

# **Biologically Active Peptides from Australian Amphibians**

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by

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# Chapter 6 Amphibian Neuropeptides

## 6.1 Introduction

### 6.1.1 Amphibian Neuropeptides

The identification and characterisation of neuropeptides in amphibians has provided invaluable understanding of not only amphibian ecology and physiology but also of mammalian physiology. In the 1960's Erspamer demonstrated that a variety of the peptides isolated from amphibian skin secretions were homologous to mammalian neurotransmitters and hormones (reviewed in [10]). Erspamer postulated that every amphibian neuropeptide would have a mammalian counterpart and as a result several were subsequently identified. For example, the discovery of amphibian bombesins lead to their identification in the GI tract and brain of mammals [394].

Neuropeptides form an integral part of an animal's defence and can assist in regulation of dermal physiology. Neuropeptides can be defined as peptidergic neurotransmitters that are produced by neurons, and can influence the immune response [395], display activities in the CNS and have various other endocrine functions [10]. Generally, neuropeptides exert their biological effects through interactions with G protein-coupled receptors distributed throughout the CNS and periphery and can affect varied activities depending on tissue type. As a result, these peptides have biological significance with possible application to medical sciences. Neuropeptides isolated from amphibians will be discussed in this chapter, with emphasis on the investigation into the biological activity of peptides isolated from several *Litoria* and *Crinia* species.

Many neurotransmitters and hormones active in the CNS are ubiquitous among all vertebrates, however, active neuropeptides from amphibian skin have limited distributions and are unique to a restricted number of species. Amphibian neuropeptides can be divided into several main groups as characterised by distinctive features: the tachykinins, bradykinins, caeruleins, bombesin-related, opioid and tryptophyllin peptides. There are also several miscellaneous peptides that do not appear to fit into the main groups (Table 6.1).

**Table 6.1:** Selected neuropeptides isolated from amphibian skin.

Family	Neuropeptide	Sequence <sup>1</sup>	Species <sup>2</sup>
<b>Tachykinins</b>			
Physalaemin-related	Physalaemin	pEADPNK <b>FYGLM</b> -NH <sub>2</sub>	a
	Uperolein	pEDPNA <b>FYGLM</b> -NH <sub>2</sub>	b
Kassinin-related	Kassinin	DVPKSDQ <b>FVGLM</b> -NH <sub>2</sub>	c
	Phyllomedusin	pENPNR <b>FVGLM</b> -NH <sub>2</sub>	d
<b>Bradykinins</b>			
	Bradykinin	<b>RPPGFSPFR</b> -OH	e
	Phyllokinin	<b>RPPGFSPFR</b> IY(SO <sub>3</sub> )-OH	f
<b>Caeruleins</b>			
	Caerulein	pEQDY(SO <sub>3</sub> ) <b>TGWMD</b> F-NH <sub>2</sub>	g
	Caerulein 1.2	pEQDY(SO <sub>3</sub> ) <b>TGWFD</b> F-NH <sub>2</sub>	h
	Phyllocaerulein	pEEY(SO <sub>3</sub> ) <b>TGWMD</b> F-NH <sub>2</sub>	i
<b>Bombesin-related</b>			
	Bombesin	pEQRLGNQ <b>WAVGHLM</b> -NH <sub>2</sub>	j
	[Phe <sup>13</sup> ]Bombesin	pEQRLGNQ <b>WAVGHFM</b> -NH <sub>2</sub>	k
	Litorin	pEQ <b>WAVGHFM</b> -NH <sub>2</sub>	l
	Phyllolitorin	pEL <b>WAVGHFM</b> -NH <sub>2</sub>	m
<b>Opioids</b>			
Dermorphins	Dermorphin	<b>Ya</b> <b>F</b> GYPS-NH <sub>2</sub>	n
Deltorphins	Deltorphin A	<b>Ym</b> <b>F</b> HLM-D-NH <sub>2</sub>	o
	Deltorphin B	<b>Ya</b> <b>F</b> EVVG-NH <sub>2</sub>	p
<b>Tryptophyllins</b>			
TPH-4		pE <b>P</b> WM-NH <sub>2</sub>	q
		pE <b>P</b> WV-NH <sub>2</sub>	q
		<b>P</b> PWL-NH <sub>2</sub>	q
Tryptophyllin L 1.2		<b>F</b> PWL-NH <sub>2</sub>	r
		<b>F</b> PPL-NH <sub>2</sub>	r
		<b>F</b> PPWL-NH <sub>2</sub>	q
TPH-5		<b>F</b> PPWL-NH <sub>2</sub>	q
		<b>F</b> PPWM-NH <sub>2</sub>	q
	Tryptophyllin L 1.3	pE <b>F</b> PWL-NH <sub>2</sub>	r
<b>Miscellaneous</b>			
	Xenopsin	pEGKRPWIL-OH	s
	TRH	pEHP-NH <sub>2</sub>	t
	<i>Crinia</i> angiotensin II	APGDRIYVHPF-OH	u

<sup>1</sup>**Sequence:** Lower case indicates D-amino acid. Sequences have been aligned to shown similarities in either the N- or C-terminals within each neuropeptide family. Identical residues are in bold.

<sup>2</sup>**Species:** (a) *Physalaemus fuscumaculatus* [396], and various other *Physalaemus* species [17]; (b) *Uperoleia rugosa*, *Uperoleia marmorata* [397]; (c) *Kassina senegalensis* [398]; (d) *Pseudophryne coriacea* [399] *Phyllomedusa bicolor* [18]; (e) various *Rana* species [400]; (f) *Phyllomedusa rohdei* [401]; (g) *Litoria caerulea* [34], various *Litoria* species [27], *Xenopus laevis* [10], *Leptodactylus labyrinthicus* [34]; (h) *Litoria splendida* [29], *Litoria citropia* [402]; (i) *Phyllomedusa sauvagei* [403]; (j) various *Bombina* and *Alytes* species [404]; (k) *Bombina orientalis* [405]; (l) *Litoria aurea* [406]; (m) various *Phyllomedusae* species [403]; (n) various *Phyllomedusa* species [272]; (o) *Phyllomedusa sauvagei* [407]; (p) *Phyllomedusa bicolor* [408]; (q) *Phyllomedusa rohdei* [409, 410]; (r) *Litoria rubella*, *Litoria electrica* [46]; (s) *Xenopus laevis* [19]; (t) *Bombina orientalis* [411]; (u) *Crinia georgiana* [412].

### 6.1.1.1 Tachykinins

Various genera of anurans produce tachykinin neuropeptides in their skin secretions. Many of these peptides are anionic and contain a N-terminal pGlu residue. Typically, these peptides have the C-terminal moiety FXGLM-NH<sub>2</sub> (where X is varied) and have mammalian counterparts, substance P and the neurokinin peptides [1]. Substance P and neurokinin have been implicated in a variety of activities in the central and peripheral nervous systems, in addition to the cardiovascular and immune systems [265].

Substance P	RPKPQQFFGLM-NH <sub>2</sub>
Neurokinin A	HKTDSFVGLM-NH <sub>2</sub>

Tachykinin neuropeptides can be subdivided into two groups, the physalaemin- and kassinin-related peptides based on the presence of an aromatic residue (Tyr or Phe) or an aliphathic residue (Val or Ile) at the fourth position from the C-terminus (residue X) (Table 6.1). The first tachykinin neuropeptide to be isolated was physalaemin from *Physalaemus fuscumaculatus* during Erspamer's initial survey [396] and is the most potent hypotensive agent isolated [403].

Tachykinin peptides produce rapid contraction of smooth muscle. In addition, tachykinins act as neurotransmitters and neuromodulators in the CNS, GI tract and cardiovascular systems [10]. An example of some of these physiological activities include potent vasodilation and hypotensive action, strong stimulation of secretions from the salivary and lachrymal glands, intense spasmogenic activity on some extravascular muscle *in vitro* and enhancement of capillary permeability [403].

Tachykinins act via G protein-coupled neurokinin receptors (NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub>) [265]. Neurokinin receptors are distributed on nerve terminals and cell bodies, smooth muscle and endocrine cells [265, 413]. Tachykinins produce their intestinal contraction by binding to the neurokinin receptors located on enteric neurones in the CNS resulting in the release of Ach [265]. Ach subsequently binds to mAChR directly on ileal smooth muscle producing contraction [414]. However in anurans, these neurokinin receptors are located directly on the smooth muscle and contraction occurs in a nerve-independent process

[265]. Typically, tachykinins of the kassinin-related subgroup display higher affinity for NK<sub>2</sub> and NK<sub>3</sub> receptors [265].

### 6.1.1.2 Bradykinins

Bradykinins have been isolated from a wide range of anuran genera including *Rana* and *Bombina* from Africa [415], Europe [416], Australasia [26] and America [417] and are the major component of the skin secretion in some species [418]. Bradykinin neuropeptides have much weaker physiological activity than the other amphibian neuropeptides, however, the animal overcomes this by secreting larger quantities of the peptides [418]. In mammals, these neuropeptides are predominantly present in the plasma [403].

The sequence of bradykinin peptides varies in the C-terminal region and with some peptides possessing additional C-terminal residues. The majority of the biologically active peptides from this group contain a C-terminal free acid [1]. One peptide in particular, phyllokinin, differs from other bradykinins as it contains a sulfated Tyr residue like caerulein peptides, yet its activity is identical to the other bradykinins [22].

Bradykinin peptides produce slow contraction of vascular smooth muscle. They also regulate blood pressure by exerting strong vasodilatory and hypotensive activity in some mammalian species. This is achieved by effectively increasing capillary permeability, decreasing arteriolar resistance, bronchoconstriction and stimulation of renal electrolyte secretion [419]. In addition, bradykinins cause intense pain when administered intravenously [403] in the periphery<sup>[2\*,3\*]</sup> but have been shown to relieve pain when present in the CNS<sup>[4\*]</sup>. In mammals, bradykinins bind to G protein-coupled B<sub>1</sub> and B<sub>2</sub> receptors. B<sub>1</sub> receptors are located on smooth muscle, whilst B<sub>2</sub> receptors are found in the CNS [419, 420]. Smooth muscle contraction occurs directly when bradykinins bind to B<sub>1</sub> receptors and indirectly when they bind to B<sub>2</sub> receptors. The decrease in blood pressure in mammals by bradykinins is primarily mediated through B<sub>2</sub> receptors and the subsequent release of NO and prostaglandins [419].

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<sup>2\*</sup> Walker, K., Perkins, M. and Dray, A. (1995) Kinins and kinin receptors in the nervous system, *Neurochem. Int.* 26, 1-16

<sup>3\*</sup> Raidoo, D.M. and Bhoola, K.D. (1998) Pathophysiology of the kallikrein-kinin system in mammalian nervous tissue, *Pharmacol Ther.* 79, 105-127

<sup>4\*</sup> Nagy, I., Paule, C., White, J. and Urban, L. (2007) Taking the sting out of pain, *Br. J. Pharmacol.* 151, 721-722

### 6.1.1.3 Caeruleins

Caerulein was first isolated from the Australian frog *Litoria caerulea* [34], however since its initial isolation it has been shown to be present in the skin secretions of numerous species in the genus *Litoria* [27], together with *Xenopus laevis* [26] and *Leptodactylus labyrinthicus* [34]. Caerulein peptides are characterised by a post-translationally modified pGlu residue, sulfated Tyr and C-terminal amide. The non-sulfated peptide is inactive, thus the sulfide group is essential for receptor recognition and biological activity [1].

Numerous cDNA clones that have been isolated from *Xenopus laevis* have indicated that the preprocaeruleins encode either a single copy or multiple copies of caerulein [269]. The sequence of these clones suggested that the preprocaeruleins are derived from a small family of genes which originated from different genes rather than being formed from one single gene [269]. Interestingly, several *Litoria* species including *L. splendida*, *L. peronii* and *L. citropa* produce caerulein in their secretions in the summer breeding months, whilst in the winter months, these frogs secrete an analogue of caerulein, caerulein 1.2 ([Phe<sup>8</sup>]caerulein) (Table 6.1) [402] (for caerulein 1.2 activity see Section 6.2.3).

Caerulein peptides possess a broad spectrum of activity analogous to that of the mammalian intestinal hormones gastrin and CCK [10]. *In vivo*, caerulein modifies satiety, sedation, thermoregulation, antinociception and enhances blood circulation. The analgesic effect of caerulein resembles that of CCK and these peptides are several thousand times more active than morphine [403]. Despite having no affinity for opioid receptors and their analgesic activity is mediated by a different receptor pathway, the effect can be blocked by naloxone [1]. Additionally, caeruleins also induce spasmogenicity in the gall bladder, stimulation of the bowels, exocrine pancreas and Brunner's glands, induces gastric and hepatic bile secretion and release calcitonin from the thyroid gland [403].

Gastrin	pEGPWLEEEEEAY(SO <sub>3</sub> )GWMDF-NH <sub>2</sub>
CCK-8	DY(SO <sub>3</sub> )MGWMDF-NH <sub>2</sub>

Caerulein contracts smooth muscle at nanomolar concentrations by binding to CCK receptors. Like its mammalian analogue CCK-8, caerulein may act directly on smooth muscle via CCK<sub>1</sub> receptors or indirectly via CCK<sub>2</sub> receptors. CCK<sub>2</sub> receptors are situated on cholinergic nerves in the myenteric plexus of the ileum and stimulate the release of

Ach, which subsequently produces contraction by binding to mAChR on ileal smooth muscle [421, 422].

#### 6.1.1.4 Bombesins

Bombesin peptides were initially isolated from the skin and gut of anurans of the genus *Bombina* [404], whilst the litorins were isolated from frogs of the genera *Litoria*, *Pseudophyrne* and *Rana* [406]. The discovery of these peptides in amphibians preceded their discovery in mammals [394]. Bombesin peptides are post-translationally modified to contain a C-terminal amide and a N-terminal pGlu residue and display sequence similarity in the last six residues at the C-terminus (Table 6.1). Similiar to tachykinins, the majority of changes in structure occur at the N-terminus, which is speculated to play a role in receptor recognition and differentiating biological activity [405, 423].

Bombesin- and litorin-related peptides have been isolated from a number of other vertebrates [424-426] and are analogous to human gastrin releasing peptide (GRP) and mammalian neuromedin B (NMB) [427]. NMB was originally isolated from porcine spinal cord, whilst GRP is widely distributed in the brain and GI tract [428].

NMB	VPLP...AGGTVLTKMYPRGNLWATGHFM-NH <sub>2</sub>
GRP	GNHWAVGHLM-NH <sub>2</sub>

Bombesin-related peptides act directly on extravascular smooth muscle and cause potent antidiuretic effects, vasoconstriction and moderate hypertension with tachyphylaxis [10]. These peptides have potent immunological modulating activity [429, 430] and stimulate the growth of normal and neoplastic tissue [10]. Depending on the route of administration, these peptides can stimulate or inhibit stimulation of GI secretions [403] and create a hyperglycaemic effect by increasing insulin secretion [431]. In addition, these peptides have a variety of physiological activities in the CNS and are known to modify thermoregulation, satiety, dipsogenia and behavioural parameters [1].

The physiological activities of bombesin peptides are mediated by high affinity binding to a number of G protein-coupled receptors including the NMB receptor (NMB-R or BB<sub>1</sub>), the GRP receptor (GRP-R or BB<sub>2</sub>) and the bombesin-like receptor subtypes 3 and 4 (BB<sub>3</sub> and BB<sub>4</sub>) [427, 428, 430]. The BB<sub>1</sub> and BB<sub>2</sub> are found in the skin and gut, and brain of vertebrates respectively. The subtype 3 has been shown to be present mainly in the brains

of a variety of vertebrate species, whilst BB<sub>4</sub> was found to be only present in anuran brains [427, 428, 430]. Like all G protein-coupled receptors, activation of bombesin receptors stimulates an increase in phospholipase C, mobilisation of intracellular Ca<sup>2+</sup> stores and the generation of inositol phosphates [430] resulting in smooth muscle contraction.

### 6.1.1.5 Tryptophyllins

One of the biggest mysteries surrounding the components of amphibian skin secretion are the tryptophyllins. Some forty tryptophyllin peptides have been isolated from numerous species of the *Phyllomedusa* and *Litoria* genera [14]. Several species *L. rubella* and *L. electrica* do not secrete antimicrobial peptides or any of the neuropeptide types previously discussed, instead they produce a variety of tryptophyllins [46]. To date, the biological activity of the majority of these peptides is unknown, however, they must display some form of host defence activities to protect the animals.

Tryptophyllin L 1.3 (Table 6.1) is the only tryptophyllin from *Litoria* species to display smooth muscle activity, however modest at micromolar concentrations. To date, no tryptophyllin tested has been shown to display antimicrobial or nNOS activity [14]. Erspamer tested many of these peptides in a wide range of physiological assays and found one tryptophyllin FPPWM-NH<sub>2</sub> (Table 6.1) to be immunoreactive to cells in rat adenohypophysis, in addition to inducing sedation and behavioural sleep in birds [432]. Tryptophyllin peptides contain sequence similarities to brain endomorphins YPWF-NH<sub>2</sub> and YPFF-NH<sub>2</sub>, which have high affinity for  $\mu$  opioid receptors [433], thus it is probable that tryptophyllins may exhibit similar activity to these brain endomorphins.

### 6.1.1.6 Amphibian Opioid Peptides

To date, amphibian opioid peptides have only been isolated from the skins of hylid frogs of the family Phyllomedusinae [434]. Interestingly, all amphibian opioid peptides isolated thus far, contain a D-amino acid at position 2. It is rare for D-amino acid to be found in organisms other than bacteria, mould and algae [2]. The most common of these are D-Ala, D-Leu and D-Met. This has proved to be essential for physiological activity as substitution of these residues with their corresponding L-enantiomer results in elimination of activity [434]. D-amino acids prolong activity and enhance stability against proteolytic degradation.



The cDNA clones encoding for these peptides have illustrated that in the translated peptide, the L-amino acids are present at position 2, thus isomerisation to the D-enantiomer occurs post-translationally [269, 407].

These peptides are capable of binding with high affinity to mammalian  $\mu$  or  $\delta$  opioid receptors and produce potent analgesic activity. Exceptional selectivity has been observed with many of these peptides. For example, the dermorphin peptides (Table 6.1) are selective  $\mu$  opioid receptor agonists [434], whilst the deltorphin peptides (Table 6.1) are  $\delta$  receptor selective [273]. Neither group of amphibian opioid peptides have been shown to bind to the  $\kappa$  receptors. Like other opioid receptor agonists, these peptides exert potent analgesia, tolerance and physical dependence [434] and catalepsy [435]. Dermorphin is several thousand times more potent than morphine and ten thousand times more potent than enkephalins as an analgesic. In GPI assays, its activity is comparable to dynorphin A (1-13) [436]. In addition, dermorphins effect antinociception, disruption of EEG patterns and numerous other behaviour effects as well as modifying thermoregulation. In the GI system, dermorphins suppress gastric acid secretion and stomach emptying [10]. In addition, deltorphins inhibit hypoglycemia-stimulated ACTH secretion [437].

The mammalian counterparts of these peptides are enkephalins and endorphins, however, dermorphins and deltorphins are distinct from these peptides as they contain D-amino acids (mammalian start sequence YGGF) [438]. Opioid peptides exert their pharmacological actions by binding to G protein-coupled opioid receptors to inhibit adenylate cyclase. This facilitates the opening of  $K^+$  channels resulting in hyperpolarisation and inhibition of the opening of  $Ca^{2+}$  channels causing subsequent inhibition of neurotransmitter release [439].

### **6.1.1.7 Miscellaneous Neuropeptides**

A number of other neuropeptides have been isolated from amphibians that do not correlate to any of the groups mentioned above (Table 6.1). Xenopsin was first isolated from *Xenopus laevis* and has been shown to be structurally homologous to neurotensins and exhibit similar hypotensive activities, lowering arterial pressure and producing tachyphylaxis [1]. This peptide is characterised by a free C-terminal and a pGlu residue at the N-terminal. Xenopsin has been shown to exert its activity by activation of neurotensin receptors [440] and  $B_2$  receptors [441].

Other neuropeptides isolated have been shown to be identical in sequence in both amphibians and mammals, however the roles of the peptides appear to differ in each. For example, the mammalian TRH was isolated in amphibian skin secretion (eg. *Bombina orientalis*) [411]. In both mammalian tissue and amphibians, TRH regulates thyrotropin levels [10]<sup>[5\*]</sup>. *Crinia* angiotensin II (Table 6.1) was isolated from *Crinia georgiana* [412], while angiotensin II is present in mammals. *Crinia* angiotensin II displays comparable activity to its mammalian analogue. It causes the release of Ach, activates the adrenergic system, elevates blood pressure, increases thirst and stimulates vasopressin release [442].

Angiotensin II            DRVYIHPF-OH

### 6.1.2 Smooth Muscle Contraction Preparations

Neuropeptides that contract smooth muscle form an essential part of the defence of the frog, in addition to having important functions in its body. A relatively simple functional assay for smooth muscle contraction uses isolated tissue preparations of the longitudinal muscle from GPI in an organ bath maintained at physiological conditions. GPI contains numerous receptors, most notably CCK receptors [264] and mAChR [443], thus can be used to characterise the pharmacology of the peptides at these receptors by means of their mechanical response. Identification of binding to these receptors can indicate other implications of the activity of the peptide within the CNS [264].

CCK receptors are widely distributed in the CNS and periphery and are associated with a wide variety of biological effects including anxiety, pain perception, gastric acid secretion and contraction of smooth muscle [264, 444]. CCK receptors contain a seven transmembrane helix domain topology and exert their actions through their ability to interact with G proteins [445]. The most characterised of this receptor family is that of rhodopsin (Figure 6.1) [446]. There are two main CCK receptor subtypes, CCK<sub>1</sub> and CCK<sub>2</sub> [444], which are characterised based on the relative affinities of agonist and antagonists. CCK<sub>1</sub> subtypes have a high affinity for sulfated CCK, a low affinity for gastrin and a high affinity for antagonist L-364,718, whilst CCK<sub>2</sub> subtypes have a high affinity for both

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<sup>5\*</sup> Galas, L., Bidaud, I., Bulant, M., Jenks, B.G., Ouwens, D.T.W.M., Jégou, S., Ladram, A., Roubos, E.W., Nicolas, P., Tonon, M.C. and Vandry, H. (2005) *In situ* hybridisation localization of TRH precursor and TRH receptor mRNAs in the brain and pituitary of *Xenopus laevis*, *Ann. NY Acad. Sci.* 1040, 86-105

agonists CCK and gastrin, a low affinity for L-316,718 and a high affinity for antagonist L-365,260 [447].

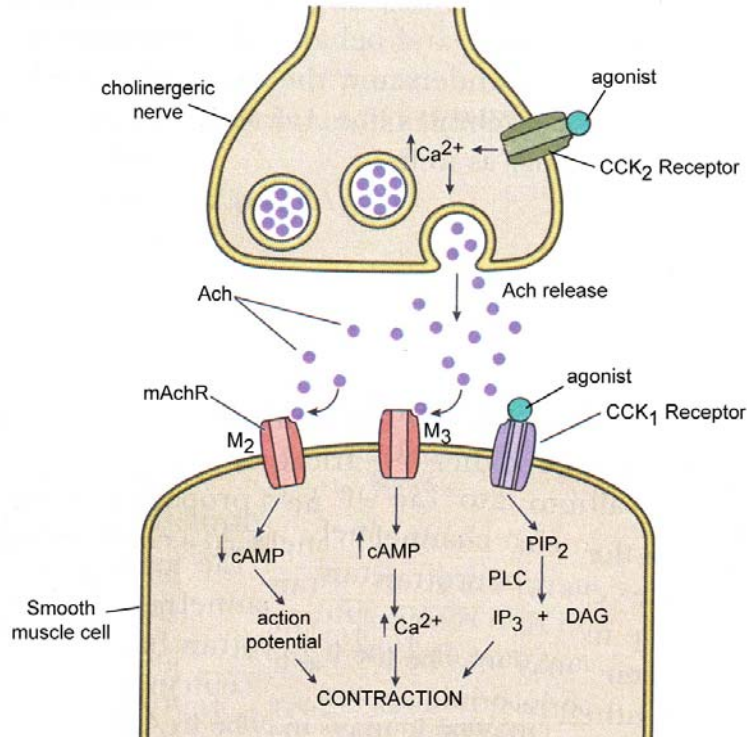
NOTE:

This figure is included on page 173 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 6.1:** 3D crystal structure of the G protein-coupled receptor rhodopsin. Adapted from [446], PDB code: 1f88.

Among the actions mediated by the CCK<sub>1</sub> receptor are the control of satiety, gallbladder contraction, pancreatic exocrine secretion, gastrin secretions and emptying and gut motility. The actions that occur through CCK<sub>2</sub> receptors include actions in the CNS such as modulation of anxiety and pain perception and actions in the periphery where it mediates gastric acid secretion, changes in renal salt absorption and smooth muscle contraction [448].

Activation of CCK receptors results in various responses depending on the tissue. In smooth muscle, contraction is achieved by CCK receptors in two ways. CCK<sub>1</sub> receptors are situated on smooth muscle and cause contraction directly. Ligand binding to CCK<sub>1</sub> receptors results in a conformation change allowing the receptor to couple to a G protein, which subsequently dissociates and activates phospholipase C (PLC). PLC cleaves phosphatidylinositol biphosphate (PIP<sub>2</sub>) to form the second messengers inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> causes the release of intracellular Ca<sup>2+</sup> which causes contraction of the smooth muscle [449]. The CCK<sub>2</sub> receptors, on the other hand, cause contraction of smooth muscle indirectly. Mobilisation of intracellular Ca<sup>2+</sup> stores results in the release of Ach from cholinergic nerves in the myenteric plexus, which subsequently activates mAChR on smooth muscle to cause contraction (Figure 6.2) [443].



