 1248 [(H_2S_2 + CO_2)] 56 and 1204 [(H_2S_2 + CO_2 + MeCHO)] 96%. Figure 2.11 displays the MS/MS/MS data obtained from the base peak of the spectrum, *m/z* 1204. The negative ion mass spectrum is complex, however, the peptide sequence can be determined by the nine α and four β cleavages.

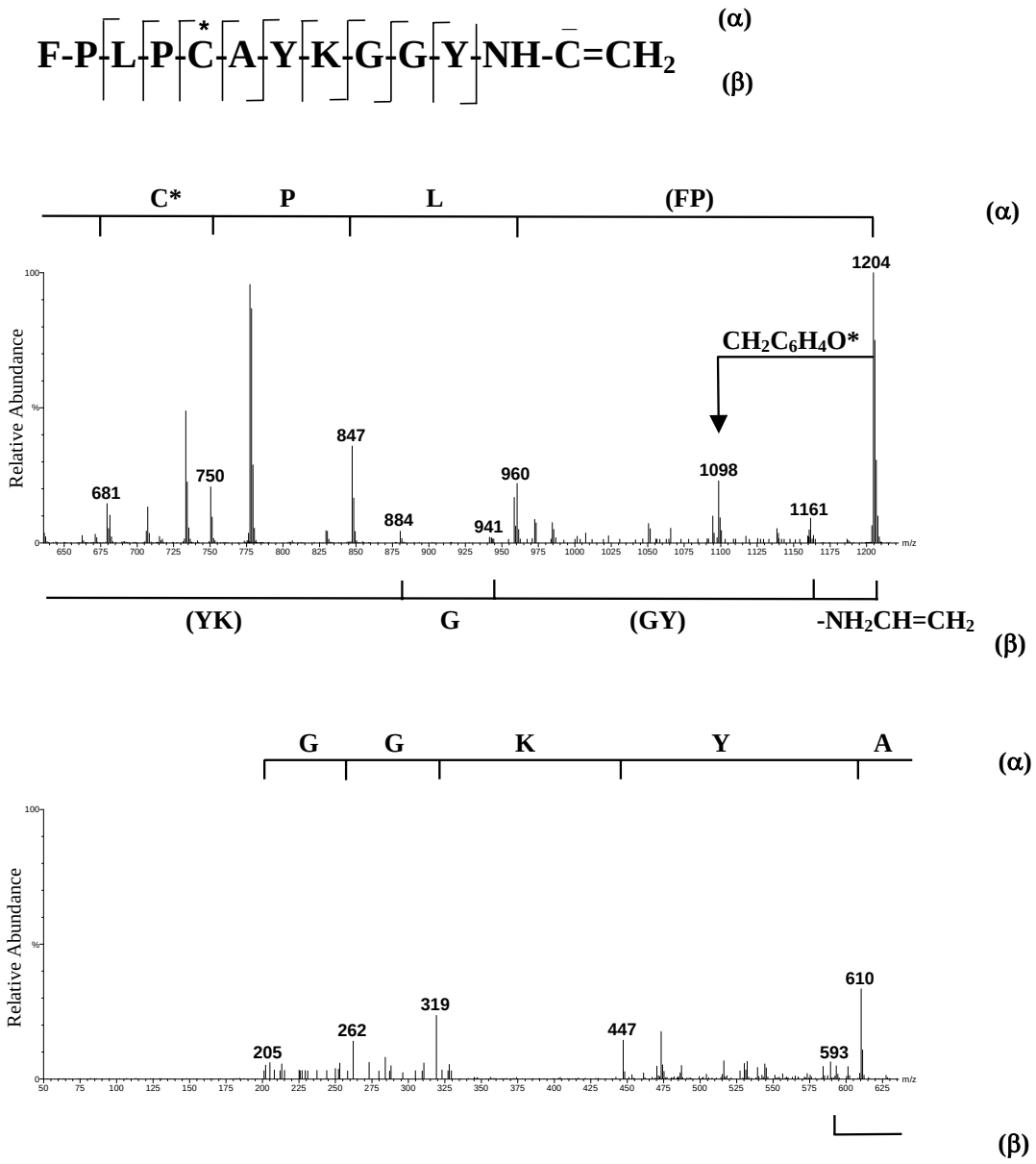


Figure 2.11. Mass spectrum (MS/MS/MS) of the $[(M-H)^- - (H_2S_2 + CO_2 + MeCHO)]^-$ fragment anion of riparin 1.3. C* is $NHC(=CH_2)CO$

Using a combination of positive and negative ion mass spectrometry and automated Edman degradation, the final sequence of riparin 1.3 is Phe Pro Leu Pro Cys Ala Tyr Lys Gly Thr Tyr Cys-OH.

The sequence data of the remaining riparin 1 group of peptides are summarised below (Table 2.4).

Table 2.4. Mass spectral data for riparin 1 peptides isolated from *Crinia riparia*

Riparin 1.2			
MH^+ 1197	Y+2 ions	*m/z*	1050, 937, 840, 743
			[FL(PP)(743)]
$(M-H)^-$ 1195	MS/MS high mass region [*m/z* (loss) relative abundance]: 1195 $(M-H)^-$ 45; 1129 (H_2S_2) 47; 1085 (H_2S_2 + CO_2) 100; 1041 (H_2S_2 + CO_2 + MeCHO) 25%		
	MS/MS/MS	*m/z*	1041
	α ions	*m/z*	781, 587, 518, 447, 284
			[(FL)(PP)C*AY(KGG-$NHCCH_2$)]
	β ions	*m/z*	998, 941, 884, 756, 593, 522, 356
			[357(PC*)AYKGG-$NHCCH_2$]

Riparin 1.2 full sequence:
FLPPCAYKGTC-OH

Riparin 1.4			
MH^+ 1344	B ions	*m/z*	602, 505, 408, 295
			[(FF)L(PP)]
	Y+2 ions	*m/z*	1197, 1050, 937, 840, 743
			[(FF)L(PP)]

$(M-H)^-$ 1342	MS/MS high mass region [*m/z* (loss) abundance]: 1342 $(M-H)^-$ 38; 1308 (H_2S) 43; 1276 (H_2S_2) 35; 1232 (H_2S_2 + CO_2) 100; 1188 (H_2S_2 + CO_2 + MeCHO) 25%		
	MS/MS/MS	*m/z*	1188
	α ions	*m/z*	587, 518, 447, 284, 156, 99 [(601)C*AYKGG(42)]
	β ions	*m/z*	1145, 1088, 1031, 903, 600, 503, 406, 293 [(FF)L(PP)(C*AY)KGG-NHCCH$_2$]

Riparin 1.4 full sequence:
FFLPPCAYKGTC-OH

Riparin 1.5

MH^+ 1318	B ions	*m/z*	602, 505, 408, 295 [FFLPP]
	Y+2 ions	*m/z*	1171, 1024, 911, 814, 717 [FFLPP]
$(M-H)^-$ 1316	MS/MS high mass region [*m/z* (loss) abundance]: 1316 $(M-H)^-$ 26; 1282 (H_2S) 42; 1250 (H_2S_2) 38; 1206 (H_2S_2 + CO_2) 100; 1162 (H_2S_2 + CO_2 + MeCHO) 21%		
	MS/MS/MS	*m/z*	1162
	α ions	*m/z*	561, 492, 421, 284 [(601)C*AH(284)]
	β ions	*m/z*	1119, 1062, 1005, 877, 740, 503, 406, 293 [(FF)LP(PC*A)HKGG-NHCCH$_2$]

Riparin 1.5 full sequence:
FFLPPCAHKGTC-OH

2.2.3a Riparin 2.1

Riparin 2.1 was purified by HPLC and analysed initially by mass spectrometry. An MH^+ peak occurred at *m/z* 1652. ESMS identified the first fourteen amino acid residues from the C-terminal end of the peptide using B fragmentations, and twelve amino acid residues from the N-terminal end were determined from Y+2 fragmentations (Figure 2.12). Lys-C digestion experiments confirmed residue four as Lys. The sequence of riparin 2.1 is `Ile Ile Glu Lys Leu Val Asn Thr Ala Leu Gly Leu Leu Ser Gly Leu-NH2`, from a combination of MS data and Edman sequencing. Details of peptide data are shown in Table 2.5.

IIEKLVNTALGLLSGL-NH_2

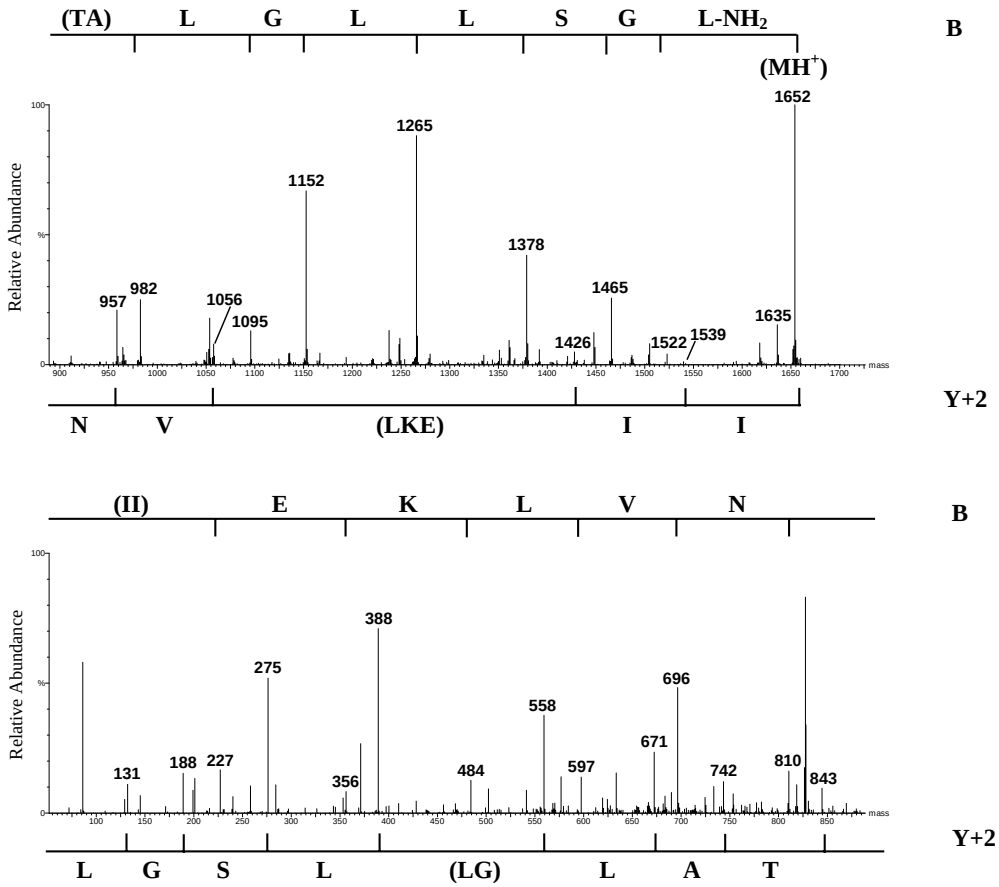
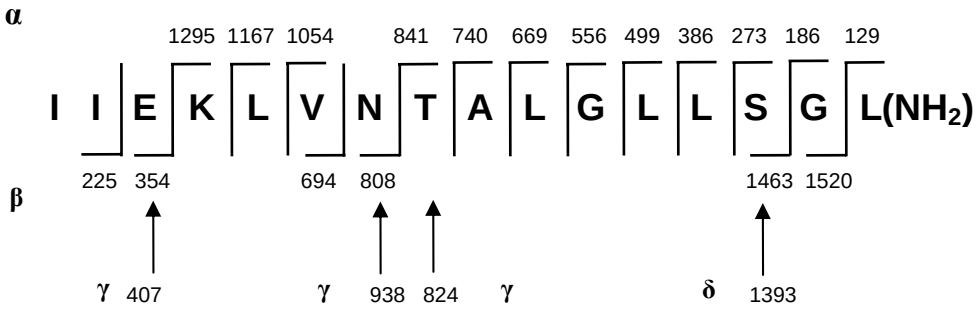


Figure 2.12. Positive ion mass spectrum (MS/MS) of riparin 2.1

Table 2.5. Sequence data of riparin 2.1

Riparin 2.1

MH^+ 1652	B ions	*m/z*	1635, 1522, 1465, 1378, 1265, 1152, 1095, 982, 810, 696, 597, 484, 356, 227
			[(II)E(K/Q)LVN(TA)LGLLSGL-NH_2]
	Y+2 ions	*m/z*	1539, 1426, 1056, 957, 843, 742, 671, 558, 388, 275, 188, 131
			[II(370)VNTAL(170)LSG(L-NH_2)]
$(M-H)^-$ 1650	MS/MS details provided on the formula below. There is no γ ion corresponding to Gln4 at *m/z* 1181: residue 4 is therefore Lys.		



Riparin 2.1 full sequence:

IIEKLVNTALGLLSGL-NH_2

2.2.4a Signiferin 3.1

The MH^+ value for signiferin 3.1 is *m/z* 1452. The positive ion mass spectrum identifies the first eleven amino acid residues from the C-terminal end of the peptide, and the first eleven amino acid residues from the N-terminal end of the peptide. Methylation experiments confirmed the C-terminal end group to be an amide. Lys-C digestion indicated residues 10 and 12 as Lys. Mass spectrometry together with Edman degradation confirmed the sequence as Gly Ile Ala Glu Phe Leu Asn Tyr Ile Lys Ser Lys Ala-NH_2. Table 2.6 lists cleavage data obtained for this peptide.

Table 2.6. Sequence data of signiferin 3.1

Signiferin 3.1			
MH^+ 1452	B ions	*m/z*	1435, 1364, 1236, 1149, 1021, 908, 745, 631, 518, 371, 242, 171 [AEFLNYIKSKA-NH_2]
	Y+2 ions	*m/z*	1395, 1282, 1211, 1082, 935, 822, 708, 545, 432, 304, 217 [GIAEFLNYIKS]

Signiferin 3.1 full sequence:
GIAEFLNYIKSKA-NH_2

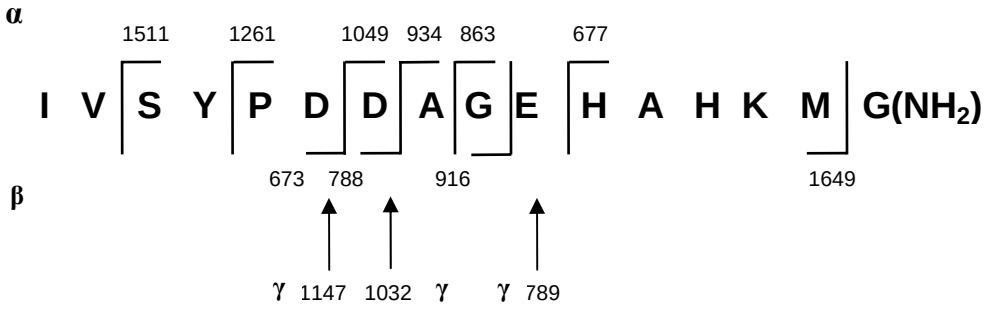
2.2.5a Riparin 5.1

Riparin 5.1 has a MH^+ value of *m/z* 1725. B cleavage ions determines the first ten amino acid residues from the C-terminal end, while Y+2 fragmentations identifies the first thirteen amino acid residues from the N-terminal end. Negative ion cleavages are shown in Table 2.7. Mass spectral data together with Edman sequencing confirmed the peptide sequence as `Ile Val Ser Tyr Pro Asp Asp Ala Gly Glu His Ala His Lys Met Gly-NH2`. Full sequencing data are shown in Table 2.7.

Table 2.7. Sequence data of riparin 5.1

Riparin 5.1

MH^+ 1725	B ions	*m/z*	1708, 1651, 1520, 1255, 1184, 861, 790, 463, 300, 213
			`[(IV)SY(327)A(323)A(265)MG-NH2]`
	Y+2 ions	*m/z*	1612, 1513, 1426, 1263, 1166, 1051, 936, 865, 808, 679, 542, 471, 334
			`[IVSYPDDAGEHAH(K/Q)(MG-NH2)]`
$(M-H)^-$ 1723	MS/MS details provided on the formula below.		



Riparin 5.1 full sequence:
`IVSYPDDAGEHAHLMG-NH2`

2.2b Discussion

The skin secretion of *Crinia riparia* has been analysed, with eight peptides isolated, seven of them novel. These peptides were named riparins [23].

The major glandular components contained unique cyclic peptides, similar to the signiferin 1 peptide isolated from *C. signifera*. Previous work indicated that signiferin 1 is a potent neuropeptide that contracts smooth muscle at a concentration of 10^{-9} M [22, 66]. Following this finding, riparin 1.1 and riparin 1.2 were also tested for pharmacological activity. Interestingly, the riparins were not active on smooth muscle cells but results demonstrate they proliferate lymphocytes at a concentration of 10^{-7} M via the CCK_2 receptor [66]. The pharmacological results of the cyclic neuropeptides are discussed in more detail in section 5.5.

The remaining peptides were tested for antibacterial activity. Generally, amphibian antibacterial peptides although active against both Gram-positive and Gram-negative organisms, show greater activity against Gram-positive organisms [26]. Antibacterial activity of a peptide can often be indicated by an Edmundson wheel projection, if it illustrates the characteristic separate hydrophilic and hydrophobic zones of amphipathic antibacterial peptides (see Figure 2.13 as an example).

Riparin 2.1 **(3)** is a narrow-spectrum antibiotic: it is active against the Gram-positive organism *Leuconostoc lactis* at MIC 25 µM. The corresponding peptides from *C. signifera*, signiferin 2.1 **(4)** and signiferin 2.2 show moderate antibacterial activity against the same organism. No potent broad-spectrum antibacterial peptides were identified from *C. riparia*.

(3) Riparin 2.1 IIEKLVNTALGLLSGL-NH_2

(4) Signiferin 2.1 IIGHLIKTALGMLGL-NH_2

The Edmundson wheel projection of riparin 2.1 displays two moderately defined zones (Figure 2.13), suggesting the possibility of biological activity.

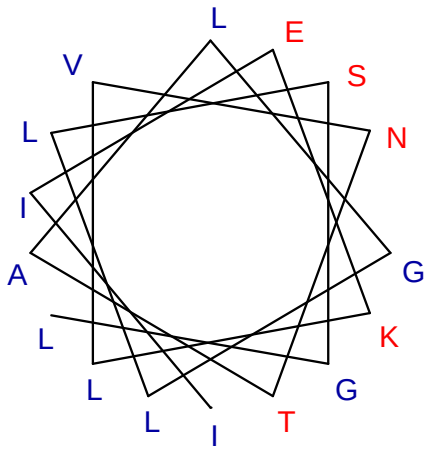


Figure 2.13. The Edmundson wheel projection of riparin 2.1. The hydrophilic zone shown in red and hydrophobic zone shown in blue

Signiferin 3.1, which is also present in the skin secretion of *C. signifera*, displays no antibacterial or anticancer activity. As this peptide must play a role in the amphibian integument, it was tested for neuronal nitric oxide synthase inhibition. There are three groups of peptide that inhibit nNOS [21], including the frenatins. Frenatin 3 **(5)** is characterised by a C-terminal acid together with two Lys residues near the C-terminus (indicated in bold), which may be significant for activity [21]. Signiferin 3.1 **(6)** also exhibits two Lys residues near the C-terminus. Signiferin 3.1 is not particularly active, inhibiting the formation of NO from nNOS at IC_{50} 81 µg/mL. Signiferin 3.1 may regulate the animal's physiology or act as a host-defence agent [67].

(5)	Frenatin 3	GLMSVLGHAVGNVLGGLF**K**P**K**-OH
(6)	Signiferin 3.1	GIAEFLNYI**K**S**K**A-NH_2

Riparin 5.1 does not exhibit sequence similarity to any other Australian amphibian peptides. It was tested for antibacterial, anticancer and pharmacological activity, but displayed no biological properties in our screening programme.

A recent study [68] investigated the cDNA cloning of the riparin 1 peptides of *C. riparia*. As an illustration, the precursor pre-pro-riparin 1.4 sequence is shown below:

pre (signal)	MKIIVVLAVLMLVSA
pro (spacer)	QVCLVSAAEMGHSSDNELSSRDLVKR
riparin 1.4	FFLPPCAYKGTC

The pre-pro sections of this precursor are very different from the conserved pre-pro sections of antibiotic peptides of *Rana* and *Litoria* species [69], suggesting the possibility that frogs of the genera *Litoria* and *Crinia* evolved from different ancestors (in the Devonian Period) [67].

2.3 Host-Defence Compounds from *Crinia deserticola*

2.3a Results

2.3.1a Isolation and Sequence Determination of Peptides

Adult specimens of *Crinia deserticola* were collected from parts of northern South Australia. Skin secretions were collected from the dorsal glands of five anurans by the surface electrical stimulation method. The HPLC chromatogram identified two peptides (Figure 2.14). Only one disulfide, signiferin 1 (fraction B), was identified. The only other fraction from which a peptide was identified was A. Material eluted prior to 30 minutes was also not investigated.

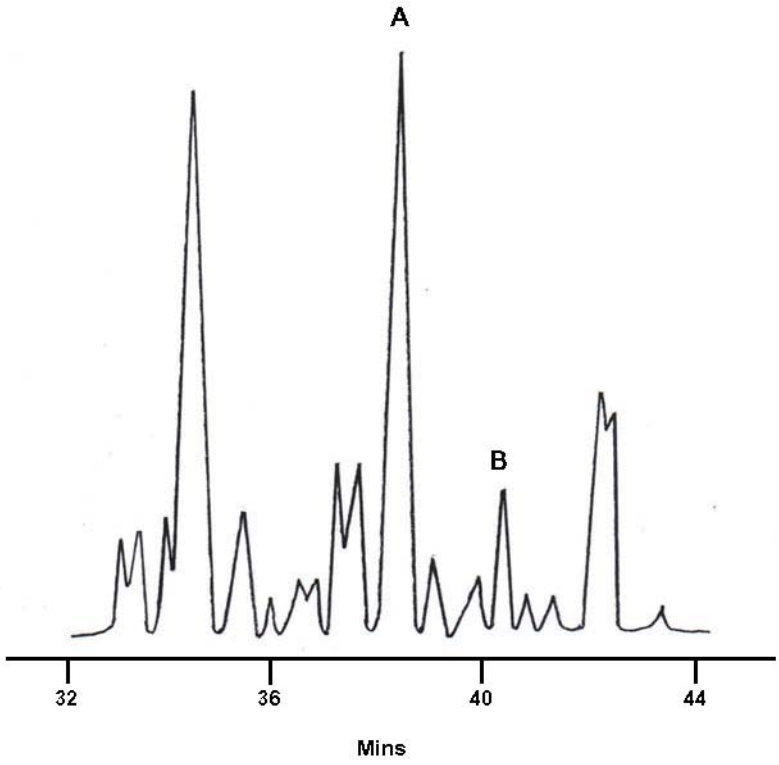


Figure 2.14. HPLC chromatogram of the glandular component from *Crinia deserticola*

The two identified peptides are listed in Table 2.8. The only disulfide, signiferin 1, is also the major glandular component in *C. signifera*.

Table 2.8. Name, sequence and activity of major peptides isolated from *Crinia deserticola*

Name	Sequence	MW	Activity	HPLC fraction
Signiferin 1	RLCIPYIIPC-OH	1187	a	B
Deserticolin 4.1	GLADFLNKAVGKVVDFVKS-NH_2	2004	b	A

(a) neuropeptide (contracts smooth muscle at 10^{-9} M); (b) not tested, but likely to be nNOS active

2.3.2a Signiferin 1

Analysis of signiferin 1 by positive ion mass spectrometry shows a MH^+ peak at *m/z* 1188 and is shown in Figure 2.15. The spectrum is difficult to interpret due to the presence of the disulfide bridge. B cleavage between Ile8 and Pro9 opens the peptide backbone, resulting in further B fragmentations to identify the residues IPYII within the disulfide moiety. A number of Y+2 fragmentations are also identified.

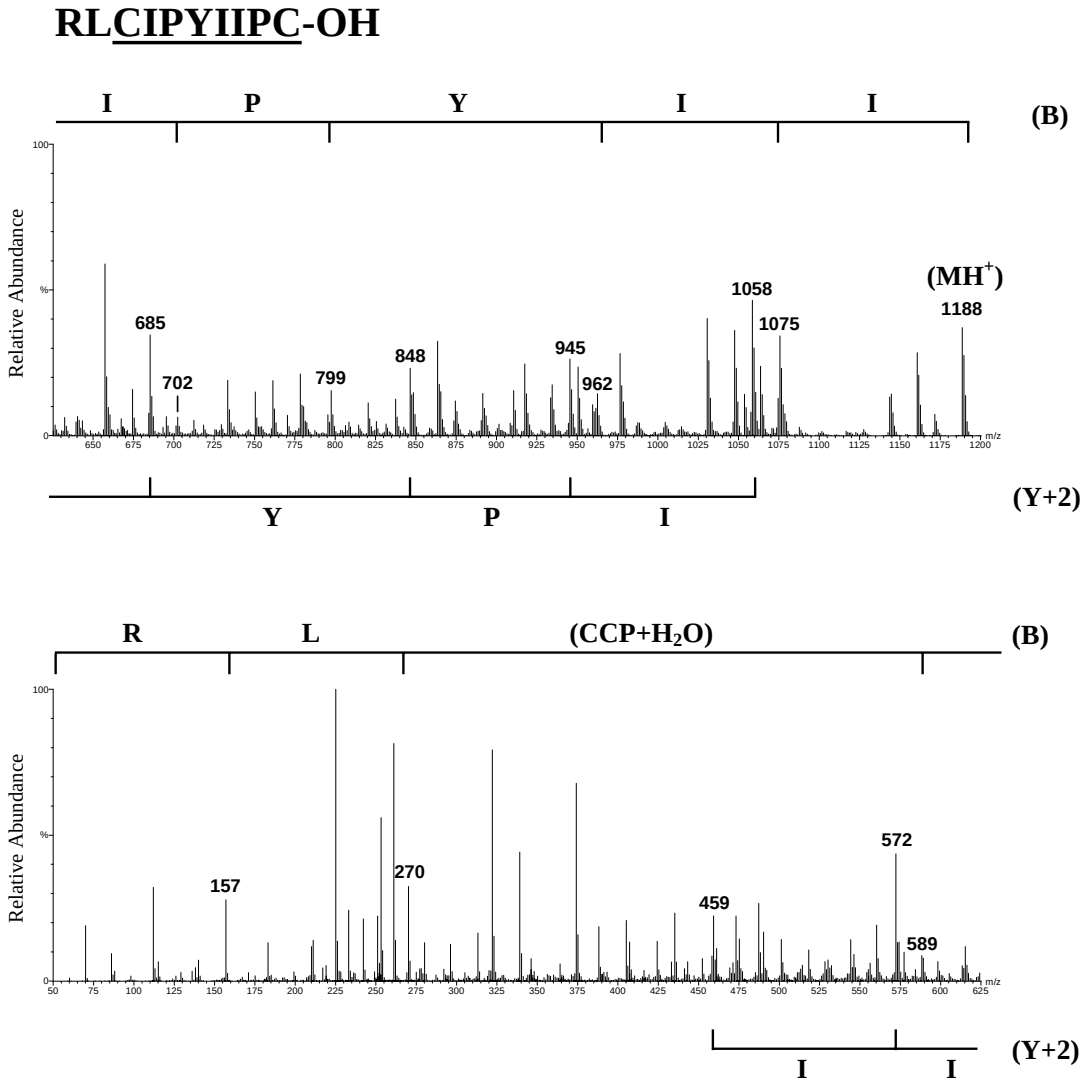


Figure 2.15. Mass spectrum (MS/MS) of the MH^+ cation of signiferin 1

The negative ion mass spectrum is also complex and contains a number of series of product ions. The presence of a disulfide link is identified by the loss of H_2S_2 from the $(M-H)^-$ anion. This fragmentation removes the disulfide link, and the resulting open-chain anion can undergo normal α and β negative ion backbone cleavages. The fragmentations of *m/z* 1186 are shown in Figure 2.16.