**Figure 6.2:** CCK receptor mediated contraction of smooth muscle. Contraction is achieved directly via CCK<sub>1</sub> receptors located on the smooth muscle cellular membrane or indirectly via CCK<sub>2</sub> receptors located on the presynaptic (cholinergic nerve) cellular membrane.

mAChR are important in the contraction of smooth muscle. Two subtypes, M<sub>2</sub> and M<sub>3</sub> are located on the cell membranes of smooth and cardiac muscle, and on the endothelium of most peripheral blood vessels [443]. Activation of the M<sub>2</sub> subtypes on the cellular membrane of smooth muscle by Ach results in a signal transduction pathway that inhibits the synthesis of cAMP producing an action potential and subsequent contraction of the cell [414]. In contrast, activation of M<sub>3</sub> subtypes cause an increase in synthesis of cAMP, mobilisation of intracellular Ca<sup>2+</sup> stores [258, 443] and a Ca<sup>2+</sup> induced contraction. Contractions mediated by mAChR will be inhibited by the antagonist atropine [261].

Amphibian neuropeptides that act as agonists for CCK receptors provide potential for new therapeutic agents that can be used in numerous medical conditions including GI abnormalities, blood pressure regulation and as non-opioid analgesic agents [395]. For example, caerulein is used therapeutically to treat a variety of GI conditions [22].

### 6.1.3 Immunomodulators

Interaction between neuropeptides in the CNS and the immune system is an area of recent interest and there is much speculation that neuropeptides affect the immune response [450, 451]. Cells in the immune system have been shown to contain specific binding sites for numerous neuropeptides including opiates, bombesins [452], ACTH, vasoactive intestinal peptide (VIP), growth hormones, insulin and substance P [453, 454]. Neuropeptides that interact with cells in the immune system and play an important role in regulating the immune response are referred to as immunomodulators.

Important cells of immune responses include lymphocytes, granulocytes, monocytes and natural killer cells [452]. Lymphocytes (T cells and B cells) are involved in the regulation of immune reactions by direct activation with other cells or indirectly through the production and secretion of soluble mediators [453]. For example, the release of lymphokines such as interleukin (IL-2) and  $\gamma$  interferon (IFN- $\gamma$ ) from lymphocytes promote and maintain the growth of T cells, whilst IFN- $\gamma$  also activates cytotoxic cells and macrophages [453]. Natural killer cells are involved in killing virus-infected and tumour cells by inhibition of virus replication and elevated antigen production [453].

Lymphocyte proliferation is often used as a parameter of immune function and indicates an elevation in immune response. Extensive research has indicated numerous factors are implicated in the suppression or promotion of lymphocyte proliferation. For example,  $\beta$  endorphins enhanced proliferation of rat splenocytes to T cell mitogen concanavalin A through a non-opioid receptor mechanism [453]. Similarly, VIP causes mitogen-induced T cell proliferation. Immune suppression has been demonstrated in the presence of  $\delta$  opioid receptor antagonists [455], morphine and other related opioid peptides [456].

One way of measuring lymphocyte proliferation is to measure the uptake of isotopically labelled nucleotides such as [ $H^3$ ]thymidine by lymphocyte cells. This is an indirect way of indicating cellular proliferation as it measures the amount of labelled nucleotide incorporated into the cellular DNA, providing an indicator of replication of cellular DNA, thus cellular proliferation [453]. An alternative method is the Alamar Blue fluorescence dye method. Cellular proliferation is measured directly by a redox indicator present in Alamar Blue. The indicator changes colour from blue to red as a consequence of chemical

reduction resulting from cellular proliferation [457]. The cells are incubated with Alamar Blue, followed by measuring the absorbance at 570 to 600 nm to quantify the extent of reduction in terms of a specific absorbance which reflects the level of proliferation [457]. The later assay is preferred as it is efficient, quick and relatively inexpensive relative to the [ $H^3$ ]thymidine assay. In addition, the Alamar Blue assay allows for cells to be monitored during the culture period and permits further analysis of cells [457].

There has been a suggestion that immunoreactive forms of CCK have a regulatory function in human lymphocytes and the immune system [458]. The identification of CCK<sub>2</sub> receptors on immune cells such as human Jurkat lymphoma cell lines [450, 451, 459], monocytes [459] and T lymphocytes [452] have supported this. In these cell lines, the genes encoding CCK<sub>2</sub> but not CCK<sub>1</sub> receptors have been identified [460]. CCK has recently been demonstrated to be widely distributed in the CNS, where it exerts a variety of behavioural effects via its neurotransmitter action [452].

CCK<sub>2</sub> receptor activation by CCK-8 or related agonists, results in activation of PLC and a subsequent rapid increase in basal intracellular Ca<sup>2+</sup> levels [461] which regulates the production of a central mediator of the immune response (eg. IL-2) [450]. IL-2 is an important modulator of proliferation in T lymphocytes with the activation, differentiation and growth of peripheral T lymphocytes being mainly controlled by IL-2 [462]. In addition to modulation by Ca<sup>2+</sup> levels, IL-2 production depends on regulation of the activator protein-1 (AP-1) responsive gene. Activation of CCK<sub>2</sub> receptors results in an increased expression of AP-1 regulated genes and subsequent increased expression of IL-2 and cell proliferation [462]. CCK-8 and other neuropeptides that result in an increase in immune response appear to have potential as therapeutic agents to help maintain immunity [395]. Furthermore, these peptides would aid the amphibian in its defence against pathogen invasion.

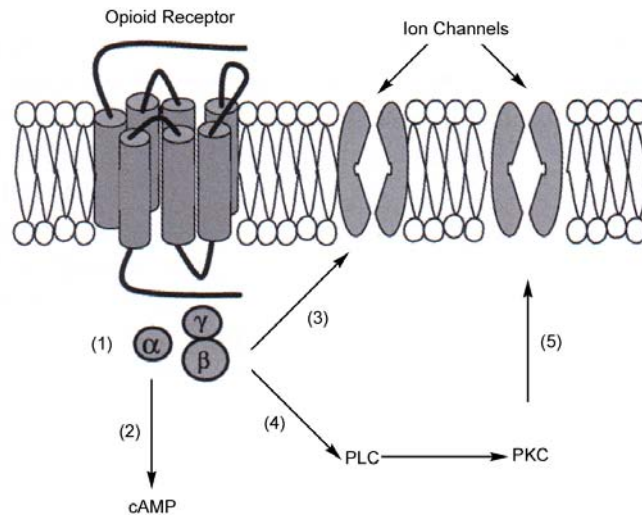
#### **6.1.4 Opioid Activity Preparations**

Opioid receptors are widely distributed throughout the brain and the periphery. They are implicated in the control of numerous physiological systems and mediate the actions of morphine-like analgesics [463]. There are three distinct classes of opioid receptors:  $\mu$ ,  $\delta$ , and  $\kappa$  receptors in which morphine, enkephalins and dynorphin selectively bind

respectively [464]. When activated by endogenous opioid peptides, these G protein-coupled receptors also regulate the body's response to pain, stress and emotions [465]. Many endogenous opioid peptides have been isolated from numerous amphibian skin secretions (deltorphins [272] and dermorphin [407]) and mammalian tissues (enkephalins [466], dynorphin [467, 468] and endomorphins [433]). Agonists of  $\mu$  opioid receptors generally are considered to be responsible for many of the typical side effects such as addiction, respiratory depression, constipation, immunosuppression and withdrawal [465].

Investigation of opioid receptor mediated responses in isolated systems is preferred in screening for opioid agonists as it allows analysis of the effect of the compound without the complications of secondary factors such as distribution, metabolism and excretion [463]. *In vitro* bioassays have been extensively used for the pharmacological characterisation of opioid agonists and peripheral tissue preparations have proved to be invaluable models. In peripheral tissue, opiates reduce impulse transmission at specific peripheral junctions in the autonomic nervous system. At neuroeffector junctions, opioid agonists act on opioid receptors contained on the presynaptic nerve terminal and inhibit electrically stimulated neurotransmitter release [463]. The most commonly used peripheral tissue bioassays are electrically stimulation of GPI or its myenteric plexus-longitudinal muscle [469, 470] and of rat vas deferens [466]. Other systems that are used are based on the inhibition of binding of labelled ligands in brain homogenates [471, 472].

GPI has been used to study both the acute effects of opioid receptors and the long-term effects of opioid tolerance and dependence. GPI preparations are used in this study as they are inexpensive, easily dissected and display long-term stability *in vitro* [463]. The specific action of opioid agonists in GPI is to depress the firing of myenteric neurons, inhibiting the presynaptic release of Ach and thereby reducing the nerve-mediated cholinergic contractions of the smooth muscle contractions [473, 474]. It has been shown that only the  $\mu$  and  $\kappa$  opioid receptor types are localised on the cholinergic neurons in GPI myenteric plexus [473, 475]. Hence, activation of the  $\mu$  and  $\kappa$  opioid receptors by agonists will increase the  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  conductance to hyperpolarize the myenteric neurons and inhibit the prejunctional release of Ach [475] and the subsequent depression of smooth muscle contraction (Figure 6.3). There is evidence that  $\delta$  receptors are also present, however these receptors do not mediate the release of Ach [463].



**Figure 6.3:** Opioid ligands induce a conformational change in the receptor that allow it to couple to G proteins. (1) The G protein complex dissociates into active  $G_{\alpha}$  and  $G_{\beta\gamma}$  subunits which can subsequently: (2) inhibit adenylyl cyclase production of cAMP; (3) decrease the conductance of voltage-gated  $\text{Ca}^{2+}$  channels, opening rectifying  $\text{K}^{+}$  channels; (4) activate the PLC/PKC pathway that (5) modulates  $\text{Ca}^{2+}$  channel activity in the plasma membrane. As a consequence, the membrane is hyperpolarized resulting in a decrease in cellular excitability, neuronal activity and the release of neurotransmitters (eg. Ach). Adapted from [476].

Inhibition of electrically stimulated smooth muscle contraction does not directly indicate that the ligand is an opioid agonist, instead specific blockade testing is required to confirm this [475]. Naloxone is commonly used to characterise the stimulated contractions. Naloxone enhances the electrically stimulated release of Ach from the myenteric plexus through antagonist effects for opioid receptor and can reverse the inhibitory action of the ligand [474]. Concentrations below 1  $\mu\text{M}$  typically inhibit only this receptor, however concentrations greater than this value result in non-specific inhibition and influences the actions of Ach, noradrenaline, adrenaline, serotonin, or histamine [477]. An example of a potent opioid peptide agonist is dynorphin A (1-13) which depresses the electrically stimulated induced contraction in GPI at low nanomolar concentrations and displays high selectivity towards  $\kappa$  opioid receptors [478]. Opioid agonists that act via  $\kappa$  and  $\delta$  opioid receptors provide possible opioid receptor agonists that may lack the typical side effects associated with  $\mu$  opioid receptors [465]. Moreover, these peptides would contribute to the host defence of amphibians against both invading pathogens and predators by effecting opioid-mediated responses.



## 6.2 Results

### 6.2.1 Disulfide Peptides

#### 6.2.1.1 Smooth Muscle Contraction Assays

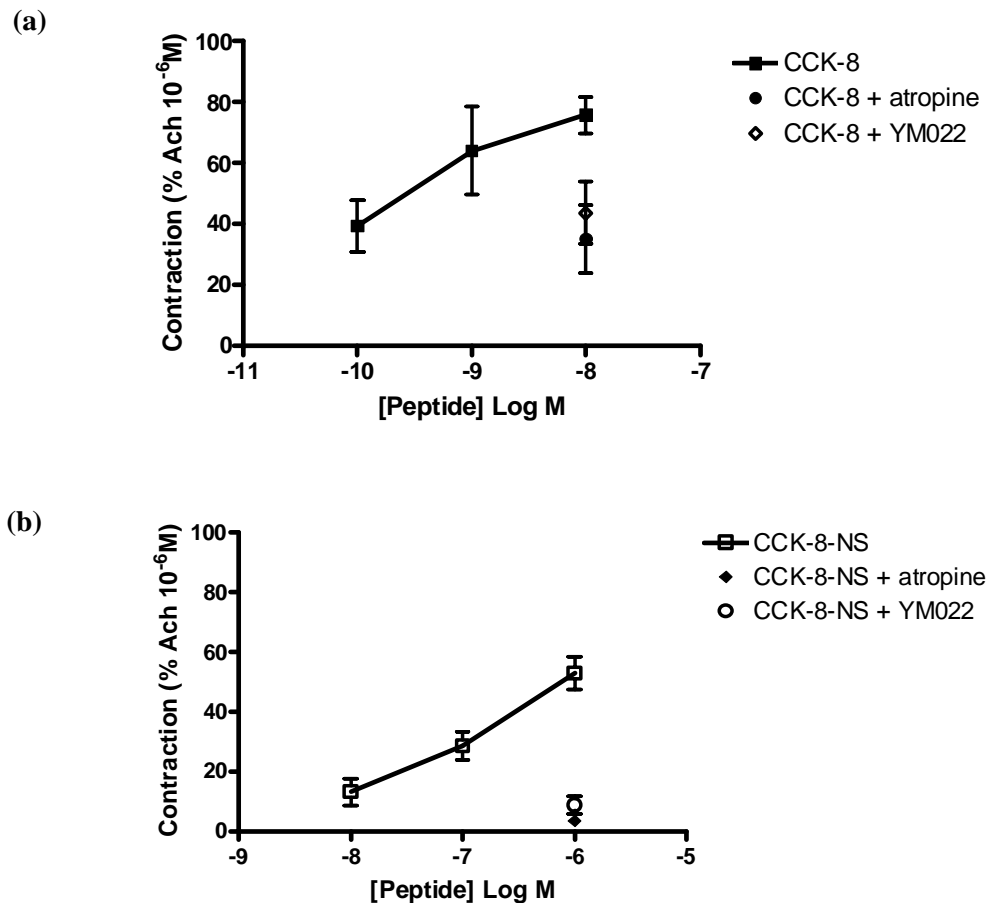
Disulfide peptides, signiferin 1, the synthetic modifications of signiferin 1 and riparin 1.1 and 1.2 (Table 6.2) were tested for smooth muscle activity on GPI. All smooth muscle contractions are described in terms of a percentage of Ach ( $10^{-6}$  M) contraction. CCK-8 and CCK-8-NS were used as standards (Figure 6.4). CCK-8 ( $10^{-8}$  M) produced a contraction that was  $60 \pm 11$  % of that produced by Ach ( $10^{-6}$  M), whilst CCK-8-NS ( $10^{-6}$  M) produced a contraction of  $32 \pm 12$  %. In the presence of atropine (mAChR antagonist), the contraction produced by CCK-8 is reduced. Similarly, YM022 (CCK<sub>2</sub> receptor antagonist) reduces the CCK-8 induced contractions. This is not surprising as CCK-8 acts via both CCK<sub>1</sub> and CCK<sub>2</sub> receptors. In contrast, the CCK-8-NS contraction is eliminated in the presence of atropine and YM022, consistent with the affinity of CCK-8-NS for CCK<sub>2</sub> receptors.

**Table 6.2:** Sequence of riparins and signiferin and its synthetic modifications.

Peptide <sup>1</sup>	Sequence	Species <sup>2</sup>
Riparin 1.1	RLCIPVIFPC-OH	a
Riparin 1.2	FLPPCAYKGTC-OH	a
Signiferin 1	RLCIPYIIPC-OH	b, c
[Gly <sup>11</sup> ]Signiferin 1	RLCIPYIIPCG-OH	d
Signiferin 1 amide	RLCIPYIIPC-NH <sub>2</sub>	d
Signiferin 1 NdiS	RLCIPYIIPC-OH	d

<sup>1</sup> NdiS indicates the absence of the disulfide linkage

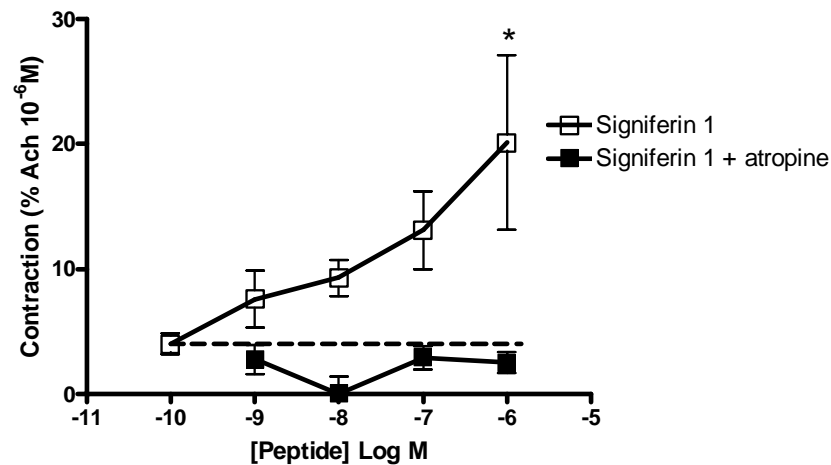
<sup>2</sup>**Species:** (a) *Crinia riparia* [44]; (b) *Crinia signifera* [47]; (c) *Crinia deserticola* [479]; (d) synthetic modification.



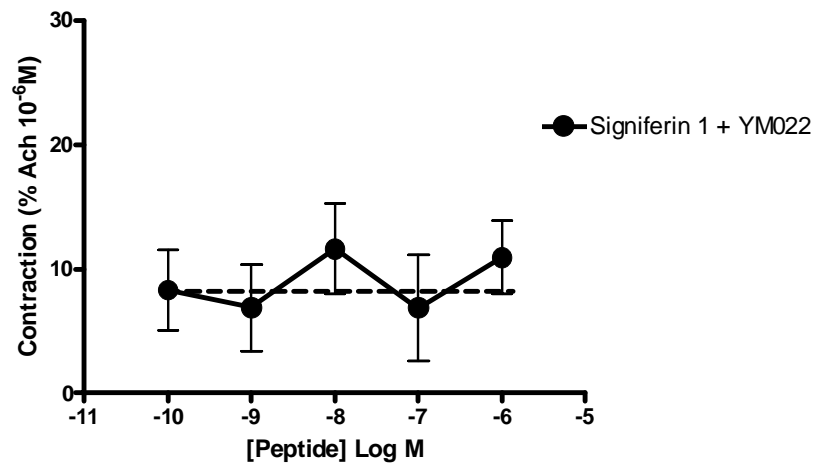
**Figure 6.4:** Smooth muscle contraction response curves of (a) CCK-8 and (b) CCK-8-NS in the presence and absence of mAChR antagonist atropine or CCK<sub>2</sub> selective antagonist YM022. Contractions are expressed as a percentage of the contraction in the presence of Ach ( $10^{-6}$  M) ( $0.16 \pm 0.02$  g,  $n=4$ ) and are shown as mean  $\pm$  SEM of three independent experiments done in duplicate.

Signiferin 1 (isolated from both *C. signifera* [47] and *C. deserticola* [479]) produced smooth muscle contraction of GPI from  $10^{-9}$  M, with maximum mean activity of 20 % at  $10^{-6}$  M (Figure 6.5). The contraction induced by signiferin 1 was blocked by atropine, suggesting that the peptide acts through a receptor that results in the release of the neurotransmitter Ach, possibly via the CCK<sub>2</sub> receptor. There is no concentration dependent increase in the contraction of signiferin 1 in the presence of atropine (Figure 6.5a). This response is emphasised by the dotted line representing the contractions at the lowest concentration of signiferin 1 ( $10^{-10}$  M) of the ileum tissue. Significant blockage of the signiferin 1-induced contraction produced by atropine eliminates the possibility of the involvement of CCK<sub>1</sub> receptor in the contraction.

(a)



(b)



**Figure 6.5:** (a) Smooth muscle contraction response curves of signiferin 1 in the presence and absence of mAChR antagonist atropine. This is an indirect assay of CCK<sub>2</sub> receptor activation, as CCK<sub>2</sub> agonists produce contraction by the release of Ach from nerve terminals. Signiferin 1 produced a significant increase in contraction ( $P < 0.05$ ) by itself. This increase in contraction was removed in the presence of atropine. (b) Smooth muscle contraction response curve of signiferin 1 in the presence of CCK<sub>2</sub> receptor antagonist YM022. The increase in contraction ( $P < 0.05$ ; see Figure 6.5a) was reduced in the presence of YM022. Contractions are expressed as a percentage of the contraction in the presence of Ach ( $10^{-6}$  M) ( $0.16 \pm 0.02$  g,  $n = 4$ ) and are shown as mean  $\pm$  SEM of three independent experiments done in duplicate. \* indicates  $P < 0.05$ . The dotted line indicates contraction at the lowest concentration.

The involvement of CCK<sub>2</sub> receptors in signiferin-induced muscle contraction is confirmed by results with the selective CCK<sub>2</sub> receptor antagonist YM022 [480]. There is no concentration dependent increase in contraction with signiferin in the presence of YM022 (Figure 6.5b). The contraction of signiferin 1 at  $10^{-6}$  M in the presence of YM022 is the same as that produced by signiferin 1 alone at  $10^{-10}$  M. The dotted line emphasises this. A combination of atropine and YM022 antagonist results confirm that signiferin 1 is inducing its smooth muscle contraction via CCK<sub>2</sub> receptor and that CCK<sub>1</sub> receptor is not involved.

Neither the riparins 1.1 and 1.2 or the synthetic modifications of signiferin 1 illustrated muscle activity below concentrations of  $10^{-5}$  M (data not shown).

Several other disulfide peptides (Table 6.3) that displayed sequence similarity to signiferin 1 and riparin 1 peptides were tested for smooth muscle activity in GPI, however these peptides did not display any activity below concentrations of  $10^{-5}$  M (data not shown).

**Table 6.3:** Sequence of disulfide peptides from *Rana* species [14].

Peptide	Sequence	Species <sup>1</sup>
Unnamed	KNLLASALDKLK <u>CKVTGC</u> -OH	a
Unnamed	FLPLLAASFACTVTKKC-OH	a
Tigerin 3	RVCYA <u>IPLPIC</u> -OH	b
Japonicin 1	FFPIGVFCKIFKTC-OH	c

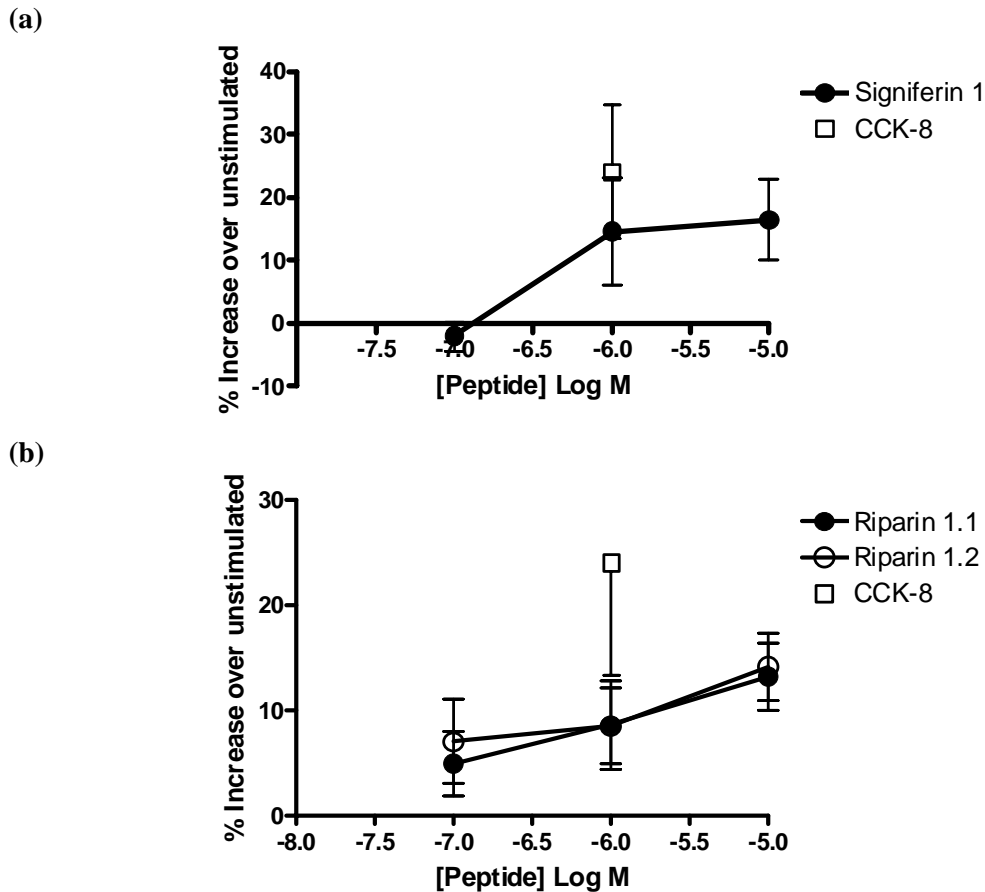
<sup>1</sup>**Species:** (a) *Rana arvalis*; (b) *Rana tigerina* [481]; (c) *Rana japonica* [482].

### 6.2.1.2 Lymphocyte Proliferation Studies

Lymphocyte proliferation was measured for mouse splenocytes using the Alamar Blue fluorescence dye method [457]. CCK-8 was used as a standard. Signiferin showed activity at  $10^{-6}$  M (Figure 6.6a), whilst the riparins 1.1 and 1.2 both showed activity at  $10^{-7}$  M (Figure 6.6b). These activities are slightly less than those observed for CCK-8 at  $10^{-6}$  M (Figure 6.6a). The activities observed for signiferin and the riparin peptides did not show a statistically significant difference from those obtained for the control CCK-8 at the highest concentration tested. Concentrations below  $10^{-7}$  M were not used because of the poor sensitivity of the Alamar Blue method at such low concentrations.

Since lymphoid cells have been shown to possess CCK<sub>2</sub> receptors exclusively [450, 460, 483, 484], it is suggested that signiferin 1 and the riparins 1.1 and 1.2 act as CCK<sub>2</sub> receptor agonists in effecting proliferation of lymphocytes. The synthetic modifications of signiferin 1 were shown to have no activity (data not shown).

In addition, the several other disulfides peptides (Table 6.3) tested were found to display no activity.



**Figure 6.6:** (a) Signiferin 1 and (b) riparin 1.1 and 1.2 concentration response curves for mouse splenocyte proliferation. The data are expressed as a percentage increase in cell proliferation over the unstimulated controls and shown as the mean  $\pm$  SEM of four independent measurements performed in quadruplicate. The response to the standard CCK-8 is shown for comparison. \* indicates  $P < 0.05$ .

### 6.2.2 Rothein 1 Peptides<sup>3</sup>

The peptide profile of the skin secretions of *Litoria rothii* varies seasonally. In the summer months, *L. rothii* produces caerulein as a major component, whilst in winter, it produces rothein 1 and caerulein 1.2 as major components [45]. The activity of caerulein 1.2 is discussed in Section 6.2.3. Rothein 1 has not shown any activity in tests conducted to date, thus it was thought that this peptide might act as a neuropeptide. Due to the unusual structure of rothein 1, a number of synthetic modifications were made and tested for biological activity to ascertain the structure activity relationship for rothein 1 (Table 6.4).

<sup>3</sup> Preliminary work on these peptides was carried out in collaboration with Dr. Vita Maselli, The University of Adelaide, Australia.

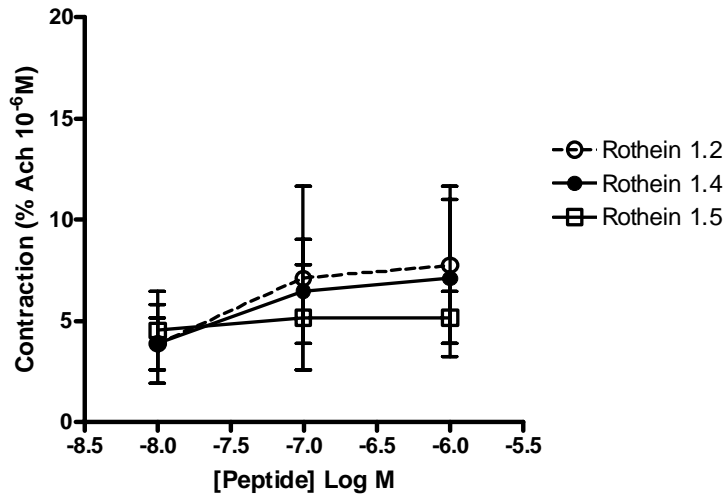
**Table 6.4:** Sequence and activities of rothein 1 and its synthetic modifications.

<b>Rothein</b>	<b>Sequence</b>	<b>Activity<sup>1</sup></b>
1	SVSNIPESIGF-OH	a, b
1.1	SVSNIPES <b>T</b> Gf-OH	a, b
1.2	<b>A</b> VSNIPE <b>S</b> IGF-OH	a, b, c
1.3	SV <b>A</b> NIPE <b>S</b> IGF-OH	a, b
1.4	SVSNIP <b>A</b> SIGF-OH	a, c
1.5	SVSNIP <b>E</b> AIGF-OH	a, c
1.6	SVSNIPESIG <b>A</b> -OH	d
1.7	SVSNIP <b>A</b> STGF-OH	d

<sup>1</sup>**Activity:** (a) lymphocyte activity; (b) secondary smooth muscle contraction; (c) smooth muscle activity; (d) not active.

### 6.2.2.1 Smooth Muscle Contraction Assays

Rothein 1 peptides were tested for smooth muscle activity in GPI. All smooth muscle contractions are described in terms of percentages of Ach ( $10^{-6}$  M) contraction. CCK-8 and CCK-8-NS were used as standards and their activities are described in Figure 6.4 and Section 6.2.1.1. Smooth muscle contractions less than 5 % of Ach ( $10^{-6}$  M) are considered to be inactive. Rothein 1 and its synthetic modifications 1.1, 1.3, 1.6 and 1.7 did not display any activity. Rothein 1.2, 1.4 and 1.5 produced smooth muscle contraction in GPI (Table 6.5). Rothein 1.2 produced a contraction in GPI from  $10^{-7}$  M, with a maximum mean activity of approximately 8 % at  $10^{-6}$  M (Figure 6.7). Rothein 1.4 produced a similar contraction to rothein 1.2. Rothein 1.5 displays very little contraction of smooth muscle with a maximum contraction of 5 %. The increase in contractions observed for the active rothein peptides were not statistically significant. This demonstrates the weak smooth muscle activity of these peptides. Due to the small contractions observed for the rothein 1 peptides, the activity of these peptides in the presence of atropine or other receptor antagonists were not considered.

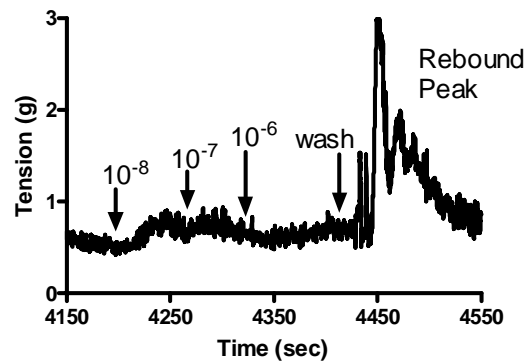


**Figure 6.7:** Smooth muscle contraction response curves of rothein 1 synthetic modifications, rothein 1.2, 1.4 and 1.5. The contractions are expressed as a percentage of the contraction in the presence of Ach ( $10^{-6}$  M) ( $1.59 \pm 0.13$  g,  $n = 4$ ) and are shown as mean  $\pm$  SEM of three independent experiments done in duplicate. \* indicates  $P < 0.05$ .

**Table 6.5:** Smooth muscle contraction response of rothein 1 and its synthetic modifications. The contractions are expressed as a percentage of the contraction in the presence of Ach ( $10^{-6}$  M) ( $1.59 \pm 0.13$  g,  $n = 4$ ) and are shown as mean  $\pm$  SEM of three independent experiments done in duplicate. The peptides that are considered to be active are indicated in bold.

Rothein	$10^{-8}$ M		$10^{-7}$ M		$10^{-6}$ M	
	Mean	SEM	Mean	SEM	Mean	SEM
1	1.94	1.29	1.29	1.29	0.00	1.94
1.1	1.94	4.52	3.23	2.58	0.00	1.94
<b>1.2</b>	<b>3.87</b>	<b>1.94</b>	<b>7.10</b>	<b>4.52</b>	<b>7.75</b>	<b>3.87</b>
1.3	2.58	0.06	3.23	1.29	3.23	1.29
<b>1.4</b>	<b>3.87</b>	<b>1.29</b>	<b>6.45</b>	<b>2.58</b>	<b>7.10</b>	<b>3.87</b>
<b>1.5</b>	<b>4.52</b>	<b>1.94</b>	<b>5.16</b>	<b>2.58</b>	<b>5.16</b>	<b>1.29</b>
1.6	0.44	0.89	0.66	0.73	1.62	1.20
1.7	1.69	1.18	1.81	1.33	1.32	0.88

In addition, the active rothein 1 peptides produced a large secondary contraction of the GPI upon being washed out of the organ bath (Figure 6.8). This contraction has not been observed for CCK-8, CCK-8-NS, Ach or any other peptides studied previously (a similar response was also seen for the fallaxidin 1 peptides, see Section 6.2.5.1). This secondary contraction was not altered by the presence of atropine (mAChR antagonist) (data not shown) which indicates that the secondary response is not mediated by mAChR or any receptors that result in the release of Ach from presynaptic nerve terminals such as CCK<sub>2</sub> receptors [264], neurokinin receptors [265] or orexin receptors [266]. In addition, Ach ( $10^{-7}$  M) was added in the presence of rothein 1 peptides to investigate whether these peptides were inhibiting mAChR. No decrease in the Ach-induced contractions was observed, thus suggesting that these peptides are not acting as antagonist to mAChR.



**Figure 6.8:** Concentration response data for rothein 1.5 illustrating the secondary smooth muscle contraction.

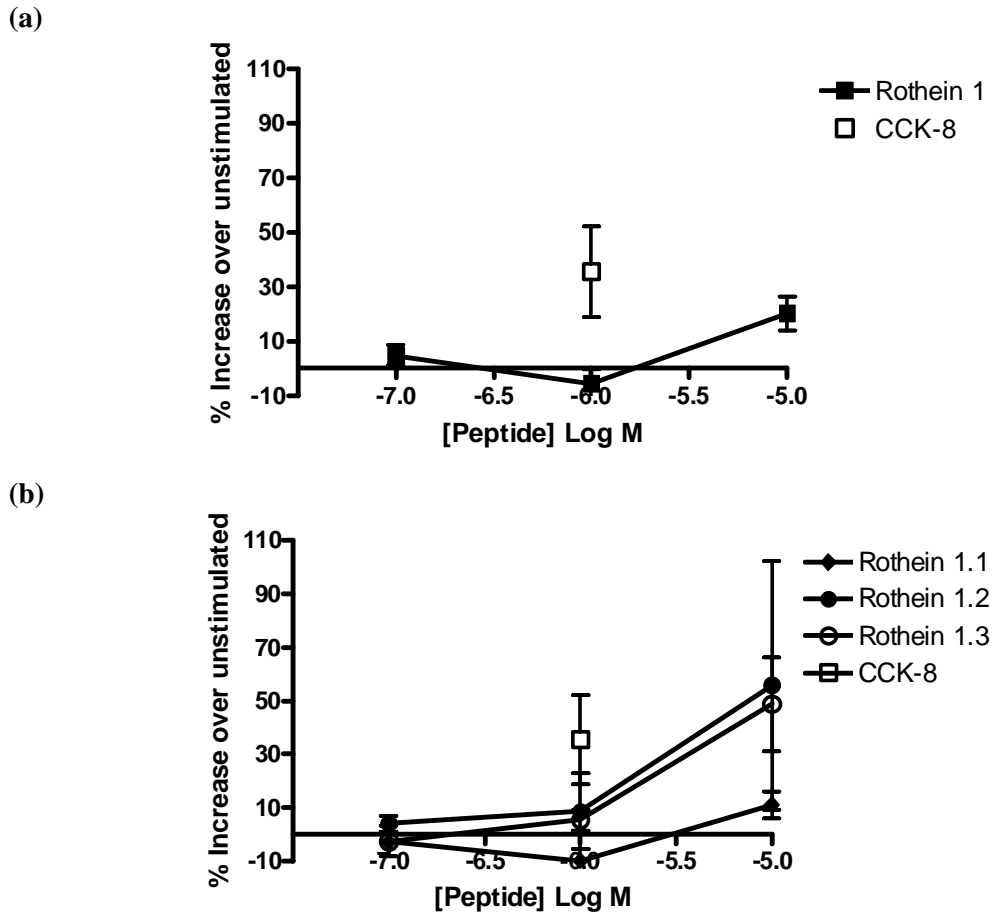
The rothein 1 peptides may be releasing an inhibitory factor in addition to the contractile response. It was suggested that NO could be a possibility as the secondary contraction occurred rapidly when the peptide was washed out and was short lived. NO is produced in the myenteric plexus by nNOS and has a rapid decay time relative to the Ach contractile effects, consistent with the secondary contraction observed [485]. To test this hypothesis, the NOS inhibitor L-NNA [262] was added. No change in the secondary contraction was observed in the presence of L-NNA, suggesting that the secondary response is not mediated by any receptors that result in the synthesis and release of cellular or neuronal NO such as B<sub>1</sub> receptors [267]. In addition, no change in the secondary contraction was observed in the presence of brethylum tosylate, which inhibits the release of noradrenaline from synaptic nerves [263]. This suggests that β adrenoceptors are not involved, in particular β<sub>3</sub> as this is predominantly present on smooth muscle in the GI tract [268]. Thus, rothein 1 peptides, in addition to the contractile activity, are acting via an unknown receptor pathway that results in relaxation of the smooth muscle.

### 6.2.2.2 Lymphocyte Proliferation Studies

Rothein 1 produced a concentration dependent increase in the proliferation of lymphocytes from a concentration of 10<sup>-5</sup> M (Figure 6.9a). Rothein 1 modifications 1.1, 1.2 and 1.3 were also active in lymphocytes, producing an increase in proliferation from concentrations of 10<sup>-5</sup>, 10<sup>-7</sup> and 10<sup>-6</sup> M respectively (Figure 6.9b). The activity of rothein 1.1 was slightly less than that of the native rothein 1 peptide, whilst rothein 1.2 and 1.3 were the most active modifications, inducing a mean increase in proliferation of around 50 %. The activities observed for the rothein peptides did not show a statistically significant difference from those obtained for the control CCK-8 at the highest concentrations tested. Rothein 1.4 – 1.7 were inactive in these assays.



It is suggested that rothein 1 and its modifications 1.1, 1.2 and 1.3 act as CCK<sub>2</sub> receptor agonists in effecting proliferation of lymphocytes as lymphoid cells have been shown to possess CCK<sub>2</sub> receptors [450, 460, 483, 484].



**Figure 6.9:** (a) Rothein 1 and (b) rothein 1 modifications, rothein 1.1, 1.2 and 1.3 concentration response curves for mouse splenocyte proliferation. The data are expressed as a percentage increase in cell proliferation over the unstimulated controls and shown as the mean  $\pm$  SEM of four independent measurements performed in quadruplicate. The response to the standard CCK-8 is shown for comparison. \* indicates  $P < 0.05$ .

### 6.2.3 Caerulein Peptides

*Litoria citropa* is an unusual species of the *Litoria* genus, in that it secretes its host defence peptides from two glands; the granular glands on the dorsal surface and the submental gland on the throat. Several antimicrobial peptides have been isolated, in addition to caerulein and its related peptides listed in Table 6.6 [402]. Caerulein has been isolated as a major neuropeptide from most *Litoria* species with the exception of *L. fallax* (Chapter 4), *L. electrica* and *L. rubella* [12]. As previously mentioned, caerulein exhibits potent smooth

muscle activity, analgesic activity several thousand times greater than morphine and is used clinically as a hormone [1, 10]. The activities of the other caerulein peptides are unknown, thus as these peptides show similarities to caerulein, their activities were investigated to see if they play a similar role in the host defence of *L. citropa*. The data presented here is preliminary.

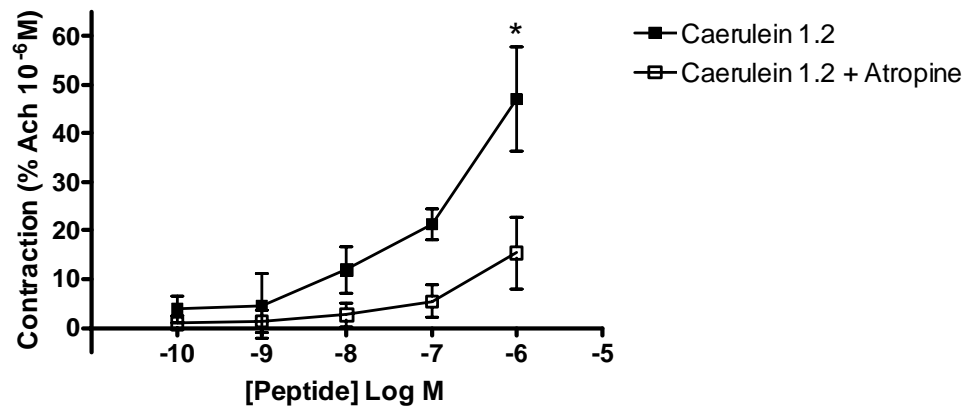
**Table 6.6:** Caerulein peptides isolated from *Litoria citropa* [402]. Gaps (-) in the peptide sequence have been inserted to illustrate sequence homology. Differences in the sequence are illustrated in bold.

Caerulein	Sequence
1.2	pEQDY(SO <sub>3</sub> )-TG- <b>WF</b> DF-NH <sub>2</sub>
2.1	pEQDY(SO <sub>3</sub> )-TG <b>AH</b> MDF-NH <sub>2</sub>
2.2	pEQDY(SO <sub>3</sub> )-TG <b>AHF</b> DF-NH <sub>2</sub>
3.1	pEQDY(SO <sub>3</sub> ) <b>GTG</b> - <b>WM</b> DF-NH <sub>2</sub>
3.2	pEQDY(SO <sub>3</sub> ) <b>GTG</b> - <b>WF</b> DF-NH <sub>2</sub>
4.1	pEQDY(SO <sub>3</sub> )-TG <b>SH</b> MDF-NH <sub>2</sub>
4.2	pEQDY(SO <sub>3</sub> )-TG <b>SHF</b> DF-NH <sub>2</sub>

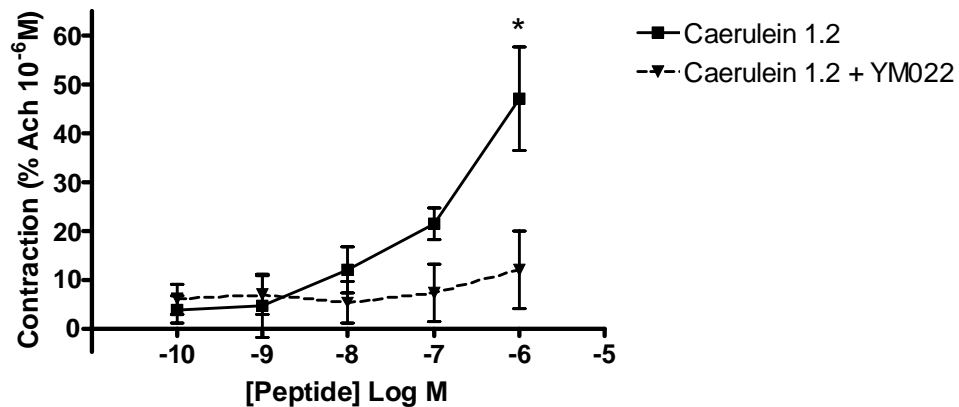
### 6.2.3.1 Smooth Muscle Contraction Assays

The caerulein peptides (Table 6.6) were tested for smooth muscle activity on GPI. All smooth muscle contractions are described in terms of a percentage of Ach (10<sup>-6</sup> M) contraction. CCK-8 and CCK-8-NS were used as standards and their activities are described in Figure 6.4 and Section 6.2.1.1. Caerulein 1.2 produced smooth muscle contraction of GPI from 10<sup>-9</sup> M, with a maximum mean activity of 50 % at 10<sup>-6</sup> M (Figure 6.10). The contraction induced by caerulein 1.2 was decreased by atropine, suggesting that the peptide acts through a receptor that results in the release of the neurotransmitter Ach, possibly the CCK<sub>2</sub> receptor. The significant decrease of the caerulein 1.2-induced contraction produced by atropine eliminates the involvement of CCK<sub>1</sub> receptors as the major receptor-mediated pathway for the contractions.

(a)



(b)



**Figure 6.10:** Smooth muscle contraction response curve of caerulein 1.2 in the presence and absence of (a) mAChR antagonist atropine and (b) CCK<sub>2</sub> receptor antagonist YM022. The increase in contraction was reduced in the presence of both antagonists. Contractions are expressed as a percentage of the contraction in the presence of Ach (10<sup>-6</sup> M) (0.16 ± 0.02 g, n = 4) and are shown as mean ± SEM of three independent experiments done in duplicate. \* indicates P < 0.05.

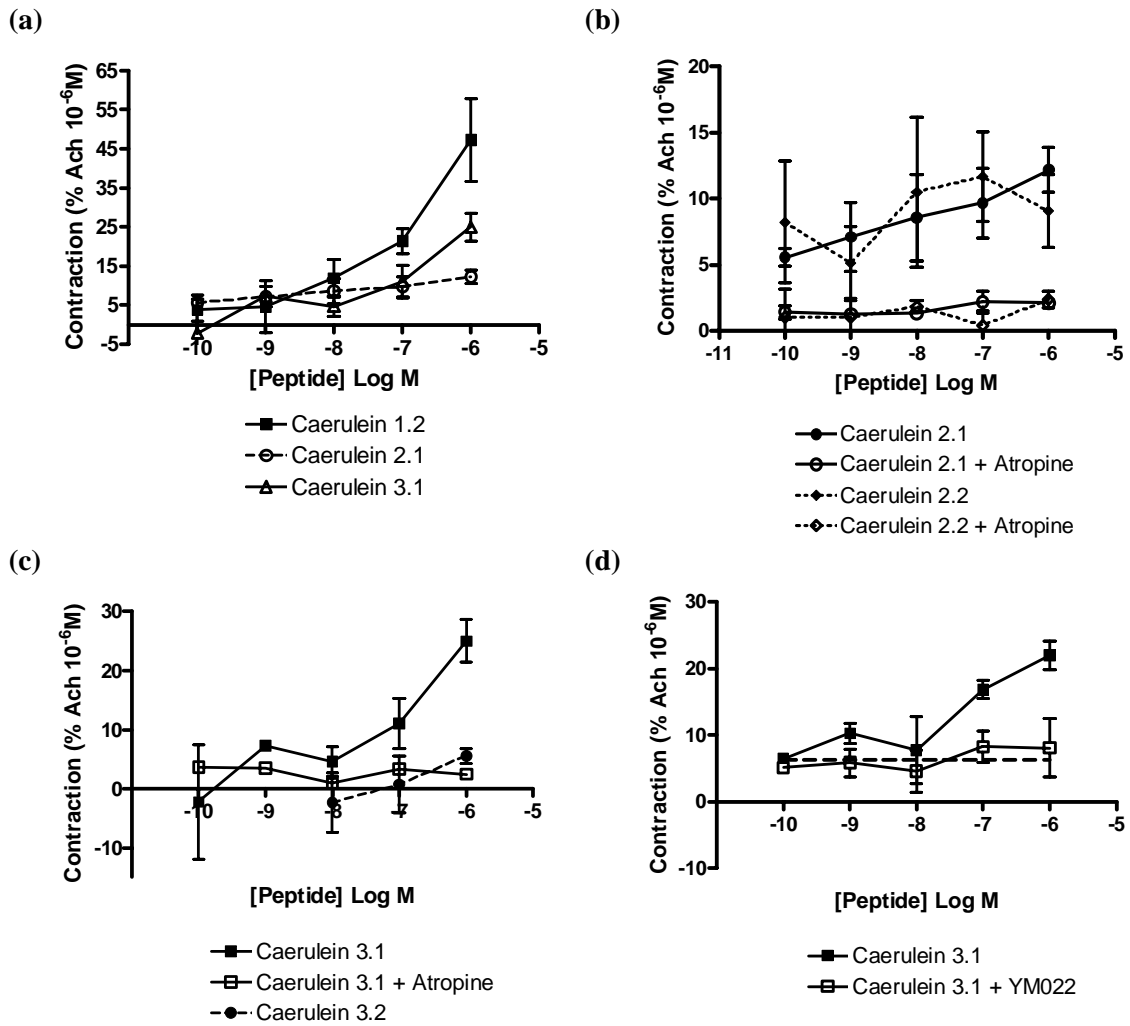
The results of caerulein 1.2-induced contractions in the presence of the selective CCK<sub>2</sub> receptor antagonist YM022 confirm the involvement of CCK<sub>2</sub> receptors. There is no concentration dependent increase in contraction with caerulein 1.2 in the presence of YM022 (Figure 6.10). This confirms that caerulein 1.2 is inducing its smooth muscle contraction via CCK<sub>2</sub> receptors and that the CCK<sub>1</sub> receptor does not display major involvement. The activity of caerulein 1.2 appears to mirror that of CCK-8-NS.

The smooth muscle contraction of the most active caerulein peptides is shown in Figure 6.11a. Caerulein 4.1 and 4.2 were not active in this assay. Caerulein 2.1 and 3.1 produced smooth muscle contraction of GPI from 10<sup>-10</sup> M and 10<sup>-9</sup> M respectively (Figure 6.11a). Caerulein 2.1 is less active than caerulein 3.1 and 1.2, with a maximum mean contraction of 12 % at 10<sup>-6</sup> M. Caerulein 3.1 produced a maximum mean contraction of 25 % at

$10^{-6}$  M. The activities of both caerulein 2.1 and 3.1 were blocked by atropine (Figure 6.11b and c). The maximum contraction of caerulein 2.1 in the presence of atropine was less than that of the peptide alone. Blockage of the caerulein-induced contractions produced by atropine eliminates the possibility of the involvement of  $CCK_1$  receptors in these contractions.

Caerulein 2.2 and 3.2 displayed remarkably different activities to their corresponding peptide pair. Caerulein 2.2 did not produce a concentration dependent increase in contraction, instead it produced a response that initially showed an increase in contraction at  $10^{-8}$  M but from  $10^{-7}$  M, a decrease in contraction was observed. The activity was blocked by atropine (Figure 6.11b). Caerulein 3.2 displayed very little activity and as the maximum contraction at  $10^{-6}$  M was 5 %, it is considered to be not active.