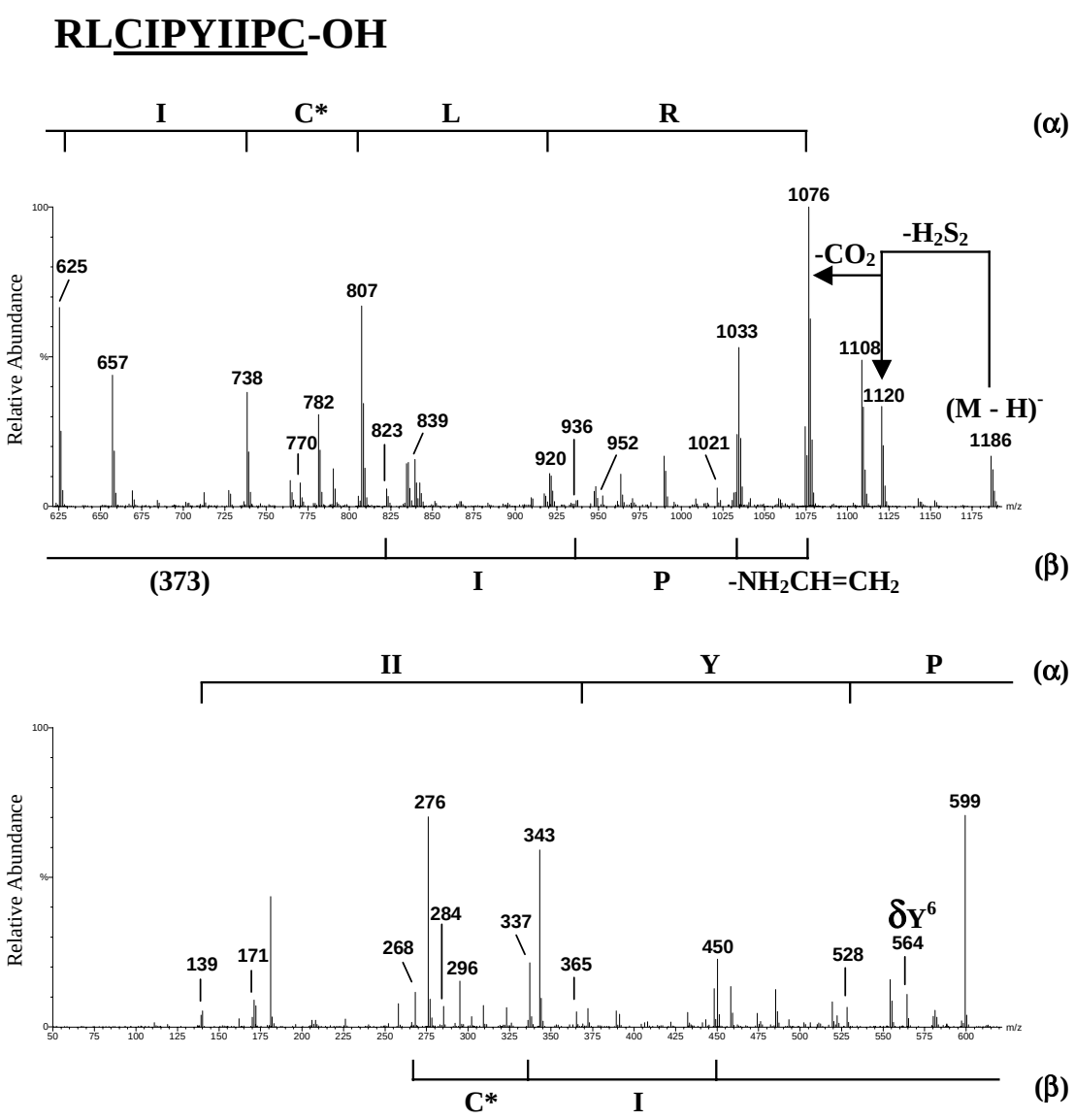


Figure 2.16. Mass spectrum (MS/MS) of the $(M-H)^-$ anion of signiferin 1. C* is $NHC(=CH_2CO)$

A combination of positive and negative ion mass spectrometry together with Edman degradation, gives the full sequence of signiferin 1 as Arg Leu Cys Ile Pro Tyr Ile Ile Pro Cys-OH.

2.3.3a Deserticolin 4.1

Deserticolin 4.1 has a MH^+ value of *m/z* 2005. B cleavage ions identifies the first sixteen amino acid residues from the C-terminal end, while Y+2 fragmentations determines the first seventeen amino acids residues from the N-terminal end of the peptide (Figure 2.17). The positive ion mass spectral data together with Edman sequencing confirmed the peptide sequence as Gly Leu Ala Asp Phe Leu Asn Lys Ala Val Gly Lys Val Val Asp Phe Val Lys Ser-NH_2.

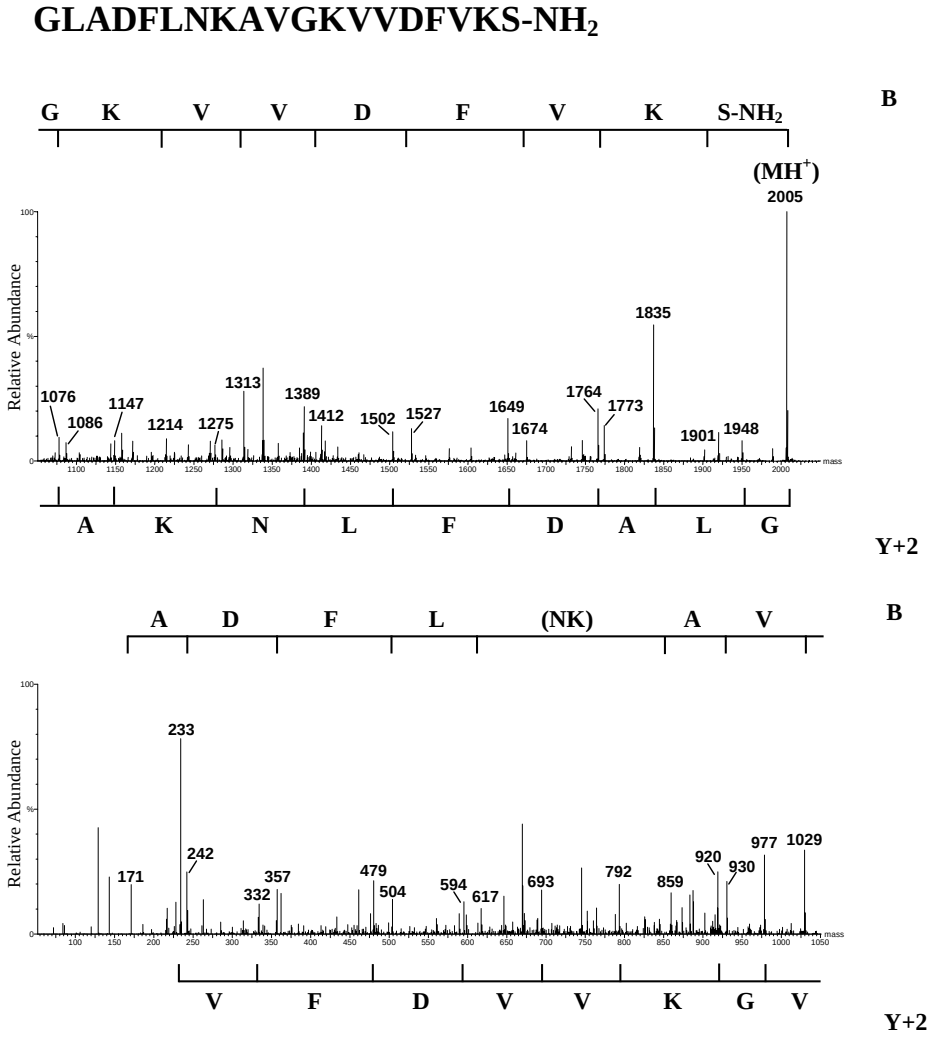


Figure 2.17. Positive ion mass spectrum (MS/MS) of deserticolin 4.1

2.3b Discussion

The glandular skin components of *Crinia deserticola* have been analysed, with two peptides isolated, one of them novel. This was named deserticolin 4.1. The remaining peptide, signiferin 1, was previously isolated from *C. signifera* [22].

The cyclic peptide, signiferin 1, is a potent neuropeptide. It contracts smooth muscle at 10^{-9} M and induces a response indirectly on smooth muscle cells via the CCK_2 receptor [66]. The pharmacological results of signiferin 1 are described in section 5.5.

Deserticolin 4.1 **(7)** has not yet been tested for biological activity, however, the peptide displays similar structural characteristics to the signiferin 4 peptides. Signiferin 4.3 **(8)** inhibits the formation of NO from nNOS at IC_{50} 40 µg/mL. It is possible that deserticolin 4.1 may also inhibit the formation of NO from nNOS.

(7) Deserticolin 4.1 GLADFLNKAVGKVVDFVKS-NH_2

(8) Signiferin 4.3 GFADLFGKAVDFIKSRV-NH_2

It may also display antibacterial activity as the Edmundson wheel projection displays two moderately defined hydrophilic and hydrophobic zones (Figure 2.18).

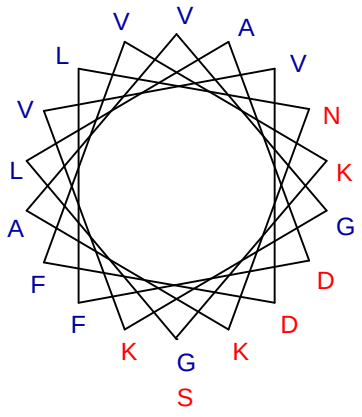


Figure 2.18. The Edmundson wheel projection of deserticolin 4.1. The hydrophilic zone shown in red and hydrophobic zone shown in blue

2.4 Conclusion

Previous studies of the glandular secretions from the Australian frog *Crinia georgiana* discovered a potent hypotensive neuropeptide that had structural similarities to human angiotensin. This prompted further investigations into the skin components of species from the same genus.

The skin components from three species of *Crinia* genus have been studied. A diverse range of compounds have been isolated and their bioactivities were investigated. Peptides displayed a broad range of actions including antibacterial, nNOS inhibition and neuropeptide activity.

Isolated peptides were unique and dissimilar to other peptides isolated from Australian anurans. Peptides with similar structural characteristics were evident within the three species. The most interesting peptides contained a disulfide bridge- a type not observed before in other Australian amphibians.

Most Australian frogs contain at least one broad-spectrum antibiotic within dermal secretions to protect it against a range of bacteria. *Crinia signifera* and *C. riparia* contain antibiotic peptides within glandular secretions. Peptides are active against the Gram-positive organism *Leuconostoc lactis*, displaying limited antibacterial activity.

Several peptides that inhibit the formation of nitric oxide (NO) by nNOS were identified. NO inhibits the growth of invading organisms in the host and is also used as a mediator in a large number of biological systems. Peptides from the *Crinia* genus have structural similarity to the frenatins.

Peptides containing disulfide bridges have been isolated from the *Rana* genus [62, 63]. Recently, Cys containing peptides have been discovered in Australian frogs from the *Crinia* genus. All species contain at least one peptide of this type, usually as the major glandular component. The peptides, named signiferin 1 and the riparins 1, are potent neuropeptides that

either contract smooth muscle tissue or proliferate lymphocytes. This occurs by peptide interaction with the CCK_2 receptor. This is described further in section 5.5.

From this study, we have found there are some differences in the peptide composition in each species. *Crinia riparia* contains a large number of powerful neuropeptides and only one antimicrobial peptide. In contrast, *C. signifera*, which is located in close proximity to *C. riparia* at the southern end of the Flinders Ranges, only contains one potent neuropeptide and a variety of antibacterial peptides. The glandular skin component of *C. deserticola* contains a neuropeptide that is also present in *C. signifera*. It is interesting to note that these neuropeptides, particularly signiferin 1 and riparin 1.1, possess different biological activities, although structurally related.

The similarities between peptides isolated from the three *Crinia* species suggest these frogs may have a common ancestor, however, differences in biological activity may have evolved due to environmental pressures like climate change and predators. The peptide profiles also aid the identification process of the frogs, which are often not distinguishable by physical appearance, as with *C. signifera* and *C. riparia*, both of which are found in the same locality in South Australia.

2.5 Experimental

2.5a Collection and Preparation of Secretions

Adult specimens of *Crinia riparia* were collected from the Flinders Ranges, South Australia, and were maintained in captivity for the duration of the study. Adult specimens of *C. deserticola* were collected from northern parts of South Australia.

Skin secretions were acquired from the dorsal glands of amphibians using the non-invasive surface electrical stimulation method (SES) [4], by Associate Professor Michael Tyler, Department of Environmental Biology, the University of Adelaide. The frog's skin was moistened with deionised water prior to the stimulation of dorsal glands that were stimulated using a bipolar electrode of 21G platinum, attached to a Palmer Student Model electrical stimulator. The electrode was rubbed in a circular manner gently over the glandular region, applying 3 V and pulse duration of 3 ms [4]. The resulting skin secretion was washed from the specimen with deionised water (50 mL), then immediately diluted with an equal volume of methanol, filtered and concentrated to a volume of 1 mL.

High performance liquid chromatography (HPLC) and electrospray ionisation mass spectrometry (ESMS) were the primary tools used to isolate and identify peptides. Sequence determination was also assisted using Lys-C digestion, methylation and Edman automated sequencing.

This work conforms with the Code Of Practice For The Care And Use Of Animals For Scientific Purposes (1990) and the Prevention Of Cruelty To Animals Act (1965), and was approved by the University of Adelaide Animals Ethics Committee.

2.5b Separation of Glandular Secretions by HPLC

Separation of crude material was achieved using a VYDAC C18 HPLC reverse phase column (5 µm, 300 A, 4.6 x 250 mm) (Separations Group, Hesperia, CA, U.S.A) equilibrated with acetonitrile/water (1:9) containing trifluoroacetic acid (0.1%). 400 µL of crude sample was injected for each HPLC run. The elution profile was generated using a linear gradient (10 -70% acetonitrile over a period of sixty minutes using a flow rate of 1 mL/min) produced by an ICI DP 800 Data Station controlling two Walters Millipore 510 and 501 HPLC pumps. The eluent was monitored by UV absorbance at 214 nm using a Walters Millipore Lambda Max 481 LC Spectrophotometer (ICI Australia, Melbourne, Australia). The HPLC profiles are shown in Figures 2.7 and 2.14. The fractions were collected before being analysed by mass spectrometry.

2.5c Analysis by Mass Spectrometry

Mass spectral data were determined by electrospray ionisation mass spectrometry (ESMS) using a Micromass Q-TOF 2 hybrid orthogonal acceleration time of flight mass spectrometer. The peptide was dissolved in water/methanol (1:1) and infused into the electrospray source at 8 µL/min. Electrospray conditions were as follows: capillary voltage 3 kV, source temperature 80ºC, desolvation temperature 150ºC and cone voltage 50 - 110 V. The collision activation mass spectral data (MS/MS) were obtained using a collision energy of 70% and argon collision gas energy set to ~50 eV to give optimal fragmentation. Peptides were analysed in both the positive and negative ion modes. ESMS data are shown in Figures 2.8 - 2.12, 2.15 -2.17.

2.5d Automated Edman Sequencing

Automated Edman sequencing was used to confirm the peptide sequence as determined by mass spectrometry and to distinguish between residues isoleucine and leucine, and glutamine and lysine. Sequencing was performed in the Department of Molecular Biosciences, the University of Adelaide, using an applied Biosystem 470A proscribe sequencer equipped with

a 900A data analysis module. The most effective results were obtained using a disc of immobilon film treated with bioprene in ethanol onto which the peptide was absorbed from aqueous acetonitrile (90%). The disc was pierced several times with a razor blade to aid the flow of solvent [53].

2.5e Determination of the C-terminal End Group

C-terminal end groups were determined by methylation, using 'acidified methanol'. Acidified methanol was prepared by adding methanol (858 µL) to a 10 mL screw top test tube. This was cooled in dry ice for five minutes. Acetyl chloride (142 µL) was then added under nitrogen before the test tube was sealed and cooled again in dry ice for five minutes. The solution was allowed to warm to 20°C over a period of one hour, flushing with nitrogen every fifteen minutes. The reagent was stored at -4°C and was usable for a maximum of five days.

Acidified methanol (~100 µL) was added to a sample of lyophilised peptide (~5 µg), and the resulting mixture heated at 45°C for thirty minutes. The resulting solution was diluted in water and analysed by mass spectrometry. The mass difference between the MH^+ of the methylated peptide and the MH^+ of the natural peptide enabled the determination of the number of CO_2H and $CONH_2$ residues in the natural peptide [55].

2.5f Enzyme Digestion using Lys-C

An aqueous ammonium hydrogen carbonate (0.1 M, 5 µL, pH=8) buffer solution and endoprotease Lys-C (1 µL made from three units dissolved in 200 µL of water) (*Lysobacter enzymogenes*, Sigma, St. Louis, M.O., U.S.A) was added to lyophilised peptide (~50 µg) in an Eppendorf tube. The resulting solution was incubated at 45°C for one hour before dilution with water and analysis by mass spectrometry [54].

2.5g Preparation of Synthetic Peptides

Synthetic peptides were prepared by Genscript (U.S.A.) using L-amino acids via the standard N-α-Fmoc method [56]. Synthetic peptides were of greater than 70% purity and were further purified as required. Each synthetic peptide was shown to be identical to the native peptide by HPLC and electrospray mass spectrometry.

2.5h Antibacterial Testing

Synthetic peptides were tested for antibacterial properties by the Microbiology Department at the Institute of Medical and Veterinary Science (IMVS), Adelaide. The method used measured the inhibition zones produced by the peptide on a thin agarose plate containing the microorganisms of interest [57]. The activities were recorded as the minimum inhibitory concentration (MIC) of peptide per mL required to totally inhibit the growth of the named microorganism.

2.5i Anticancer Testing

Anticancer properties of synthetic peptides were tested by the National Cancer Institute, Washington, D.C., U.S.A, by assessing the *in vitro* chemosensitivity of tumour cell lines to synthetic peptides. The three human tumour cell lines used were- MCF7 (breast), NCI-H 460 (lung) and SF-268 (central nervous system). Chosen active peptides were then tested against a full panel of 55 cancer cell lines. Assays were conducted using a standard protocol [58].

2.5j Neuronal Nitric Oxide Synthase Inhibition Testing

Peptides were routinely tested for inhibition against the enzyme neuronal nitric oxide synthase. Tests were carried out by the Australian Institute of Marine Science, Queensland. Inhibition was measured by monitoring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline using a standard method [21].

2.5k Smooth Muscle Contraction Assay

Peptides were tested for their ability to contract guinea-pig ileal smooth muscle tissue using the method described in section 5.7. Their response was monitored against the standard CCK-8 and CCK-8-NS peptides.

2.5l Lymphocyte Proliferation Assay

Peptides were tested for immune functionality using a standard lymphocyte proliferation assay, as described in section 5.7.

Chapter 3- Peptides from *Litoria dentata*

3.1 Introduction

The *Litoria* genus belongs to the family Hylidae, and consists of numerous species that are distributed throughout Australia. *Litoria dentata*, otherwise known as the Bleating Tree Frog (Figure 3.1), is found on the coastal regions of southern New South Wales [8] (Figure 3.2). It can be found around coastal lagoons and swamps, preferring low vegetation and urban bushland, where it shelters beneath loose bark or stones near breeding sites. *L. dentata* is an attractive elongate frog and is commonly pale brown or cream in colour. Males range in length from 32.0 - 40.0 mm and females are between 39.0 - 44.0 mm in length [8].

Figure 3.1. *Litoria dentata*

Figure 3.2. The distribution of *Litoria dentata*

The skin components from anurans of the *Litoria* genus have been extensively studied, with a number of peptides exhibiting both antibacterial activity and pharmacological activity identified.

Litoria dentata is related to the Australian anurans *L. rubella* and *L. electrica*, and to *L. capitula* (of Irian Jaya) and *L. congenita* (from New Guinea) [8]. Skin secretions from both *L. rubella* and *L. electrica* have previously been studied [70]. *Litoria rubella* (Red Tree Frog) (Figure 3.3) is widely distributed throughout central and northern Australia. Its ability to adapt to different climates has furthered the evolution of a number of specific populations within this area [71]. The related frog, *L. electrica* (Buzzing Tree Frog) (Figure 3.4), is located in northern Australia in a specific region below the Gulf of Carpentaria [70].

Figure 3.3. *Litoria rubella*

Figure 3.4. *Litoria electrica*

Examination of glandular components of *L. rubella* and *L. electrica* identified peptide profiles not exhibited by other *Litoria* species. No compounds displaying antibacterial activity were isolated, however, a number of other peptides were produced. The most abundant belonging to the tryptophyllin family (Table 3.1) [70].

Table 3.1. Tryptophyllin L peptides isolated from *Litoria* species

Tryptophyllin L	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	IPWL-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* [71]; (b) *L. electrica* [70]

The tryptophyllins were first discovered in the South American hylid frog, *Phyllomedusa rohdei* [72]. Tryptophyllins share similar structural characteristics to two human brain endomorphins **(1,2)** which have a high affinity for the γ-receptor [73]. Therefore, they may have roles as neurotransmitters.

(1) Endomorphin 1 YPWF-NH_2

(2) Tyr-W-M1F1 YPWG-NH_2

Until recently, the biological activity and role of tryptophyllins in anuran skin remained uncertain. Previous tryptophyllins exhibited either minimal or no smooth muscle activity, however, the peptide FPPWM-NH_2 induced sedation and behavioural sleep in birds [20]. The recently isolated tryptophyllin, PdT-1 **(3)** [74], is the first amphibian skin tryptophyllin to possess pharmacological activity and is a potent myoactive agent at nanomolar concentrations [74].

(3) PdT-1 KPHypAWVP-NH_2

This chapter examines the peptide profile of *Litoria dentata* by:

i) describing the isolation and structure determination of skin peptides, and
ii) comparing the structures of peptides with those identified from other *Litoria* species.

3.2 Results

3.2a Isolation of Active Peptides

Specimens of *Litoria dentata* were collected from southern parts of New South Wales. The surface electrical stimulation method [4] was employed to acquire skin secretions from the dorsal glands of five amphibians. The HPLC chromatogram identified six major peptides (fractions labelled A - F) within skin secretions, with retention times greater than fifteen minutes (Figure 3.5). Material eluted prior to fifteen minutes has not been investigated. Mass spectrometry and automated Edman sequencing identified the amino acid sequences summarised in Table 3.2, which have been named dentatins. The dentatins are classed into four structural groups: the structure determination of each peptide is described later in this chapter. A peptide from each group was synthesised and found to be identical to native peptides by HPLC and mass spectrometry.

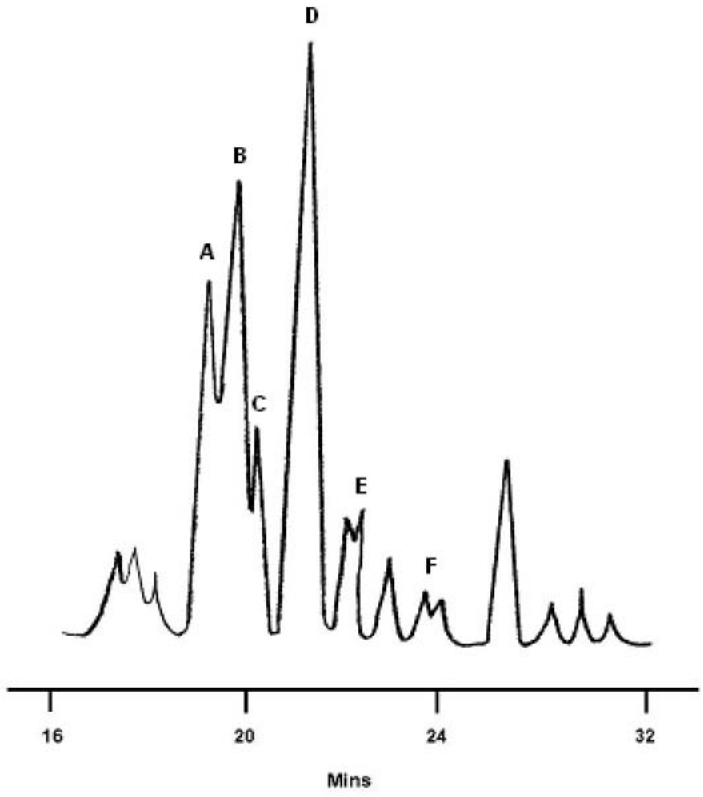


Figure 3.5. HPLC chromatogram of the glandular secretion from *Litoria dentata*

Table 3.2. Name, sequence and activity of major peptides isolated from *Litoria dentata*

Name	Sequence	MW	Activity	HPLC trace
Tryptophyllin L 1.3	pEFPWL-NH_2	671	a	D
Dentatin 1.1	WSPFWD-NH_2	835	b	A
Dentatin 1.2	WSPFGER-NH_2	876	b	C
Dentatin 2.1	GLLDFL-NH_2	675	b	E
Dentatin 2.2	GLLDFLGI-OH	846	b	F
Dentatin 3.1	FNPFMI-NH_2	766	b	B

(a) neuropeptide (contracts smooth muscle at 10^{-8} M); (b) unknown

3.2b Sequence Determination of Peptides

All peptides isolated are unique to *Litoria dentata*, except tryptophyllin L 1.3, which has been previously isolated from *L. rubella*.

Primary structures of peptides were principally determined by electrospray ionisation mass spectrometry (ESMS), methylation and enzymatic digest. The peptides are post-translationally modified at the C-terminal end group, except dentatin 2.2. This was confirmed by conversion of peptides into methyl esters and determining the appropriate molecular weight by mass spectrometry. Automated Edman sequencing confirmed the primary peptide sequence and, in particular, differentiated between leucine and isoleucine.