Due to the small contractions observed for caerulein 2 peptides (maximum contraction < 15 %), the activity of these peptides in the presence of the selective  $CCK_2$  receptor antagonist YM022 were not considered. Instead the activity of the caerulein 3.1 in the presence of YM022 was tested. There was no concentration dependent increase in the caerulein 3.1-induced contraction in the presence of YM022 (Figure 6.11d) and this is emphasised by the dotted line. The combination of the antagonist results here confirms that caerulein 3.1 is also acting via  $CCK_2$  receptors.

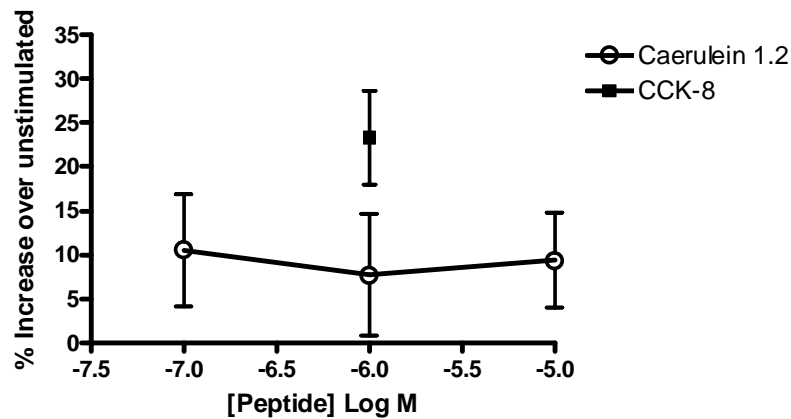


**Figure 6.11:** Smooth muscle contraction response curve of (a) caerulein 1.2, 2.1 and 3.1; (b) caerulein 2.1 and 2.2 in the presence and absence of mAChR antagonist atropine. This is an indirect assay of CCK<sub>2</sub> receptor activation, as CCK<sub>2</sub> agonists produce contraction by the release of Ach from nerve terminals. The contraction of both peptides was removed in the presence of atropine; (c) caerulein 3.1 and 3.2 in the presence and absence of atropine. The increase in contraction observed by caerulein 3.1 was removed in the presence of atropine; (d) caerulein 3.1 in the presence and absence of CCK<sub>2</sub> receptor antagonist YM022. The increase in contraction was reduced in the presence of YM022. The dotted line shows the minimum contraction of caerulein 3.1 in the absence of YM022. Contractions are expressed as a percentage of the contraction in the presence of Ach ( $10^{-6}$  M) ( $0.16 \pm 0.02$  g,  $n = 4$ ) and are shown as mean  $\pm$  SEM of three independent experiments done in duplicate. \* indicates  $P < 0.05$ .

### 6.2.3.2 Lymphocyte Proliferation Studies

The most active peptide of the caeruleins was chosen to determine its activity in lymphocyte proliferation of mouse splenocytes. Caerulein 1.2 produced an increase in the proliferation of lymphocytes from a concentration of  $10^{-7}$  M (Figure 6.12). The response was not concentration dependent, instead at all concentrations, an increase in lymphocyte

proliferation over the unstimulated control of approximately 10 % was observed. Concentrations below  $10^{-7}$  M were not used because of poor sensitivity of the Alamar Blue method at such low concentrations. Since lymphoid cells have been shown to possess CCK<sub>2</sub> receptors exclusively [450, 460, 483, 484], it is most likely that caerulein 1.2 is acting as a CCK<sub>2</sub> receptor agonist to effect proliferation of lymphocytes.



**Figure 6.12:** Concentration response curve for caerulein 1.2 for mouse splenocyte proliferation. The data is expressed as a percentage increase in cell proliferation over the unstimulated controls and shown as the mean  $\pm$  SEM of three independent measurements performed in quadruplicate. The response to the standard CCK-8 is shown for comparison.

### 6.2.3.3 Opioid Activity Studies

Caerulein 1.2 was tested for its activity against electrically stimulated GPI to ascertain its effect on opioid receptors. The GPI with its myenteric plexus intact was stimulated electrically to produce Ach-induced contractions of the smooth muscle (see Section 6.1.4). The electrically-stimulated contractions were inhibited in a concentration-dependent manner by the addition of atropine (data not shown). While caerulein 1.2 decreased the peak height under the test conditions used, it is suggested that physiological antagonism occurred, rather than an action at opioid receptors. Pre-treatment of the ileum segments with CCK<sub>1</sub> and CCK<sub>2</sub> antagonists derazepide and YM022, resulted in elimination of both basal tone and the fall in electrically-stimulated peak height. Thus, it can be concluded that the observed activity was merely physiological antagonism produced by an underlying smooth muscle contraction induced by caerulein 1.2 via CCK receptors.

## 6.2.4 Tryptophyllin Peptides

There are a number of tryptophyllin peptides that have been isolated from numerous amphibian species [10, 14] in which the biological activities are unknown. Preliminary activity testing on some of these peptides (Table 6.7) was undertaken in an attempt to ascertain some insight into the role of these tryptophyllins in the animal's host defence system. None of the peptides tested displayed any contractile activity of GPI smooth muscle (> 5 % Ach ( $10^{-6}$  M) contraction).

**Table 6.7:** The sequence and activity of tryptophyllin peptides isolated from *Litoria* species and their synthetic modifications.

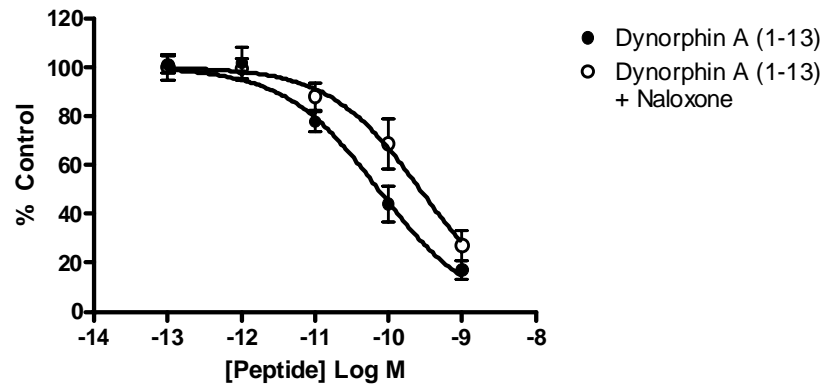
Tryptophyllin	Sequence	Species <sup>1</sup>	Activity <sup>2</sup>
L 1.2	FPWL-NH <sub>2</sub>	a, b	1
[Phe <sup>5</sup> ]L 1.3	pEFPWF-NH <sub>2</sub>	c	2
L 1.4	FPFPWP-NH <sub>2</sub>	a	2
[Leu <sup>6</sup> ]L 1.4	FPFPWL-NH <sub>2</sub>	c	2
L 3.1	FPWP-NH <sub>2</sub>	a, b	2
[Arg <sup>4</sup> ]L 3.1	FPWR-NH <sub>2</sub>	c	1
L 4.2	FLPWY-NH <sub>2</sub>	a	2

<sup>1</sup>Species: (a) *Litoria rubella* [46]; (b) *Litoria electrica* [39]; (c) synthetic modification.

<sup>2</sup>Activity: (1) opioid activity; (2) not active.

### 6.2.4.1 Opioid Activity Studies

Some tryptophyllins (Table 6.7) were tested for activity against electrically stimulated myenteric plexus-longitudinal muscle preparation of GPI. All activities are described as a percentage of the stimulated control contractions (basal contraction: 100 % control). Dynorphin A (1-13) was used as a peptide standard (Figure 6.13). Dynorphin A (1-13) resulted in a depression of the stimulated contraction at  $10^{-11}$  M, with an IC<sub>50</sub> value of  $7.2 \times 10^{-11}$  M. In the presence of naloxone the concentration response was significantly shifted by half a log unit to the right (IC<sub>50</sub>  $2.7 \times 10^{-10}$  M). This is consistent with dynorphin A (1-13) binding selectively to opioid receptors.

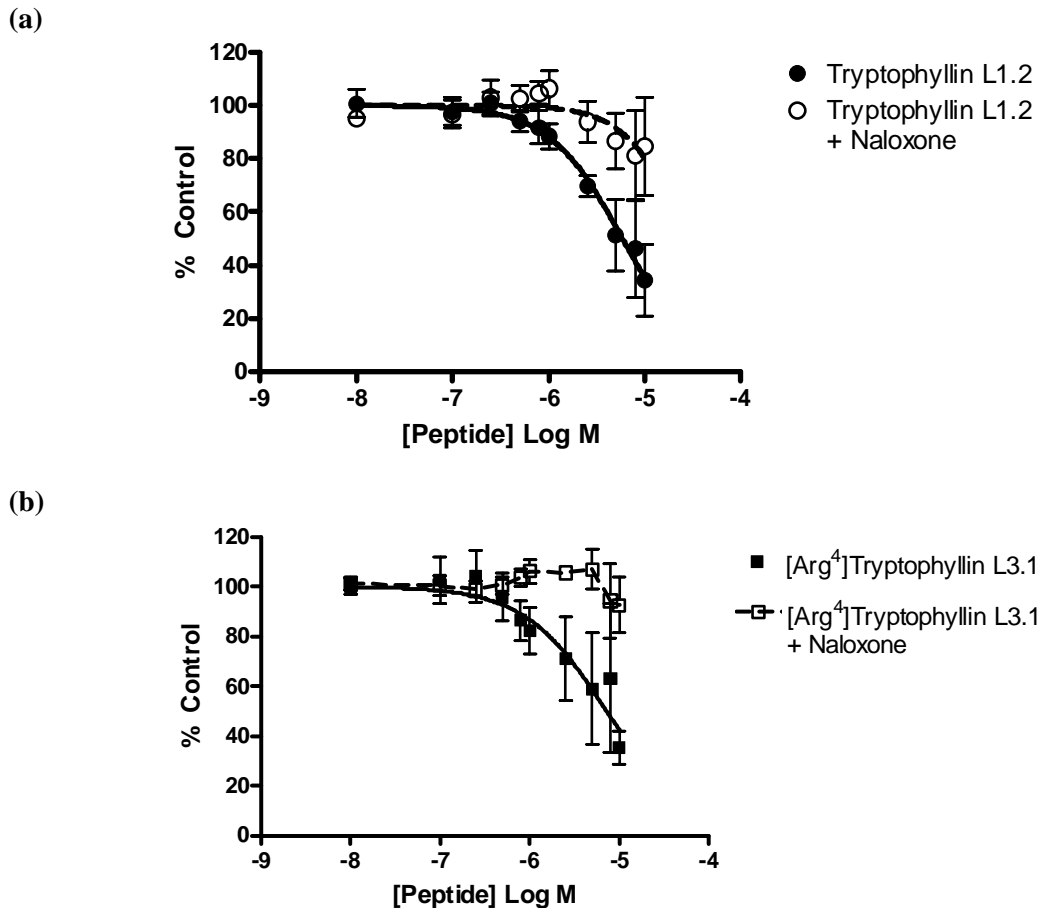


**Figure 6.13:** Inhibition of stimulated ileum contraction response curve of dynorphin A (1-13) in the presence and absence of naloxone. The response curve is significantly shifted in the presence of naloxone. The activity is expressed as a percentage of the stimulated ileum basal contraction of the control (100 %) with inhibition of contraction indicated by a decrease in % control. The data are shown as mean  $\pm$  SEM of four independent experiments done in duplicate.

Tryptophyllin L 1.2 produced a depression of the stimulated contraction of GPI from  $5 \times 10^{-7}$  M with an  $IC_{50}$  value of  $5.9 \times 10^{-6}$  M (Figure 6.14a). The tryptophyllin L 1.2-induced depression of contraction was significantly shifted by a log unit to the right in the presence of naloxone ( $IC_{50}$   $2.7 \times 10^{-5}$  M), suggesting that this peptide is acting through opioid receptors. Similarly,  $[Arg^4]$ tryptophyllin L 3.1 produced a depression of the stimulated contraction of GPI with an  $IC_{50}$  of  $7.2 \times 10^{-6}$  M (Figure 6.14b). This depression of contraction was significantly removed in the presence of naloxone. This suggests that, like tryptophyllin L 1.2,  $[Arg^4]$ tryptophyllin L 3.1 is acting through opioid receptors.

The apparent dissociation constant  $K_d$  for  $[Arg^4]$ tryptophyllin L 3.1 could not be determined as the concentration response curve in the presence of naloxone did not fall far enough for an  $IC_{50}$  value to be calculated. The  $K_d$  values (Table 6.8) indicate that naloxone is slightly more effective in blocking tryptophyllin L 1.2 receptor binding as it is dynorphin A (1-13) receptor binding.





**Figure 6.14:** Inhibition of stimulated ileum contraction response curve of (a) tryptophyllin L 1.2 and (b) [Arg<sup>4</sup>]tryptophyllin L 3.1 in the presence and absence of naloxone. The response curves of both peptides is significantly shifted in the presence of naloxone. The activity is expressed as a percentage of the stimulated ileum basal contraction of the control (100 %) with inhibition of contraction indicated by a decrease in % control. The data are shown as mean  $\pm$  SEM of four independent experiments done in duplicate.

**Table 6.8:** The effects of tryptophyllins and dynorphin A (1-13) in stimulated myenteric plexus-longitudinal GPI preparations.

Peptide	IC <sub>50</sub> (M)	SEM (log units)	Naloxone K <sub>d</sub>
Dynorphin A (1-13)	7.2 x 10 <sup>-11</sup>	0.10	6.9 $\pm$ 2.1 x 10 <sup>-8</sup> M
Tryptophyllin L 1.2	5.9 x 10 <sup>-6</sup>	0.06	2.4 $\pm$ 1.0 x 10 <sup>-8</sup> M
[Arg <sup>4</sup> ]Tryptophyllin L 3.1	7.2 x 10 <sup>-6</sup>	0.12	*

IC<sub>50</sub> values were obtained from assays spanning the 50 % inhibition response (Figure 6.13 and 6.14). Naloxone K<sub>d</sub> is the apparent dissociation constant of the antagonist, computed from the equation  $K_d = C/(DR-1)$  derived from the mass law for competitive antagonism, where C is the concentration of naloxone (1 x 10<sup>-7</sup> M) and DR is the dose ratio of the agonist (the ratio of IC<sub>50</sub> values in the presence and absence of the antagonist) [469].

\*Naloxone K<sub>d</sub> for [Arg<sup>4</sup>]tryptophyllin L 3.1 could not be determined as the concentration response curve in the presence of naloxone was blocked, thus no IC<sub>50</sub> value could be calculated.

## 6.2.5 Miscellaneous Peptides

Numerous small peptides have been isolated as the major components of the skin secretions from various amphibian species, however, the biological activities of many of these peptides is unknown [14]. Given the high concentration of these peptides in the skin secretions, it is likely that they have a defined role in the animal's host defence. Preliminary testing of a selection of these peptides (Table 6.9) was undertaken to provide some insight into their host defence roles. Many of these peptides were not active in any of the assays tested and their activities are still unknown.

**Table 6.9:** Sequence of miscellaneous peptides from hylid frogs and their activities.

Peptide	Sequence	Species <sup>1</sup>	Activity <sup>2</sup>
Fallaxidin 1.1	YFPIPI-NH <sub>2</sub>	a	1, 2
Fallaxidin 1.2	YFPIPF-NH <sub>2</sub>	a	1, 2
Fallaxidin 1.3	YHPF-NH <sub>2</sub>	a	1, 2
Unnamed	IVFFP-OH	b	1
Unnamed	IVFFP-NH <sub>2</sub>	b	1
Dentatidin 1.1	WSPFWD-NH <sub>2</sub>	c	1
Dentatidin 1.1mod	WSPFWD-OH	d	1
Dentatidin 1.2	WSPFWR-NH <sub>2</sub>	c	1
Peronein 1.1	pEPWLPFG-NH <sub>2</sub>	e	2, 3
Peronein 1.2	pEPWIPFV-NH <sub>2</sub>	e	1
Peronein 1.3	pEPWLPFV-NH <sub>2</sub>	e	1
Peronein 1.4	pETWLPFV-NH <sub>2</sub>	e	3
Unnamed	FLPFFP-OH	f	1
Unnamed	FLPFFP-NH <sub>2</sub>	f	1

<sup>1</sup>**Species:** (a) *Litoria fallax*; (b) *Litoria ewingi* [486]; (c) *Litoria dentata* [487]; (d) synthetic modification; (e) *Litoria peronii* [140]; (f) *Hyla arborea* [488].

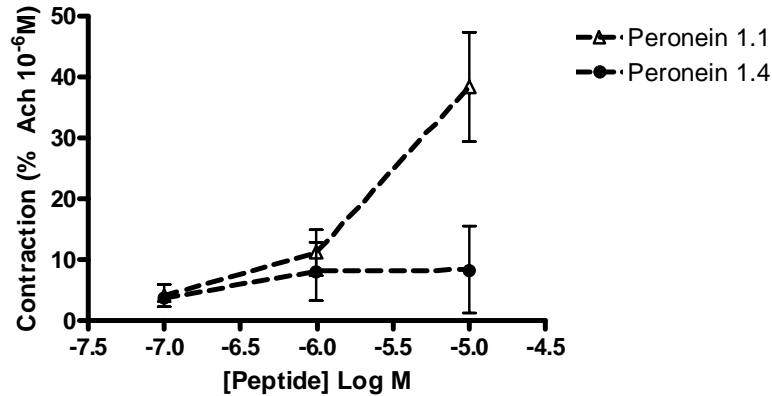
<sup>2</sup>**Activity:** (1) not active; (2) secondary smooth muscle contraction; (3) smooth muscle active.

### 6.2.5.1 Smooth Muscle Contraction Assays

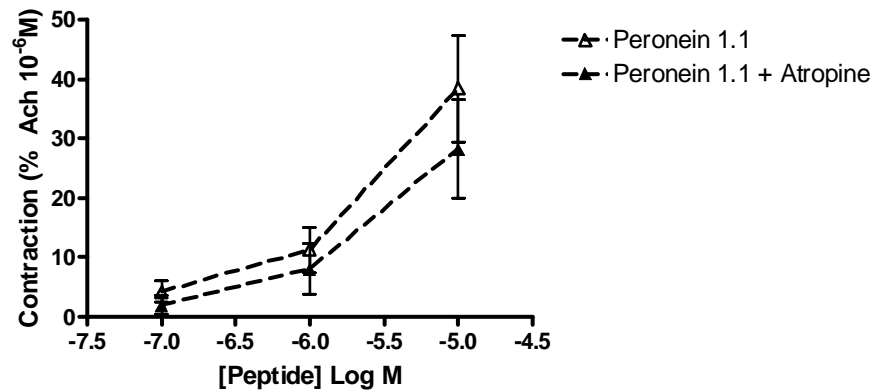
Peronein 1.1 and 1.4 were found to contract smooth muscle in GPI. Peronein 1.1 produced contractions of GPI from 10<sup>-6</sup> M, with a maximum mean contraction of approximately 40 % at 10<sup>-5</sup> M (Figure 6.15a). Peronein 1.4 produced weaker contractions of GPI from 10<sup>-6</sup> M and a maximum mean contraction of approximately 9 % (Figure 6.15a). Due to the small contractions observed for peronein 1.4, the activity of this peptide in the presence of

receptor antagonists was not tested. Instead, only peronein 1.1 was tested in the presence of atropine (Figure 6.15b) and no significant reduction in contraction was observed. This suggests that peronein 1.1 is not producing contraction of smooth muscle through mAChR or any other receptor that results in the release of Ach (eg. CCK<sub>2</sub> receptors).

(a)



(b)



**Figure 6.15:** Smooth muscle contraction response curve of (a) peronein 1.1 and 1.4 (b) peronein 1.1 in the presence and absence of mAChR antagonist atropine. The increase in contraction was not reduced in the presence of atropine. Contractions are expressed as a percentage of the contraction in the presence of Ach ( $10^{-6}$  M) ( $0.16 \pm 0.02$  g,  $n = 4$ ) and are shown as mean  $\pm$  SEM of three independent experiments done in duplicate. \* indicates  $P < 0.05$ .

Like the rothein 1 peptides, a large secondary contraction of the GPI was observed for the fallaxidin 1 peptides and peronein 1.1 when they were washed out of the organ bath. Similar to the rothein 1 peptides, these responses were not altered by atropine or Ach, suggesting that these peptides are not acting through the mAChR. Again no change in secondary contraction was seen in the presence of L-NNA (NOS inhibitor) or brethylum tosylate (inhibits release of noradrenaline from synaptic nerves), thus they are not acting through any receptors that result in the release of NO or noradrenaline.

## 6.3 Discussion

### 6.3.1 Disulfide Peptides

The host defence peptides profiles of the three *Crinia* species that have been studied are varied. *C. signifera* and *C. deserticola* both are well protected against predators, producing the disulfide peptide signiferin 1 in addition to several antimicrobial peptides and nNOS inhibitors [47, 479], whilst *C. riparia* produces a number of disulfide peptides including riparin 1.1 and 1.2 [44]. Signiferin 1 has been shown to contract smooth muscle at  $10^{-9}$  M and induce proliferation of lymphocytes at  $10^{-6}$  M. Riparin 1.1 and 1.2 lack smooth muscle activity, but act to proliferate lymphocytes by binding to CCK<sub>2</sub> receptors at  $10^{-7}$  M concentrations. It is interesting that these peptides do not display antimicrobial activity despite having sequence similarity to many antimicrobial disulfide peptides [14].

Signiferin 1 is suggested to act through CCK<sub>2</sub> receptors in both smooth muscle and lymphocytes. In smooth muscle the reduction of contraction following the addition of atropine (a mAChR antagonist) and YM022 (a CCK<sub>2</sub> antagonist) indicates that signiferin 1 is operating through CCK<sub>2</sub> receptors. Signiferin 1 also enhanced proliferation of lymphocytes through interactions with CCK<sub>2</sub> receptors. The smooth muscle and lymphocyte activities of signiferin 1 require the presence of both the disulfide link and the C-terminal Cys carboxyl group. This was confirmed experimentally as the synthetic modifications of signiferin 1; signiferin 1 amide, the reduced form of signiferin 1 and [Gly<sup>11</sup>]signiferin 1 (Table 6.2) shown no activity in smooth muscle or lymphocyte assays at concentrations below  $10^{-5}$  M.

Given that signiferin 1 and riparin 1.1 have similar sequences, the very different activities of the two peptides is unusual. It therefore must be asked: what structural features are responsible for the different activities? The solution structure of both peptides has been determined in membrane mimicking environments [376] and several significant differences are observed (Figure 6.16). Firstly the overall shape of the disulfide rings is varied resulting in different orientations of the Arg side chains. Arg side chains being long and flexible and have the capability to interact with the receptor at multiple directions, however although some disorder is observed in the N-terminus, there appears to be a consistent directional difference. Therefore, Arg occupies different regions of space and as

this residue is important for receptor binding, may contribute to the differences in activity. Secondly, the different orientation of the aromatic residues Tyr6 and Phe8 in the  $\beta$  turn of signiferin 1 and riparin 1.1 respectively may also be important. A number of aromatic residues (Tyr189, Tyr351 and Phe347) in CCK<sub>2</sub> receptors have been shown to be critical for binding and signal transduction [422]. Therefore, the entirely different projections of the peptide aromatic residues may alter the ability of these residues to establish  $\pi$ - $\pi$  interactions with the aromatic residues of the receptor and may contribute to the differences in the biological responses. As a consequence, these structural differences may cause the peptides to bind differently across the membrane-spanning domains of the receptor, thus exerting different biological responses with the same receptor in different tissues.

NOTE:

This figure is included on page 199 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 6.16:** Solution structures of (a) signiferin 1 and (b) riparin 1.1 as determined in aqueous TFE [376].

This finding that the cyclic peptides signiferin 1 and riparin 1.1 are selective agonists of CCK<sub>2</sub> receptors is not without precedent. The majority of previous studies in this area have been done with CCK peptide analogues. Initial observations showed that in aqueous solutions, CCK-8 displays some folding [489, 490]. This led to the synthesis of peptide analogues cyclised through amide bond formation between Asp1 and Lys4 side chains to give compounds that display high potency and selective CCK<sub>2</sub> receptor agonist activity [491, 492]. More recent NMR results (using aqueous solutions of micelles) indicate that the binding of gastrin and other CCK peptides to CCK<sub>2</sub> receptors may involve a  $\beta$  turn towards the C-terminal end of the peptides [493].

### 6.3.2 Rothein 1 Peptides

In the winter months, *L. rothii* produces caerulein 1.2 and rothein 1 as the major components in its skin secretions, whilst in the summer months the animal secretes the active neuropeptide caerulein [45]. Caerulein contracts smooth muscle at nanomolar concentrations, enhances blood circulation, and modifies satiety, sedation and thermoregulation. It is also an analgesic agent several thousand times more active than morphine [10]. Rothein 1 was thought to act as a neuropeptide as it lacks antimicrobial and nNOS inhibitory activities. It has an unusual structure; dissimilar to other peptides isolated from Australian species. Rothein 1 does not contract smooth muscle, but it does effect the proliferation of mouse splenocytes. Caerulein 1.2 contracts smooth muscle and induces proliferation of lymphocytes (Section 6.3.3). It is likely that the animal's production of the rothein 1 and caerulein 1.2 peptide combination in the winter months displays a similar spectrum of activity as that of caerulein, however at a much lower potency. Furthermore, due to the weak activity of rothein 1, it is likely that the peptide has another role in the host defence that has not yet been determined.

Due to its unusual primary structure, a number of synthetic modifications of rothein 1 were prepared in order to see what structural features favoured improved activity in smooth muscle and splenocyte proliferation. Ala was used to replace the N- and C-terminal residues and all of the hydrophilic residues sequentially. In addition, in two peptide modifications, the hydrophobic Ile9 was replaced by a hydrophilic Thr residue.

In smooth muscle assays, the replacement of Ile9 with Thr did not improve the smooth muscle contraction activity of rothein 1. Similarly, the same results were observed with the replacement of Ser3 and Phe11 with Ala. Substitution of Ser8 with Ala created an increase in activity, while the replacement of Ser1 or Glu7 with Ala produced the most active synthetic modifications, suggesting a more hydrophobic residue at these position improved receptor recognition. Furthermore, when Glu7 was replaced with Ala, this modified peptide was only active when Ile9 was present and not the Thr substitution, possibly highlighting the requirement for the long, hydrophobic side chain for receptor binding and smooth muscle contraction.

For splenocyte proliferation, the replacement of any of Glu7, Ser8 or Phe11 with Ala removes splenocyte activity altogether. Very little difference in activity was seen when Ile9 was replaced by Thr (as in rothein 1.1), suggesting that the hydrophobic residue at this position was not essential for receptor recognition. Replacement of either Ser1 or Ser3 with Ala resulted in improved activity, suggesting a more hydrophobic N-terminal results in improved receptor recognition. It implies that, with the exception of Ser1 and Ser3, all other hydrophilic residues and the aromatic Phe11 residue are necessary for splenocyte activity.

### 6.3.3 Caerulein Peptides

Caerulein and a number of its related peptides (Table 6.6) were identified as major components in the skin secretion of *L. citropa* [402]. Caerulein is not an unusual component of frog skin secretion, with it present in most frogs of the genus *Litoria* [27]. As previously discussed (Section 6.1.1.3), caerulein has a broad spectrum of activities in the CNS, with potencies analogous to CCK [10]. Caerulein binds to CCK receptors to exert its activities. Caerulein may contract smooth muscle directly by binding to CCK<sub>1</sub> receptors or indirectly by binding to CCK<sub>2</sub> receptors, stimulating the release of Ach from cholinergic nerves, which subsequently contracts smooth muscle via mAChR. As the activity of caerulein is comparable to CCK-8 [10] and a peptide sample of caerulein was unavailable, it is not unreasonable to compare the activities of its related peptides to that of CCK-8 as a means of comparison to caerulein.

Caerulein 1.2 is produced by not only *L. citropa*, but also by *L. rothii* as an analogue of caerulein in the winter months. Caerulein 1.2 differs from caerulein by the substitution of Met8 with Phe. CCK-8 produced a contraction of smooth muscle of  $60 \pm 11$  % (Ach  $10^{-6}$  M) at  $10^{-8}$  M, whilst at the same concentration caerulein 1.2 produced a contraction of  $13 \pm 6$  %. The maximum contraction of caerulein 1.2 occurred at  $10^{-6}$  M, thus a two log unit shift in activity was observed between the two peptides. The activity of caerulein 1.2 appears to resemble that of CCK-8-NS. Antagonist studies in the presence of atropine and YM022 confirm that caerulein 1.2 produces the majority of its contractile response through CCK<sub>2</sub> receptors. In addition, caerulein 1.2 was shown to enhance proliferation of lymphocytes, but to a lesser extent than CCK-8. Caerulein 1.2 must display additional

activity, as it seems unusual that these animals would substitute a potent peptide for a less active analogue.

It appears that although the substitution of Met8 with Phe has reduced the potency of the smooth muscle activity, this substitution has resulted in almost exclusive CCK<sub>2</sub> receptor selectivity. This is not surprising as a number of aromatic residues (Tyr189, Tyr351 and Phe347) (Figure 6.17b) have been shown to be critical for CCK<sub>2</sub> receptor binding and signal transduction [422]. Thus, by providing an additional aromatic side chain available for  $\pi$ - $\pi$  interactions, the interaction between the peptide and the receptor is strengthened. It has been shown that Met6 of CCK-8 (corresponding to Met8 of caerulein) interacts with a hydrophobic pocket of CCK<sub>1</sub> receptor (Met121, Val125 and Ile352) (Figure 6.17a) [448, 494]. It is likely that substitution of this residue with Phe8 (in caerulein 1.2) weakens the interaction of the peptide with this hydrophobic pocket, reducing the CCK<sub>1</sub> receptor binding affinity.

NOTE:

This figure is included on page 202 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 6.17:** The 2D representation of CCK-9 interacting with the binding site in the (a) CCK<sub>1</sub> and (b) CCK<sub>2</sub> receptor. Residues in the CCK<sub>1</sub> and CCK<sub>2</sub> receptors that have been shown to be important for interactions with CCK-9 are shown in pink, whilst CCK-9 is illustrated in black [494].

In the smooth muscle assays, the activities of caerulein 2 peptides decreased significantly with respect to caerulein and caerulein 1.2. The addition of Ala at position 7 and the



substitution of Trp for His8 reduced the affinity of the peptide for its receptor (most likely CCK<sub>2</sub> receptor). Similarly, the replacement of Ala7 to Ser in caerulein 4 peptides eliminated the smooth muscle activity. It appears that the Trp residue is important for receptor recognition as demonstrated by the activity of caerulein 3.1, as this peptide had improved activity relative to the caerulein 2 and 4 peptides. However, the activity was approximately half that of caerulein 1.2. Caerulein 3.2 was inactive. It is important to note that Trp residues are proposed to interact with the membrane interfacial region and may act to anchor the peptide at the membrane and improve receptor binding<sup>[6\*]</sup>. Furthermore, the insertion of a Gly at position 5 suggests that the length of the chain between the sulfated Tyr4 and the C-terminal amide is important for receptor binding and activation. This is consistent with results from previously studies with CCK analogues and CCK<sub>1</sub> receptors [448]. These caerulein analogues must have some other activity as they comprise the host defence of *L. citropa* and it is energetically unfavourable for anurans to produce secretory peptides that are inactive.

In addition, caerulein 1.2 was shown to cause a decrease in the stimulated contractions of GPI, suggesting it may act to produce an effect similar to opioid agonists. However, pre-treatment of the ileum tissue with CCK<sub>1</sub> and CCK<sub>2</sub> antagonists derazepide and YM022, resulted in elimination of this activity, thus it can be concluded that the observed activity was merely physiological antagonism produced by an underlining smooth muscle contraction induced by caerulein 1.2 and not opioid activity. This is not surprising as the analgesic activity of caerulein is induced in an opioid-independent method, as caerulein has no affinity for opioid receptors [495]. Instead it is believed to be through a CCK receptor mediated pathway [1]. Thus, if like caerulein, caerulein 1.2 does display analgesic activity it will be through a CCK receptor mediated pathway.

### 6.3.4 Tryptophyllins

The presence of opioid receptors on the myenteric plexus of GPI longitudinal muscle has been well established [469, 470, 473]. In this tissue preparation, determination of potencies is not complicated by degradation because any loss of twitch inhibitions can be observed directly. In addition, it has been shown that the relative potencies of ligands in these *in vitro* assays correlate to the potencies of the ligands *in vivo* [496]. Agonists of the  $\mu$  and  $\kappa$

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<sup>6\*</sup> Killian, J.A. and von Heijne, G. (2000) How proteins adapt to a membrane-water interface, *Trends Biochem. Sci.* 25, 429-434

opioid receptors will depress the firing of myenteric neurons, inhibit the release of Ach and reduce nerve-mediated cholinergic contractions of longitudinal muscle [473, 477]. In these experiments, dynorphin A (1-13) depressed the stimulated contractions at an  $IC_{50}$  value of  $7.2 \times 10^{-11}$  M. This is comparable to previous experiments ( $IC_{50}$  values  $6.3 \times 10^{-10}$  M [468] and  $3.4 \times 10^{-10}$  M [497]). This effect is reversed and blocked by the opioid antagonist naloxone. Although dynorphin A (1-13) is a selective  $\kappa$  opioid receptor agonist [478], it has been shown to also bind to both  $\mu$  and  $\delta$  opioid receptors with lower affinity [433]. Thus, it is not surprising that in the presence of naloxone the concentration response was shifted to the right. Naloxone has been reported to be more selective for  $\mu$  opioid receptors in GPI [474], and display a 10-fold preference for  $\mu$  over  $\kappa$  receptors [498].

Dynorphin A (1-13) is extraordinarily potent: approximately  $10^5$  times more potent than both active tryptophyllins. Naloxone is slightly more efficient (by half a log unit) at blocking the receptor binding of tryptophyllin L 1.2 and dynorphin A (1-13). In addition, naloxone was able to almost eliminate the response of the tryptophyllin peptides. This suggests that the tryptophyllins are most likely more selective for the  $\mu$  receptor subtype, however, tests in rat vas deferens would be necessary to ascertain if the tryptophyllins have affinity for  $\delta$  receptors. Tryptophyllin L 1.2 and [Arg<sup>4</sup>]tryptophyllin L 3.1 show some sequence similarities to endomorphins, however, the tryptophyllin peptides are approximately 200 times less potent [499]. The animal overcomes this weak activity by secreting large quantities of these peptides. It is likely that these tryptophyllin peptides have a role in the host defence similar to the biological effect exerted by endomorphins, but more testing would be needed to confirm this.

It is evident by the substitution of the C-terminal Pro residue in tryptophyllin L 3.1 with the aliphatic side chain of Leu in tryptophyllin L 1.2 and Arg in [Arg<sup>4</sup>]tryptophyllin L 3.1 that a long side chain is required in these peptides for opioid receptor recognition and binding. As the relative potencies of the tryptophyllin L 1.2 and [Arg<sup>4</sup>]tryptophyllin L 3.1 were approximately equal, it is unclear whether a charged aliphatic side chain improves receptor binding a great deal. The long side chain may assist the peptide in binding to the  $\mu$  opioid receptor active site by interacting with residues on the side of the binding pocket. This would enable the C-terminal of the peptide to interact with aromatic residues (His297, Tyr326 and Trp318) that are important for binding [500, 501] and position the Trp3

residue of the peptide in a favourable position for interaction with Asp residues of the binding pocket.

### 6.3.5 Peronein 1 Peptides

*L. peronii* produces seasonal secretions; with antimicrobial caerin peptides in the winter, and caerulein and a series of peronein peptides in both seasons. Of the four peronein peptides tested in our assays, only peronein 1.1 and 1.4 showed preliminary activity. Peronein 1.1 produced a two fold response in smooth muscle assays; a primary contraction from  $10^{-6}$  M, with a strong contraction at  $10^{-5}$  M, and a secondary smooth muscle contraction (Section 6.3.6). Peronein 1.4 is less active. This suggests that the C-terminal Gly residue is important for receptor binding and recognition. The activity of peronein 1.1 showed no significant reduction in the presence of atropine, indicating that the peptide is not contracting smooth muscle via mAChR or receptors that result in the release of Ach from cholinergic nerves. It is likely that the peronein peptides not active in our assays have some other activity that assists the defence of *L. peronii* against predators and pathogens. More investigation is required to ascertain the exact roles of these peptides.

### 6.3.6 Secondary Smooth Muscle Contraction

The rothein 1 peptides, fallaxidin 1 peptides and peronein 1.1 produced an unusual secondary contraction of GPI upon being washed out of the organ bath. This contraction has not been observed for CCK-8, CCK-8-NS or any other peptides previously studied. As the observed response reflects the net effect mediated by various receptors, it is likely that these peptides are acting via a receptor pathway that results in relaxation of smooth muscle, in addition to their contractile response. A similar response has been observed for cannabinoid systems [502, 503].

Smooth muscle contraction is mediated by an increase in intracellular  $\text{Ca}^{2+}$  concentrations, permitting the interaction between the contractile proteins, actin and myosin, whilst relaxation results from a decrease in intracellular  $\text{Ca}^{2+}$  concentration [504]. The two intracellular second messengers involved in smooth muscle relaxation are cAMP and cGMP, which are produced after the stimulation of a number of receptors. In general, cAMP-dependent smooth muscle relaxation is stimulated by  $\beta$  adrenoceptors, whilst

cGMP-mediated relaxation is activated by NO and natriuretic peptides [504]. The NOS inhibitor L-NNA did not alter the secondary response, thus, it can be concluded that the secondary response was not mediated by any receptors that result in the release of NO or its activation of cGMP, such as B<sub>1</sub> receptors [267]. The addition of brethylum tosylate did not result in any change in the response, indicating that noradrenaline and  $\beta$  adrenoceptors were not involved [268], thus it is unlikely that the relaxation is cAMP-mediated.

The observed secondary response could also be mediated by the activation of an excitatory neuron and an inhibitory neuron simultaneously. It has been reported that the myenteric plexus of GPI contains both excitatory and inhibitory nerves. The excitatory nerves contain Ach and tachykinins such as substance P and neurokinin A, while the inhibitory nerves contain VIP, peptide histidine methionine, peptide histidine isoleucine [505] and ATP [502]. As no change in the secondary contraction was observed in the presence of atropine, the response was not mediated by the excitatory cholinergic nerve release of Ach. To test this hypothesis further, the effects of excitatory and inhibitory neurotransmitters antagonists could be studied. The results here are preliminary and at present, it is unclear what role this activity would have in the animal's host defence.

## 6.4 Experimental

All experimental procedures were approved by The University of Adelaide Animal Ethics Committee.

Ach, atropine, CCK-8, N<sup>ω</sup>-nitro-L-arginine (L-NNA) and porcine dynorphin A (1-13) were purchased from Sigma-Aldrich (Sydney, New South Wales, Australia). CCK-8-NS, YM022 ((*R*)-*N*-[2,3-dihydro-1-[2-(2-methylphenyl)-2-oxoethyl]-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-*N'*-(3-methylphenyl)-urea) and derazepide (N-[(3*S*)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-1*H*-indole-2-carboxamide) were bought from Tocris Bioscience (Ellisville, Missouri, USA). Alamar Blue was purchased from Astral Scientific (Caringbar, New South Wales, Australia).

All peptides used in experimental procedures were commercially synthesised with L-amino acids using the standard N- $\alpha$ -Foc method, by either Mimotopes (Clayton, Victoria, Australia) or GenScript Corp (Piscataway, New Jersey, USA) (for full details refer to Maeji *et al.*) [279]. The caerulein peptides were provided by Dr. P. Boontheung (Department of Chemistry, The University of Adelaide).

### 6.4.1 Smooth Muscle Contraction Assay

Male guinea pigs (~ 300 g) were used in these experiments. Immediately prior to the experiment, the guinea pigs were killed by stunning and subsequent decapitation. The ileum was extracted, mesenteric tissue was removed and the tissue cleaned by rinsing with physiological salt solution (Kreb's solution; comprising of in mM: KCl 2.7, CaCl<sub>2</sub> 1.0, NaHCO<sub>3</sub> 13.0, NaH<sub>2</sub>PO<sub>4</sub> 3.2, NaCl 137, glucose 5.5; pH 7.4). 2 cm segments were suspended in 10 mL organ baths containing Kreb's solution and gassed with 95 % O<sub>2</sub> and 5 % CO<sub>2</sub>. The ileum segments were connected to an isometric force-displacement transducer and a tissue holder. The tension was recorded using MACLAB (version 3.0). The Kreb's solution was replaced several times to ensure that the ileum segments were washed thoroughly, and allowed to equilibrate for a period of 30 min under a resting tension of 2 g. Solution supply reservoirs and organ baths were maintained at 37 °C and gassed with O<sub>2</sub>/CO<sub>2</sub> as described above.

Following the equilibration time, the bath solution was replaced several times until a stable tension baseline was reached. The tension was readjusted to 2 g. Ach (10<sup>-8</sup> – 10<sup>-6</sup> M) was

added to each organ bath to constrict the ileum tissue. This was washed out by replacing the Krebs's solution several times. A second addition of Ach ( $10^{-6}$  M) was added to ensure that the response was stable. The Ach was washed out and ileum tissue left for 5 min until a stable baseline was achieved. A cumulative concentration response curve to CCK-8 ( $10^{-10}$  –  $10^{-8}$  M) was then completed. Following a wash, the tissue was again left for 5 min until a stable baseline was achieved. The cumulative concentration response curve was repeated for CCK-8-NS ( $10^{-9}$  –  $10^{-7}$  M) or peptide samples ( $10^{-10}$  –  $10^{-5}$  M). In several experiments, following the wash, ileum tissues were pretreated with atropine ( $3 \times 10^{-7}$  M), YM022 ( $1 \times 10^{-6}$  M) or L-NNA ( $2 \times 10^{-4}$  M) and allowed to equilibrate for 15 min until a stable baseline was reached. Ach, CCK-8, CCK-8-NS and peptide samples were reapplied as outlined above.

To investigate the nature of the secondary contraction response observed for rothein 1, fallaxidin 1 peptides and peronein 1.1, following the wash, the ileum was pretreated with atropine ( $3 \times 10^{-7}$  M), L-NNA ( $2 \times 10^{-4}$  M) and brethylum tosylate ( $2 \times 10^{-5}$  M) and allowed to equilibrate for 15 min until a stable baseline was reached. Like Ach, KCl (2 M) was used as an internal standard to ensure the response was stable. The KCl was washed out, atropine and brethylum tosylate reapplied, and the ileum tissue left for 5 min until a stable baseline was once again achieved. The peptide samples were reapplied as outlined above.

To compare the potencies of different peptides and to measure the changes in potency produced by receptor antagonists, the contractions of the ileum were expressed as a percentage of Ach ( $10^{-6}$  M) contraction. Data are expressed as mean  $\pm$  SEM. Differences between data sets were evaluated by performing analysis of variance (ANOVA) followed by Dunnett's test. A level of  $P < 0.05$  was considered to be significant.

### 6.4.2 Lymphocyte Proliferation Studies

Male Balb/C mice (30 g) aged 6 – 8 weeks were used. Lymphocytes<sup>4</sup> were prepared as previously described [506] with minor modifications. Aseptic methods were used during lymphocyte preparation. The mice were killed by cervical dislocation, immediately followed by extraction of the spleen. A single-cell suspension was prepared from the

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<sup>4</sup> A reviewer has proposed the use of lymphoid cell lines that are enriched for expression of CCK receptors to provide more sensitive data. CCK-enriched cell lines were not available for testing. Appropriate tissue cell lines have been extensively investigated, such as Jurkat cell lines (unpublished observations), and found to be more insensitive than the methodology described here.

spleen by massaging and washing it through a nylon mesh into RPMI 1640 (Hepes modification, 0.3 mg.mL<sup>-1</sup> of L-glutamine and 5 mL of penicillin/streptomycin solution per L) (15 mL). The cell suspension was centrifuged (5 min, 4 °C, 100 g), the supernatant discarded and the cells resuspended in 1 mL of media. Ice-cold lysis buffer (10 mL) (1 mL of 20.56 g.mL<sup>-1</sup> tris base (pH 7.65), 9 mL of 0.83 % aqueous NH<sub>4</sub>Cl, mixed prior to addition to cells) was added, the suspension placed on ice for 4 min, centrifuged (5 min, 100 g) and the supernatant discarded. The cells were resuspended in media (10 mL), centrifuged (5 min, 100 g), the supernatant removed and resuspended in enriched RPMI 1640 (RPMI 1640 enriched with 10 % fetal bovine serum) (5 mL).

The number of viable lymphocytes was counted using trypan blue and a haemocytometer. Cells were diluted in enriched media to 1 x 10<sup>6</sup> cells.mL<sup>-1</sup> and 100 µL of this diluted suspension was added to each well of 96 multiwell plates (TTP, Zurich, Switzerland) to give a final volume of 200 µL and a final diluted cell count of 50,000 cells per well. 10 µL of RPMI 1640 medium containing the test sample (CCK-8, CCK-8-NS or peptide samples) was added to the plates to obtain a final concentration of peptide of 10<sup>-7</sup> – 10<sup>-5</sup> M.

The plates were incubated (37 °C, 5 % CO<sub>2</sub>) in a humidified incubator (Thermoline, Sydney, New South Wales, Australia) for 24 hrs<sup>5</sup>. 25 µL of mitochondrial activity indicator dye Alamar Blue [457] was then added to give a final concentration of 2.5 µg.mL<sup>-1</sup> and the plates were incubated for an additional 4 hrs in the above conditions. 175 µL aliquots were placed in each well of a white 96 well plate and the fluorescence measured in a Polestar Galaxy (BMG Labtechnologies, Durham, NC, USA) fluorescent plate reader (excitation 544 nm, emission 590 nm).

To compare the potencies of different peptides, the response was presented as a percentage increase over the unstimulated control. Data are expressed as mean ± SEM. Differences between data sets were evaluated by performing analysis of variance (ANOVA) followed by Dunnett's test. A level of P < 0.05 was considered to be significant.

### 6.4.3 Opioid Activity Studies

Male guinea pigs (~ 300 g) were used in these experiments. Immediately prior to the experiment, the guinea pigs were killed by stunning and subsequent decapitation. The

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<sup>5</sup> This incubation time is standard methodology [457, 506].

ileum was extracted approximately 10 cm from the ileo-caecal junction and strips of longitudinal muscle with attached myenteric plexus were removed and cleaned by rinsing with physiological salt solution (Kreb's solution). The tissue segments were prepared as per the smooth muscle contraction assay described in Section 6.4.1.

Following the 30 min equilibration time, the bath solution was replaced several times until a stable baseline tension was obtained. The tension was readjusted to 2 g. Ach ( $10^{-8}$  –  $10^{-6}$  M) was added to each organ bath to constrict the ileum tissue. This was washed out by replacing the Kreb's solution several times. To electrically stimulate neurons in the myenteric plexus, the tissue preparation was passed between a pair of platinum electrodes, which were connected to the output of a Grass stimulator (West Warwick, USA). The tissue was stimulated at 60 V, 0.1 Hz with pulses of 2 ms duration. L-NNA ( $2 \times 10^{-4}$  M) was added and the tissue preparation equilibrated for a further 15 min until a stable contraction response was achieved. In each tissue preparation the concentration response curve for dynorphin A (1-13) ( $10^{-12}$  –  $10^{-10}$  M) were constructed using serial application. Following a wash, the L-NNA ( $2 \times 10^{-4}$  M) was reapplied and the tissue was again left for 5 min until a stable contraction was achieved. The cumulative concentration response curve was repeated for the peptide samples ( $10^{-10}$  –  $10^{-5}$  M). In several experiments, following the wash, ileum tissues were pretreated with naloxone ( $1 \times 10^{-7}$  M) in addition to L-NNA ( $2 \times 10^{-4}$  M) and allowed to equilibrate for 15 min until a stable contraction was reached. Dynorphin A (1-13) and the peptides samples were reapplied as outlined above.

To compare the potencies of different peptides and to measure the changes in potency produced by naloxone, the concentrations of agonist which decrease the electrically stimulated contractions by 50 % ( $IC_{50}$ ) were determined. The apparent dissociation constant  $K_d$  of naloxone was also calculated for each peptide using the equation  $K_d = C/(DR-1)$  derived from the mass law for competitive antagonism, where C is the concentration of naloxone ( $1 \times 10^{-7}$  M) and DR is the dose ratio of the agonist (the ratio of  $IC_{50}$  values in the presence and absence of the antagonist) [469]. Differences between data sets were evaluated by performing ANOVA, followed by Dunnett's test. A level of  $P < 0.05$  was considered significant.



# Chapter 7 Cloning of Precursor cDNAs From Australian Amphibians

## 7.1 Introduction

### 7.1.1 Amphibian Precursors

The skin secretions of amphibians contains an array of biologically active material including peptides, proteins, biogenic amines and alkaloids that comprise the animal's primary defence system (Section 1.1) [11]. Traditionally, the components were isolated from dried skin extractions, however, due to the declining amphibian numbers, the skin secretions are now collected from the skin granular glands using non-invasive methods including pharmacological stimulation or transdermal electrical stimulation [53, 83] (Section 1.3). As a result, many peptides have been identified due to their abundance in the secretion, allowing for their isolation and sequencing, followed by the discovery of their biological activity.

In the last two decades, the application of newly developed screening methods has allowed the analysis of the cDNA sequences of the genes that code for the secretory peptides. As a consequence, the cDNA clones have predicted the occurrence of some peptides prior to their actual isolation [507-509]. For example, deltorphins were first identified as putative peptides encoded within the dermorphin cDNA [508]. Likewise, the antimicrobial peptidyl-glycine-leucine carboxamide (PGLa) was identified in the open-reading frame of preprocaerulein [507].

Amphibian skin secretions are known to contain a variety of biologically active peptides, some of which share common structural features with endogenous vertebrate regulatory peptides, whilst other display broad spectrum antimicrobial, antifungal and antitumor activity and illustrate diverse primary structures. Despite the obvious differences in peptide primary structure, the cDNA clones isolated from various amphibians within distinct genera worldwide have indicated that there is some homology among the putative signal peptides and the first three residues in the acidic spacer region [25]. This suggests that one

exon encoded the signal peptide for a number of unrelated peptide precursors and was present in the evolution of the amphibian. This will be discussed in more detail in Section 7.1.4.