3.2c Tryptophyllin L 1.3

Tryptophyllin L 1.3 is the major glandular component in *L. dentata* skin secretions and was previously isolated from *L. rubella* [36]. Positive ion mass spectrometry showed an MH^+ peak at *m/z* 672. The MS/MS of the MH^+ parent ion is shown in Figure 3.6. ESMS identified the first three amino acid residues from the C-terminal end of the peptide using B fragmentations. All five amino acid residues could be determined from Y+2 cleavages from the N-terminal end of the peptide. Tryptophyllin L 1.3 has a primary C-terminal amide group and an N-terminal pyroglutamate residue. Edman degradation could not be used to confirm the peptide sequence due to the presence of the N-terminal pGlu. Mass spectral data identified the sequence of tryptophyllin L 1.3 as pGlu Phe Pro Trp Leu/Ile-NH_2. The peptide pGlu Phe Pro Trp Leu-NH_2 was synthesised and was identical (HPLC and MS/MS) with the native peptide.

pEFPWL-NH_2

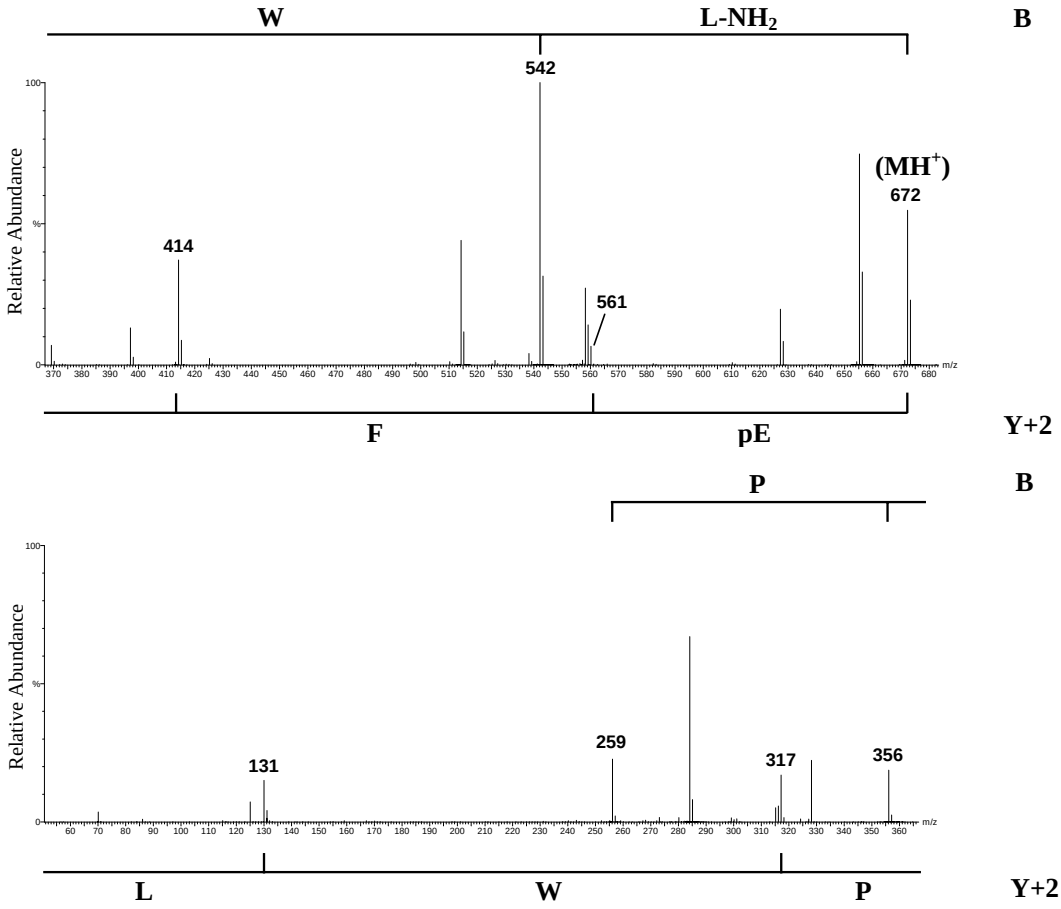


Figure 3.6. Mass spectrum (MS/MS) of the MH^+ parent ion of tryptophyllin L 1.3

3.2d Dentatins 1

The dentatins 1 were present as two minor glandular components. Analysis of dentatin 1.1 by ESMS indicated a MH^+ peak at *m/z* 836. The MS/MS spectrum is shown in Figure 3.7. The first six amino acid residues from the C-terminal end of the peptide were identified from B fragmentations, and the first four amino acid residues from the N-terminal end were determined from Y+2 cleavages. Mass spectrometry together with methylation experiments indicated a primary amide C-terminal end group. This information together with automated Edman sequencing confirmed the sequence of dentatin 1.1 as Trp Ser Pro Phe Trp Asp-NH_2. Sequencing data for the related peptide dentatin 1.2 are summarised in Table 3.3.

Table 3.3. Sequence data of dentatin 1.2

Dentatin 1.2			
MH^+ 877	B ions	*m/z*	704, 575, 518, 371, 274, 187 [WSPFGER-NH_2]
	Y+2 ions	*m/z*	691, 604, 507, 360 [WSPF(358)-NH_2]

Dentatin 1.2 full sequence:
WSPFGER-NH_2

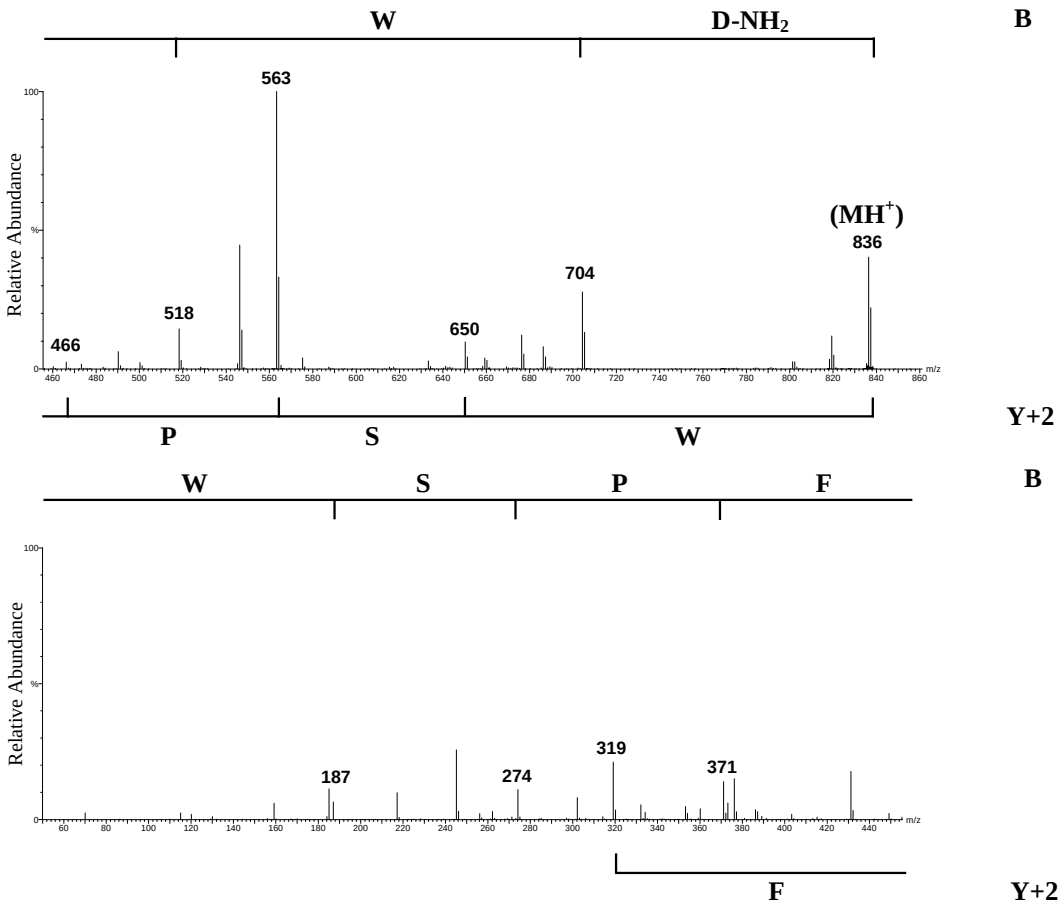


Figure 3.7. Positive ion mass spectrum (MS/MS) of dentatin 1.1

3.2e Dentatins 2

Dentatin 2.1 was purified by HPLC and analysed by mass spectrometry. A MH^+ ion was evident at *m/z* 676 and is illustrated in Figure 3.8. B cleavages enable five amino acid residues from the C-terminal end of the peptide to be identified. Only three amino acid residues can be determined from Y+2 cleavages. Together, these provide only a partial peptide sequence. Methylation experiments confirmed the C-terminal end group to be an amide. A combination of data from the mass spectrum and Edman sequence gave a final sequence of dentatin 2.1 as Gly Leu Leu Asp Phe Leu-NH_2. Full data for the related dentatin 2.2 peptide are shown in Table 3.4.

Table 3.4. Sequence data of dentatin 2.2

Dentatin 2.2			
MH^+ 847	B ions	*m/z*	716, 659, 546, 399, 284, 171
			[(GL)LDFLGI-OH]
	Y+2 ions	*m/z*	677, 564, 449, 302, 189
			[(GL)LDFL(GI)-OH]

Dentatin 2.2 full sequence:
GLLDFLGI-OH

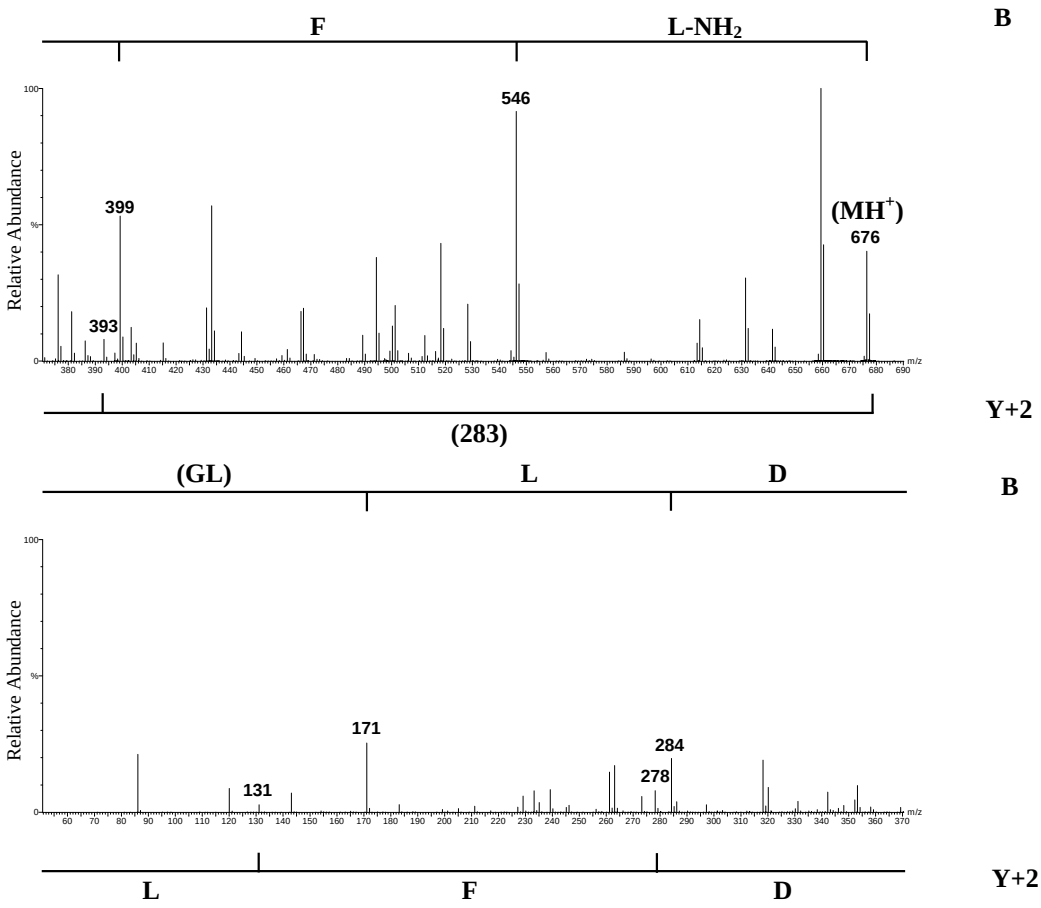


Figure 3.8. Positive ion mass spectrum (MS/MS) of dentatin 2.1

3.2f Dentatin 3.1

Dentatin 3.1 has a MH^+ value of *m/z* 767. Four amino acid residues from the C-terminal end of the peptide were identified from B fragmentations, and all six amino acid residues were identified from Y+2 cleavages. An amide C-terminal end group was evident from methylation experimental data. The sequence was confirmed by Edman sequencing and mass spectrometry to give Phe Asn Pro Phe Met Ile-NH_2. The peptide data for dentatin 3.1 are shown in Table 3.5.

Table 3.5. Sequence data of dentatin 3.1

Dentatin 3.1			
MH^+ 767	B ions	*m/z*	637, 506, 359, 262 [(261)PFMI-NH_2]
	Y+2 ions	*m/z*	620, 506, 409, 262, 131 [FNPFMI-NH_2]

Dentatin 3.1 full sequence:
FNPFMI-NH_2

3.3 Discussion

The glandular skin components of *Litoria dentata* were analysed. Six peptides were isolated and identified, five of them novel. These peptides were named dentatins.

Previous studies of Australian green tree frogs have shown that the glandular skin secretions contain a powerful neuropeptide (like caerulein) and several antibiotic peptides. In contrast, *Litoria rubella* and *L. electrica* (which are related to *L. dentata*) contain neither caerulein-type neuropeptides nor any antibiotic peptides. However, they do possess a number of tryptophyllin peptides that show no significant antibiotic or smooth muscle activity.

The skin peptides isolated from *Litoria dentata* confirm its relationship to *L. rubella* and *L. electrica*. The major glandular secretion in *L. dentata* contained the peptide tryptophyllin L 1.3, which is also present in *L. rubella* [71]. Preliminary results indicate tryptophyllin L 1.3 contracts smooth muscle at 10^{-8} M [75].

The remaining peptides, which do not show structural similarity to other isolated peptides, have not been tested for pharmacological activity at the time of writing.

3.4 Experimental

3.4a Collection of Secretions

Adult specimens of *Litoria dentata* were collected from southern parts of New South Wales and maintained in captivity for the duration of the experiment.

Skin secretions were obtained from the dorsal glands of amphibians using the non-invasive surface electrical stimulation method [4] by Associate Professor Michael Tyler, Department of Environmental Biology, the University of Adelaide. Details of this method are outlined in section 2.5a.

High performance liquid chromatography and electrospray ionisation mass spectrometry were the primary tools used to isolate peptides. Sequence determination was assisted using methylation and automated Edman sequencing.

This work conforms with the Code Of Practice For The Care And Use Of Animals For Scientific Purposes (1990) and the Prevention Of Cruelty To Animals Act SA (1965), and was approved by the University of Adelaide Animals Ethics Committee.

3.4b Separation of Secretions by HPLC

Separation of crude secretions was achieved using a VYDAC C18 HPLC reverse phase column equilibrated with acetonitrile/water (1:9) containing trifluoroacetic acid (0.1%). 200 µL of crude sample was injected in each HPLC run. The elution profile was generated using a linear gradient (10 - 70% acetonitrile over a period of thirty minutes using a flow rate of 1 mL/min). The eluent was monitored by UV absorbance at 214 nm. The HPLC profile is shown in Figure 3.5. The fractions were collected and analysed by mass spectrometry.

3.4c Analysis by Mass Spectrometry

Mass spectral data were determined by electrospray ionisation mass spectrometry using a Micromass Q-TOF 2 hybrid orthogonal acceleration time of flight mass spectrometer. The peptide was dissolved in water/methanol (1:1) and infused into the electrospray source at 8 µL/min. Electrospray conditions are detailed in section 2.5c. Mass spectral data are shown in Figures 3.6 - 3.8.

3.4d Automated Edman Sequencing

Edman sequencing was used to confirm the peptide sequence as determined by mass spectrometry and to distinguish between residues isoleucine and leucine, and glutamine and lysine. Sequencing was performed in the Department of Molecular Biosciences, the University of Adelaide, using an applied Biosystem 470A proscribe sequencer [53].

3.4e Determination of the C-terminal End Group

C-terminal end groups were determined by methylation using 'acidified methanol'. The preparation of acidified methanol is outlined in section 2.5e. The mass difference between the MH^+ of the methylated peptide and the MH^+ of the natural peptide enabled the determination of the number of CO_2H and $CONH_2$ residues in the natural peptide [55].

3.4f Preparation of Synthetic Peptides

Synthetic peptides were prepared by Genscript (U.S.A.) using L-amino acids via the standard N-α-Fmoc method [56]. Synthetic peptides were of > 80% purity and were shown to be identical to the native peptide by HPLC and electrospray mass spectrometry.

3.4g Smooth Muscle Contraction Assay

See section 5.7.

Chapter 4- Antimalarial Activity of Anuran Peptides

4.1 Introduction *

4.1a General

The parasitic disease malaria was discovered in 1897 by Ross [30]. Today, it is considered to be one of the most serious diseases in the world, with the number of people affected escalating. The disease is responsible for over one million deaths per year. Unfortunately, the majority of these are children [30]. Malaria is transferred by mosquitoes to humans through the blood stream and is predominant in tropical and subtropical areas (shaded in Figure 4.1) [76].

Figure 4.1. The distribution of malaria, which is predominant in tropical and subtropical areas

* A summary of malarial nomenclature is provided in section 4.6

The sudden growth in fatalities has arisen from malarial resistance to current therapeutic drugs. Resistance to antimalarial compounds is due to two main factors [30]:

i) the adaptability of the parasite *Plasmodium*, and
ii) the use of antimalarials for inadequate routine treatment and undiagnosed fevers in endemic areas.

The development of antimalarial compounds is imperative in order to prevent the disease. However, the biochemical pathways of the parasite and its mode of action are poorly understood, making novel treatments difficult to produce. Currently, the drug chloroquine (Figure 4.2) is the most effective control measure against the disease. New treatments investigate the potential of animal venoms and secretions as pharmacological tools, with a diverse range of biologically active compounds isolated from many species. This is discussed further in section 4.2.

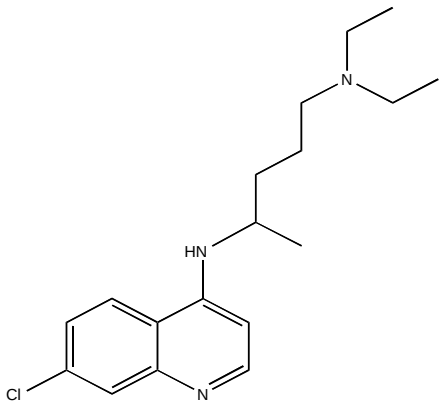


Figure 4.2. The molecular structure of chloroquine

4.1b The Malaria Parasite

Malaria is caused by an intracellular protozoan of the genus *Plasmodium*. The four species of *Plasmodium* are [30]:

i) *Plasmodium vivax*
ii) *Plasmodium ovale*
iii) *Plasmodium malariae*, and
iv) *Plasmodium falciparum*.

The most harmful of the parasitic species to infect humans is *P. falciparum*.

The parasite requires two hosts- a female *Anopheles* mosquito (Figure 4.3) and a human.

Figure 4.3. A female *Anopheles* mosquito feeding on the human host

The parasitic life cycle is categorised into four phases as follows [30, 77] (Figure 4.4):

Phase 1: **Fertilisation**- a female mosquito, *Anopheles*, withdraws blood from an infected host that contains intraerythrocytic parasites. The parasites develop into sexual stage gametocytes in eight to ten days, which undergo fertilisation in the gut of the insect. The resulting invasive ookinete burrows into the stomach wall of the mosquito.

Phase 2: **Sporogony**- development of the oocyst continues until the mature cyst bursts. Free sporozoites migrate into the salivary glands of the mosquito, ready to be transmitted to another individual.

Phase 3: **Hepatic schizogony**- the now infected female mosquito feeds again, both withdrawing blood from the human host while injecting sporozoite-containing saliva into the capillaries of the skin. The sporozoites invade liver cells and multiply to form merozoites. After five days the infected liver cell bursts, releasing merozoites into the blood stream.

Phase 4: **Erythrocyte schizogony**- Merozoites enter red blood cells where they develop through ring, trophozoite and schizont stages. The human erythrocytes provide the parasite with a safe environment, where it can grow and mature. The parasite divides to produce 16 - 20 daughter merozoites during a forty-eight hour cycle. When mature, the red blood cell bursts, releasing merozoites to invade new red blood cells. The cycle then continues.

Phase 1 is sexual whilst the other phases are asexual.

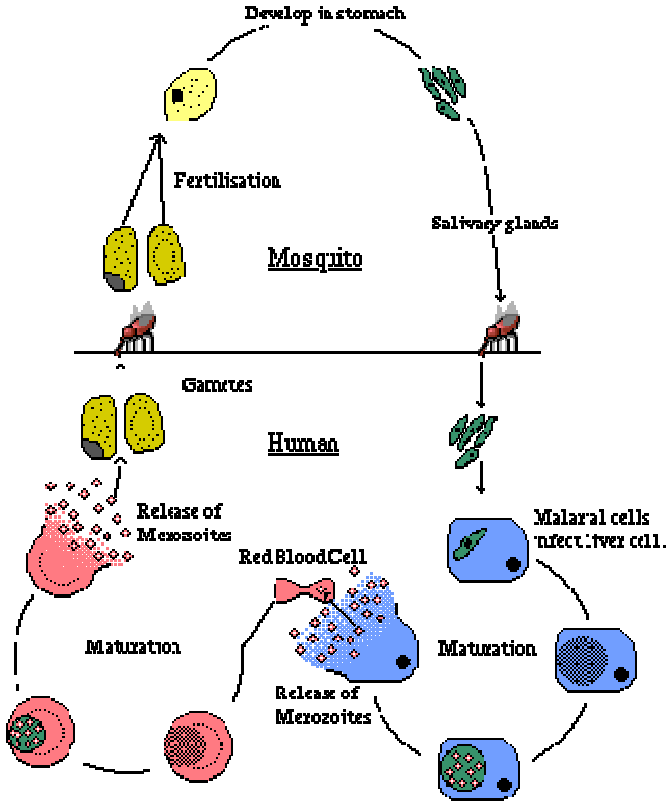


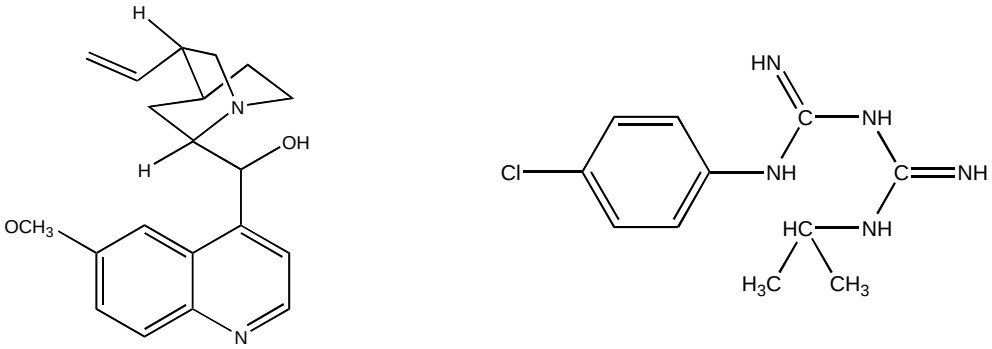
Figure 4.4. The life cycle of the malaria parasite

Symptoms of malaria include fever, convulsions and fits, which if untreated results in a coma and eventual death [30]. The final stages result from infected red blood cells adhering to the vascular endothelium of post-capillary venules in the brain [77]. At this point, the parasites are most susceptible to antimalarial agents [77].

4.2 Antimalarial Peptides

4.2a Classical Malarial Drugs

Two classes of drugs, antifolates and quinoline-containing drugs, have traditionally been used as a classic approach to antimalarial chemotherapy (Table 4.1) [78, 79]. Each compound affects different stages of the malaria life cycle and produces a different action on the cell and malarial pigment. Quinoline-containing compounds have been invaluable in the treatment and control of malaria [77]. The most successful compound is chloroquine (see Figure 4.2), which was first introduced in the 1940s. Its effectiveness and low risk have resulted in this compound being widely used. In consequence, chloroquine is subject to malarial resistance and is now ineffective in some areas [77]. Parasite resistance of quinine and other antimalarials like proguanil (Figure 4.5), has prompted the development of new and effective drugs against existing targets [79].



(a) **(b)**

Figure 4.5. Two commonly used antimalarial drugs- **(a)** quinine and **(b)** proguanil

Table 4.1. The four categories of antimalarial compounds and their effect on the life cycle of the malaria parasite

Group	Compound types	Stage of life cycle affected	Action on cells	Action on malarial pigment
1	Chloroquine Mepacrine 4-Aminoquinolines	All asexual stages	-	Rapid coarse clumping
2	Quinine Mefloquine Primaquine 8-Aminoquinolines	All stages except mature gametocytes of *P. falciparum*	- Degeneration of nuclei - Vacuolation of cytoplasm	Slow, fine clumping
3	Antifolates: - Proguanil - Pyrimethamine - Sulphonamides	Schizogony	Maturation arrest producing large non-viable parasites	-
4	Sesquiterpenes: -Artemisinine	Schizogony	-	-

The development of novel chemotherapeutics would be assisted by understanding the mode of action of current treatments, the life cycle of *Plasmodium* and the interaction with its host. Classic strategies are based on modifying known antimalarial agents or seeking new biological targets [80]. Theories concerning mechanism of action have emerged through investigating various parasitic stages in the erythrocyte, including inhibition of the parasite feeding process and transport systems.

Chloroquine is active against the *Plasmodium* parasite during the intra-erythrocytic mature stage of the cycle [77]. In this stage, the merozoite rapidly develops by ingesting haemoglobin and host-cell cytoplasm through specialised membrane areas [30]. As haemoglobin is digested, iron-haem pigment (haemozoin) accumulates in food vacuoles. Studies indicate that chloroquine interferes with the parasite feeding process by forming molecular complexes with the pigment, producing irregular masses and clumping with the cell [30] (Figure 4.6).

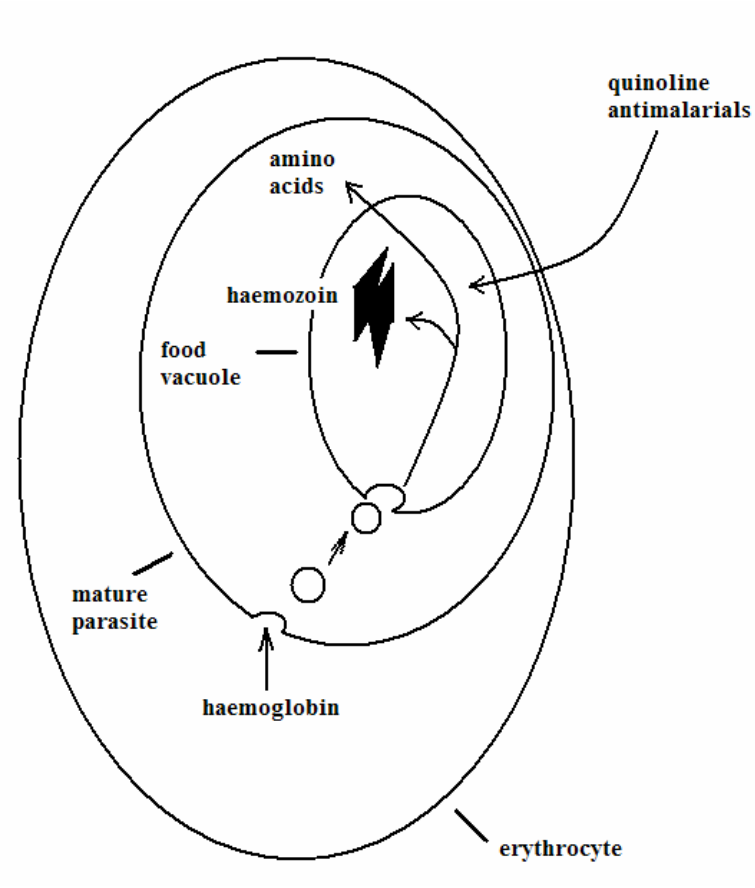
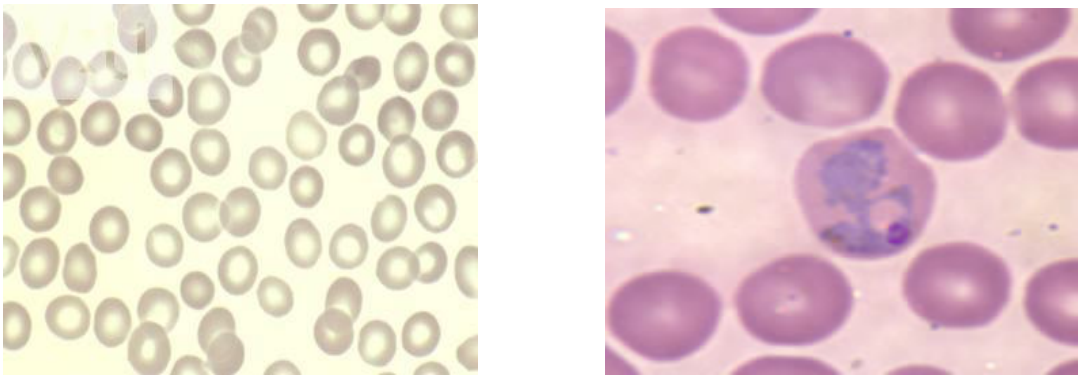


Figure 4.6. The action of antimalarial peptides on the parasite feeding process in the infected erythrocyte

Transport systems may also be exploited as targets for antimalarial drugs. Infected erythrocytes are permeable to a wide range of solutes due to parasite-induced permeation pathways [79]. This allows proteins to pass through the parasitic membrane.

The alteration in membrane structure of infected erythrocytes predisposes the host-cell membrane to interactions with lytic compounds [32]. The normal discoid shape of the host-cell (Figure 4.7a) is modified and knobs on the membrane appear (Figure 4.7b) [30, 77, 81]. These knobs are involved in the cytoadherence of infected cells to the walls of capillaries and venules [82].



(a) **(b)**

Figure 4.7. The **(a)** normal discoid shape of the host erythrocyte and **(b)** an infected erythrocyte with the appearance of knobs on the membrane

4.2b Anuran Antimalarial Compounds

Antimalarial compounds, particularly those coupled with reduced haemolytic activity, have invoked interest as potential antimalarial agents [32].

Amphibians possess a range of host-defence compounds in the glandular skin secretion that exhibit either neuropeptide or antimicrobial activity [60]. Particularly, amphibians from the genus *Phyllomedusa* contain a group of structurally and functionally related peptides in the skin known as dermaseptins [32, 78, 83]. These are linear polycationic peptides composed of 28 - 34 amino acid residues in length and have a characteristic α-helical amphipathic structure [78]. They are active against a broad spectrum of pathogenic microorganisms [84].

The naturally occurring dermaseptins S3 and S4 (Table 4.2) exhibit antiplasmodial activity [81] that occurs by permeabilisation of the host-cell plasma membrane [83]. However, dermaseptin S4 has pronounced haemolytic activity against host erythrocytes [78], therefore, a range of substitution and deletion peptides derived from dermaseptin S4 were investigated (Table 4.2) [32, 85]. Several derivatives displayed both enhanced antibacterial activity and reduced haemolytic activity, making them ideal potential antimalarial products.

Table 4.2. The antimalarial activity of dermaseptin S4 and some derivatives

Name	Sequence	Antimalarial activity IC_{50} (μM)
Dermaseptin S4	ALWMTLLKKVLKAAAKAALNAVLVGANA-OH	0.3-2.2
Dermaseptin S3	ALWKNMLKGIGKLAGKAALGAVKKLVGAES-OH	0.8
K_4-K_{20}-S4	ALWKTLLKKVLKAAAKAALKAVLVGANA-OH	0.2
K_4-S4(1-16)	ALWKTLLKKVLKAAAK-OH	20
K_4-S4(1-16)a	ALWKTLLKKVLKAAAK-NH_2	2

Parasite inhibition is dependent on the nature of the peptide. Structural differences include:

i) a shortened peptide of dermaseptin S4 that has rapid antimalarial activity and is less haemolytic to erythrocytes,
ii) replacement of residues with charged species to increase antiplasmodial activity,
iii) deletion of residues from the C-terminal end to decrease activity, and
iv) amidation of the C-terminal end to increase antimalarial potency.

Studies using confocal microscopy indicate peptides can pass through the complex series of host-cell and parasite membranes to interact directly with the intracellular parasite [32]. This may occur via pore formation or ion channels.

Australian frogs from the *Litoria* genus contain a number of antimicrobial, anticancer and antifungal peptides within their skin secretion (Table 4.3) [67].

Table 4.3. A selection of peptides isolated from the *Litoria* genus that exhibit a range of activities

Peptide	Sequence	MW	Species	Activity
Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	2582	a,b,c	1,2,3,4
Caerin 1.8	GLFKVLGSVAKHLLPHVVPVIAEKL-NH_2	2662	d	1,2,3,4
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	1613	e	1,2,3,4
Aurein 3.2	GLFDIVKKIAGHIASSI-NH_2	1766	f,g	1,2

Species: (a) *Litoria splendida* [12]; (b) *L. caerulea* [86]; (c) *L. gilleni* [87]; (d) *L. chloris* [36]; (e) *L. citropa* [10]; (f) *L. aurea* [26]; (g) *L. raniformis* [26]

Activity: (1) antibiotic activity; (2) anticancer activity; (3) fungicide activity; (4) nNOS inhibitor

Aurein 3.2 and citropin 1.1 belong to a group of antibacterial peptides that are less than 20 residues in length. Characteristic properties include a cationic nature- possessing at least two basic residues at position 7 and 8. They have a well-defined amphipathic α-helical structure (Figure 4.8) [10, 26], and NMR-studies indicate they interact with cell membranes via the carpet mechanism [88].

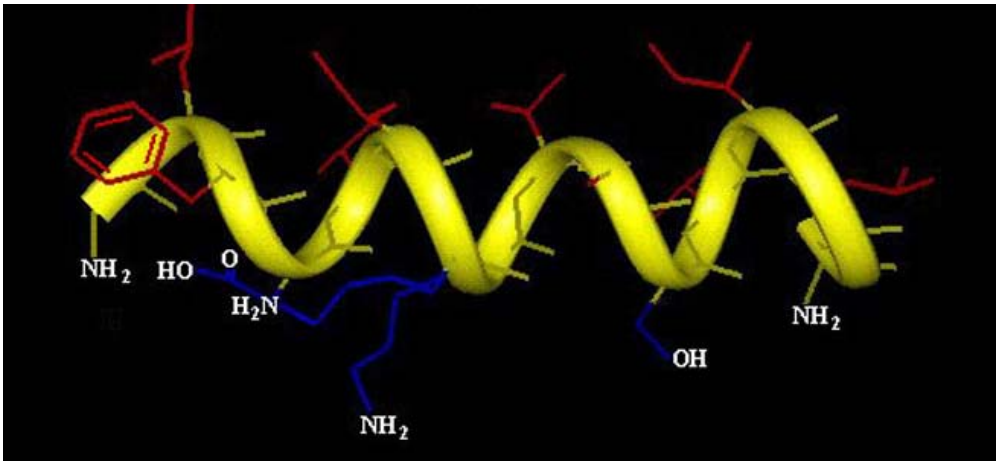


Figure 4.8. The solution structure of citropin 1.1 illustrating a well-defined amphipathic α-helical structure

The largest group of antibacterial peptides isolated from Australian amphibians are the caerin 1 peptides [67], which are predominantly active against Gram-positive bacteria. Solution structures suggest they form two amphipathic helices separated by a flexible hinge region initiated by Pro15 (Figure 4.9) [89]. The hinge assists the peptides interaction with the membrane, which is also via the carpet mechanism.

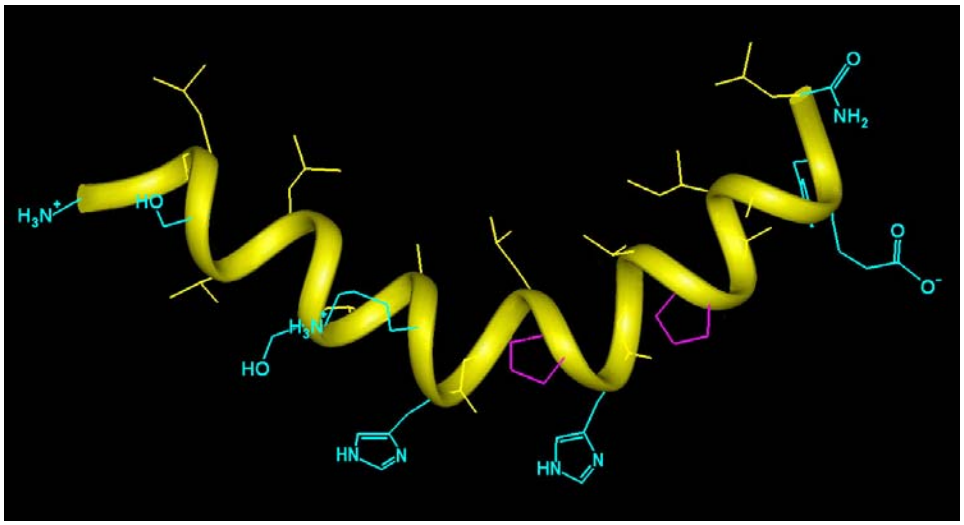


Figure 4.9. The solution structure of caerin 1.1 illustrating two amphipathic helices separated by a flexible hinge region

This research investigated the potential of amphibian peptides and their derivatives as antiplasmodial agents. The objective was to:

i) assess the effect of peptides on the malaria parasite *Plasmodium falciparum* in order to find the most potent antimalarial peptide, and

ii) investigate the mechanism of antimalarial action.

4.3 Results

A number of anuran peptides and their modifications were tested for potential antiplasmodial activity (Table 4.4), including cationic modifications and isomers. Haemolysis tests were conducted by V. Maselli (Department of Chemistry, the University of Adelaide). Parasite inhibition and parasite plasma membrane dissipation assays were conducted at La Trobe University, Bundoora, Melbourne, by Dr L. Tilley and research members. Some of these were conducted with V. Maselli in attendance.

Table 4.4. Anuran peptides and modifications tested for antimalarial activity

Peptide	Sequence	MW
Aurein 3.2	GLFDIVKKIAGHIASSI-NH_2	1766
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	1613
4 Citropin (cationic modification)	GLFKVIKKVAKVIKKL-NH_2	1809
Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	2581
D-caerin 1.1	Gllsvlgsvakhvlphvvpviaehl-NH_2	2581
2 (i)	GLLKVLGSVAKHVLPHVVPVIAAAL-NH_2	2498
2 (ii)	GLLKKLKKVAKKVLPKVVPVIAEKL-NH_2	2736
3 (i)	GLLSVLGSVAKHVLPHVVGVIAEHL-NH_2	2541
3 (ii)	GLLSVLGSVAKHVLGHVVPVIAEHL-NH_2	2541
Caerin 1.8	GLFKVLGSVAKHLLPHVVPVIAEKL-NH_2	2661

4.3a Antimalarial Activity of Peptides against *Plasmodium falciparum*

Antimalarial activity was assessed by measuring the incorporation of [^{3}H]-hypoxanthine into the parasite's nucleic acids of *P. falciparum*-infected human red blood cells. Synchronised cultures at the trophozoite stage were cultured in the presence of peptide and [^{3}H]-hypoxanthine. The cell-associated radioactivity was determined. Inhibition of growth was calculated from controls (without peptide) and recorded as an IC_{50} value. This value represents the concentration of peptide required to inhibit parasite growth by 50%. The smaller the IC_{50} value, the more potent the peptide.

Amphibian peptides inhibited the growth of *P. falciparum* in the micromolar range (Figure 4.10). The caerin 1 peptides and analogues were among the most active, with caerin 1.1 and caerin 1.8 displaying an IC_{50} value of 0.67 and 1.4 µM respectively. Citropin 1.1, the cationic modification and aurein 3.2 displayed IC_{50} values greater than 10 µM. An increase in charge in citropin 1.1 and the cationic modification did not result in a significant change in antimalarial activity despite this observation in the dermaseptins [32].

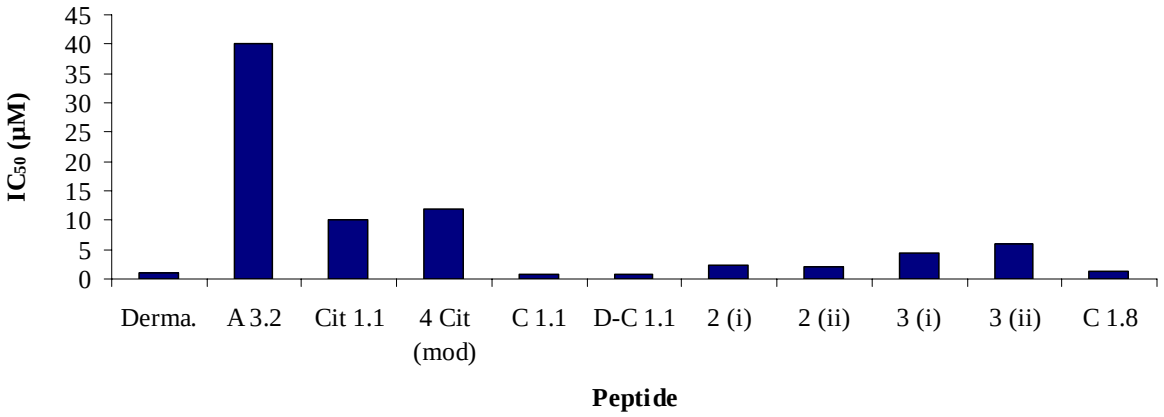


Figure 4.10. Antiplasmodial activity of anuran peptides. Caerin 1.1 and caerin 1.8 are the most active, whilst aurein 3.2 and citropin 1.1 show little antimalarial activity

4.3b Haemolytic Activity versus Antimalarial Activity

To further investigate the mechanism of antimalarial activity, peptides were tested for haemolytic activity. Haemolysis of normal red blood cells was determined by measuring the specific absorbance of haemoglobin in the supernatants after 30 minutes of exposure to peptides at a concentration 0 – 1 mg/ml. Figure 4.11 displays the concentration of peptide required to achieve 50% haemolysis of cells (HU_{50}) (n=3). From the data, there is no correlation between malaria parasite inhibition and cell lysis. However, citropin 1.1 and aurein 3.2 lysed cells at a greater peptide concentration compared with the caerins.

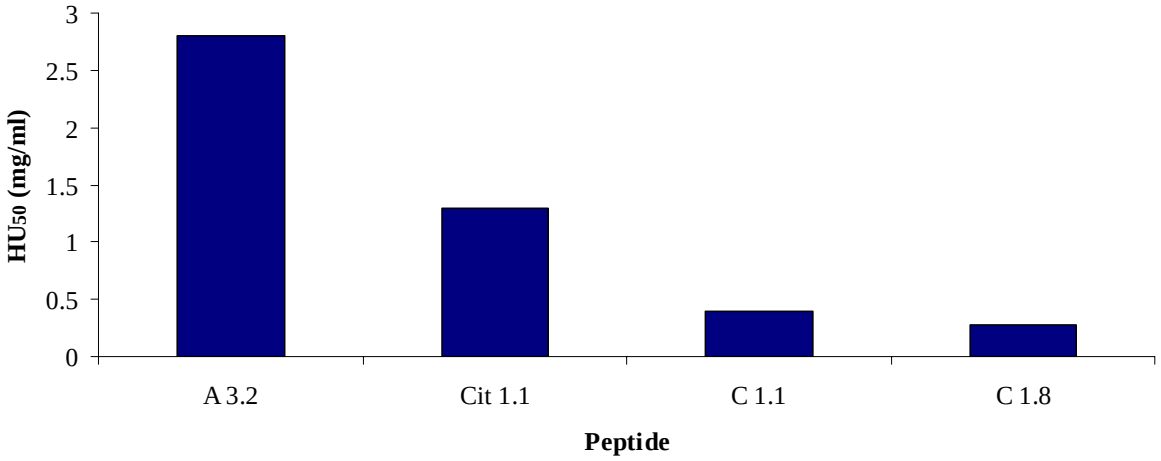


Figure 4.11. Haemolytic activity of amphibian peptides

4.3c Dissipation of the Parasite Plasma Membrane Potential

To assess whether the peptide can gain access to the parasite membrane, the dissipation of R123 fluorescence was monitored. The fluorescent dye rhodamine 123 accumulates inside cells in proportion to the membrane potential ($\Delta\Psi$). Permeabilisation of the parasite membrane by the peptide is expected to reduce $\Delta\Psi$ and therefore dye accumulation. The study ascertained that fluorescence measurement dissipates $\Delta\Psi$, as measured by reduced accumulation of the dye using the ionophores nigericin and monesin (data not shown). Results demonstrate that each anuran peptide can permeabilise the cell by crossing the host-cell membrane and interacting with the plasma membrane to reduce $\Delta\Psi$, therefore releasing the fluorescent dye R123 (Figure 4.12).

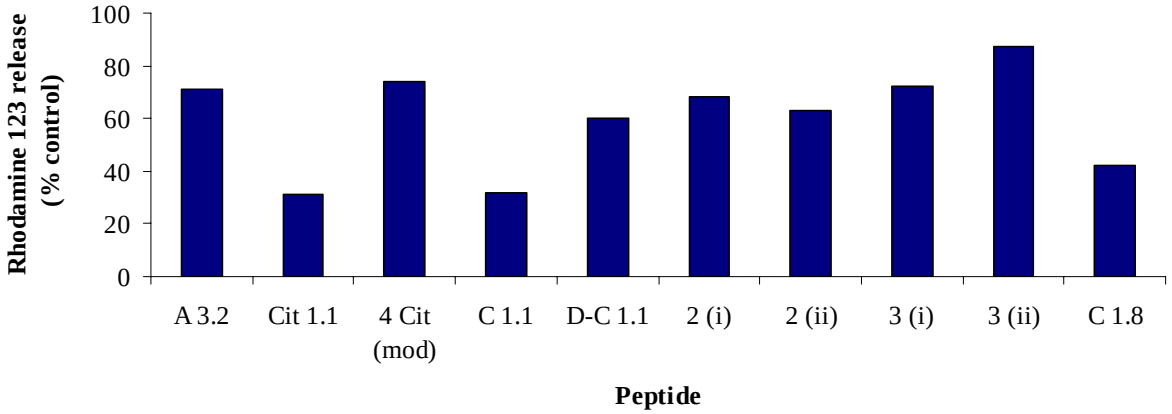


Figure 4.12. Dissipation of the membrane potential by relative fluorescence

4.3d D- versus L- isomers

Optical isomerism plays a vital role in living cells. Almost all amino acids are optically active, but only one of the isomers is biologically usable [90]. The organism distinguishes one optical isomer from the other through its enzymes. A specific portion of the enzyme provides the asymmetric environment to allow one of the isomers to bind and undergo reaction [90]. To verify the possibility that antimalarial activity may depend upon specific interactions of the peptides with a chiral molecule, such as receptors or enzymes, the D-amino acid isomer of caerin 1.1 was assayed in parallel to the L-amino acid counterpart.

The D-isomer of caerin 1.1 displayed a profile identical to that of the L-isomer with respect to its ability to induce lysis of normal red blood cells and inhibition of parasite growth. This implies that the mechanism of their activity is not mediated by particular peptide interactions with specific chiral molecules like receptors or enzymes.

4.4 Discussion

Antimicrobial peptides have been isolated from both vertebrates and invertebrates and are an essential component of their defence system [32]. Due to the rapid resistance of current antimalarial drugs, various animal-derived peptide antimicrobials have been investigated as potential antiplasmodial agents. New compounds are aimed at specifically and selectively affecting the parasite with minimal toxicity to the host.

It has been previously demonstrated that dermaseptin S4 derivatives, originally isolated from the anuran genus *Phyllomedusa*, exhibit potential antiplasmodial activity. Inhibition of growth depends on the nature of the peptides. The antiplasmodium effect was amplified as a result of increased hydrophobicity, however, this generally resulted in an increase in haemolysis of the host-cell [91]. Furthermore, as peptides increased in chain length, antimalarial activity increased whilst haemolytic activity decreased [91].

The results show that aurein 3.2, citropin 1.1 and citropin cationic modification inhibited the development of *Plasmodium falciparum* in erythrocytes in vitro with IC_{50} values greater than 10 µM. Antiplasmodial activity was most effective with the caerin peptides, in particular caerin 1.1 and caerin 1.8, which had IC_{50} values of 0.67 and 1.4 µM respectively. Although the IC_{50} values may be influenced by experimental parameters, such as the source of serum and red blood cells, and how long the erythrocyte had been stored before infection, the values remained in the low micromolar range [80]. There was no correlation between parasite inhibition, red blood cell lysis of uninfected cells and parasite membrane permeabilisation. However, the majority of peptides had a low red blood cell lysis value, indicating they may not disrupt the host-cell membrane.

Further investigations considered the mechanics of peptides inhibiting parasite growth. It was proposed that peptides induce the death of the parasites by lysing their membranes. As the lipid compositions of the host and parasite membrane are different [92], introducing the peptide into the host-cell would expose it to both membranes and enable it to exert its effect

on the more susceptible membrane [91]. Peptide permeability through the host-cell membrane is influenced by its charge and amphipathicity [32]. All anuran peptides displayed a fluorescence value greater than 30%, indicating they are able to cross the host-cell membrane to effectively interact with the parasite membrane.

Caerin 1.1 and citropin 1.1 are broad-spectrum antibiotics, mainly active against Gram-positive organisms. Their effect on membrane cells is exerted by pore-formation. The difference in the peptides' structures may potentially contribute to their difference in antimalarial activity. Their specificities and selectivities rely on variances in the charge, hydrophobicity and electrostatic potential distributions.

Citropin 1.1 is cationic and consists of a clear amphipathic α-helical structure (see Figure 4.8, section 4.2b). Citropin 1.1 may only selectively bind to red blood cell membrane receptors, contributing to its low haemolytic value. Further, its structure may circumvent its ability to interact sufficiently with the lipid bilayer of the erythrocyte membrane. Similarly, this may also occur with respect to the parasite membrane. This results in the parasite inhibition value of citropin 1.1.

The hinge present in caerin 1.1 (see Figure 4.9, section 4.2b) is likely to have an important role in antimalarial activity. The D- and L- isomers of caerin 1.1 displayed similar activity and therefore action is not mediated by specific interactions with a chiral center.

The difference in antiplasmodial activity may arise from peptide interactions with specific target receptors. Both peptides may target the same receptor but react with a different site to produce a specific function. They may also bind to different targets. Either peptide may bind to a parasite protein targeted on the erythrocyte membrane or a protein that is present on healthy red blood cells but is inactive until activated by infection [93].

The unique biochemical features and ability to inhibit parasite growth with minimal haemolytic activity make these peptides very promising tools for further malaria research. Their activities on other strains of *P. falciparum* and other *Plasmodium* species should also be tested.

4.5 Experimental

4.5a Isolation and Synthesis of Peptides

Peptides were isolated from various Australian amphibia and sequenced using mass spectrometry. Synthetic peptides were prepared by Mimotopes (Victoria, Australia).

4.5b Determination of Haemolytic Potential

Human blood from a healthy single donor was rinsed three times in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) by centrifugation (2700 rpm for 1 min). Peptides in the concentration range 0 - 1 mg/mL were incubated with washed human erythrocytes (2×10^7 cells) in phosphate-buffered saline, pH 7.4 (100 µL) for 30 min at 37°C. After centrifugation (5000 rpm for 4 min), the absorbance at 412 nm of the supernatant was measured. A freeze/thaw control was carried out to determine the absorbance associated with 100% haemolysis. The HU_{50} value was taken as the mean concentration of peptide producing 50% haemolysis (n=3) [83].

4.5c Determination of Antimalarial Activity by IC_{50}

D10 is a chloroquine-sensitive strain of *P. falciparum*. Malaria parasites were plated at 1% parasitemia (2% hematocrit) (1 infected cell per 50 red blood cells), in 96-well trays in the presence of different concentrations of peptides. Parasites were incubated for 72 h, with daily replacements of the drug-supplemented medium. Growth curves based on the uptake of [^{3}H]-hypoxanthine was obtained in duplicate. The concentration of peptide required to produce 50% inhibition of growth (IC_{50}) was determined [32].

4.5d Peptide-Mediated Dissipation of the Parasite Membrane Potential

Trophozoite stage culture in modified growth medium (wash medium containing 10 mM bicarbonate and 7% plasma) at 0.5% hematocrit was incubated in the presence of 1 µM rhodamine 123 for 30 min at 37ºC. Rhodamine 123 accumulates inside cells in proportion to the membrane potential ($\Delta\psi$) and has been shown to respond to the dissipation of the plasma membrane $\Delta\psi$ in *P. falciparum*. Aliquots of this culture were then exposed to peptides, or a mixture of 10 µM nigericin (K^+:H^+ exchanger) and 10 µM monensin (Na^+:H^+ exchanger) to dissipate the ion gradient across membranes (positive control). The samples were taken at different time intervals and the cells were washed in PBS and resuspended in the original sample volume of PBS. Aliquots of 120 µL were placed in a 96-well plate and read in a fluorescence reader (excitation wavelength λ_{ex} = 530 nm, emission wavelength λ_{em} = 585 nm). Relative fluorescence (as a percentage of that of the untreated control at the same time) was recorded [83].

4.6 Glossary

Gametocyte- A cell capable of dividing to produce gametes eg. a spermatocyte or oocyte

Ookinete- The motile, worm-shaped zygote of ce rtain protozoa, such as *Plasmodium*, which is found in the insect vector

Sporogony- Reproduction by multiple fission of a spore or zygote characteristic of many sporozoans. Results in the production of sporozoites

Sporozoites- A stage in the life cycle of the malaria parasite. Sporozoites are produced in the mosquito and migrate to the mosquito's salivary glands. They can be inoculated into a human host when the mosquito takes a blood meal on the human. In the human, the sporozoites enter liver cells where they develop into the next stage of the malaria parasite life cycle

Schizogony- The division of cells, especially of protozoans, in nonsexual stages of the life history of the organism

Merozoites- Stage in the life cycle of the malaria parasite. Merozoites are formed during the asexual division of the schizont and are released to invade other cells

Ring stage- Early trophozoites

Trophozoite stage- A developmental form during the blood stage of malaria parasites. After merozoites have invaded the red blood cell, they develop into trophozoites, which develop into schizonts

Schizont stage- A developmental form of the malaria parasite that contains many merozoites. Schizonts are seen in the liver-stage and blood-stage parasites

Venules- Small veins

Chapter 5- Amphibian Neuropeptides

5.1 Introduction

5.1a General

Anurans secrete host-defence compounds onto their skin surface that play a role in defending the animal from a variety of environmental threats ranging from bacteria to predators [13]. Isolated peptides exhibit two types of activity [13]:

i) pharmacological activity, and
ii) antibiotic activity.

Those with pharmacological activity may be divided into eleven groups and are discussed further within section 5.1.

Vittorio Erspamer and his research group commenced the investigation into the defence chemistry of amphibian neuropeptides in the 1960s. A number of reviews outlining the pharmacology and biological activity of peptides have been published since this early work [60, 67, 94].

Neuropeptides are peptidergic neurotransmitters that are produced by neurons and are an integral part of the host-defence system of an animal [95]. The study of amphibian neuropeptides is important in understanding both the ecology and physiology of the animal and has provided important information about mammalian physiology that may lead to the development of new therapeutics [40].

Amphibians synthesise and store neuropeptides and hormones within granular glands located on the dorsal surface of the animal. Generally, peptides are similar in structure to those isolated from higher vertebrates, including mammalian sources [1, 96], but may also be novel analogues of known peptide families or display structural variations with diverse pharmacological properties [97]. Isolated host-defence compounds are subjected to biological screening assays to test activity on a variety of smooth muscle tissue types, systemic blood pressure, exocrine secretion, anterior pituitary secretion and the central nervous system [40].

The aim of this research was to:

i) test a range of anuran neuropeptides for pharmacological activity, and
ii) determine the mechanism of action.