Recently, the precursor characteristics of antimicrobial peptides have been extensively investigated with hope for possible therapeutic applications, as the peptides display broad spectrum activity, act rapidly and do not display the same resistances as current antibiotics [11]. Amphibians from different families, genera and species produce a distinct set of antimicrobial peptides with varying chain length, charge, hydrophobicity and biological spectrum of action. Generally, the precursors code for a single copy of the mature antimicrobial peptide at the C-terminus of the sequence [250]. Interestingly, the precursors of antimicrobial peptides have displayed prepro sequences at the N-terminal of approximately 50 residues, which are highly conserved among species despite significant diversity in the C-terminal sequences encoding for the antimicrobial peptides (Table 7.1) [25, 510]. The conserved N-terminal consists of a 22 residue signal peptide followed by an acidic spacer of 16 to 25 residues that concludes in the prohormone processing site -Lys-Arg- [250]. It has been suggested that the conserved region may be important in the biology of the expressing cell [25].

Some precursors encode for more than one biologically active peptide, with some examples of precursors encoding for as many as eight different bioactive peptides [511]. Many of these peptides may act together to coordinate complex behavioural responses. Generally it has been shown that these precursors encode for: (i) duplications of an identical peptide sequence; (ii) related peptide sequences; and (iii) peptides with different biological functions; all separated by acidic spacer domains [511]. The precursors that encode tandem repeats of individual peptides produce peptides that act as neuropeptides. For example, preprocaerulein encodes for multiple caerulein peptides separated by conserved acidic spacer regions [508]. Similarly, multiple copies of the opioid peptides dermorphin and deltophin are produced from their corresponding precursors [407, 512].

The structural information encoded within precursors directs the nature and extent of proteolytic processing to produce the biologically active mature peptide. Processing can include proteolytic cleavages, hydroxylation, amidation, pGlu formation and glycosylation [513].

**Table 7.1:** Selected translated sequences isolated from hydrid and ranid amphibians.

Name*	Signal Peptide	Acidic Spacer	Peptide
Aurein 2.2	MAFLKKSFLVLVFLGLVSLSLIC	EKEKRQNEEDEDENEAAANHEEGSEEKR	GLFDIVKKVVGALGSLG
Aurein 2.3	MAFLKKSFLVLVFLGLVSLSLIC	EKEKRQNGEDEDENEAAANHEEGSEEKR	GLFDIVKKVVGAIGSLG
Aurein 2.5	MAFLKKSFLVLVFLGLVSLSLIC	EKEKRQNEEDEDENEAAANHEEGSEEKR	GLFDIVKKVVGAFGSLG
Aurein 3.1	MAFLKKSFLVLVFLGLVSLSLIC	EKEKRQNEEDEDENEAAANHEEGSEEKR	GLFDIVKKIAGHIAGSIG
Aurein 5.3	MAFLKKSFLVLVFLGLVSLSLIC	EQEKRQEENQEEDEEENEAASEEKR	GLMSSIGKALGGLIVNVLPKPTPAS
Brevinin-2Ef	MFTMKKSLLLIFFLGTISLSLC	QEERNADDDDGEMTEEEKR	GIMDTLKNLAKTAGKGALQSLVKMASCKLSGQC
Brevinin-1E	MFTLKKSMLLLFFLGTINLSLC	EEERDADEEERRDNPDESEVEVEKR	FLPLLAGLAANFLPKIFCKITRKC
Brevinin-2Ta	MFTMKKSLLLFFLGTISLSLC	QEERNADEDDGEMTEEEKR	GILDTLKNLAKTAGKGILKSLVNTASCKLSGQC
Brevinin-2Tb	MFTMKKSLLLFFLGTISLSLC	QEERNADEDDGEMTEEEKR	GILDTLKHLAKTAGKGALQSLLNHASCKLSGQC
Caerin 1.1	MASLKKSLFVLVLLGFVSVSIC	EEEKRQDEDEDEHEEEEGESQEEGSEEKR	GLLSVLGSAKHVLPVVPVIAEHLG
Caerin 1.11	MASLKKSLFVLVFLGFVSVSIC	EEEKRQDEDEDEHEEEEGENQEEGSEEKR	GLFSVLGSAKHVVPRVVPVIAEHLG
Caerin 1.12	MAFLKKSFLVLVFLGLVSVSIC	EEEKRQDEDEDEHEEEEGENQEEGSFTEEKR	GLFGILGSAKHVLPVVPVIAEHSG
Caerin 1.13	MASLKKSLFVLVFLGFVSVSIC	EEEKRQDEDEDENEEEEGENQEEGSEEKR	GLLSVLGSLKLI VPHVVPVIAEHLG
Caerin 1.14	MASLKKSLFVLVFLGFVSVSIC	EEEKRQDEDEDENEEEEGENQEEGSEEKR	SVLGKSAKHLPVVPVIAEKTG
Caerin 1.15	MASLKKSLFVLVFLGFVSVSIC	EEEKRQDEDEDENEEEEGENQEEGSEEKR	GLFGLAKGSAKPHVVPVISOVLG
Cancrin	MFTLKKSLLLVFI LGMVSLCLC	MRESSDDEEPNVTENEETGNEGDVEVR	GSAQPYKQLHKVVNWDPYG
Dermaseptin	MAFLKKSFLVLVFLAIVPLSIC	EEEKREEEENEKQEDDDQSEKR	GLVSGLLNTAGGLLGDLGSLGSLSGGES
Dermaseptine B3	MAFLKKSFLVLVFLGLVSLSLIC	EEEKREEEENEKQEDDEQSEKR	ALWKNMLKGIKLAGQAALGAVKTLVGAE
Dermaseptine B4	MAFLKKSFLVLVFLGLVSLSLIC	EEEKRENEKDEIEQEDDEQSEKR	ALWKDILKNVGAAGKAVLNTVTDVMVNQGEQ
Dermaseptine B6	MAFLKKSFLVLVFLGLVSLSVC	EEEKRENEDEMEQEDDEQSEKR	ALWKDILKNAGKAALNEINQLVNQGEL
Dermatoxin DRT-B	MAFLKKSFLVLVFLGLVPLSLC	ESEKREGENEEEQEDDQSEKR	SLGSFLKGVGTTLASVGVVSDQFGKLLQAGQG
Dermatoxin DRT-S	MAFLKKSFLVILVFLGLVPLSFC	ENDKREGENEEEQDDDQSEKR	ALGTFLKGVGSAVATVGMVADQFGKLLQAGQG
DRP-AC-1	MAFLKKSLLLVLVFLGLVSLSLIC	EEEKRENEDEEKQEDDDQSENKR	GLLSGILNTAGGLLGNLIGSLSNGES
DRP-AC-2	MAFLKKSLLLVLVFLALVPLSIC	EEEKREEEDEEKQEDDDQSENKR	GLLSGILNSAGGLLGNLIGSLSNGES
DRP-AC-3	MAFLKKSLLLVLVFLGLVSLSLIC	EEEKRENEDEEEQEDDEQSEMRR	SVLSTITDMAKAAGRAALNAITGLVNQGEQ
Esculentin 1b	MFTLKKPLLLIVLLGMISLSLC	EQERNADEEEEGSEIKR	GIFSKLAGKKLKLNLLISGLKNVKGKEVGMVVRTGIDIAGCKIKGEC
Frenatin 1.1	MAFLKKSFLVLVFLGLVSLSLIC	EKEKKEQDEDEDENEKEESEEGSEEKR	GLLDTLGGILGLGR
Frenatin 3	MHFLKKSIFVLVFLGLVSLSLIC	EKEKREDQNEEEVDENESESEEKR	GLMSVLGHAVGNLGLGFKPKS
Frenatin 3.1	MHFLKKSIFVLVFLGLVSLSLIC	EKEKREDQNEEEVDENEASEEKR	GLMSILGKVAGNVLGGLFKPKENVQKM
Frenatin 4.1	MAFLKKSFLVLVFLGLVNLISLC	EEEKREENKEEEDENEALSEVVKR	GFLEKLTGAKDFASAFVNSIKGT
Gaegurin-4	MFTMKKSLLLFFLGTISLSLC	EEERSADEDDGEMTEEEVVKR	GILDTLKQFAKGVGKDLVKGAAQGVSTVSCKLAKTC
Gaegurin-5	MFTLKKSLLLFFLGTISLSLC	EEERNADEEEKRDVEVEKR	DVEVEKRFGLGALFKVASKVLPVVFCAITKKC
Grahamin-1	MLFTKKSQLLLFFPGTINLSLC	QDETNAEEERRDEEVAKMEEIKR	GLLSGILGAGKHIVCGLSGLC
Grahamin-2	MLFTKKSMLLLFFLGTISLSLC	EQERNADEEEERRDEEVAKMEEIKR	GLLSGILGAGKNIVCGLSGLC
Nigrocin-2S	MFTLKKSI LLLFFLGTINLSLC	QDETNAEEERRDEEVAKMEEIKR	GILSGILGAGKSLVCGLSGLC

**Table 7.1: continues.**

Name*	Signal Peptide	Acidic Spacer	Peptide
Nigrocin-2	MFTTKKSILLFFFLGMVSLALC	EQERDANEEERRDELDERDVEAIKR	GLLSKVLGVGKKVLCGVSGLC
PBN1	MAFLKKSFLVLFVFLGLVLSLCSIC	EEEKRETEEKEYDQGEDDKSEEKR	FLSLIPHIVSGVAALAKHLG
PBN2	MAFLKKSFLVLFVFLALVPLSIC	EKKKSEEEENEEKQEDDQSEEKR	GLVTSLIKGAGKLLGGLFGSVTGGQS
Phylloxin PLX-B	MVFLKKSLLLVLVFLVGLVLSLCSIC	EENKREEHEEIEENKEKAEKKR	GWMSKIASGIGTFLSGMQQG
Phylloxin PLX-S	MVFLKKSLLLVLVFLVGLVLSLCSIC	EENKREEHEEVEENAEEKAEKKR	GWMSKIASGIGTFLSGVQQG
Ranalexin	MFTLKKSLLLLFFFLGTINLSLC	EEERNAEEERRDNPDERDVEVEKR	FLGGLIKIVPAMICAVTKKC
Ranatuerin 2P	MFTMKKSLLLLFFFLGTISLSLC	EQERGADEDDGVEITEEVKVR	GLMDTVKKNVAKNLAGHMLDKLKCKITGC
Ranatuerin 2Pa	MFTLKKSLLLLFFFLGTISLSLC	QREADDDQGEVQQEVKVR	GFLSTVKNLATNVAGTVIDTIKCKVTGGC
Temporin-1SKa	MFTLKKSLLLLFFFLGTINLSLC	EEERNAEEERRDDPEERDVEVEKR	FLPVILPVIGKLLNGILG
Temporin-1SKc	MFTLKKSLLLLRRLGIINLSLC	EEERDADEEERRDDPEEGDVEEKR	AVDLAKIANIANKVLSSLFG
Temporin B	MFTLKKSLLLLFFFLGTINLSLC	EEERNAEEERRDEPDERDVQVEKR	LLPIVGNLLKSLLG
Temporin G	MFTLKKSLLLLFFFLGTINLSLC	EEERDADEEERRDDLEERDVEVEKR	FFPVIGRILNGILG
Temporin H	MFTLKKSLLLLFFFLGTINLSLC	EEERNAEEERRDEPDERDVQVEKR	LSPNLLKSLLG

\*Auerins from *Litoria aurea* [251]; brevinin-1s from *Rana esculenta* [514]; brevinin-2s from *Rana temporaria* (AJ251567); caerins from *Litoria caerulea* [25]; cancrin from *Rana cancrivora* [515]; dermaseptin from *Ascaphus annae* [516]; dermaseptines from *Phyllomedusa bicolor* [51]; dermatoxins DRT-S and phylloxin PLX-S from *Phyllomedusa sauvagei* [517]; dermatoxins DRT-B and phylloxin PLX-B from *Phyllomedusa bicolor* [518, 519]; DRP-ACs from *Ascaphus callidryas* [25]; esculentins from *Rana esculenta* [514]; frenatins from *Litoria infrafrenata* [252]; gaegurins from *Rana rugosa* [520]; grahamins from *Rana grahmi* [521]; nigrocin-2s from *Oncomelania schmackeri* (AM494476); nigrocin-2 from *Rana nigromaculata* (AM494062); PBNs from *Phyllomedusa bicolor* [25]; ranalexin from *Rana catesbeiana* [522]; ranatuerins from *Rana pipiens* [523]; temporin-1s from *Rana sakuraii* [524]; temporins from *Rana temporaria* [525].

## 7.1.2 Peptide Biosynthesis

Peptides are formed by covalently linking the  $\alpha$  nitrogen of one amino acid to the  $\alpha$  carbonyl carbon of another amino acid to form a chain. The main differentiation between peptides and proteins is the length of the polypeptide chain, with peptides generally considered to have less than 50 amino acids [439]. Despite this classification, insulin which contains 50 residues is considered to be the smallest protein.

Peptides are synthesised by a process that involves the genes encoding DNA being transcribed into the intermediate messenger RNA (mRNA) molecule, followed by translation of mRNA into a polypeptide chain [255]. Transcription is catalysed by RNA polymerase in the nucleus and the resulting mRNA is complementary to the template DNA strand and carries the same information [526]. The mRNA then undergoes post-transcriptional processing prior to exiting the nucleus, involving RNA capping and polyadenylation. These modifications increase the stability of mRNA molecule from enzymatic degradation by exonucleases and aid in its export from the nucleus to the cytoplasm [527]. Translation of the mRNA is catalysed by a ribosome enzyme assembly which translates the base sequences coded by the mRNA (see Appendix C for the Genetic Code) into the appropriate amino acid sequence [528]. Typically, translation of secretory peptides occurs on the endoplasmic reticulum [529] immediately following transcription. It is an energetically unfavoured process, as the mRNA is degraded readily [255]. In order to make it efficient for the cell, multiple ribosomal complexes are involved in translating each mRNA strand and result in the synthesis of multiple polypeptide chains from each mRNA molecule [528]. The biologically active peptides are initially formed as part of a larger precursor protein, often referred to as the prepropeptide.

Typically the precursor protein is usually around 100 to 250 residues long and comprises of a N-terminal signal peptide, followed by an acidic spacer of variable sequence and regions that encode the peptide sequence. Several different peptides can be encoded by one precursor, with each peptide separated by an acidic spacer region [439, 530]. The signal and spacer regions are important to direct the transport and processing of the precursor.

The signal peptide is highly hydrophobic and allows the insertion of the precursor into the endoplasmic reticulum. It usually contains a region of residues with bulky side chains, followed by amino acids with small, neutral side chains [529]. It is cleaved early in peptide biosynthesis in the rough endoplasmic reticulum by proteolytic enzymes and results in the formation of the propeptide [530]. The endoprotease acts preferentially on amino acids with small neutral side chains (Ala, Cys, Ser and Thr) that appear at the junction between the signal and acidic spacer [531]. This cleavage can occur by three proposed mechanisms; the pore model [532], the loop model [529, 533] and the trigger hypothesis [534].

In the pore model, the nascent polypeptide binds to a receptor and causes an aggregation of the protein subunits within the membrane to form pores, through which the translated polypeptide chain leaving the ribosome passes. The signal region is then cleaved cotranslationally as the peptide chain enters the cisternal space of the endoplasmic reticulum [532, 535]. Alternatively, in the loop model, the hydrophobic signal region enters the apolar membrane, leaving the more hydrophilic propeptide on the ribosome side of the membrane forming a loop (Figure 7.1). The signal region is then cleaved on the inner membrane surface and the propeptide chain is transferred across the membrane as it folds [529, 533]. The final model, the trigger hypothesis, is similar to the pore model, however, the segregation of protein subunits does not occur cotranslationally, instead it is a result of a conformational change that occurs in the newly synthesised precursor upon contact with the membrane prior to insertion across the lipid bilayer [534].

NOTE:

This figure is included on page 216 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 7.1:** Loop model for presecretory peptide interactions with membranes. The signal peptide region is cleaved from the precursor molecule [531].

The resulting propeptide is then transported to the Golgi apparatus [530], packaged and stored in secretory granules until it is required. The pro region of the precursor protein is hydrophilic, contains a high proportion of acidic residues and varies in length and primary structure. It has been proposed that the acidic spacer is required to provide the minimum critical length for segregation and transport to the Golgi apparatus [529]. Additionally, it is required to ensure correct folding and to protect the peptide from enzymatic degradation [531]. Generally the propeptide displays little biological activity, thus the acidic spacer region is cleaved by proteolytic processing to release the mature peptide. The site of cleavage of the acidic spacer pro sequence is usually characterised by a pair of basic residues, providing cleavage points for various trypsin-like endoproteases enabling the peptide to be liberated (Section 7.1.3.1) [439].

Lastly, prior to secretion by the exocrine mechanism, the peptide undergoes post-translational modifications such as acetylation, sulfation, glycosylation, the formation of disulfide bridges, amidation and isomerisation of L-amino acids to the D-form (Section 7.1.3.2). Typically these modifications are essential to display maximum biological activity. In many cases however, the active peptide is cytotoxic to the host [27]. To overcome this problem, many peptides are stored as inactive precursors and the acidic spacer is removed and post-translation modifications occur upon release [5].

### **7.1.3 Post-Translational Processing**

As previously discussed, peptides and proteins are synthesised as larger precursors, which are cleaved in a sequence-specific manner [536, 537]. A variety of highly regulated and specific post-translational processes and reactions are required to liberate the biologically active peptide from the large precursor molecule. Post-translational processing may include proteolytic cleavage or the addition of modifying groups to amino acids in the peptide. These processing events occur during intracellular transport or in the secretory vesicles just prior to exocytosis.

#### **7.1.3.1 Propeptide Cleavages**

Many peptides are initially synthesised as larger, inactive, precursor molecules and undergo several post-translational processing cleavage events required to liberate the

biologically active peptide. While each precursor has a distinct primary sequence, proteolytic cleavage generally occurs at dibasic residue sites that are located at the N- and C-terminus of the peptides within their precursor sequence.

The most common dibasic cleavage sites occur at residues Lys-Arg↓ (KR↓), however Lys-Lys↓, Arg-Arg↓ and occasionally Arg-Lys↓ dibasic residues sites also occur but are less frequent [530, 538-540]. Additionally, some cleavages occur at sites containing two basic amino acids separated by two, four or six other residues (Arg-X<sub>n</sub>-Arg where n is 2, 4 or 6 residues) [538]. Despite the prevalence of dibasic residue processing sites, cleavage can occur at monobasic Arg sites, such as in the processing of provasopressin [535] and procaerulein [508]. Processing at multi-basic residue sites has also been shown to occur, in addition to the occasional cleavage at nonbasic residues such as between two Trp (W↓W) and other hydrophobic, short chain aliphatic and acidic residues [530, 538].

The enzymes involved in these processing events include endoproteases such as proprotein convertases [539-541], and exopeptidases such as carboxypeptidases and aminopeptidases [536]. Endoproteases cleave the propeptide backbone at the dibasic residues site. The basic amino acids of this cleavage site are removed from the N-terminal by aminopeptidases and from the C-terminal by carboxypeptidases. Normally, enzyme processing of the precursor molecule occurs in the secretory granules just prior to secretion. However, there is evidence that some cleavages by endoproteases occur in the *trans*-Golgi apparatus prior to packaging into granules [439, 536]. There is also evidence that some propeptide cleavages occur extracellularly on the skin of the animal following release by exocytosis. This is observed with some neuropeptides [542] and is evident in the skin secretion of *L. peronii*, with some unprocessed properoneins present in the secretion [140]. This requires enzymes to be co-released and processing to occur immediately on the skin. This has also been well characterised in the cases of angiotensin and neurotensin [542].

### 7.1.3.2 Post-Translational Modifications

Peptide precursors show great diversity in terms of their molecular weight and the extent of post-translational modifications to residues in the polypeptide chain. Post-translational modifications are covalent processing events that can change both the physical and functional properties of a peptide by the addition of a modifying group to particular



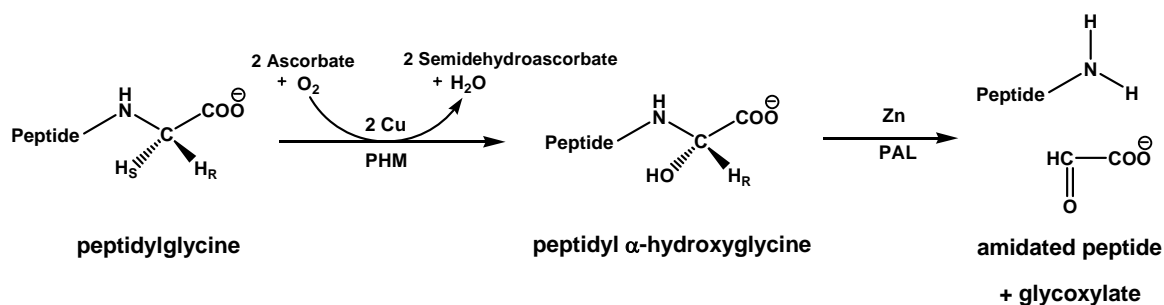
residues. These modifications include the formation of cysteine bridges, blocking of C- and N-terminals by amidation and acetylation respectively, formation of pGlu and O-sulfation of Tyr residues, and have been shown to be essential for biological activity of the peptides [142].

Only the modifications present in the mature peptides that are encoded by the precursors isolated in this chapter will be discussed here.

#### **7.1.3.2.1 C-Terminal Amidation**

Amidation of peptides is a ubiquitous post-translational modification, widespread in vertebrates, invertebrates and plants [543]. An estimated half of all known bioactive peptides contain a C-terminal amide moiety [538, 544]. This modification is generally considered to be essential for the biological activity and stability of the peptide within the cell [545]. In the early 1980's it was concluded that the C-terminal amide group was not incorporated into the polypeptide chain during translation in peptide biosynthesis, but instead was generated as a result of post-translational processing [546].

An exposed Gly residue at the C-terminal is mandatory for amidation, acting as a signal and the biosynthetic precursor [546]. C-terminal amidation occurs under physiological conditions by a single multifunctional protein with two distinct enzyme activities known as peptidyl-glycine- $\alpha$ -amidating monooxygenase (PAM) [513]. The N-terminal domain is a hydroxylating monooxygenase that oxidises the  $\alpha$  carbon of Gly, in a reaction that uses copper, oxygen and ascorbate as cofactors [538, 544, 545]. The C-terminal lyase domain releases glyoxylate, leaving behind the nitrogen of Gly as a C-terminal amide (Figure 7.2) [538].



**Figure 7.2:** Two step conversion of peptidylglycine substrates into the corresponding amidated peptide. Peptidylglycine- $\alpha$ -hydroxylating monooxygenase (PHM); peptidyl- $\alpha$ -hydroxyglycine  $\alpha$ -amidating lyase (PAL) [544].

For amidated peptides, the C-terminal amide is normally required for full biological activity. The amide group prevents ionisation of the C-terminus and increases the hydrophilicity of the peptide, thus improving receptor recognition [544]. For example, studies of G-protein coupled interactions illustrate that the amide moiety is an essential determinant for receptor interaction [544]. Typically, antimicrobial peptides possess a C-terminal amide, a moiety considered to be essential for its activity [50].

### 7.1.3.1.2 Disulfide Bridges

Disulfide bridges are crucial to the folding and stability of many proteins and are most commonly found in extracellular proteins and peptides. In solution, many peptides exist as an equilibrium of different conformers, however covalent disulfide links constrain the solution state to a more limited equilibrium of conformers and increase the effective concentration of the bioactive conformation [547].

Disulfide bonds form between two Cys thiol groups and have been shown to form in a protein spontaneously in the presence of oxygen. It has been suggested that spontaneous oxidation may not proceed at a physiologically relevant rate [548]. *In vitro* reformation of disulfide bonds occurs at a slow rate and the bonds formed are not always in correct combinations. In contrast, *in vivo* disulfide bond formation is rapid and accurate [549], with enzymes such as disulfide oxidoreductases (in particular thioredoxin) and protein disulfide isomerase implicated in catalysing the formation of disulfide bonds [548].

### 7.1.4 Evolutionary Insights

Many classical perspectives of amphibian evolution and phylogenetic relationships are based upon factors including morphological characteristics and fossil records [524]. Incomplete records have resulted in much speculation, but recently, these perspectives have been rejected on the basis of phylogenetic analysis of nucleotide sequences of orthologous genes [524]. The structure of precursors of secretory peptides can yield information about the evolution of hormones and peptides. On the basis of common features, the peptides can be grouped together into collections that share a common evolutionary origin.

Analysis of the cDNA clones of the antimicrobial peptides from South American and Australian hylids and Asian, European and North American ranids has indicated that they all derived from a single multigene family (preprodermaseptin) originating from a common ancestor [25, 51, 510]. As the mature peptide and the conserved signal and spacer regions are encoded in the same gene, there is no possibility that the preproregion could be added to the gene by post-translational events. Furthermore, the immense numbers of distinct peptides encoded by this gene family reveal an unparalleled degree of gene diversification between organisms. This has also been observed within the genes encoding for immunoglobulins [550] and venom-derived toxins [551] from other vertebrates. As a consequence, the evolution of large variable gene families can be studied by looking at the precursor genes for amphibian antimicrobial peptides [25].

Diversification of the antimicrobial peptide loci is part of an optimal evolutionary strategy developed by amphibian species to combat novel ecological situations in which there are rapid changes in the microbial predators [250]. In innate (non-adaptive) immunity, gene-encoded antimicrobial peptides form the frontline host defence against harmful microorganisms. These peptides can be either inducible or constitutive and can kill a wide spectrum of microorganisms by disruption and permeation of the target cell membrane. In contrast, in mammals the distinct sets of antimicrobial peptides appear to have diversified in a species-specific manner due to recent gene duplication prior to evolutionary divergence [552].