5.1b Bombesins and Litorins

The bombesin peptides are isolated from the skin and gut of frogs of the *Bombina* genus. The related litorin peptides are generated by species of the *Litoria*, *Pseudophyrne* and *Rana* genera [20]. Structural features characteristic of this group include a C-terminal amide and an N-terminal pyroglutamate residue. They display a similar spectrum of physiological activity that includes smooth muscle contraction, the stimulation of normal and neoplastic tissues, enhancement of secretions like gastrin and a variety of effects in the central nervous system [20, 98]. They also exhibit potent immunomodulatory properties [99, 100]. A selective range of peptides are shown in Table 5.1, with activity recorded using a smooth muscle contraction assay.

Table 5.1. Smooth muscle contraction activities of bombesin and litorin type neuropeptides

Name	Sequence	MW	Activity* % of bombesin	Activity % of litorin	Species
Bombesin [101]	pEQRLGNQWAVGHLM-NH_2	1618	100		a,b,c
[pGlu[1]]bombesin (6-14) [101]	pEQWAVGHLM-NH_2	1050	290		a
Litorin [101, 102]	pEQWAVGHFM-NH_2	1084	130	100	d
Rohdei litorin [103]	pELWATGHFM-NH_2	1071		16	e
Phyllolitorin [103]	pELWAVGSFM-NH_2	1019		2	e,f
Ranatensin [103, 104]	pEVPQWAVGHFM-NH_2	1280		85	c

***** Threshold concentration of the various peptides in producing guinea-pig colon smooth muscle contraction as a % of the threshold concentration of either bombesin (0.06 to 0.3 nM) or litorin. Value shown is the mean of the range of response for that peptide

(a) *Bombina bombina*; (b) *B. orientalis*; (c) *Rana pipiens*; (d) *Litoria aurea*; (e) *Phyllomedusa rohdei*; (f) *P. sauvagei*

Bombesin is similar in sequence to the C-terminal end of human gastrin-releasing peptide (GRP) **(1)** and neuromedin B (NMB) **(2)** (indicated in bold text) [98].

(1) GRP VPLPAGGGTVLTKMYPRGNH**WAVGHLM**-NH_2

(2) NMB GNL**WATGHFM**-NH_2

The peptides in Table 5.1 elicit activity by binding to one or more of four types of G protein- coupled receptors [98, 105, 106], namely:

i) NMB receptor (NMB-R or BB_1) found in skin and gut,

ii) GRP receptor (GRP-R or BB_2) found in brain,

iii) bombesin-like receptor subtype 3 (BB_3), and

iv) bombesin-like receptor subtype 4 (BB_4) found only in brains of anurans.

5.1c Caeruleins

Caerulein is the most studied of all amphibian neuropeptides; initially isolated from skin secretions of *Litoria caerulea*. It has since been isolated from other species of *Litoria* together with *Xenopus laevis* and *Leptodactylus labyrinthicus* [67]. Caerulein **(3)** is often the major neuropeptide present in the glandular skin component. The caerulein concentration may vary seasonally according to the reproduction and hibernation periods of the animal [12].

Caerulein contains pyroglutamate and tyrosine sulfate residues together with a C-terminal $CONH_2$ group. The tyrosine sulfate residue is essential for maximum biological activity.

(3) Caerulein pEQDY(SO_3)TGWMDF-NH_2

The biological activity of caerulein is similar to those of the mammalian intestinal peptide hormones gastrin and cholecystokinin. Caerulein contracts smooth muscle at the nanomolar concentration (10^{-9} M) [20] and may act directly on smooth muscle via the CCK_1 receptor or indirectly via the CCK_2 receptor (for details on receptors see section 5.3). Other biological activities of caerulein include the enhancement of blood circulation, sedation, thermoregulation and it is an analgaesic [20].

5.1d Tachykinins

The tachykinins are present in a number of anuran genera and are classified into various groups depending on the nature of the peptide (Table 5.2). The tachykinins are generally anionic in nature with some containing an N-terminal pyroglutamate residue and ending with the C-terminal consensus FYGLM-NH_2.

The major activity of the tachykinins is smooth muscle contraction, however, they may also act in the central nervous system as neurotransmitters and neuromodulators. Tachykinin peptides produce intestinal contraction through receptors located on enteric neurons via an

indirect mechanism, or by acting directly on smooth muscle receptors. The tachykinin mammalian counterparts substance P **(4)** and the neurokinins **(5)** act via the G protein-coupled NK_1, NK_2 and NK_3 receptors [94]. The receptors are widely distributed on nerve terminals and on a variety of smooth muscle.

(4) Substance P RPKPQQFFGLM-NH_2

(5) Neurokinin A HKTDSFVGLM-NH_2

Table 5.2 displays the activity of a selection of tachykinins using a smooth muscle contraction assay. Activity is recorded as an EC_{50} value, which represents the concentration of peptide required to achieve 50% tissue contraction.

Table 5.2. Smooth muscle contraction activities of a selection of tachykinin peptides

Name	Sequence	MW	EC_{50} (M)	Species
Substance P like:				
Xenopus SP [107]	KPRPDQFYGLM-NH_2	1349	10^{-9}	a
Xenopus NKA [107]	TLTTGKDFVGLM-NH_2	1280	10^{-9}	a
Ranakinin [108]	KPNPERFLYGLM-NH_2	1462	10^{-9}	b
Ranatachykinin A [108, 109]	KPSPDRFYGLM-NH_2	1309	10^{-9}	c
Aromatic:				
Physalaemin [108]	pEADPNKFYGLM-NH_2	1264	10^{-9}	d,e
Uperolein [108]	pEPDPNAFYGLM-NH_2	1233	10^{-10}	f,g
Ranatachykinin B [109]	YKSDSKFYGL-NH_2	1206	10^{-8}	c
Ranatachykinin C [109]	KPNPERFYAPM-NH_2	1348	10^{-8}	c
Aliphatic:				
Kassinin [108, 110, 111]	DVPKSDQFVGLM-NH_2	1333	10^{-9}	h,i

(a) *Xenopus laevis*; (b) *Rana ridibunda*; (c) *R. catesbeina*; (d) *Physalamus bilogonigerus*; (e) *P. fuscumacalatus*; (f) *Uperoleia rugosa*; (g) *U. marmorata*; (h) *Kassina senegalensis*; (i) *Neoceratodus forsteri*

5.1e Bradykinins

The bradykinins exist in a number of amphibian genera. The bioactivities are less effective and potent than those of the other neuropeptide groups. Activity includes smooth muscle contraction, the relaxation of intestinal, urogenital and respiratory tracts, and the regulation of blood pressure [112]. The actions of bradykinin are achieved via the G protein-coupled B_1 and B_2 receptors [112, 113]. Table 5.3 displays the smooth muscle contraction activity of a selection of bradykinins. Activity is recorded as an EC_{50} value, indicating the concentration of peptide required to achieve 50% tissue contraction.

Table 5.3. Smooth muscle contraction activities of a selection of bradykinin neuropeptides

Name	Sequence	MW	EC_{50} (M)	Species
Bradykinin [114-116]	RPPGFSPFR-OH	1059	10^{-8}	a,b,c
[Thr^6]bradykinin [116]	RPPGFTPFR-OH	1073	10^{-6}	c,d
Kinestatin [117]	pEIPGLGPLR-NH_2	932	10^{-8}	e

(a) *Rana temporaria*; (b) *R. palustris*; (c) *Bombina orientalis*; (d) *R. rugosa*; (e) *B. maxima*

5.1f Tryptophyllins

The tryptophyllins have been isolated from frogs of the *Phyllomedusa* and *Litoria* genera. They usually exist as major glandular components. Until recently, tryptophyllins were found to exhibit neither antimicrobial nor nNOS activity and their role in the host-defence chemistry of the animal remained uncertain [70, 71]. The recently isolated peptide PdT-1 (Table 5.4) and tryptophyllin L 1.3 (Table 5.4) are the first amphibian tryptophyllins to display smooth muscle activity. Biological activity is recorded as an EC_{50} value.

Table 5.4. Smooth muscle contraction activities of a selection of tryptophyllins

Name	Sequence	MW	EC_{50} (M)	Species
Tryptophyllin L 1.2 [70, 71]	FPWL-NH_2	560	-	a,b
Tryptophyllin L 1.3 [71]	pEFPWL-NH_2	671	10^{-6}	a
PdT-1 [74]	KPHypAWVP-NH_2	809	10^{-8}	c

(a) *Litoria rubella*; (b) *L. electrica*; (c) *Pachymedusa dacnicolor*

The tryptophyllins show some sequence similarity to human brain endomorphins (example, endomorphin 1, **(6)**) that have affinity for the γ-receptor [73].

(6) Endomorphin 1 YPWF-NH_2

5.1g Dermorphins and Deltorphins

The pharmacological properties of the dermorphins and deltorphins have been extensively studied [20, 118-120]. The uncharacteristic D-amino acid residue at position 2 is essential for biological activity (Table 5.5). D-amino acids form a different molecular structure to the L-amino acid form, with this structural element being crucial for the interaction of these peptides with opiate receptors [121].

The built-in analgaesic system in the body is dependent on the presence of opiate receptors (Figure 5.1). Analgaesic neurotransmitters, including endogenous opiates like morphine, are released from a descending analgaesic pathway and bind with opiate receptors on the afferent pain fibre terminal. The analgaesic effect is due to the activation of μ and δ opioid receptors [122]. This binding suppresses the release of neurotransmitters like acetylcholine, norepinephrine, dopamine, serotonin and substance P, thereby blocking further transmission of the pain signal [18]. Opioid receptors are widely distributed in the brain, spinal cord and peripheral nervous system and in a variety of immune cells [118].

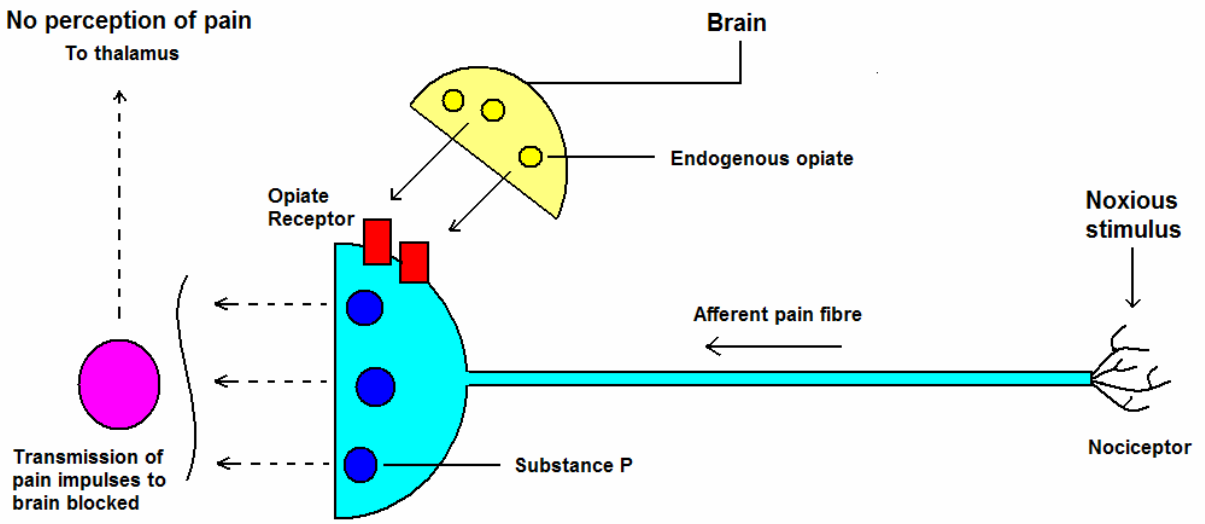


Figure 5.1. The proposed analgaesic pathway. Endogenous opiates bind to opiate receptors to suppress the release of neurotransmitters like substance P, thereby blocking the transmission of pain impulses to the brain

Table 5.5. Opioid activities of dermorphin and deltorphin neuropeptides

Name	Sequence	MW	EC_{50} (M)*	Species
Dermorphins				
Dermorphin [123, 124]	Y(D-Ala)FGYPS-NH_2	802	10^{-11}	a,b,c,d
[Hyp^6]dermorphin [124, 125]	Y(D-Ala)FGYHypS-NH_2	818	10^{-9}	a,b,c
Deltorphin				
[a2]deltorphin 1 [119, 120]	Y(D-Ala)FDVVG-NH_2	768	10^{-6}	e
[a2]deltorphin 2 [119, 120]	Y(D-Ala)FEVVG-NH_2	782	10^{-6}	e

* EC_{50} is reported for inhibition of twitch responses in electrically stimulated mouse vas Deferens, an index of μ opioid activation

(a) *Agalychnis callidryas*; (b) *Phyllomedusa rohdei*; (c) *P. sauvagei*; (d) *P. burmeisteri*; (e) *P. bicolor*
Amino acid: Hyp- hydroxyproline (MW 131)

5.1h Miscellaneous Neuropeptides

A number of isolated amphibian peptides exhibiting a range of activity have not been categorised into one of the above groups and are listed in Table 5.6. These include rothein 1 and the disulfide-containing peptides isolated from the *Crinia* genus. The activities of these peptides are discussed in section 5.4 and 5.5 respectively.

Table 5.6. Activities of miscellaneous neuropeptides

Name	Sequence	MW	EC_{50} (M)	Species
Temporin 1Gb [126]	SILPTIVSFLSKFL-NH_2	1562	Smooth muscle relaxant (10^{-6})	a
Brevinin 1 [127]	FLPVLAGIAAKVVPALFCKITKKC-OH	2526	Insulin release agent (10^{-6})	b
Pipinin 1 [128]	FLPIIAGVAAKVFPKICAISKKC-OH	2562	Histamine release agent (10^{-6})	c
pLR [129]	LVRGCWTKSYPPKPCFVR-OH	2136	Histamine release agent (10^{-6})	c
Sauvagine [40]	pEGPPISIDLSLELLRKMIEIEKQEKEKQ QAAWNRLLLDTI-NH_2	4600	Trypsin inhibitor	d

(a) *Rana grylio*; (b) *R. palustris*; (c) *R. pipiens*; (d) *Phyllomedusa sauvagei*

5.2 Immunomodulatory Peptides

5.2a General

The environment contains many potentially harmful microorganisms. Over the course of evolution, organisms have developed a variety of defences to overcome would-be invaders. Barriers include structural properties like skin or specific responses like the immune system [130].

The immune system is designed to protect the host from invading pathogens and to eliminate disease. It is composed of a number of specialised cell types that have unique functions together with particular sets of chemical messengers [131]. Protection from infection and disease is provided by two major components (Figure 5.2) [130]:

i) the innate immune system, and
ii) the adaptive immune system.

The innate immune system is the first line of defence against an antigen and includes three components [18]:

i) physical,
ii) biochemical, and
iii) cellular.

The adaptive immune system is activated when the innate immune system is inadequate to cope with infection. The adaptive immune system functions by integrating the lymphatic, circulatory and endocrine systems. The most important of these is the lymphatic system, which consists of both B and T lymphocytes [130]. Each type of lymphocyte plays a specific role in the immune system. The cells are typed according to their function and their immunologically defined cell surface markers [131].

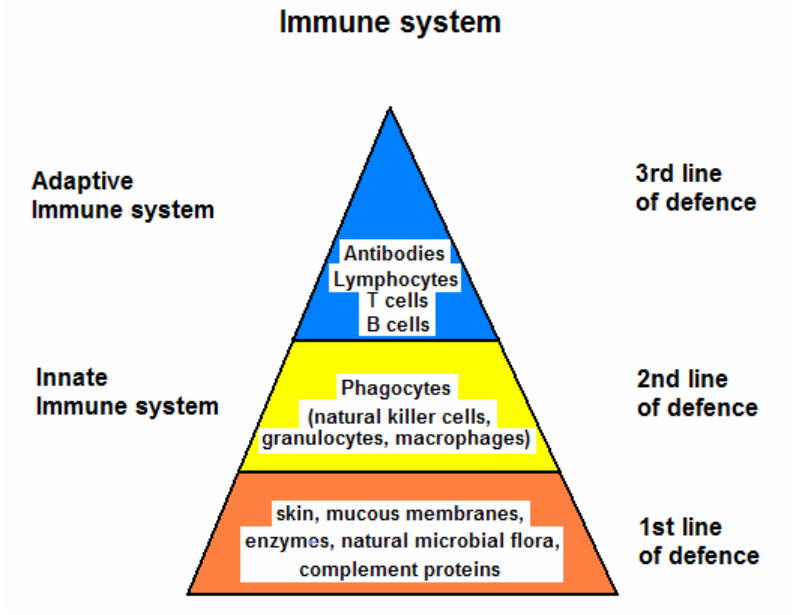


Figure 5.2. The three lines of defence involved in the immune system

The first major class of cells, T lymphocytes, are located in the thymus gland. They provide two important functions [131]:

i) the destruction of host-cells with altered surfaces, and
ii) the regulation of immune reactions by direct interaction with other cells or indirectly through the production and release of soluble mediators.

T cells are responsive to T cell mitogens (substances that induce cell division) like concanavalin A [131]. Upon interaction with T cell mitogens, T lymphocytes undergo a blastogenic response (transformation of small lymphocytes into larger cells that are capable of undergoing mitosis) to produce lymphokines. This includes interleukin 2 that promotes and maintains the growth of T lymphocytes [131].

The second major class of lymphocytes is B cells, which are located within bone marrow. B cells are active primarily against viruses and bacteria and are involved in the synthesis of immunoglobulin [131].

5.2b Immunomodulators and T Cell Activation

Hormones and neuropeptides like substance P (see **(4)**, section 5.1d) and neuropeptide Y **(7)** influence immune cell activity by controlling the proliferation, differentiation and function of various tissues and neoplastic cells [132, 133]. Neuropeptides directly affect immune function through common mediators and receptors [95], which allow communication between these two systems [131, 134, 135] .

(7) Neuropeptide Y YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRY-NH_2

T cells exclusively have CCK_2 receptors (Figure 5.3) [136]. The neuropeptides CCK-8 and CCK-8-NS (see section 5.3 for peptide sequences) are CCK_2 receptor agonists that produce:

i) the growth and division of cells,
ii) chemotaxis, and
iii) superoxide production.

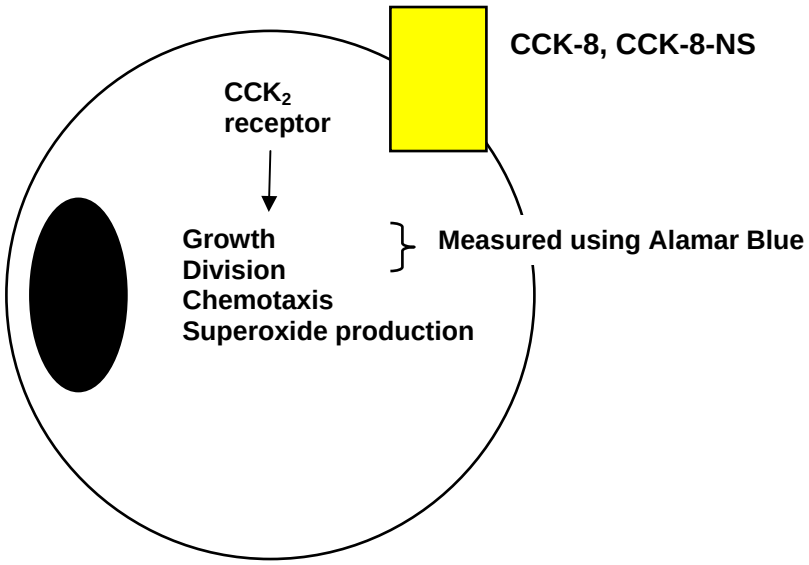


Figure 5.3. Activation of CCK_2 receptors induces lymphocyte proliferation

The Alamar Blue method, which measures the proliferation of lymphocytes, is a common assay in immunological research [137]. This one step assay only requires the addition of the fluorescent dye Alamar Blue to cultures. Alamar Blue contains Redox indicator, with the oxidized form being dark blue in colour. As cellular growth induces the chemical reduction of media, the indicator changes to red. Following cell incubation, assessment of cell growth is measured by determining the fluorescence value using an ELISA fluorescent plate reader (see experimental section 5.7).

The Alamar Blue method has a number of advantages including [137]:

i) a simple protocol that saves time and reduces errors,
ii) a rapid assessment of proliferation,
iii) monitoring cell proliferation during the culture period,
iv) less costs, and
v) permitting additional analysis of proliferating cells.

This research tests a number of amphibian peptides and their modifications for CCK_2 receptor agonism by measuring cell proliferation using the Alamar Blue method.

5.3 Cholecystokinin

5.3a General

The peptide cholecystokinin (CCK) was originally discovered in the porcine gastrointestinal tract [138] and is considered the most widespread and abundant neuropeptide in the central nervous system (CNS) [139, 140]. There are a number of biologically active CCK peptides that are derived from the 115 amino acid precursor. These include CCK-58, CCK-33, CCK-8 (sulfated **(8)** and nonsulfated **(9)**), CCK-7 (sulfated and nonsulfated) and CCK-4 **(10)**. They all share a common carboxyl-terminal heptapeptide domain that is required to elicit a response with full efficacy and potency [141]. The post-translational addition of sulfate to the tyrosine residue in CCK-8 is critical for high-affinity binding and biological activity of CCK-8 at the receptor site [142]. Desulfation of CCK-8 results in a loss in affinity and potency [143].

(8) CCK-8 DY(SO_3)MGTMDF-NH_2

(9) CCK-8-NS DYMGTMDF-NH_2

(10) CCK-4 TMDF-NH_2

Cholecystokinin-8 is released as a hormone from the mucosa of the small intestine and as a neurotransmitter-neuromodulator from nerve terminals of the myenteric plexus. CCK-8 exerts a variety of pharmacological effects through receptors, classified as CCK_1 and CCK_2 [140]. These include stimulation of pancreatic enzyme secretion [144], smooth muscle contraction and increase in blood circulation [142, 145]. Each receptor plays a specific role in the physiological and pharmacological responses to CCK peptides (see section 5.3b) [140]. The use of selective agonists and/or antagonists separates the specific pharmacological responses mediated by each CCK receptor.

5.3b Cholecystokinin Receptors

The cholecystokinin (CCK) receptors differ in anatomical location and action [136]. The receptors have been classified into two subtypes based on ligand binding assays and the use of selective antagonists [139]. CCK_1 receptors:

i) occur in peripheral tissues,
ii) are present on smooth muscle and neuronal cells, and
iii) have a high affinity for sulfated CCK.

In contrast, CCK_2 receptors [143]:

i) are distributed in the central nervous system,
ii) are present on T lymphocytes, and
iii) have a high affinity for both sulfated and non-sulfated forms of CCK-related peptides.

The sequences of both receptors are known and representations of their 3D structures have been reported (Figure 5.4) [139, 146]. NMR and other experimental data have been used to determine where CCK-8 binds to receptors [147, 148].

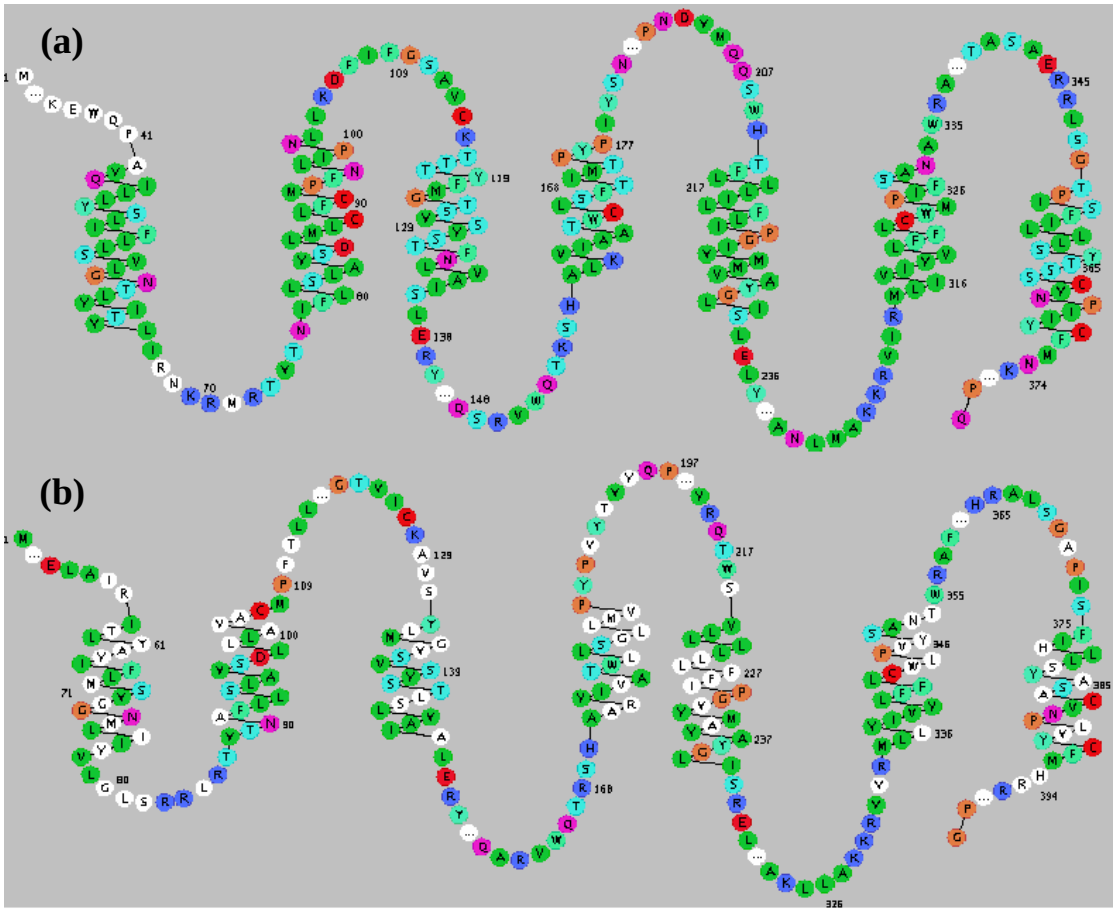


Figure 5.4. The **(a)** CCK_1 receptor and **(b)** CCK_2 receptor shown as snake diagrams. Residues are shown in CPK-like colours. Source: [149, 150]

Peptides regulate smooth muscle function in mammalian species by initiating stimulatory or inhibitory effects [151]. This can be measured using a simple functional assay for CCK receptors. Guinea-pig ileum longitudinal muscle contains both receptor subtypes. CCK receptor agonists elicit contraction of smooth muscle in two ways. CCK_1 receptors present on smooth muscle contract smooth muscle directly (Figure 5.5). In contrast, CCK_2 receptors act indirectly, by causing the release of acetylcholine from cholinergic nerves in the myenteric plexus, which activates muscarinic receptors on smooth muscle (Figure 5.5) [152, 153].

Muscarinic receptors play an important role in the contraction of many types of smooth muscle [154]. The receptors M_2 and M_3 are located on cell membranes (smooth muscle, cardiac muscle and glands) and on the endothelium of most peripheral blood vessels [155]. Atropine blocks the effect of acetylcholine at muscarinic receptors [156].

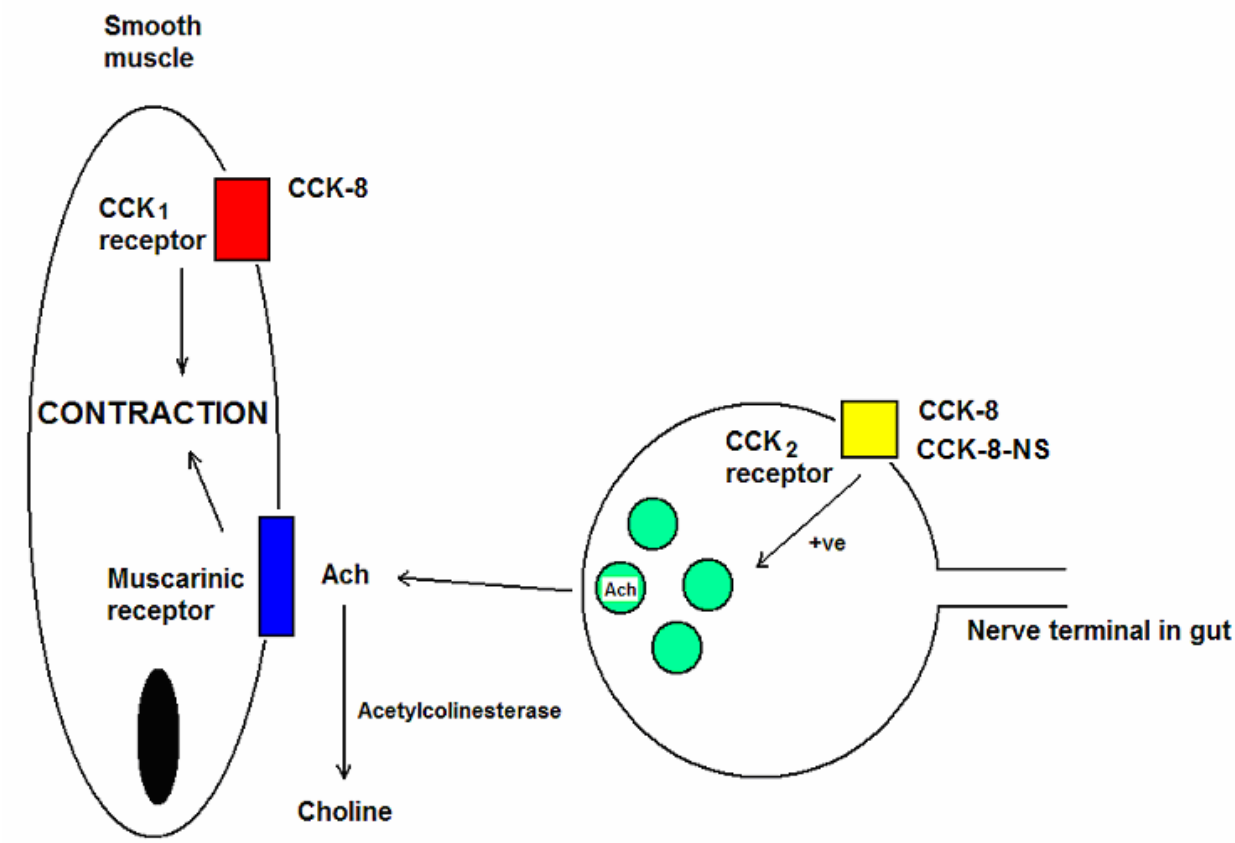


Figure 5.5. Diagram of a muscarinic nerve/smooth muscle interaction in the ileum. CCK peptides cause smooth muscle contraction by direct activation of CCK_1 receptors on smooth muscle and indirectly by activation of CCK_2 receptors on muscarinic nerve terminals, with subsequent release of acetylcholine (Ach)

Activation of various receptor types can be manipulated by compounds that selectively enhance (agonists) or block (antagonists) responses at each of the receptor types. The CCK_2 receptor antagonist LY-288,513 (Figure 5.6) was used to test peptides for CCK_2 receptor selectivity. In the presence of LY, reduced biological activity was recorded for peptides acting via CCK_2 receptors.

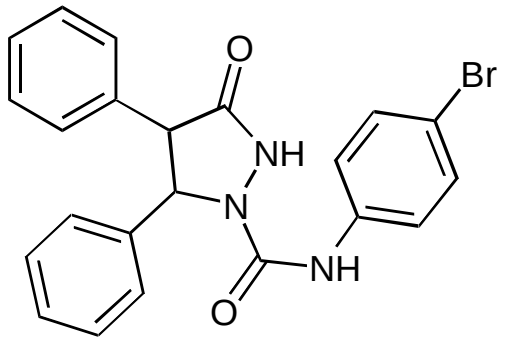


Figure 5.6. The CCK_2 receptor antagonist LY-288,513

5.3c Peptide-Receptor Interactions

CCK receptors belong to the largest group of plasma membrane receptors known as the G protein-coupled receptor (GPCR) family [142]. GPCRs regulate a wide range of physiological processes by transmitting signals into cells in response to stimuli such as peptides, proteins and hormones [157, 158]. The receptors are integral membrane proteins consisting of seven transmembrane helices that are connected by intracellular and extracellular loops, an N-terminal extracellular domain and a C-terminal intracellular region (Figure 5.7) [158].

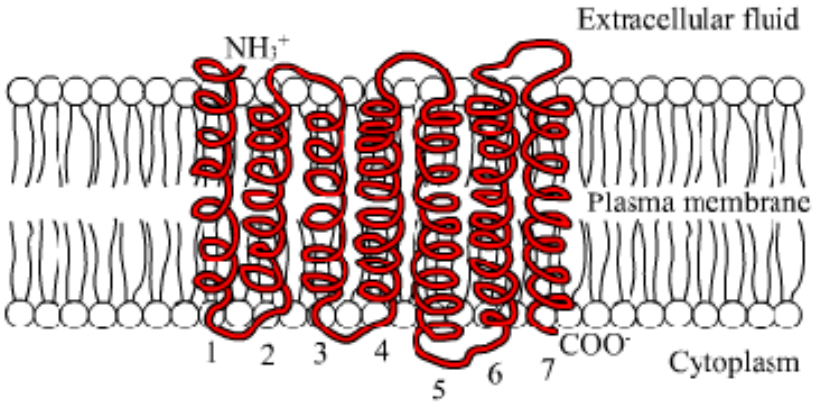


Figure 5.7. The seven transmembrane α-helix structure of a G protein-coupled receptor [159]

A knowledge of receptor binding sites is required for the understanding of ligand action [158]. CCK_1 and CCK_2 receptors have distinct binding sites [139], which are composed of amino acids of the first and second extracellular loops and amino acids of the upper third transmembrane helix [158].

The interactions between a peptide and its receptor are critically important [142]. The binding specificity and affinity arise from interactions due to the general size, shape and hydrophobicity of the ligand. Structurally related ligands generally dock in a similar region of the receptor [142].

Many compounds exhibiting diverse structures are capable of binding and activating GPCRs. A peptide may behave as a full agonist, a partial agonist or an antagonist depending on the species, tissue or biological event [158]. Partial agonists are of therapeutic interest due to their ability to produce low side-effects and desensitise their target [160]. Understanding the molecular basis of ligand-receptor binding and the conformational changes involved in reaching active or inactive receptor states will greatly facilitate the design of novel therapeutics [142, 161].

5.4 The Rothein Peptides

5.4a Introduction

The host-defence peptides from skin secretions of *Litoria rothii* (Roth's Tree Frog) (Figure 5.8) have been investigated [162]. *L. rothii* is distributed across the north of Australia from the top of Western Australia across to the Northern Territory and down the north eastern coastal area of Queensland [8].

Figure 5.8. *Litoria rothii*

The peptide content of skin secretions varies seasonally. During the winter season (or the inactive period of the animal) the major glandular components are the neuropeptides rothein 1 (Table 5.7) and caerulein 1.2. In contrast, the major components of the summer secretion (or the reproductive period of the animal) are caerulein together with a number of potent antibiotic caerin peptides and an nNOS inhibitor [162].

The novel peptide rothein 1 was sequenced using a combination of positive ion and negative ion mass spectrometry [162]. It exhibits neither antibiotic, anticancer nor nNOS activity. Rothein 1 was tested for pharmacological activity using a smooth muscle contraction assay and a lymphocyte proliferation assay. The structure of rothein 1 (Figure 5.9) was determined using two-dimensional nuclear magnetic resonance [163].

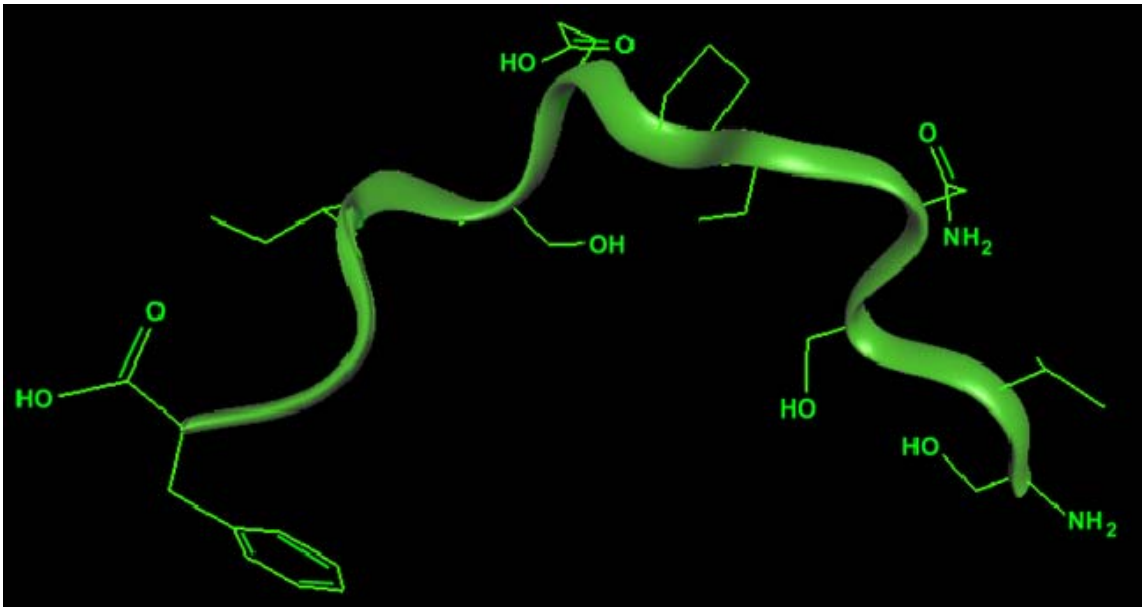


Figure 5.9. The structure of rothein 1

This section investigates the:

i) pharmacological activity of rothein 1 and some modifications, and
ii) peptide-receptor binding.

5.4b Results

Rothein 1 and a number of rothein 1 synthetic modifications were tested for biological activity (Table 5.7). Rothein 1 was modified by:

i) substituting the hydrophobic residue Ile for the hydrophilic residue Thr at the C-terminal end of rothein 1.1,
ii) substituting residue one for the hydrophobic residue Ala in rothein 1.2,
iii) substituting Ser for Ala in position 3 of rothein 1.3, and
iv) substituting a hydrophilic residue for a hydrophobic residue in the centre of the peptide in rotheins 1.4 and 1.5.

Several other synthetic modifications have also been made; however their pharmacological activities were not available at the time of writing this thesis.

Table 5.7. Sequences of rothein 1 and rothein 1 synthetic modification peptides

Name	Sequence	MW
Rothein 1	SVSNIPESIGF-OH	1148
Rothein 1.1	SVSNIPESTGF-OH	1136
Rothein 1.2	AVSNIPESIGF-OH	1133
Rothein 1.3	SVANIPESIGF-OH	1133
Rothein 1.4	SVSNIPASIGF-OH	1090
Rothein 1.5	SVSNIPEAIGF-OH	1133

Two biological assays were used to ascertain the biological activity of rothein peptides.

5.4.1b Contraction Studies

Acetylcholine produced a concentration-dependent increase in contraction of isolated guinea-pig ileum segments (Figure 5.10). Cholecystokinin-8 (CCK-8) was used as the standard mixed CCK_1CCK_2 receptor agonist and produced an increase in contraction. The maximum contractile response of CCK-8 occurred at 10^{-9} M (Figure 5.11). This was 60% of the response produced by the maximally effective concentration of acetylcholine (see Figure 5.10 and Figure 5.11). Cholecystokinin-8 nonsulfated (CCK-8-NS) was used as the standard CCK_2 receptor agonist and produced an increase in contraction that was less potent and less effective than CCK-8 (Figure 5.11). These results are consistent with previous studies [152].

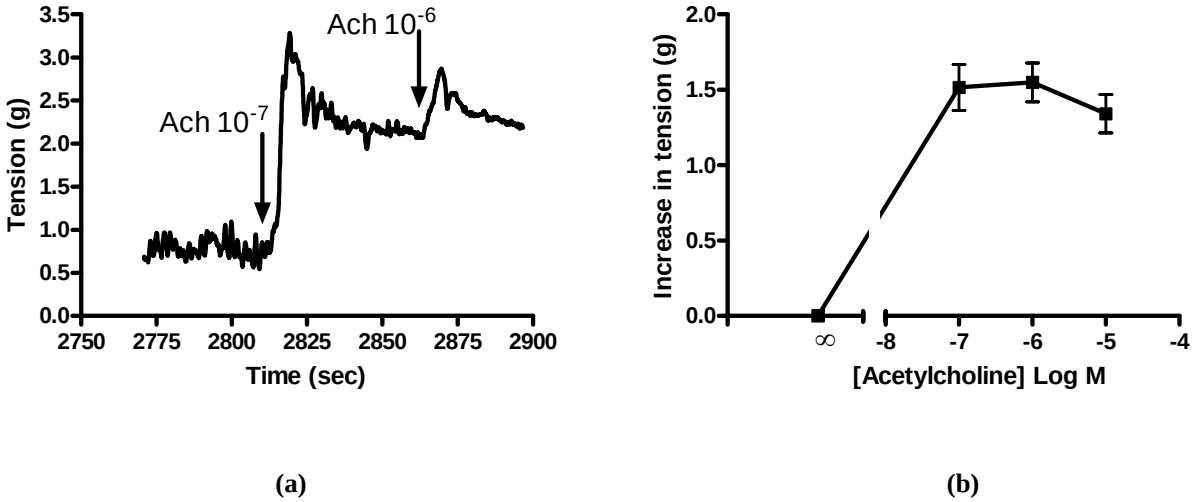


Figure 5.10. (a) Individual trace illustrating the contraction of ileum produced by acetylcholine, and **(b)** concentration-response curve to acetylcholine in guinea-pig ileum showing mean ± SEM for four independent experiments

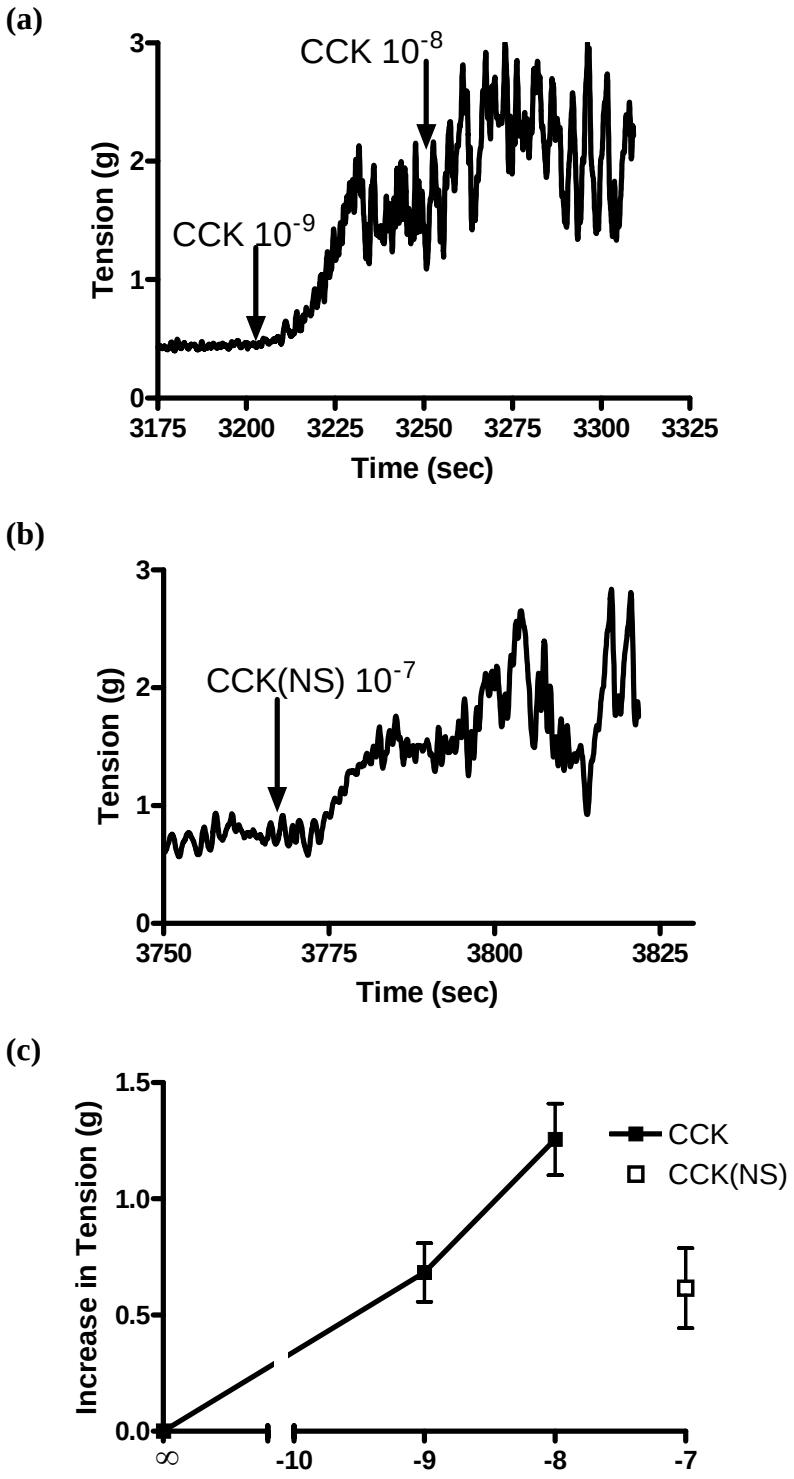


Figure 5.11. Individual traces of guinea-pig ileum contraction to **(a)** CCK-8 and **(b)** CCK-8-NS, and **(c)** CCK-8 and CCK-8-NS concentration-response curves. Mean ± SEM of four independent experiments are shown

The rothein peptides displayed two separate responses. The peptides produced a slight contraction of the ileum segments when applied directly (the direct response, Figure 5.12a,b and Table 5.8), but upon washout, a much larger contraction was produced (Figure 5.12a,c). This "rebound response" was not seen with CCK-8, CCK-8-NS or the other peptides studied to date.

This "rebound response" could be seen after washout of a single concentration (10^{-7} M) of rothein (data not shown). While all the rothein peptides produced this "rebound response", rothein modifications 1.2 and 1.3 produced the smallest rebound response (Figure 5.12c). Rothein peptide modifications 1.4 and 1.5 produced the largest direct response.

Table 5.8. Smooth muscle contraction activities of rothein peptides

Peptide	**Activity [Min. Conc. (M)]**
Rothein 1	10^{-7}
Rothein 1.1	10^{-8}
Rothein 1.2	10^{-8}
Rothein 1.3	10^{-8}
Rothein 1.4	10^{-8}
Rothein 1.5	10^{-8}

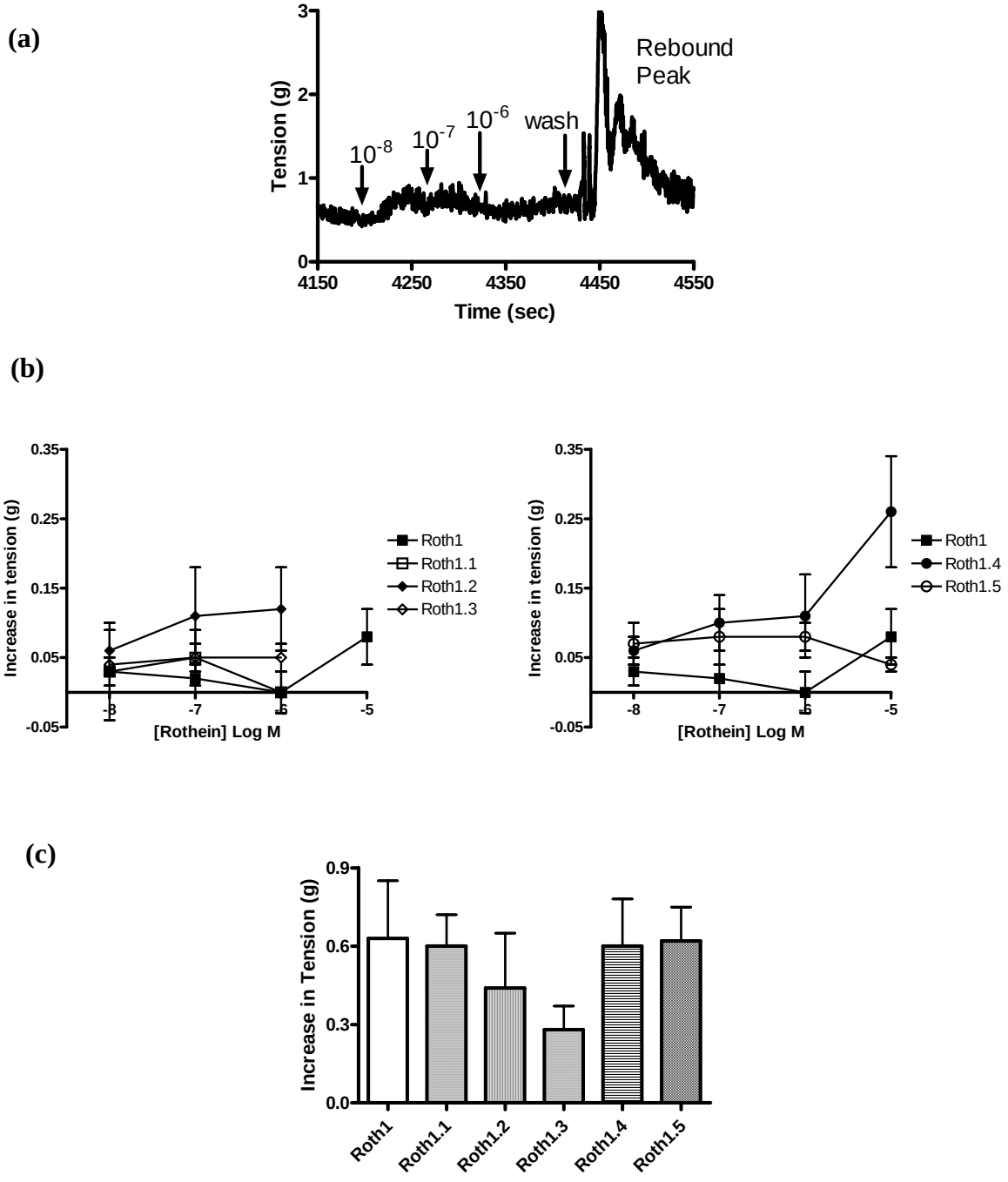


Figure 5.12. The bimodal response of rothein peptides- **(a)** individual trace of rothein peptide displaying the "rebound response", **(b)** concentration-response curves of rothein peptides, and **(c)** contraction of ileal segments produced by rothein peptides during the "rebound response". Mean ± SEM of three independent experiments are shown. (Note the "rebound response" is much higher than the direct response to all peptides)

Both the direct response and the "rebound response" to the rotheins were largely removed by atropine, suggesting that the release of acetylcholine from neuronal stores is responsible for the contraction of the ileum. To see if the "rebound" was due to the rotheins themselves inhibiting muscarinic receptors, acetylcholine (10^{-7} M) was added in the presence of rothein. No diminution of the acetylcholine-induced contraction was seen (data not shown). This suggests that the rotheins are not an antagonist to muscarinic receptors. However, the rotheins may be releasing an inhibitory factor in addition to acetylcholine. As a contraction response occurred rapidly when the peptides were washed out, the inhibitory compound must be short lived. A possible candidate is nitric oxide, an inhibitor of acetylcholine-induced contraction. Nitric oxide is produced by neuronal nitric oxide synthase in the myenteric plexus of the central nervous system and has a very rapid decay time relative to acetylcholine's contractile effects, consistent with the "rebound response" seen above [164].

The effects of rothein 1 and rothein 1.4 were determined in the presence of the CCK_2 receptor antagonist LY-288,513 (10^{-6} M). The contractile response slightly decreased for rothein 1 and was substantially diminished for rothein 1.4 (control 0.6 ± 0.18 g vs 0.12 ± 0.18 g in the presence of LY, n=4), suggesting the peptides act via CCK_2 receptors (Figure 5.13).

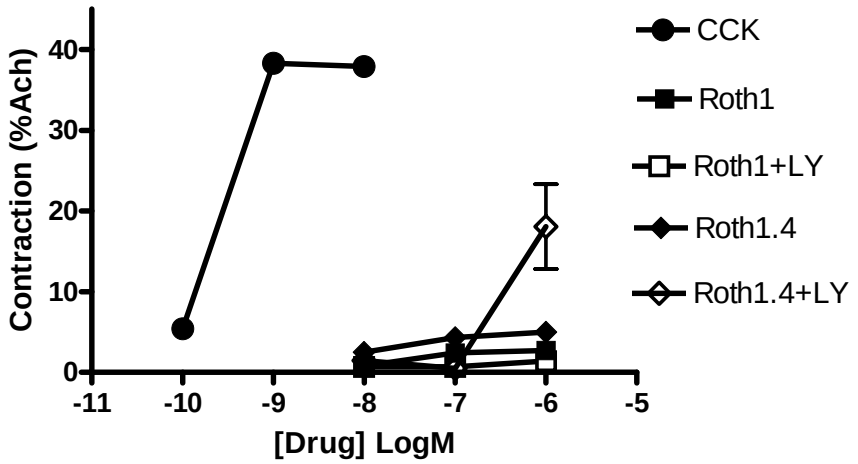


Figure 5.13. Guinea-pig ileum contraction produced by rothein 1 and rothein 1.4 in the presence and absence of the CCK_2 receptor antagonist LY

5.4.2b Lymphocyte Proliferation Studies

The rotheins were tested as potential CCK_2 agonists. Lymphocytes possess CCK_2 receptors exclusively on their surface that proliferate upon stimulation [136]. Spleen derived lymphocyte proliferation was assessed using the Alamar Blue fluorescence dye method [165].

The standard CCK-8 produced a concentration-dependent increase in lymphocyte proliferation in the presence (Figure 5.14) and absence (data not shown) of the mitogen concanavalin A. CCK-8-NS was less effective (Figure 5.14). This is consistent with previous studies [166, 167].

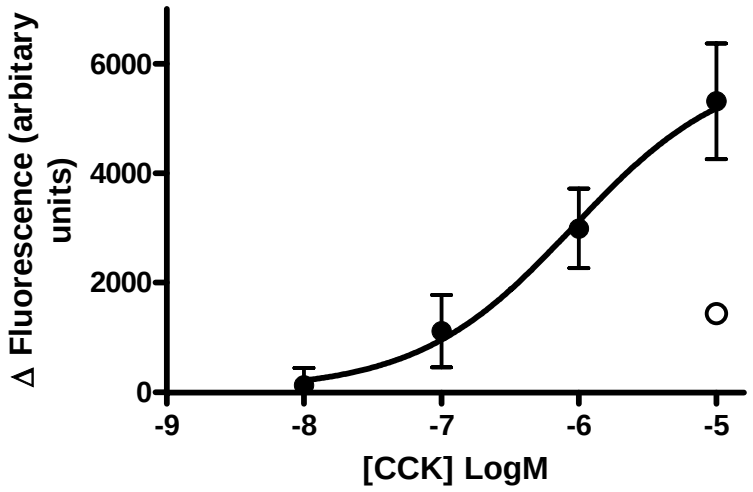


Figure 5.14. CCK-8 (●) and CCK-8-NS (○) concentration-response curves in mouse splenocytes. The values shown indicate an increase in fluorescence over the unstimulated controls; they are a mean ± SEM of two independent experiments performed in quadruplicate

Rothein 1 and rothein modifications 1.1, 1.2 and 1.3 produced a concentration-dependent increase in lymphocyte proliferation that was similar in potency to CCK-8-NS (Figure 5.15 and Table 5.9). The results indicate that the rotheins may be acting as CCK_2 receptor agonists. Rotheins 1.4 and 1.5 were inactive in this assay.