Amphibian dermal glands synthesise and store antimicrobial peptides that are released onto the outer layer of the skin in response to stimuli, to provide an effective and rapid defence against noxious microbials [5, 27]. Amphibian host antimicrobial activity is associated with a substantial degree of peptide polymorphism. Animals belonging to different species and sub-species secrete a distinct set of antimicrobial peptides that differ in their structural characteristics and spectrum of action. No two species have been shown to produce identical skin peptide profiles. This suggests that if the genes responsible for synthesis of these peptides have come from the same gene family, they have been subjected to diversifying selection as observed in other immune-related genes [51].

At a molecular level, the evolution of the antimicrobial genes has not occurred randomly. There is suggestion that after a putative ancestral gene surfaced in the common lineage of Hylidae and Ranidae, it then diversified within these groups with numerous duplication and divergence events [250]. It is likely that evolutionary pressure has acted to conserve the nucleotide sequence encoding the signal and acidic spacer peptides, whilst the peptide-encoding region is hypervariable. The conservation of the prepro sequence may be due to ensuring that the correct proteolytic processing occurs, in addition to correct targeting and transport of the mature peptide to the secretory glands [553].

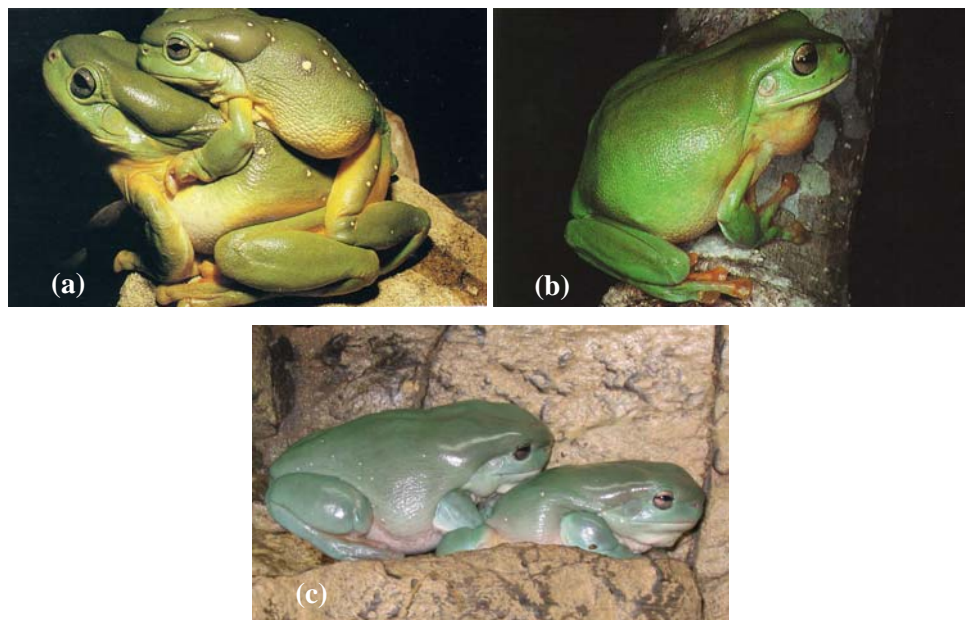
### 7.1.5 Australian Amphibians

During the last decade, our research group has investigated and structurally characterised among 150 peptides from the skin secretions of more than 25 different Australian amphibians from the genera *Litoria*, *Crinia*, *Uperoleia* and *Limnodynastes* [27]. Despite the isolation of a number of different classes of bioactive peptides, the only clones of cDNA-deduced precursor structures that have been reported, have been for the caerins from *L. caerulea* [25] and *L. splendida* [372], aureins from *L. aurea* [251] and frenatins from *L. infrafrenata* [252]. Until recently, the acquisition of the skin secretions required the sacrifice of the animal. Due to the rapid decline of amphibian populations in Australia as a result of habitat destruction and contamination, disease and climate change [6, 8, 554, 555], this technique is highly undesirable. However, cDNA library construction from the lyophilised skin secretions [251] have permitted investigations into the precursor structures.

The cloning of precursor cDNAs from lyophilised skin secretions of several Australian amphibians; a hybrid formed from interbreeding of *L. splendida* and *L. caerulea* animals, *C. riparia* and *L. fallax* will be discussed in this chapter. As *L. fallax* has been discussed in Chapter 4 it will not be discussed in this introduction. The aim of this study was two fold: (i) to clone and characterise the precursor proteins of the major secretory peptides of the hybrid, *C. riparia* and *L. fallax*; and (ii) to indicate the evolutionary significance of the genes of these Australian species from the genera *Litoria* and *Crinia*, relative to other hylids and ranids studied worldwide.

### 7.1.5.1 Hybrid

Many species of amphibians hybridise naturally, with several species forming extensive zones of hybridisation [556-560]. Interspecific female hybrids between a female magnificent tree frog *L. splendida* and male green tree frog *L. caerulea* [372] were produced in captive populations (Figure 7.3). The parent species are two closely related species from the Australian *Litoria caerulea* species Group (family: Hylidae, Pelodyadinae subfamily) [561].



**Figure 7.3:** (a) *L. splendida* (male and female animals are shown in amplexus), (b) *L. caerulea* and (c) hybrid.

The hybrid secretes a mixture of host defence peptides, which include several peptides also produced by the parent species, in addition to several novel peptides not identified in either

parent species (Table 7.2). Caerins 1.1, 1.6, 1.10 and 1.20 all display wide spectrum antimicrobial activity, whilst caerins 2.6, 2.7, 3.1 and 4.2 are narrow spectrum antibiotics. Caerin 1.1 and 1.10 show only modest nNOS inhibitory action. Additionally, the ubiquitous neuropeptide caerulein peptide is a potent smooth muscle and analgesic agent.

**Table 7.2:** Peptides isolated from the skin secretion of the hybrid of female *L. splendida* and male *L. caerulea* [372].

Peptide	Sequence	Activity <sup>1</sup>
Caerulein	pEQDY(SO <sub>3</sub> )TGWMDF-NH <sub>2</sub>	a, b
Caeridin 1	GLLDGLLGTGL-NH <sub>2</sub>	c
Caeridin 5	GLLGMVGSLLGGLGL-NH <sub>2</sub>	c
Caerin 1.1	GLLSVLGSAKHVLPVVPVIAEHL-NH <sub>2</sub>	d, e*
Caerin 1.6	GLFSVLGAVAKHVLPVVPVIAEKL-NH <sub>2</sub>	d, e
Caerin 1.10	GLLSVLGSAKHVLPVVPVIAEKL-NH <sub>2</sub>	d
Caerin 1.20	GLFGILGSAKHVLPVVPVIAEHL-NH <sub>2</sub>	d, e*
Caerin 2.2	GLVSSIGRALGLLADVKSKEQPA-OH	f
Caerin 2.6	GLVSSIGKVLGLLADVKSKEQPA-OH	e, f
Caerin 2.7	GLVSSIGKALGLLADVKSKEQPA-OH	f
Caerin 3.1	GLWQKIKDKASELVSGIVEGVK-NH <sub>2</sub>	f
Caerin 3.5	GLWEKVKEKANELVSGIVEGVK-NH <sub>2</sub>	f
Caerin 4.2	GLWQKIKSAAGDLASGIVEAIKS-NH <sub>2</sub>	f
Caerin 5.1	AEILFGDVRPPWMPPIFPEMP-OH	c

<sup>1</sup>**Activity:** (a) neuropeptide; (b) analgesic agent; (c) activity unknown; (d) wide spectrum antimicrobial; (e) nNOS inhibitor, \*indicates only moderate activity (f) narrow spectrum antimicrobial.

### 7.1.5.2 *Crinia riparia*

*Crinia riparia* is a member of the family Myobatrachidae, which is comprised of fifteen species distributed throughout most of Australia [243]. Typically, *C. riparia* animals are small with a polymorphic appearance (Figure 7.4). The animal has a geographical distribution that is confined entirely to the Flinder's Rangers, South Australia and is often found aggregated in groups of up to forty individuals in moist shallow rock spaces [562].



**Figure 7.4:** *Crinia riparia*.

The skin secretion of *C. riparia* has been investigated, with many novel disulfide peptides characterised [44] and named the riparin peptide group (Table 7.3). The riparin peptide family include peptides which display a number of biological activities including neurological, nNOS inhibitory and antimicrobial activity. The riparin peptides are quite unique and show little structural similarity to those previously isolated from other Australian amphibians and possess no neuropeptides analogous to the caeruleins or uperoleins [27].

**Table 7.3:** Riparin peptides isolated from the skin secretion of *Crinia riparia* [44].

Peptide	Sequence	Activity <sup>1</sup>
Riparin 1.1	RLCIPVIFPC-OH	a
Riparin 1.2	FLPPCAYKGTC-OH	a
Riparin 1.3	FPLPCAYKGTYC-OH	a
Riparin 1.4	FFLPPCAYKGTC-OH	a
Riparin 1.5	FFLPPCAHKGTC-OH	a
Riparin 2.1	IIIEKLVNTALGGLSGL-NH <sub>2</sub>	b
Riparin 3.1	IVSYPDDAGEHAHKMG-NH <sub>2</sub>	c

<sup>1</sup>Activity: (a) neuropeptide; (b) narrow spectrum antimicrobial; (c) activity unknown.

## 7.2 Results

### 7.2.1 Precursor cDNA Cloning from the Hybrid Frog

The analysis of the skin secretion of an adult female hybrid bred from a captive population of a female *L. splendida* and a male *L. caerulea* was conducted using a primer based on the 5'-untranslated region of preprocaerin 1.1 previously cloned from *L. caerulea* skin. Two different cDNA clones encoding for caerin 1.1 were revealed. Each preprocaerin 1.1 cDNA encoded a single copy of the previously characterised caerin 1.1 (Figure 7.5).

Alignment of both full-length nucleic acid sequences (Figure 7.6) and the translated open-reading frame amino acid sequence (Figure 7.7) reveal that the two clones of preprocaerin 1.1 exhibit a high degree of both nucleotide and primary structure homology with the preprocaerin 1.1 cDNA clones isolated from the parent species *L. splendida* [372] and *L. caerulea* [25]. The putative signal peptide sequences were almost identical in structure. Interestingly, a high degree of homology was also observed for the acidic spacer. All of the acidic spacer regions were flanked N-terminally with a propeptide convertase processing site -Lys-Arg- (-KR-) for a protease cleavage. The location of this site has been shown to be highly conserved in all of the *Litoria* species studied to date [25, 251, 252, 372]. All of the open-reading frames for caerin 1.1 precursors terminate in an amide donor Gly residue, confirming the presence of the C-terminal amide in the mature peptide.



```

          M  A  F  L  K  K  S  L  F  L  V
1  GGCTTGTAAGACCAAGCAT GGCGTTCCTA AAGAAATCTC TTTCCTTGT
          T  T
                                     G
                                     G

          L  F  L  G  F  V  S  V  S  I  C  E  E  E  K  R
51 ACTATTCCTT GGATTCGTTT CTGTTTCCAT CTGTGAAGAA GAGAAAAGAC
          T      G

          Q  E  D  E  D  E  H  V  E  E  G  E  N  Q  E  E  G
101 AAGAGGACGA GGATGAACAT GTGGAAGAGG GTGAAAATCA GGAAGAGGGA
          A          AT      A
                   D

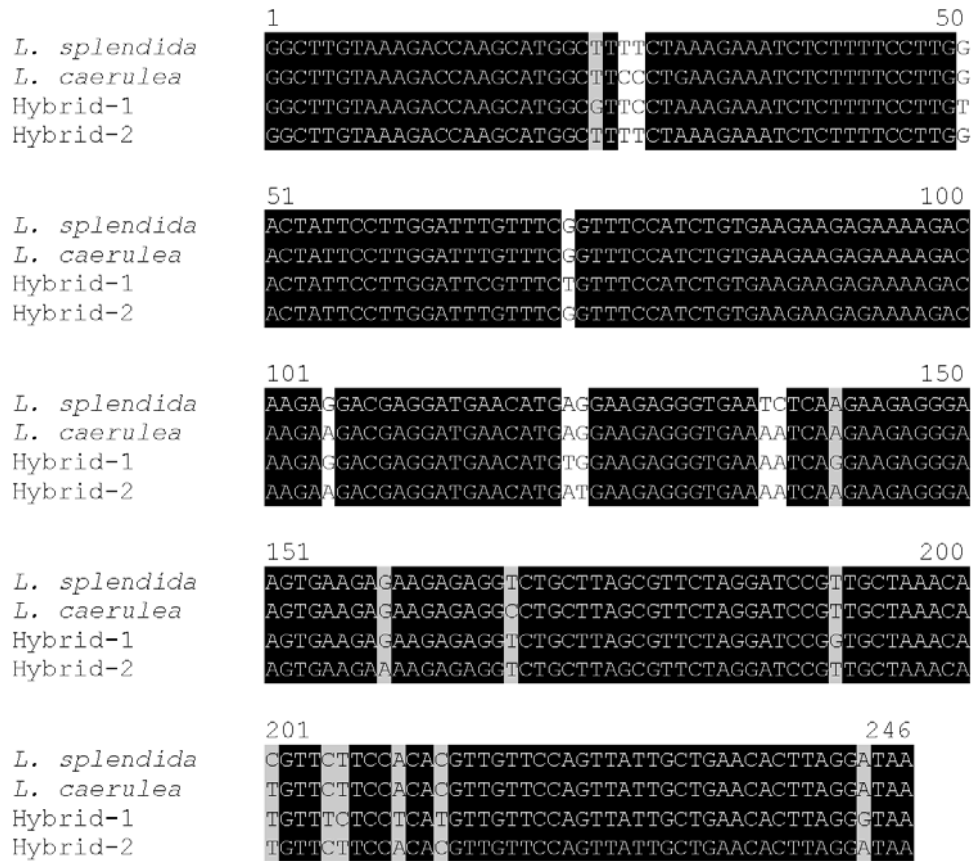
          S  E  E  K  R  G  L  L  S  V  L  G  S  V  A  K  H
151 AGTGAAGAGA AGAGAGGTCT GCTTAGCGTT CTAGGATCCG GTGCTAAACA
          A          T

          V  L  P  H  V  V  P  V  I  A  E  H  L  G  *
201 TGTTTCTCCT CATGTTGTTC CAGTTATTGC TGAACACTTA GGGTAA
          CT  A  C          A

```

**Figure 7.5:** Nucleotide sequences of the cDNA clones encoding the open-reading frame of caerin 1.1 cloned from the hybrid skin secretion. Putative signal peptide sequences are double-underlined, mature caerin 1.1 sequence is single-underlined and the stop codon is indicated by \*. Differences between the two clones are indicated by the nucleotides below the full nucleotide sequence. Likewise, any differences in amino acid sequence are indicated below the nucleotide sequence.

A BLAST search in the EMBL Protein Sequence Database indicated that the signal region of the caerin 1.1 precursors exhibited a high degree of structural similarity to other signal peptides of antimicrobial peptide precursors reported from hybrid and ranid frogs. In particular, a comparison of the inferred amino acid sequence of the two caerin 1.1 precursors with those preprodermaspetins from the South American hybrid frogs has revealed that the signal peptides (90 % identical) are highly homologous. This suggests that the precursor genes of the hybrid caerin 1.1 belong to the same preprodermaspetin family. The nucleotide sequences have been deposited into the EMBL Nucleotide Sequence Database under accession codes EU856538 and EU856539.



**Figure 7.6:** Alignment of nucleotide sequences of the caerin 1.1 cDNA clones from hybrid, *L. splendida* and *L. caerulea*. Identical bases are boxed in black and consensus bases are shaded in grey.



**Figure 7.7:** Alignment of translated prepro region of the caerin 1.1 precursor open-reading frames. Identical amino acid residues are boxed in black.

### 7.2.2 Preproriparin cDNA Cloning from *Crinia riparia*

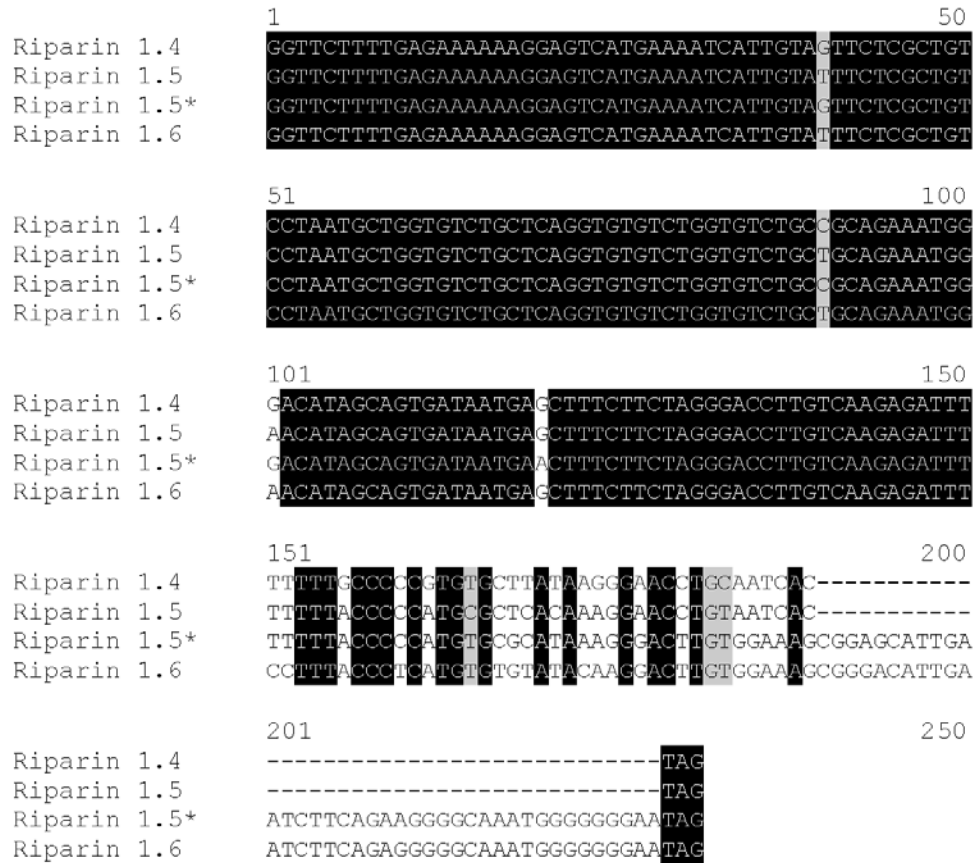
Four different preproriparin cDNAs were consistently cloned from the skin secretion of a specimen of *C. riparia*. These preproriparin cDNAs encoded for a single copy of the previously characterised riparin 1.4, 1.5 and its amide, and a novel peptide named riparin 1.6 (Figure 7.8). Alignment of the nucleic acid sequences (Figure 7.9) and the translated open-reading frame amino acid sequence (Figure 7.10) reveal that the clones of preproriparins exhibit a high degree of both nucleic acid and primary structure homology. The putative signal and acidic spacer peptides display sequences that are almost identical in primary structure. The acidic spacer regions were flanked C-terminally with a propeptide convertase processing site -Lys-Arg- (-KR-) for protease cleavage. The topographical localisation of this site, together with the cleavage site located between the signal and acidic spacer domains was highly conserved among the preproriparins clones isolated here. However, a clear difference can be seen when compared to the preprocaerins and preproaureins previously cloned from *L. caerulea* [25] and *L. aurea* [251].

The open-reading frames of the riparin 1.5(NH<sub>2</sub>) and riparin 1.6 precursors terminated in an amide donor Gly residue that indicates the presence of a C-terminal amide in the mature peptide. It is interesting that this animal produces two separate clones of riparin 1.5, one terminating in a free acid, the other with a post-translationally modified C-terminal amide.



**Figure 7.8:** Nucleotide sequences of the cDNA clones encoding the open-reading frame of (a) riparin 1.4; (b) riparin 1.5; (c) riparin 1.5(NH<sub>2</sub>); and (d) riparin 1.6 cloned from the skin secretion of *C. riparia*. Putative signal peptide sequences are double-underlined, mature riparin sequences are single-underlined and the stop codon is indicated by \*.

A BLAST search in the EMBL Protein Sequence Database indicated that the signal region of the riparin precursors show very little structural similarities to other precursors isolated from amphibians. The nucleotides sequences have been deposited into the EMBL Nucleotide Sequence Database under accession codes EF550516 - EF550519.



**Figure 7.9:** Alignment of nucleotide sequences of the cloned riparin cDNAs from *C. riparia*. Identical bases are boxed in black and consensus bases are shaded in grey. \* indicates that riparin 1.5 peptide is C-terminally amidated.



**Figure 7.10:** Alignment of translated open-reading frames of the riparin precursor cDNA clones. Identical amino acid residues are boxed in black.

### 7.2.3 Preprofallaxidin cDNA Cloning from *Litoria fallax*

Nine different preprofallaxidin cDNA sequences were cloned from the lyophilised skin secretion of *L. fallax* (Figure 7.11). Each clone encoded previously characterised fallaxidins, in addition to several novel peptides, named fallaxidin 1.4, 2.3, 3.2, and 4.1 – 4.3 (Table 4.1). The deduced molecular masses of the predicted primary structures of the five fallaxidins encoded within the open-reading frames of the cDNA clones were used to locate these peptides within the reverse phase HPLC fractions that had been subjected to MS analysis. Only fallaxidin 4.1 (725 Da, + Na<sup>+</sup> salt), and 4.2 (1310 Da) could be located (Figure 4.3).

Many of the novel fallaxidin peptides have resulted from several point mutations in the gene nucleotide sequence. Consequently, the corresponding mature peptides have only one or two different amino acids in their sequences. For example, the nucleotide sequences of the precursors of fallaxidin 3.1 and 3.2 differ by only three bases, resulting in two substitutions in the amino acid sequences (Leu<sup>3</sup> to Phe and His<sup>15</sup> to Pro).

Each cDNA clone encoded either a single peptide or multiple distinct peptides. Several clones contained multiple copies of the smaller peptides such as the fallaxidin 1 peptides. Interestingly, these clones also code for antimicrobial peptides in addition to these smaller peptides. This has been noted in other examples, including preprocearulein, which contains multiple copies of caerulein peptide interspersed by an antimicrobial peptide [269, 563]. Similarly, the xenopsin precursor fragment encoded in the precursor for the xenopsin hormone, displays antimicrobial activity [563].

Alignment of the nucleic acid sequences (Figure 7.12) and the translated open-reading frame amino acid sequence (Figure 7.13) reveal that the clones of preprofallaxidins exhibit a high degree of both nucleic acid and primary structure homology. The putative signal and acidic spacer peptides display sequences that are highly conserved. The acidic spacer regions were flanked C-terminally with a propeptide convertase processing site, -Lys-Arg- (-KR-), for a protease cleavage. The topographical location of this site, together with the cleavage site (-C-) between the signal and acidic spacer regions, was highly conserved between preprofallaxidins, in addition to previously cloned precursors from frogs of the *Litoria* genus [25, 251, 252].

In the preprofallaxidin clones that code for multiple peptides, the same protease cleavage site flanks each of the peptides and these sites are separated by the same -Ser-Glu-Glu-(-SEE-) motif. Both the nucleotide sequences and the primary structure of the regions separating the mature fallaxidin peptides are highly conserved. The open-reading frames of the fallaxidin 1, 2 and 4 peptide precursors terminated in an amide donor Gly residue that indicates the presence of a C-terminal amide in the mature peptide. In the open-reading frame of clones encoding fallaxidin 3 peptides, this Gly residue was absent, consistent with the presence of a C-terminal free acid in the primary sequence of the mature peptide.

A BLAST search in the EMBL Protein Sequence Database indicated that the signal region of the fallaxidin precursors exhibited a high degree of structural similarity to other signal peptides of antimicrobial peptide precursors reported from hylid and ranid frogs. The nucleotides sequences have been deposited into the EMBL Nucleotide Sequence Database under accession codes EU912528 – EU912536.