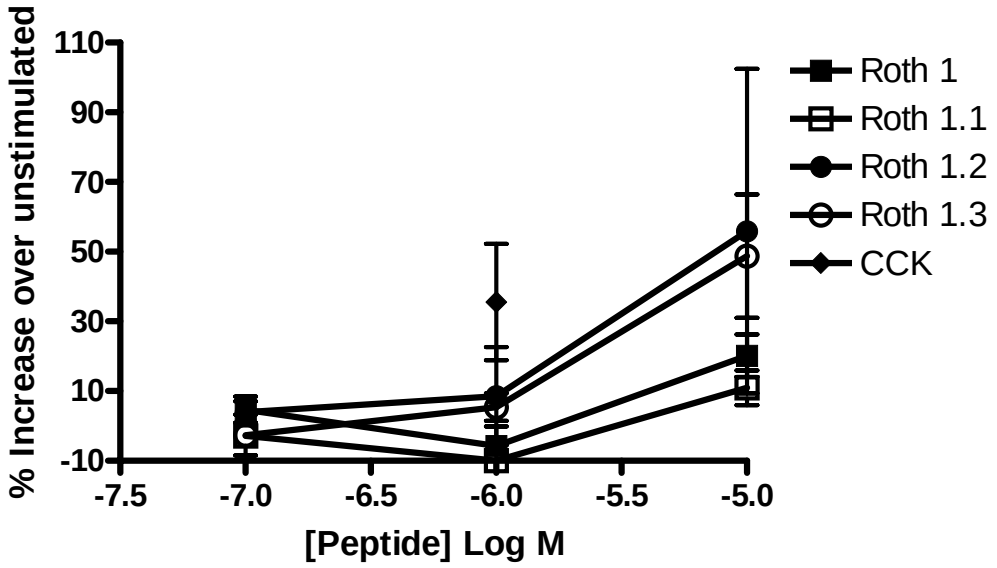


Figure 5.15. Rothein concentration-response curves in mouse lymphocytes. Data are given as the percent increase over the unstimulated controls and are shown as mean ± SEM of two independent experiments performed in quadruplicate. The response to CCK is shown for comparison

Table 5.9. Lymphocyte proliferation activities of rothein peptides

Peptide	**Activity [Min. Conc. (M)]**
Rothein 1	10^{-7}
Rothein 1.1	10^{-5}
Rothein 1.2	10^{-7}
Rothein 1.3	10^{-7}
Rothein 1.4	Inactive
Rothein 1.5	Inactive

5.4c Discussion

The amphibian neuropeptide caerulein and the mammalian gastrin-like neuropeptide cholecystokinin-8 (CCK-8) are agonists for the receptor subtypes CCK_1 and CCK_2 [136]. The CCK receptor sequences have been reported [139] together with the binding site of CCK-8 on the receptors [139, 147, 148]. CCK receptor agonists act to cause contraction of guinea-pig ileum smooth muscle [152]. Smooth muscle is contracted directly by CCK_1 receptors present on the tissue, and contracted indirectly by CCK_2 receptors that cause the release of acetylcholine from cholinergic nerves in the myenteric plexus, activating muscarinic receptors located on smooth muscle [152]. In these experiments, CCK-8 and CCK-8-NS were used as standards to calibrate smooth muscle and lymphocyte proliferation responses. The standard neuropeptide CCK-8, which activates both CCK_1 and CCK_2 receptors, produced a concentration-dependent increase in contraction of guinea-pig ileal segments (see Figure 5.11). The CCK_2 receptor agonist, CCK-8-NS, produced a concentration-dependent increase in contraction of guinea-pig ileal segments that was less potent and less effective than CCK-8 (see Figure 5.11).

Rothein 1 and peptide modifications were tested for agonist activity via one or both CCK receptor subtypes. The rothein peptides exhibited two separate responses. Prior to washout, the peptides themselves produced only a slight contractile effect on smooth muscle that was comparable to that of CCK-8-NS. Upon washout, a large contractile response was observed. This suggests that the rotheins may be activating an unknown receptor to release an inhibitory factor simultaneously with the activation of CCK_2 receptors to produce smooth muscle contraction (Figure 5.16). A possible inhibitor of acetylcholine-induced contraction may be nitric oxide (NO). NO would be eliminated rapidly by diffusion following washout, therefore releasing acetylcholine to produce the stimulation and contraction of smooth muscle tissue [168]. A similar response has been reported for the cannabinoid system [169, 170]. To further investigate the inhibitory effect of NO, the use of a non-selective NOS inhibitor like L-NAME (N omega-nitro-L-arginine methyl ester) could be used [168]. A stimulatory effect would only

be observed if the peptides are activating an unknown receptor to release NO as the inhibitory compound. At present, the significance of the “rebound response” is uncertain. This effect needs to be further investigated.

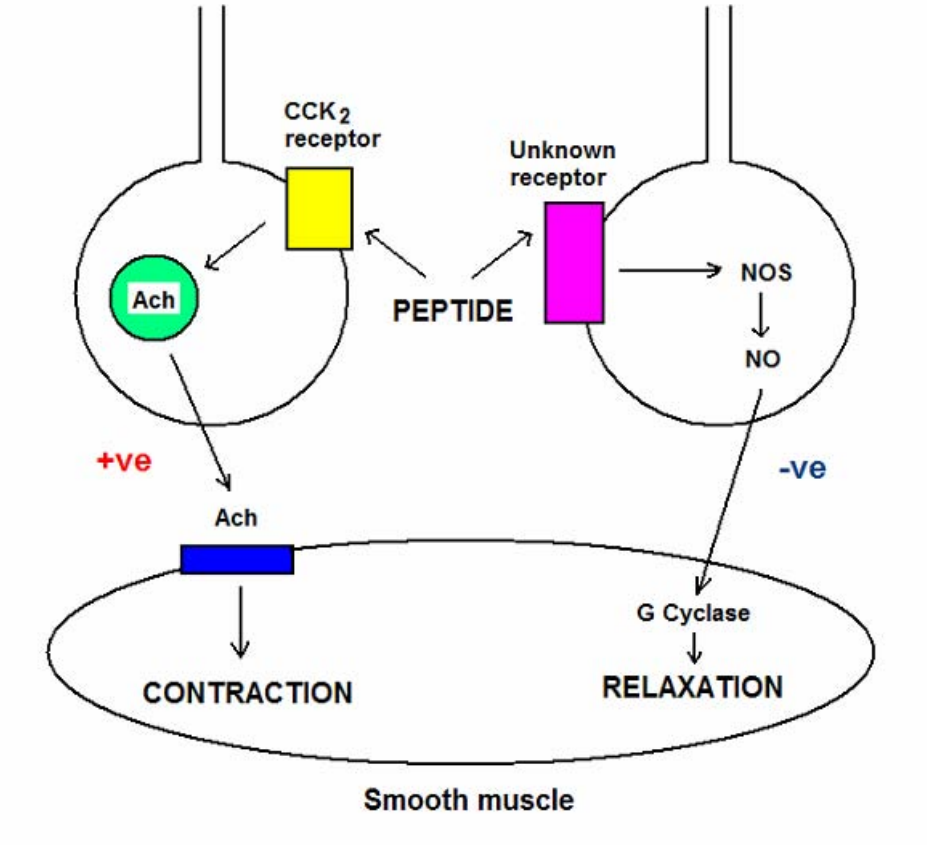


Figure 5.16. The stimulatory and inhibitory effects of peptides on smooth muscle

The smooth muscle response of rothein 1 and rothein 1.4 were investigated in the presence of the CCK_2 receptor antagonist LY-288,513. The contractile response of both peptides decreased in potency, indicating that the rotheins are selective CCK_2 receptor agonists.

Peptides were tested for their ability to proliferate lymphocytes. Lymphoid cells exclusively have CCK_2 receptors [136], and exposure of mouse lymphocytes [166] to CCK_2 agonists results in lymphocyte proliferation. In this study, the standard CCK-8 produced a concentration-dependent increase in proliferation of mouse splenocytes, as measured by the Alamar Blue assay [137]. CCK-8-NS produced a proliferation response that was less potent than CCK-8. Rothein 1.2 and rothein 1.3 induced proliferation of lymphocytes and were similar in potency to CCK-8-NS. Rothein 1 and rothein 1.1 were less effective. This suggests that these peptides may be CCK_2 receptor agonists. The remaining rothein peptides were inactive in this assay.

Based on the experimental data, the bioactivities of rothein 1 and synthetic modifications occur via activation of CCK_2 receptors. As rothein 1 produces both inhibitory and stimulatory effects of smooth muscle tissue, it seems unlikely that the major role of this peptide involves smooth muscle contraction. In contrast, rothein 1 is a CCK_2 receptor agonist in T lymphocytes, indicating a possible role in immune function. The three-dimensional structure of rothein 1 (Figure 5.9) displays a high degree of conformational flexibility in solution. This flexibility may aid peptide-receptor binding affinity.

Rothein 1 displayed cell proliferation and smooth muscle activities that were similar in potency to CCK-8-NS. The bioactivity of rothein 1.1 (Table 5.7) differed in the two biological systems studied. Rothein 1.1 contracted smooth muscle at 10^{-8} M but proliferated lymphocytes at 10^{-5} M. Therefore, by making the C-terminal end of the peptide more hydrophilic, the peptide has a greater affinity for CCK_2 receptors present on smooth muscle cells. Rotheins 1.2 and 1.3 were structurally modified at the N-terminal end of the peptide. This modification did not result in a significant change in biological activity, suggesting that the N-terminal end of the peptide may not be important for peptide-receptor binding. Rothein 1.4 and 1.5 also differed in the two biological systems studied. Rothein 1.4 and 1.5 were modified in the centre

of the peptide. This did not result in a loss of biological activity for CCK_2 receptor binding on smooth muscle cells, however, modification of the central part of the peptide resulted in the complete loss of biological activity for lymphocyte activation. The central hydrophilic residues of the peptide are thus necessary for lymphocyte activity. The difference in biological activity may arise from peptides binding to different receptors on lymphocytes and smooth muscle cells.

Further studies are required in order to determine the role of rothein in anuran skin, particularly in the induction of lymphocyte proliferation. This will involve testing peptides for activity against a range of other G protein-coupled receptors from known neuropeptide families. Photoaffinity labelling is a biochemical approach used to identify and characterise receptors for further structural investigations [157, 171]. It is a technique in which a photochemically reactive molecular entity, specifically associated with a biomolecule, is photoexcited in order to covalently attach a label to the biomolecule, usually via intermediates [172, 173]. The assistance of photoaffinity labelling of ligands will provide structural analysis of ligand-peptide complexes.

5.5 The Bioactivities of Disulfide Neuropeptides from *Crinia*

5.5a Introduction

Cyclic peptides are present in various mammalian and non-mammalian species and exhibit both pharmacological and antibacterial properties. Cys containing peptides have been isolated from anurans of the *Rana* and *Crinia* genera [22, 23, 62, 127].

North American and European frogs belonging to the family *Ranidae* contain a number of novel antimicrobial peptides within granular gland secretions [174]. Many of these contain two Cys residues towards the C-terminal end of the peptide linked via a disulfide bridge [62, 175, 176]. Isolated host-defence compounds from the *Rana* genus are grouped into eight families based on structural similarities. These include peptides from the esculentin, brevinin and ranateurin families (Table 5.10). Structural features of these basic peptides include a heptapeptide loop linked by a disulfide bridge at the carboxyl terminus and an N-terminal section that varies in sequence and length. The presence of a disulfide bridge does not appear to be critical for antimicrobial activity [62]. The final family, the tigerinins (Figure 5.17), possess a disulfide bridge composed of nine amino acid residues. In this case, the absence of the disulfide bridge results in the loss of antibacterial activity [63].

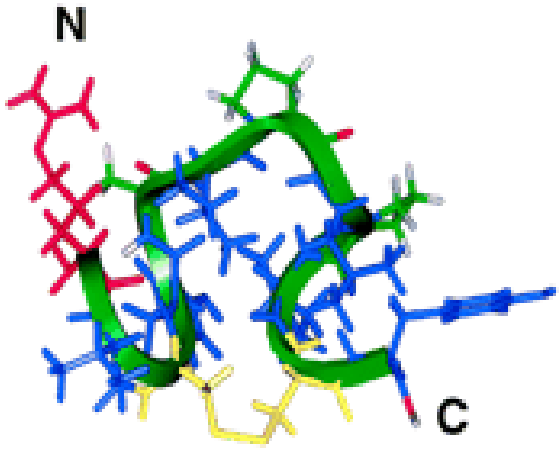


Figure 5.17. The structure of tigerinin 3. Hydrophilic residues are indicated in red and hydrophobic residues are indicated in blue. Cysteine side chains are in yellow [63]

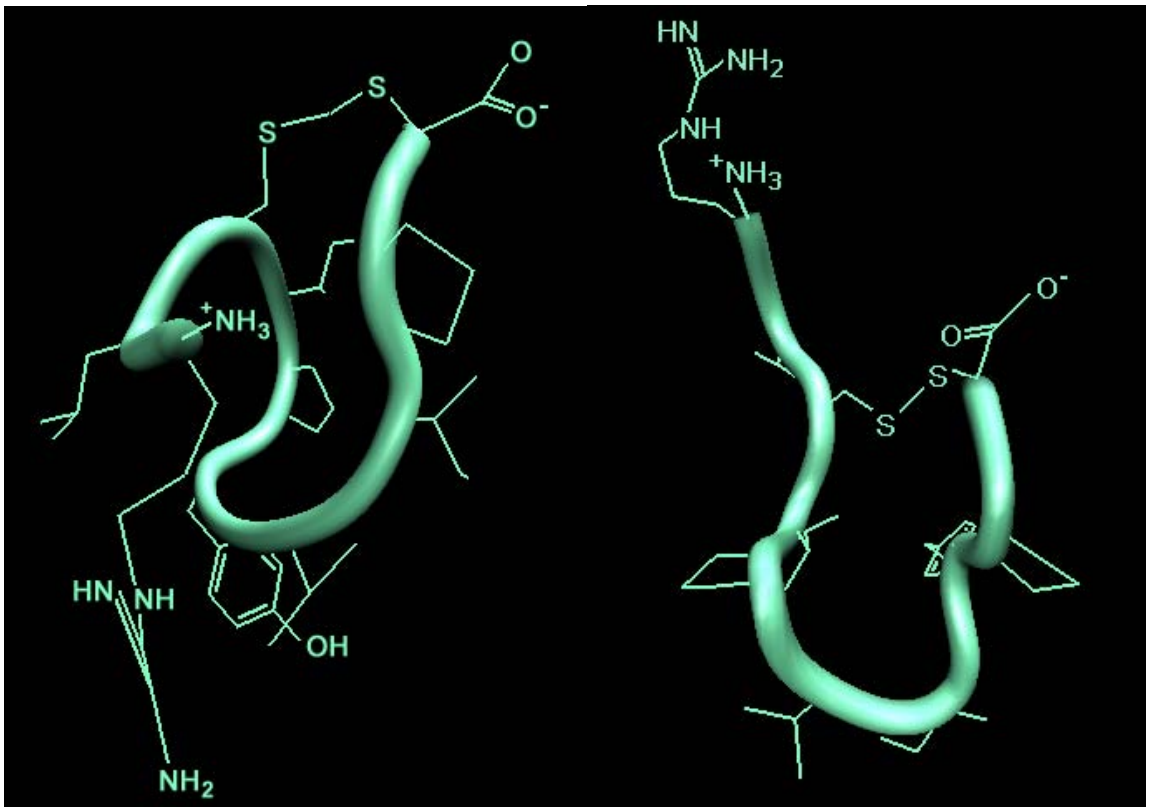
Table 5.10. A selection of disulfide peptides and their activities isolated from the *Rana* genus

Name	Sequence	MW	Activity	Species
Esculentin-1a [177]	GIFSKLAGKKIKNLLISGLKNVGKEV GMDVVRTGIDIAGCKIKGEC-OH	4805	a	a
Brevinin-1 [175]	FLPVLAGIAAKVVPALFCKITKKC-OH	2526	a	b
Ranateurin-2 [176]	GLFLDTLKGAAKDVAGKLEGLKCKITGCKLP-OH	3183	a	c
Peptide-LR [129]	LVRGCWTKSYPPKPCFVR-OH	2136	b	d
Neuromedin-N [96]	HLRRCGKKPYILMACS-OH	1873	c	e

Activity: (a) antimicrobial; (b) histamine release agent; (c) smooth muscle contraction

Species: (a) *Rana esculenta*; (b) *R. brevipoda porsa*; (c) *R. catesbeiana*; (d) *R. pipiens*; (e) *R. palustris*

The peptides signiferin 1 and riparin 1.1 (Table 5.11) were isolated from anurans of the *Crinia* genus. Neither displayed antibiotic nor anticancer activity, hence, they were tested for pharmacological properties. Their three-dimensional structures were determined by 2D NMR techniques (Figure 5.18) [178].



(a) **(b)**

Figure 5.18. The structures of **(a)** signiferin 1 and **(b)** riparin 1.1

This section investigates the:

i) pharmacological activity of disulfide peptides and their modifications, and

ii) peptide-receptor interactions.

5.5b Results

A number of disulfide containing peptides were tested for biological activity (Table 5.11).

Table 5.11. Disulfide-containing peptides tested for pharmacological activity

Name	Sequence	MW
Signiferin 1	RLCIPYIIPC-OH	1187
Signiferin 1-NS	RLCIPYIIPC-OH	1190
Signiferin 1 (modification)	RLCIPYIIPCG-OH	1245
Signiferin 1 (amide)	RLCIPYIIPC-NH_2	1186
Riparin 1.1	RLCIPVIFPC-OH	1157
Riparin 1.2	FLPPCAYKGTC-OH	1196

Two biological assays were used to determine the biological activity of the disulfide peptides.

5.5.1b Contraction Studies

The standard peptides CCK-8 and CCK-8-NS both produced a potent increase in contraction of guinea-pig ileum (Figure 5.19). This is consistent with previous studies [152]. Signiferin 1 produced an increase in contraction that was equieffective and equipotent with CCK-8-NS at 10^{-9} M (Figure 5.19). This result suggests that signiferin 1 may be acting as a CCK_2 receptor agonist. The use of the selective CCK_2 receptor antagonist LY-288,513 (10^{-6} M) substantially reduced the contraction produced by signiferin 1 at 10^{-7} M (Figure 5.19), indicating that this neuropeptide is acting via CCK_2 receptors. This result needs to be repeated to confirm initial results.

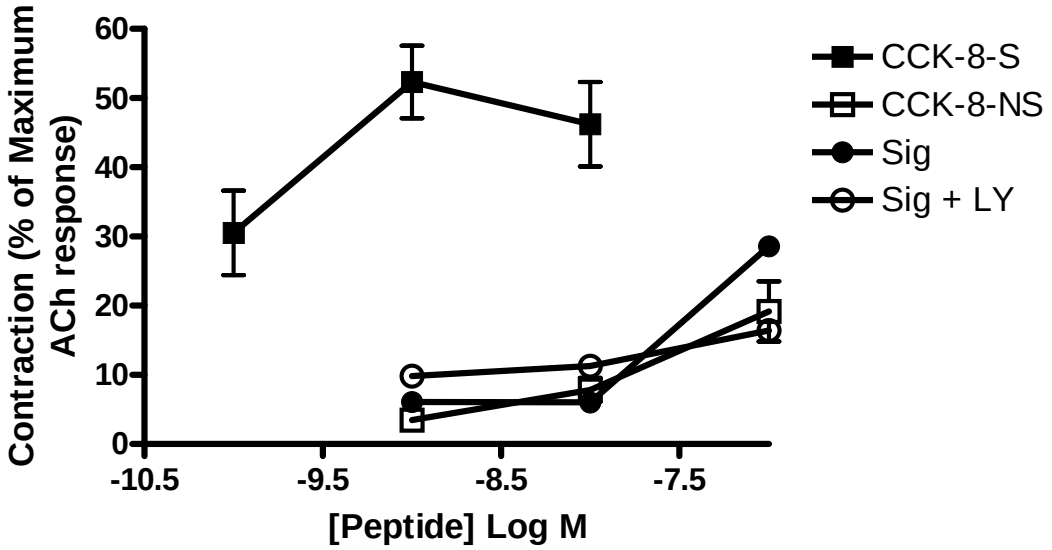


Figure 5.19. Concentration-response curves of signiferin 1 in the presence and absence of LY, in comparison with CCK-8 and CCK-8-NS (n=1)

The remaining signiferin 1 modification peptides and the riparins were inactive in this assay.

5.5.2b Lymphocyte Proliferation Studies

The disulfides listed in Table 5.11 were tested as potential CCK_2 receptor agonists. The lymphocyte surface possesses CCK_2 receptors exclusively that produce lymphocyte proliferation when stimulated [136]. Spleen derived lymphocyte proliferation was assessed using the Alamar Blue fluorescence dye method [165].

CCK-8 produced a concentration-dependent increase in lymphocyte proliferation in the presence (see Figure 5.14) and absence (data not shown) of the mitogen concanavalin A. The enhancement of peptides in both assays was parallel and in the range 5 – 10%. CCK-8-NS was less effective (see Figure 5.14). This is consistent with previous studies [166, 167].

Riparin 1.1 (control 385.5 ± 1.6, n=4) produced a concentration-dependent increase in lymphocyte proliferation in both the presence (Figure 5.20) and absence (data not shown) of concanavalin A (10^{-6} M) [66]. Riparin 1.2 (control 423.7 ± 30.9, n=4) also produced an increase in lymphocyte proliferation that was slightly more effective than riparin 1.1 (Figure 5.20). The results indicate that the riparins may be acting as CCK_2 receptor agonists.

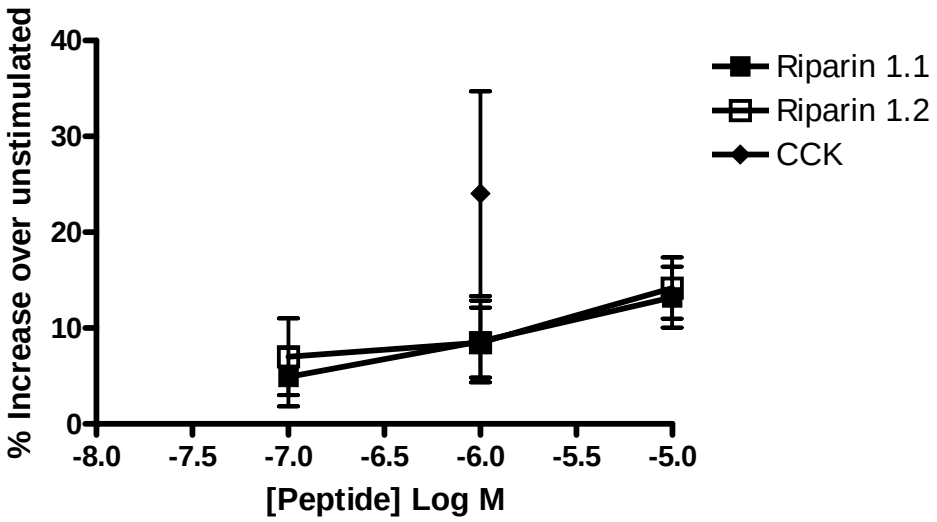


Figure 5.20. Riparin 1.1 and riparin 1.2 concentration-response curves in mouse lymphocytes. Data are given as the percent increase over the unstimulated controls and are shown as mean ± SEM of four independent experiments performed in quadruplicate. The response to CCK is shown for comparison

The signiferin peptides were inactive in this assay.

5.5c Discussion

Based on the experimental data, the disulfide peptides are potent neuropeptides that play significantly different roles in the amphibian integument. Signiferin 1, from *Crinia signifera*, contracts smooth muscle tissue, whilst the riparins may be immunomodulators. Both bioactivities occur by activation of CCK_2 receptors. *Crinia signifera* has potent antibiotic peptides in its skin secretion as well as signiferin 1. The activity of signiferin 1 may reflect the environmental conditions surrounding the anuran. If ingested by a predator, the release of signiferin 1 would cause an oesophagus reflex action, therefore, enabling the frog to escape. In contrast, *Crinia riparia* contains no potent antimicrobial peptides in its skin secretion. Instead, it produces the riparins 1, which act as CCK_2 receptor agonists in the immune system. *Crinia riparia* inhabits areas of static water infused with bacterial organisms, requiring the riparins 1 to induce immune protection through the proliferation of T cells. The role of the riparins to the frog is extremely important for protection against microorganisms, particularly in view of the lack of potent antibacterial compounds in the skin secretion of *C. riparia*.

Signiferin 1 and riparin 1.1 differ in structure in the centre of the peptide. The cyclic nature of these peptides (Figure 5.18) may provide the necessary structural requirements to bind to specific binding domains on CCK_2 receptors, however, the presence of certain amino acid residues are important for initiating biological activity depending on the biological system investigated. Signiferin 1.1 contains the hydrophilic residue tyrosine that may be important for binding to CCK_2 receptors on smooth muscle cells, whereas, the hydrophobic residues present in riparin 1.1 may be important for lymphocyte proliferation. Both peptides may also initiate different biological actions as a result of different receptor affinities. Further investigations are required into peptide-receptor binding, together with defining a mechanism of action for riparin 1.1 in the induction of lymphocyte proliferation.

We investigated several modifications to determine how structure influences activity. The disulfide modifications displayed no pharmacological activity. The absence of a disulfide bridge of signiferin 1 resulted in a loss of biological activity. Similarly, the addition of Gly in signiferin 1 (modification) destroyed the pharmacological response, indicating the C-terminal

end Cys is important for receptor activation. We also investigated the effect of C-terminal end groups. Conversion of the carboxyl terminus from an acid to an amide eliminated the contractile response.

The investigation of the bioactivity of disulfide peptides has provided insight into the roles of host-defence compounds for the *Crinia* genus. Further studies are required to determine if peptides bind to other known G protein-coupled receptors from the various other neuropeptide families. Photoaffinity labelling of ligands will provide structural insight into the location and nature of the agonist with G protein receptor binding sites.

5.6 Conclusion

Neuropeptides play an important role in the host-defence chemistry of investigated amphibians of the genera *Litoria* and *Crinia*. Bioactivity occurs via the activation of G protein-coupled receptors.

Rothein 1, isolated from *Litoria rothii*, is a potent neuropeptide that proliferates T cells through the activation of CCK_2 receptors, indicating a possible role in immune function. The high degree of conformational flexibility of the peptide may be necessary for receptor binding.

Disulfide containing peptides have been isolated from frogs belonging to the *Rana* family and more recently from Australian frogs of the *Crinia* genus. Signiferin 1 and riparin 1.1, although displaying similar secondary structural characteristics and activating CCK_2 receptors, played different roles in the amphibian integument. Signiferin 1 is a potent neuropeptide that contracts smooth muscle, whereas, riparin 1.1 proliferates T lymphocytes. The cyclic nature of both peptides may enable the peptide to bind to specific binding domains of the receptor.

5.7 Experimental

5.7a Smooth Muscle Contraction Assay

This work was approved by the University of Adelaide Animal Ethics Committee.

Acetylcholine, atropine, concanavalin A, CCK-8 and CCK-8-NS were obtained from Sigma-Aldrich. Alamar Blue was obtained from Astral Scientific (Caringbar, New South Wales, Australia).

Guinea-pigs weighing approximately 300 g were used. Immediately before the experiment, the guinea-pigs were killed by stunning and subsequent decapitation. The ileum was dissected free and was cleansed by rinsing with physiological salt solution (composition in mM: KCl 2.7, $CaCl_2$ 1.0, $NaHCO_3$ 13.0, NaH_2PO_4 3.2, NaCl 137, glucose 5.5, (pH 7.4)), and mesenteric tissue was removed. Segments of about 3 cm were cut, which were suspended in 20 mL organ baths containing the physiological salt solution and were gassed with 95% O_2 and 5% CO_2. Segments were connected to a tissue holder and to an isometric force-displacement transducer. Tension was recorded via a MACLAB v 3.0. Segments were washed thoroughly by replacing the physiological salt solution repeatedly, and were then allowed to equilibrate for a period of 30 min under 2 g of resting tension. Supply reservoirs and organ baths were maintained at 37°C and were gassed with O_2/CO_2 as outlined above.

Following the 30 min equilibration period, the tissue-bathing solution was replaced repeatedly with fresh drug-free physiological salt solution until a stable baseline tension was achieved. The tension was then readjusted to 2 g. All segment preparations were then constricted with acetylcholine (10^{-8} – 10^{-6} M). After washout, acetylcholine (10^{-7} M) was used again to check that the response was stable. After 5 min washout and achievement of a stable baseline, a cumulative response curve to CCK-8 (10^{-10} - 10^{-8} M) was performed. After another 5 min

washout and achievement of a stable baseline, a cumulative concentration response curve to the peptide (10^{-9} - 10^{-7} M) or CCK-8-NS (10^{-9} - 10^{-7} M) was performed. In some experiments, following washout, tissues were either pre-treated with atropine or vehicle and CCK-8 or the peptide reapplied.

5.7b Lymphocyte Proliferation Assay

Male Balb/C mice aged 6 - 8 weeks were used. Aseptic techniques were used during preparation of the lymphocytes. Mice were killed by cervical dislocation followed by prompt removal of the spleen. The spleen was prepared as a single-cell suspension by massaging and washing through a nylon mesh into a 15 mL tube with up to 15 mL of RPMI 1640 (Hepes modification, 0.3 mg/mL of L-glutamine and 5 mL of penicillin/streptomycin solution per litre). The cells were centrifuged at 4°C for 5 min at 100 g, the supernatant material discarded and the cells resuspended in 1 mL of media followed by the addition of 10 mL of ice-cold lysis buffer (1 mL of 20.56 g/L tris base (pH 7.65), 9 mL of 0.83% NH_4Cl in water, mixed just prior to addition of cells). The suspension was placed on ice for 4 min, centrifuged (5 min at 100 g) and the supernatant material discarded. The suspensions of cells were pooled and were resuspended in 10 mL of media followed by centrifugation (5 min at 100 g), removal of supernatant material and resuspended in 5 mL of enriched RPMI 1640 (RPMI 1640 enriched with 10% fetal bovine serum). The number of viable lymphocytes in the suspension was counted using trypan blue and a haemocytometer. Cells were then diluted in enriched media to 1 x 10^{-6} cells/mL and 100 µL of this suspension was added to each well of the 96 multiwell plates (TTP, Zurich, Switzerland) to give a final volume of 200 µL, and final cell count to 50,000 cells per well.

Either vehicle or the mitogen concanavalin A (90 µl) (2.5 µg/mL final concentration) was added to the wells, and then 10 µL of RPMI 1640 medium containing either CCK-8, CCK-8-NS or the peptide (to produce final concentrations of 10^{-7}- 10^{-5} M) was added to the plate. Plates were incubated at 37°C, using 5% CO_2 in a humidified incubator (Thermoline, Sydney, New South Wales, Australia) for 24 h. The mitochondrial activity indicator dye

Alamar Blue (25 µL) was then added to give a final concentration of 2.5 µg/mL, and the plates incubated as above for a further 4 h. After this, 175 µL aliquots were pipetted from each well into a white 96 well plate, and fluorescence measured in a Polestar Galaxy (BMG Labtechnologies, Durham, NC, U.S.A) fluorescent plate reader (excitation 544 nm, emission 590 nm).

Chapter 6- Eugenin- a Marsupial Neuropeptide

6.1 Introduction

6.1a Marsupials and their Defence System

Marsupials are born at an immature stage of development after only a short gestation period [179, 180]. Gestation times vary from as little as eleven days in the striped-faced dunnart to thirty-five days in the koala [181]. Developmental changes that occur during the early lactation phase take place during pouch life [182]. After the short period of intrauterine development, the young marsupial crawls unaided to the mother's pouch, attaches to a teat and undergoes further development in an aerial environment [183] (Figure 6.1). During this time, both the milk composition and pouch young undergo changes. Physiological changes in the young include intermittent suckling, growing fur and opening of the eyes, before they attempt to leave the pouch [179].

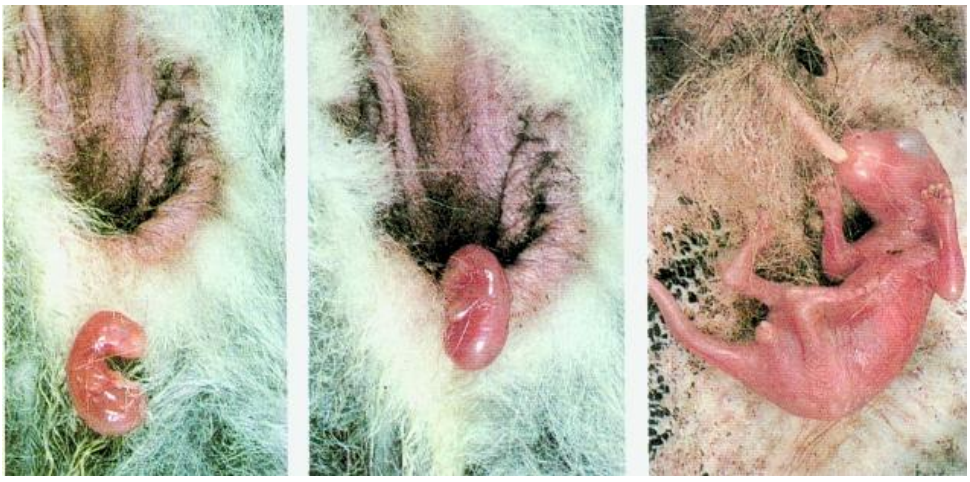


Figure 6.1. The developmental changes of the marsupial young in the pouch

The immune system of newborns matures shortly after birth, however, they initially rely on that provided by the mother [180, 182]. The young receive immunity passively either through the placenta and/or the mammary gland. This provides protection against pathogens until their own immune system has matured and they have acquired an immune system of their own [184]. The specialised mammary gland secretion colostrum is produced less than forty-eight hours after birth. Colostrum contains a number of components that are essential for immune protection, including lymphoid cells, cytokines, immunomodulating substances, growth factors, hormones and immunoglobulins [185]. The milk composition also contains the nutrients required for the developing young [179], which primarily occurs through immunoglobulins [180].

The pouch in diprotodonts, like the Tammar wallaby, is a deep moist environment [180]. The pouch microclimate is characterised by high humidity and a constant temperature that is close to maternal body temperatures [186]. The pouch is a favourable environment for microorganisms- containing a variety of Gram-positive and Gram-negative bacteria [187, 188]. Immediately prior to the birth of the young, the mother licks around the pouch cleaning it. This maternal action is believed to reduce bacterial flora [179]. Yadav, in 1973, reported a possible relationship between birth of young and changes in the pouch flora [187]. He postulated two mechanisms for this change: maternal cleansing and pouch secretions.

Whilst the details of marsupial pouch secretions have not yet been studied, preliminary studies have revealed antimicrobial peptides are present in the pouch of the koala, *Phascolarctos cinereus* [189]. Furthermore, bioactive peptides form part of the host-defence system of a variety of animals. Therefore, marsupial mothers may provide active peptides in their pouches to protect their young during the first weeks of occupancy.

Eugenin is a neuropeptide that was recently isolated from the pouch of the Tammar wallaby [190]. Aims of this research were to:

i) isolate and determine the structures of any active peptides from the pouch secretion of a number of marsupials, including the potoroo- *Potorous tridactylus*, bettong-

Bettongio penicillata, hairy-nosed wombat- *Vombatus ursinus* and red kangaroo- *Macropus rufus*,

ii) determine the bioactivity of any isolated peptides, and

iii) conduct pharmacological tests on the isolated marsupial peptide, eugenin.

6.1b Tammar Wallaby

The Tammar wallaby, *Macropus eugenii* (Figure 6.2), is located on the mainland of South Australia and Western Australia, as well as on some offshore islands including Kangaroo Island [191] (Figure 6.3). It lives in coastal scrub, dry forest and woodland, which provides shelter during the daytime. The wallaby generally feeds in open grassy areas.

Figure 6.2. Tammar wallaby

Figure 6.3. The distribution of *Macropus eugenii*

Wallaby young are born between late January and March. A mere few hours after birth, the female mates again. The new embryo remains passive during the lactation period. The first young remains resident in the pouch for approximately 8 - 9 months before embarking on life in the outside world around September or October. The passive embryo is reactivated in mid December and develops in the pouch, with the young born approximately forty days later [191].

6.2 Eugenin

6.2a Isolation and Sequence Analysis

Previous work identified a maternal defence compound from the pouch of the Tammar wallaby, named eugenin [190, 192].

Swab samples were subjected to analysis by HPLC and electrospray mass spectrometry using a Micromass Q-TOF 2 mass spectrometer. Eugenin was isolated in microgram amounts from pouch swabs taken from early lactating females during the first two weeks of the occupancy of young in the pouch. However, the compound was not detected in pouch swabs from either females without young or females bearing young subsequent to the early lactation period. Consequently, the hypothesis that eugenin is involved in protection of the young was put forth [190].

Eugenin **(1)** was sequenced using a combination of positive ion and negative ion mass spectrometry. The peptide is post-translationally modified, with a pyroglutamate at the N-terminal end and an amide moiety at the C-terminal end of the peptide. It also contains a sulfated tyrosine residue [190].

(1) Eugenin pEQDY(SO_3)VFMHPF-NH_2

6.2b Neuropeptide Activity

Eugenin has similar structural elements to the mammalian intestinal neuropeptide cholecystokinin-8 (CCK-8) **(2)** and the amphibian neuropeptide caerulein **(3)**.

(2) Cholecystokinin-8 DY(SO_3)MGWMDF-NH_2

(3) Caerulein pEQDY(SO_3)TGWMDF-NH_2

CCK-8 and caerulein act directly on smooth muscle via the CCK_1 receptor, or indirectly via the CCK_2 receptor [139, 152] (for details on receptor subtypes, see section 5.3). Both neuropeptides also stimulate the proliferation of lymphocytes via a similar mechanism (see section 5.2).

As the above peptides were known CCK receptor agonists, we performed biological activity screening tests of eugenin in tissues with well-characterised CCK responses. This involved a smooth muscle contraction assay and a lymphocyte proliferation study.

6.3 Results

Two biological assays were used to determine the biological activity of eugenin.

6.3a Contraction Studies

A concentration-dependent increase in contraction in guinea-pig ileal segments was produced by acetylcholine. The standard CCK-8 (mixed CCK_1CCK_2 receptor agonist) produced potent increases in contraction to 60% of the maximally effective concentration of acetylcholine (Figure 6.4). This maximum contractile response occurred at 10^{-9} M. The standard CCK-8-NS (CCK_2 receptor agonist) also produced an increase in contraction but was less potent and less effective than CCK-8 (Figure 6.4). These results are consistent with previous studies [152].

Eugenin produced an increase in contraction that was equieffective and equipotent with CCK-8-NS (Figure 6.4). This observation suggests that eugenin may be acting as a CCK_2 receptor agonist.

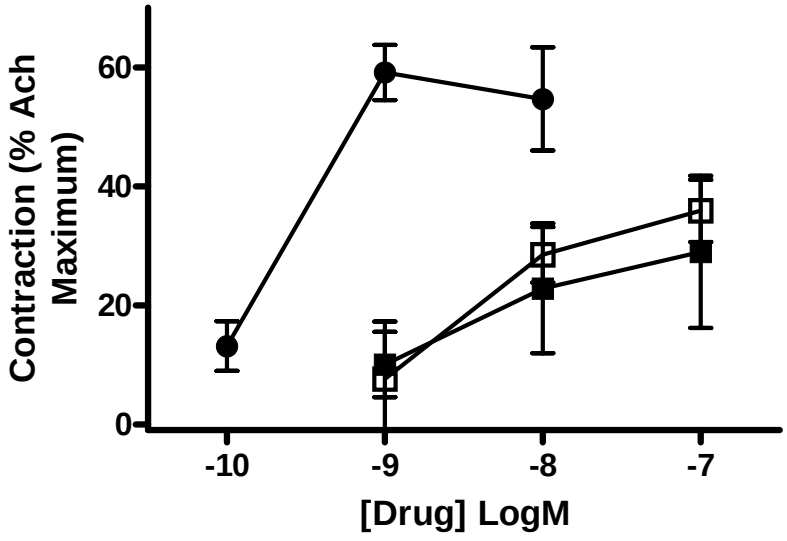


Figure 6.4. CCK-8 (●), CCK-8-NS (□) and eugenin (■) concentration-response curves in guinea-pig ileum

The contractions produced by CCK_2 agonists are due to the release of acetylcholine from cholinergic nerve terminals. Therefore, the effects of eugenin and CCK-8 were determined in the presence of atropine (10^{-6} M). At this concentration of atropine, the maximally effective concentration of acetylcholine was completely blocked. Atropine had no effect on the contraction produced by CCK-8, however, it did completely stop the contraction of ileal tissue produced by 10^{-8} M eugenin and reduced the contraction produced by 10^{-7} M eugenin [190] (Figure 6.5).

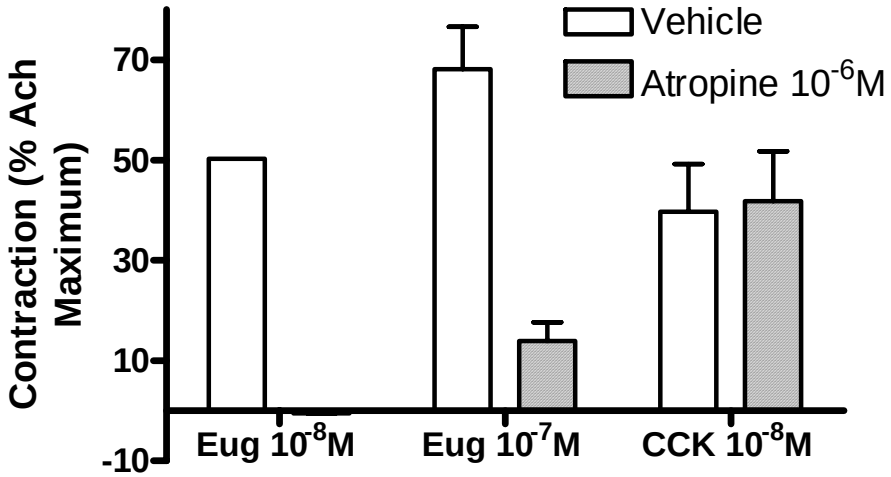


Figure 6.5. The effect of atropine on contractions produced by CCK-8 and eugenin in guinea-pig ileum

6.3b Lymphocyte Proliferation Studies

Eugenin has important implications for the maternal defence of pouch young by acting as a CCK_2 agonist. Lymphocytes possess CCK_2 receptors on their surface that proliferate when stimulated. Spleen derived lymphocyte proliferation was assessed using the Alamar Blue fluorescence dye method [165].

CCK-8 produced a concentration-dependent increase in lymphocyte proliferation in the presence (Figure 6.6) and absence (data not shown) of the mitogen concanavalin A. CCK-8-NS was less effective (Figure 6.6). This is consistent with previous studies [166, 167].

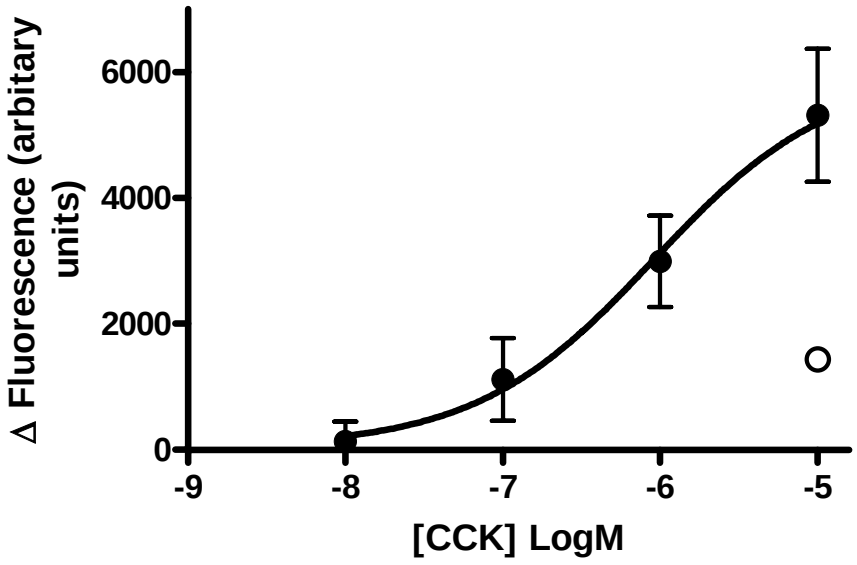


Figure 6.6. CCK-8 (●) and CCK-8-NS (○) concentration-response curves in mouse splenocytes

Both eugenin and desulfated eugenin (although to a lesser extent) produced a concentration-dependent increase in lymphocyte proliferation in both the presence (Figure 6.7) and absence (data not shown) of concanavalin A [190].

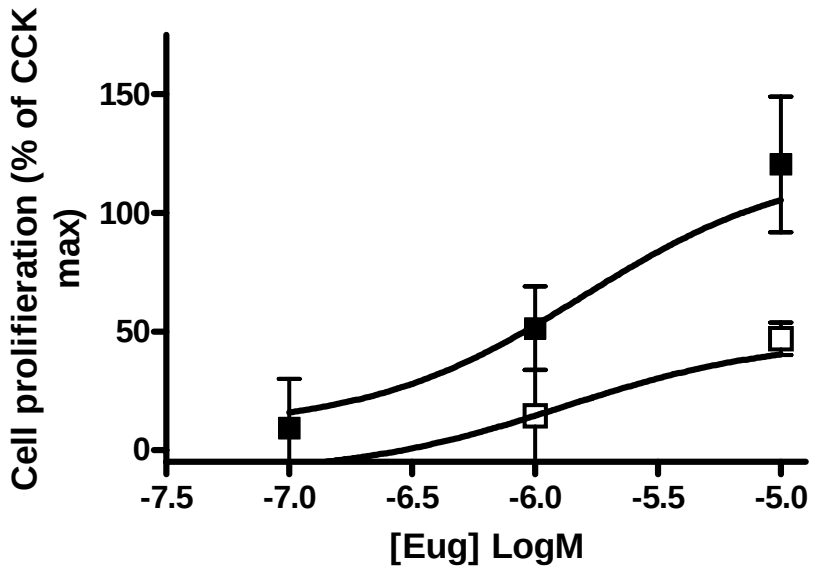


Figure 6.7. Eugenin (■) and eugenin-NS (□) concentration-response curves in mouse splenocytes

6.4 Discussion

Young marsupials are born at an early stage of development, independent of an immune system. This renders the young vulnerable to the pouch microclimate, which is a favourable environment for bacteria. Accordingly, pouch young are reliant on the vital immune protection provided by the mother to combat the potentially harmful microorganisms.

There are three possible scenarios which may explain how the female wallaby protects the young during the early period of occupancy in the pouch:

i) she may have antimicrobial and other biologically active agents in her milk,
ii) there may be host-defence compounds in the secretion contained in the pouch, or
iii) there may be host-defence compounds in the saliva, which she deposits when licking the pouch.

Pouch swabs taken from the Tammar wallaby during the first two weeks of lactation contained small amounts of eugenin. Eugenin has a sequence related to the mammalian gastrin-like neuropeptide CCK-8 [193] and the amphibian neuropeptide caerulein [19]. Eugenin contains the same first four residues as caerulein, but the sequence proceeding the Tyr(SO_3) residue is different from those of CCK-8 and caerulein. All peptides contain a sulfated tyrosine residue. This residue is critical for biological activity, with activity decreasing if the residue is hydrolysed [20].

As CCK-8 and caerulein activate both CCK_1 and CCK_2 receptors, we proposed that eugenin may act via one or both CCK receptor subtypes. The standard neuropeptide CCK-8, which activates the CCK_1 and CCK_2 receptors, produced a concentration-dependent increase in contraction of guinea-pig ileal segments. The selective CCK_2 receptor agonist, CCK-8-NS, also produced a concentration-dependent increase in contraction of ileal segments, but was less potent and less effective than CCK-8. Eugenin produced a concentration-dependent contraction of smooth muscle that was similar in potency to CCK-8-NS [190].

The effect of the muscarinic blocker atropine was examined to determine if eugenin acts via CCK_2 receptors. Atropine had no effect on the response of the standard CCK-8, but reduced the response of eugenin, indicating that eugenin does act indirectly on smooth muscle via CCK_2 receptors.

The role of eugenin in the wallaby pouch was further investigated through monitoring the interaction of the peptide with CCK_2 receptors. Lymphoid cells exclusively have CCK_2 receptors [136], and exposure of mouse lymphocytes [166] or lymphoid tumour cell lines [167] to CCK agonists results in lymphocyte proliferation. In this study, CCK-8 caused a concentration-dependent increase in proliferation of mouse splenocytes, as measured by the Alamar Blue assay [137]. CCK-8-NS produced a less potent proliferation response than CCK-8. Eugenin produced a proliferation response that was equal in potency to CCK-8, while desulfated eugenin produced a diminished response. These results are consistent with eugenin being a CCK_2 receptor agonist [190]. This result can be confirmed by testing cell proliferation in the presence of the CCK_2 receptor antagonist LY-288,512. *

The results show that eugenin may act as an immunomodulator in the wallaby's pouch. Eugenin is observed in the pouch within the first two weeks of lactation when the young has no immune system of its own and there is a considerable decrease in the amount of microbial flora in the pouch [194]. However, eugenin has no antibacterial activity. The skin, an active immune tissue containing CCK_2 receptors, can be stimulated by eugenin to produce immune cells [166, 167]. The activation of CCK_2 receptors also increases the production of host-defence compounds such as interleukins and the secretion of immunoglobulins on the mucosal surface of the intestine [195, 196].

* An examiner has suggested that skin epithelial cells may have T cell like receptors that can recognise pathogens. They may then recruit dendritic cells that travel to the draining lymph nodes to present antigens to lymphocytes. Perhaps eugenin may enhance some part of this pathway

Therefore, based on the experimental data described, we submit that eugenin stimulates immune cells in the pouch of the Tammar wallaby during the early lactation period. This reduces bacterial flora numbers in the pouch [190] and provides necessary immune protection of the pouch young.

However, the Tammar wallaby may be unique in this regard as we were unable to isolate any active components from pouches of other marsupials. Similarly, we tested the pouches of the bettong, potoroo, hairy-nosed wombat and red kangaroo. This list is far from exhaustive and therefore further research is required in order to establish a sound inference on the role of eugenin in marsupial defence systems.

6.5 Experimental

6.5a Collection of Pouch Material

Cotton wool swabs were taken from the pouches of various marsupials at different time intervals. Each swab was shaken with deionised water (50 mL), the mixture diluted with an equal volume of methanol, filtered through a Millex HV filter unit (0.45 µm) and lyophilised, before being separated by HPLC.

6.5b Separation of Material by HPLC

Crude material was separated using HPLC, and was achieved using a VYDAC C18 HPLC reverse phase column (5µ, 300A, 4.6 x 250 mm) (Separations Group, Hesperia, CA, USA) equilibrated with 10% acetonitrile/aqueous 0.1% TFA. The lyophilised mixture was dissolved and injected into the column. The elution profile was generated using a linear gradient produced by an ICI DP 800 Data Station controlled by two LC1100 HPLC pumps. The gradient increased from 10 - 75% acetonitrile over a period of 60 min at a flow rate of 1 mL/min. The eluant was monitored by ultraviolet absorbance at 214 nm. Fractions were collected and analysed by mass spectrometry.

6.5c Contraction Studies

Eugenin was tested for neuropeptide activity by monitoring smooth muscle contraction in guinea-pig ileal tissue using the method described in section 5.7. The response was monitored against the standards CCK-8 and CCK-8-NS.

6.5d Lymphocyte Proliferation Assay

Eugenin was tested for immune functionality using the standard lymphocyte proliferation assay described in section 5.7.

Summary and Future Directions

Skin secretions from amphibians of the *Crinia* genus were investigated. A number of novel compounds exhibiting a diverse range of antibacterial and pharmacological properties were isolated. The peptides were unique in structure compared to those isolated from other Australian amphibians, however, peptides with similar structural characteristics were evident within the three species. The major peptide components were potent disulfide containing neuropeptides of a type not observed in other Australian amphibians that have been previously investigated. Several antimicrobial peptides active against Gram-positive organisms were also isolated, together with peptides that inhibit the formation of nitric oxide by nNOS.

The cyclic neuropeptides, signiferin 1 and the riparins 1, demonstrate different pharmacological activities. Signiferin 1 contracts smooth muscle tissue, whereas riparin 1.1 proliferates lymphocytes. Both biological processes occur by peptide interaction with the CCK_2 receptor. The different role of the disulfides in the amphibian integument may reflect the diversity of their environmental conditions. Further studies using photoaffinity labelling may provide greater insight into receptor binding domains. Peptides should also be tested for binding affinity to other G protein-coupled receptors.

Skin components from anurans of the *Litoria* genus have been extensively studied. Peptides displaying both antibacterial activity and pharmacological activity have been isolated. Six peptides were identified within skin secretions from *L. dentata*. The neuropeptide tryptophyllin L 1.3 has been previously isolated from the related frog *L. rubella*. Preliminary results indicate that tryptophyllin L 1.3 contracts smooth muscle at 10^{-8} M via CCK_2 receptors. Other components also need to be tested for biological activity.

The host-defence peptides isolated from *L. rothii* include both neuropeptides and antibacterial peptides. Rothein 1 is the major peptide found in the winter frog secretion. Rothein 1 proliferates T cells via CCK_2 receptors, indicating a possible role in immune function. The

rothein peptides also display a bimodal contractile response following washout. This may be produced by an inhibitory compound like nitric oxide. Further investigations into the “rebound response” produced are required.

The disease malaria is responsible for millions of deaths per year. Due to the increase in resistance of current antimalarial compounds, the development of new treatments from various animal-derived peptide antimicrobials have been studied. Amphibian peptides and their derivatives inhibited the growth of the malaria parasite *Plasmodium falciparum* with minimal haemolytic activity, making them promising tools for malaria research. Further studies using fluorescent binding will determine the inhibitory stage of the parasite in the cell. Other strains of *Plasmodium* should also be investigated.

Young marsupials rely on immune protection provided by the mother. The host-defence compound eugenin was isolated from pouch secretions of the Tammar wallaby. Eugenin activates CCK_2 receptors resulting in lymphocyte proliferation, therefore, providing vital immune protection of the pouch young. Host-defence compounds from other marsupials should be investigated using a different technique for the collection and separation of crude material.

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