(a)

```

1      M A S L K K S L F L V
   GAATTCACTA GTGATTATGG CTTCTCTAAA GAAATCTCTT TTCCTTGTGC
51    L F L G L V S L S I C E E K K R E
   TATTCCTTGG ATTAGTCTCC CTGTCCATCT GTGAAGAAAA GAAAAGAGAG
101   N E D D A E D E N H E E E S E E K
   AATGAAGATG ATGCAGAGGA TGAAAACCAT GAAGAGGAAA GTGAAGAGAA
151   R G L L D F A K H V I G I A S K
   GAGAGGTCTG CTTGATTTTG CAAAACATGT TATTGGAATT GCATCAAAGC
201   L G K R S E E K R Y H P F G K R S
   TAGGAAAACG AAGTGAAGAG AAGAGATACC ATCCTTTTGG GAAAAGAAGT
251   E E K R Y F P I P F G K R S E E K
   GAAGAGAAGA GATATTTTCC TATTCCTTTT GGGAAAAGAA GTGAAGAGAA
301   R Y F P I P I G K R S E E K R Y
   GAGATATTTT CCTATTCTTA TTGGAAAAAG AAGTGAAGAG AAGAGATATT
351   F P I P I G K K
   TTCTTATTCC TATTGGGAAA AAAAAAAAAAAAAAAAAAAAAA

```

(b)

```

1      M A S L K K S L F L V L F
   GAATTCGATT ATGGCTTCCC TAAAGAAATC TCTTTCCTT GTACTATTCC
51    L G F V S L S I C E E E K R E N E
   TTGGATTTGT CTCCCTGTCC ATTTGTGAAG AAGAGAAGAG AGAAAATGAA
101   G N E N E E E D E N H E E G S E E
   GGAATGAAA ATGAGGAAGA GGATGAAAAC CATGAAGAGG GAAGTGAAGA
151   K R G L L D L A K H V I G I A S
   GAAGAGAGGT CTGCTTGACC TTGCAAAACA TGTTATTGGA ATTGCATCAA
201   K L G K R S E E K R Y H P F G K R
   AGCTAGGAAA AAGAAGTGAA GAGAAGAGAT ACCATCCTTT TGGGAAAAGA
251   S E E K R Y H P F G K R S E E K R
   AGTGAAGAGA AGAGATACCA TCCTTTTGA AAAAGAAGTG AAGAGAAGAG
301   Y P P I G K *
   ATACCCCCCT ATTGGAAAAT GA

```

(c)

```

1      M A S L K K S L F L V
   GAATTCACTA GTGATTATGG CTTCCCTAAA AAAATCTCTT TTCCTTGTGC
51    L F L G L V S L S I C E K E K R E
   TATTCCTTGG ATTAGTCTCC CTGTCTATCT GTGAAAAAGA GAAGAGAGAG
101   N E G N E N E E E E E N H E E G S
   AATGAAGGGA ATGAAAATGA GGAAGAGGAG GAAAACCATG AAGAGGGAAG
151   E E K R G L L D L A K H V I G I
   TGAAGAGAAG AGAGGTCTGC TTGACCTTGC AAAACATGTT ATTGGAATAG
201   A S K L G K R S E E K R G L L D F
   CTTCAAAGCT AGGAAAAAGA AGTGAAGAGA AGAGAGGTCT GCTTGATTTT
251   A K H V I G I A S K L G K *
   GCAAAACATG TTATTGGAAT TGCATCAAAG CTAGGAAAAT GA

```

Figure 7.11: continues next page.



(d)

```

1   GAATTCGATT  M A S L K K S F F L V L F
           ATGGCTTCCT TGAAGAAATC TTTTCTTCTT GTGCTATTCC
51  L G L V S L S M C E E K K R E N E
           TGGGATTAGT TTCCCTGTCC ATGTGTGAAG AAAAGAAAAG AGAGAATGAA
101 D D A E D G N H E E E S E E K R G
           GATGATGCGG AGGATGGAAA CCATGAAGAG GAAAGTGAAG AGAAGAGAGG
151 L V D F A K H V I G I A S K L G
           TTTGGTTGAT TTTGCAAAAC ATGTTATTGG AATTGCATCA AAGCTGGGAA
201 K R S E E K R Y H P F G K R S E E
           AAAGAAAGTGA GGAGAAGAGA TACCATCCTT TTGGGAAAAG AAGTGAAGAG
251 K R Y F P I P F G K R S E E K R Y
           AAGAGATATT TTCCAATTCC TTTTGGGAAA AGAAGTGAAG AGAAGAGATA
301 F P I P I G K R S E E K R Y F P
           TTTTCTTATT CCTATTGGAA AAAGAAGTGA AGAGAAGAGA TATTTTCTTA
351 I P I G K R S E E K R Y F P I P I
           TTCTTATTGG GAAAAGAAGT GAAGAGAAGA GATATTTTCC TATTCCTATA
401 G K *
           GGAAAATGA

```

(e)

```

1   ACTAGTGATT  M A S L K K S L F L V L F
           ATGGCTTCTC TGAAGAAGTC TCTATTCCTT GTACTATTCC
51  L G L L S L S I C E E Q K R E N E
           TTGGATTACT CTCCCTGTCC ATCTGTGAAG AACAGAAGAG AGAGAATGAA
101 E D A E D E N H E E E S E E K R G
           GAGGATGCTG AGGATGAAAA CCATGAAGAG GAAAGTGAAG AGAAGAGAGG
151 L L D F A K H V I G I A S K L G
           TCTGCTAGAC TTTGCAAAAC ATGTTATTGG AATTGCATCA AAGCTAGGAA
201 K R S E E K R F W P F M G K R S E
           AAAGAAGTGA AGAGAAGAGA TTCTGGCCTT TTATGGGAAA AAGAAGTGA
251 E K R F W P F M G K R S E E K R F
           GAGAAGAGAT TCTGGCCTTT TATGGGAAA AGAAGTGAAG AGAAAAGATT
301 F R V L A K L G K L A K *
           TTTCCGTGTG CTTGCTAAAT TAGGAAAATT AGCAAAATGA

```

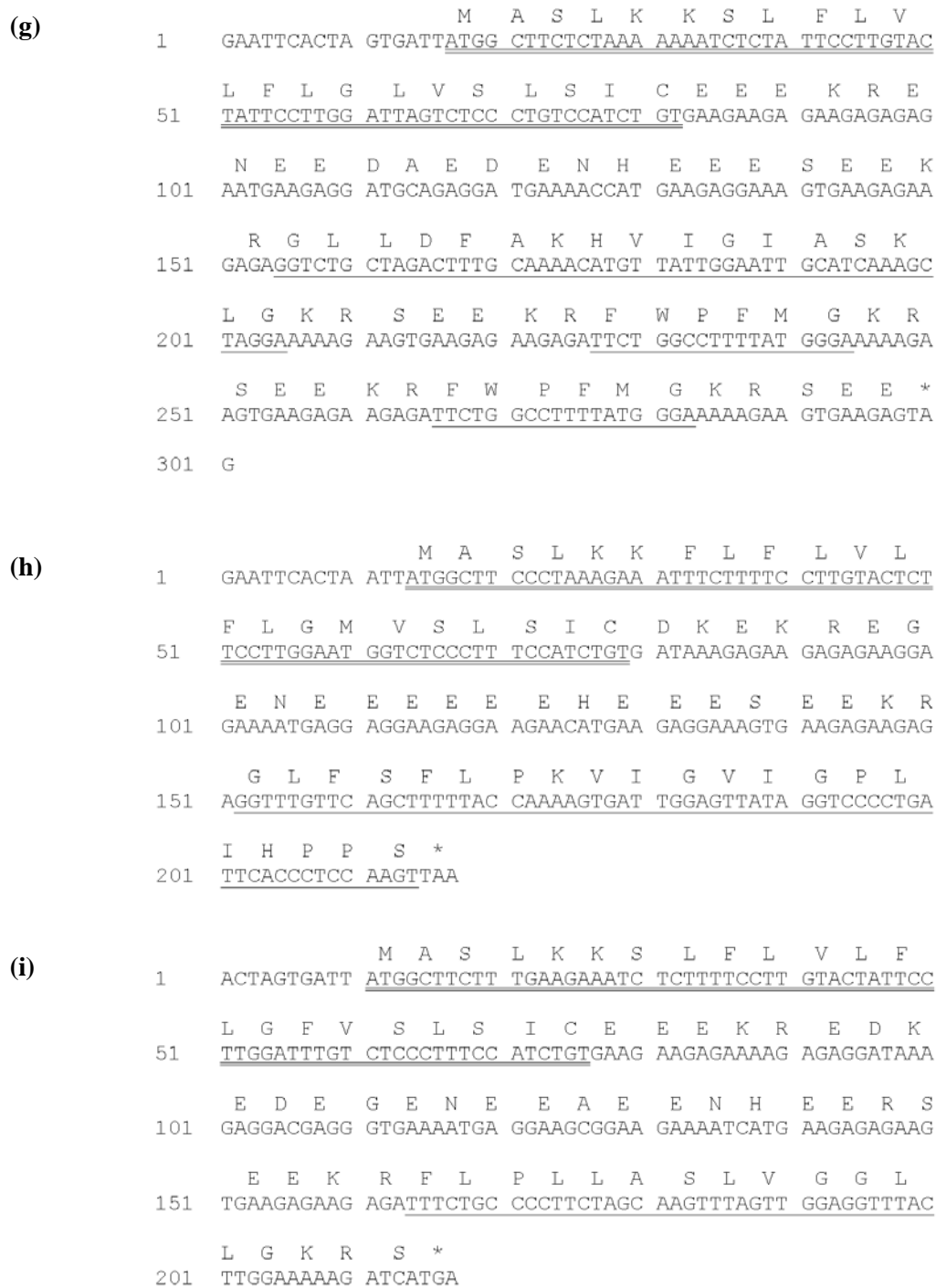
(f)

```

1   GAATTCACTA  M A S L K K S L F L V
           GTGATTATGG CTTCTTGAA AAAATCTCTT TTCCTTGATC
51  L F L G M V S L S I C D K E K R E
           TATTCCTTGG AATGGTCTCC CTTTCCATCT GTGATAAAGA GAAGAGAGAA
101 G E N E E E E E E H E E E S E E K
           GGAGAAAATG AGGAGGAAGA GGAAGAACAT GAAGAGGAAA GTGAAGAGAA
151 R G L L S F L P K V I G V I G H
           GAGAGGTCTG CTCAGCTTTT TACCAAAAGT GATTGGAGTT ATAGGTCACC
201 L I H P P S *
           TGATTCACCC TCCAAGTTAA

```

Figure 7.11: continues next page.



**Figure 7.11:** Nucleotide sequences of the cDNA clones encoding the open-reading frame of (a) preprofallaxidin-1 containing fallaxidins 1.1 (2 copies), 1.2, 1.3 and 2.2; (b) preprofallaxidin-2 containing fallaxidins 1.3 (2 copies), 1.4 and 2.1; (c) fallaxidin 2; (d) preprofallaxidin-3 containing fallaxidins 1.1 (3 copies), 1.2, 1.3 and 2.3; (e) preprofallaxidin-4 containing fallaxidins 2.2, 4.1 (2 copies) and 4.3; (f) fallaxidin 3.1; (g) preprofallaxidin-5 containing fallaxidins 2.2 and 4.1 (2 copies); (h) fallaxidin 3.2; and (i) fallaxidin 4.2; cloned from the skin secretion of *L. fallax*. Putative signal peptide sequences are double-underlined, mature fallaxidin sequences are single-underlined and the stop codon is indicated by \*.

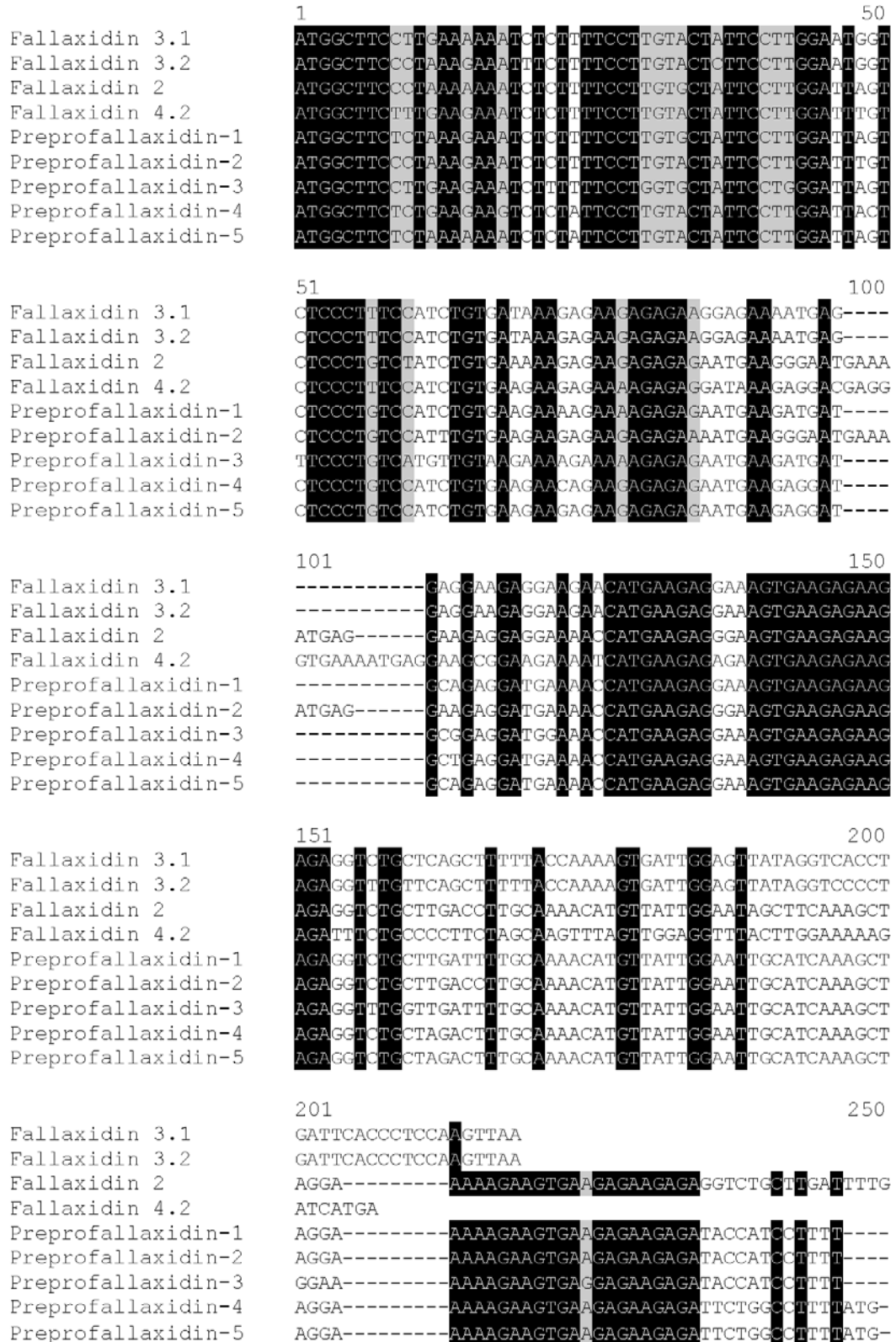


Figure 7.12: continues next page.



```

1                                     50
Fallaxidin 3.1  MASLKKSLFLVLFLGMVSLSDKREKREGE-----NEEEEEHHEESEEK
Fallaxidin 3.2  MASLKKFLFLVLFLGMVSLSDKREKREGE-----NEEEEEHHEESEEK
Fallaxidin 2    MASLKKSLFLVLFLGLVSLSDKREKRENE--NEEEENHEEGSEEK
Fallaxidin 4.2  MASLKKSLFLVLFLGFVSLSDKREKREDEGENEAAEENHEERSEEK
Preprofallaxidin-1 MASLKKSLFLVLFLGLVSLSDKREKRENE-----DDAEDENHEESEEK
Preprofallaxidin-2 MASLKKSLFLVLFLGFVSLSDKREKRENE--NEEEDENHEEGSEEK
Preprofallaxidin-3 MASLKKSFVLVFLGLVSLSMCEEKRENE-----DDAEDGNHEESEEK
Preprofallaxidin-4 MASLKKSLFLVLFLGLVSLSDKREKRENE-----EDAEDENHEESEEK
Preprofallaxidin-5 MASLKKSLFLVLFLGLVSLSDKREKRENE-----EDAEDENHEESEEK

51                                     100
Fallaxidin 3.1  RGLLSFLPKVIGVIGHLIHPPS*
Fallaxidin 3.2  RGLFSFLPKVIGVIGPLIHPPS*
Fallaxidin 2    RGLLDLAKHVIGIASKLG---KRSEEKRGLLDFAKHVIGIASKLGK*
Fallaxidin 4.2  RFLPLLASLVGGLLKRS*
Preprofallaxidin-1 RGLLDFAKHVIGIASKLG---KRSEEKYHPF-----GKRSE
Preprofallaxidin-2 RGLLDLAKHVIGIASKLG---KRSEEKYHPF-----GKRSE
Preprofallaxidin-3 RGLVDFAKHVIGIASKLG---KRSEEKYHPF-----GKRSE
Preprofallaxidin-4 RGLLDFAKHVIGIASKLG---KRSEEKFWPFM-----GKRSE
Preprofallaxidin-5 RGLLDFAKHVIGIASKLG---KRSEEKFWPFM-----GKRSE

101                                     150
Preprofallaxidin-1 EKRYFPIPEGKRSEEKRYFPIPIG----KRSEEKYFPIPIGK
Preprofallaxidin-2 EKRYHP--FGKRSEEKRY-P-PIG----K*
Preprofallaxidin-3 EKRYFPIPEGKRSEEKRYFPIPIG----KRSEEKYFPIPIGKRSEEK
Preprofallaxidin-4 EKRFWPFM-GKRSEEKRFRVLAKLGKLA*
Preprofallaxidin-5 EKRFWPFM-GKRSE*

151
Preprofallaxidin-3 YFPIPIGK*

```

**Figure 7.13:** Alignment of translated open-reading frames of the fallaxidin precursor cDNA clones. Identical amino acid residues are boxed in black. The peptides sequences are underlined.

## 7.3 Discussion

### 7.3.1 Hybrid

Interspecific breeding of two species of Australian amphibians has produced five healthy adult female hybrids. The extreme rarity of the healthy interspecies breeding in captivity, suggests that it is likely that the hybrids were produced from a single mating. The parents were identified as a female *Litoria splendida* and a male *L. caerulea* from mitochondrial DNA and morphological analysis [376].

Comparisons of gene expression between interspecific hybrids and their parental taxa have illustrated that differences in expression [564], misexpression [564, 565] and novel gene expression patterns [565] can occur in first generation hybrids. In order to establish whether altered regulation of expression has occurred in the hybrid offspring, direct comparison of DNA data for the hybrid and parents is necessary. Despite knowledge of the gender and identification of species of each parent, the identity of the individual animals is not known. As a consequence, a direct comparison of DNA data is not possible. A comparison of the cDNA data of the precursor of the major antimicrobial caerin 1.1 that is expressed by the hybrid and both parent species provides some insight into their genetic origins.

The preprocaerin 1.1 sequences were similar in all cases (Figure 7.6). In total, only 19 sites were variable among the 246 aligned nucleotides of the caerin 1.1 precursor for the hybrid and parent species. Of these variable sites, only 7 resulted in a change in the amino acid residue (5 out of 49 amino acid sites varied). It is interesting that clones with two different sequences for preprocaerin 1.1 were identified for an individual hybrid animal, whilst only one sequence has been identified among clones for each of the parent species *L. splendida* [372] and *L. caerulea* [25]. This is consistent with, but does not alone prove, interspecies breeding as the offspring may inherit a different DNA sequence from each parent.

Without any knowledge of the underlying inheritance of the skin peptides, at least two mechanisms can explain the occurrence of two distinct precursor clones for caerin 1.1 in the hybrid offspring: (i) a single locus containing two or more different alleles that encode for caerin 1.1 in each of the parent species, creating heterozygosity of the caerin 1.1

precursor in the hybrid. Segregation of these alleles could have led to the hybrid offspring inheriting a distinct allele for preprocaerin 1.1 from each parent species; (ii) more than one locus could be present in both parent species, containing alleles that encode for caerin 1.1. Inheritance of these different loci that are slightly divergent could result in the occurrence of two different precursor clones in the hybrid. However, at present there are not enough data to determine which locus is associated with which particular species. An additional complication would be polymorphism at each locus in one or both parent species.

The mostly likely explanation is the first, however, the information available at present does not allow rigorous distinction between the two explanations. Polymorphism is likely as extensive variation has been reported in the peptide profile of *L. caerulea* among different geographical populations [27, 372, 566] and it is probable that the male *L. caerulea* parent did not come from the same population as the animal studied by Vanhoye *et al.* [25]. In order to unequivocally establish the possible explanation, extensive cDNA and breeding experiments are required. However, due to the death of the hybrid offspring, this is not possible, and as a result, extensive cross breeding experiments with wild *L. splendida* and *L. caerulea* animals would need to be carried out.

## 7.3.2 Evolutionary Significance

### 7.3.2.1 *Crinia riparia*

A common feature of the disulfide peptides from *Crinia* and the antibiotic disulfide-containing peptides from *Rana* species from the northern hemisphere is the presence of a similar disulfide linkage at the C-terminal of each peptide. This poses the question, did these two types of peptides evolve from a common gene or have they evolved from different ancestral precursor genes? It has been shown that despite the wide variation present in the sequences and activities of peptides from American and European *Rana* species and Australian hylids [25, 372], the signal regions are largely conserved.

Alignment of the inferred amino acid sequences of the riparin 1.4 precursors with the several prepropeptide sequences from American and European *Rana* species and hylid frogs from Australia (*Litoria*) and South America (*Agalychnis*, *Phyllomedusa* and

*Pachymedusa*) (Figure 7.14) shows that riparin 1.4 has little sequence homology with the ranid and hylid precursor sequences. Similarly, the signal region displays only minimal homology with only 5 of the 22 aligned residues sharing identity with those of the preprodermaseptin type peptides (Figure 7.14). Both the acidic spacer region and the active peptide sequences of the riparin 1 peptides show virtually no sequence similarity with those of the ranid and hylid prepropeptides illustrated in Figure 7.14.

Recent comprehensive molecular phylogenetic analyses of the relationships among all anuran families illustrated that the myobatrachids (which include the *Crinia* genus) are the sister lineage to a hylid group. In addition, the ranids are a more distant relative of both groups [567]. The lack of sequence homology overall and the low similarity in the signal region (which is relatively conserved among ranid and hylid frogs [25]) suggests that either the riparin 1 precursors: (i) have converged to a similar structure and function as the ranid and hylid prepropeptides which were initially lost from the myobatrachid lineage, or (ii) that the prepropeptides from all three groups were derived from a single ancestral gene that has remain relatively conserved in the ranoid and hylid lineages but has undergone significant divergent evolution in the myobatrachids. At present the results obtained in this study are not sufficient to determine which of the two explanations is the more likely.

### 7.3.2.2 *Litoria fallax*

The precursor cDNAs cloned from *L. fallax* has lead to the identification of fourteen fallaxidin peptides from nine difference precursor clones, with four of these peptides not identified in the skin peptide profile. The open reading frame of the preprofallaxidin cDNAs encoding the putative signal peptide and the first three residues of the acidic spacer peptide domain, share about 50 to 90 % identity with the corresponding regions of the antimicrobial peptide cDNAs from other amphibian species (Figure 7.14). These antimicrobial peptides include caerin, esculatin, brevinin, temporin and gaegurin cDNAs, to mention a few. The comparison of the inferred amino acid sequences of these genes encoding the antimicrobial peptides suggests that they have arisen from a common ancestral origin in the early stages of amphibian evolution. The duplication and recombination events that have promoted the association of such a homologous secretory exon with genes encoding for a variety of end products in diverse amphibian species remains to be explained, but it is most likely to have occurred at the very early stages of



species radiation [51]. This is believed to have occurred some 150 million years ago [25] and is consistent with the known behaviour of Gondwanaland which dispersed to result in the isolation of Australasia around 100 to 90 million years ago [568].

The conserved preproregion of the dermaseptin type precursors has striking similarities with the precursors of preprodermorphin [407] and preprodeltorphins [512]. These precursors encode for multiple copies of the opioid peptides dermorphin and deltorphin, which were isolated from the skin of *Phyllomedusa* species. While the C-terminal of the precursors of these peptides vary from those of the antimicrobial peptides, comparison of the N-terminal signal and acidic propeptides has indicated that at the amino acid level, there are large similarities [11, 510]. This is also evident in the precursor sequences of the fallaxidin peptides isolated from *L. fallax*. The precursors of fallaxidin peptides encode for a variety of biological peptides, yet the N-terminal signal and acidic preproregion is highly conserved. This suggests that the highly conserved dermaseptin type precursor preproregion may be present in genes that correspond to amphibian peptides with numerous biological functions, not just antimicrobial peptides. This is consistent with the proposal that the topogenic sequence of the conserved signal peptide may help to ensure that the correct proteolytic maturation of the precursor or targeting of the mature peptide to the secretory granules occurs [510].

	Pre	Pro	Peptide
Brevinin-2Ef	MFTMKKSLLLIFFLGTISLSLCEEE-RNADDDDG-----EMT--EEE-KR--GIMDTLK--NLAK--TAG-----KGALQSLVKM-ASCKLSGQC--		
Gaegurin-4	MFTMKKSLFLFFLGTISLSLCEEE-RTADEDDGG----EMT--EEEVKR--GILDTLK--QFAK--GVGKDLV-----KGAAQGVST-VSCKLAKTC--		
Esculentin-1B	MFTLKKPLLLIVLLGMISLCEEE-RNADEEEG-----SEIKR--GIFSKLAGKLLKNLLISGLKNV--GKEVGMDDVVRTGIDIAGCKIKGEC--		
Ranalexin	MFTLKKSLLLFFLGTINLSLCEEE-RNAEEE-RRDNP-DER-DVEVEKRFLGGL-----I-----KIVPAMI-----CAVTKKC--		
Temporin-1Ska	MFTLKKSLLLFFLGTINLSLCEEE-RNAEEE-RRDDP-EER-DVEVEKRFLP-----VILP----VIGKLL-----NGILG----		
Nigrocin-2S	MFTLKKSIALLFFLGTINLSLCEEE-TNAEEE-RRDE---EVAKMEEIKR--GILSGILG--AGK-----SLV-----CGLSGLC--		
Caerin 1.1	MASLKKSLFLVLLLVSVSICEEEKR-QEDEDEHEEEEGESQEEGSEEKR--GLLSVLGS--VAKH--VLPHVVP-----VIAEHLG----		
Fallaxidin 3.1	MASLKKSLFLVFLGMVSLSDICEKRE-GEN-EEEE-EH-EESEEKR--GLLSFLP----K--VIG-----VIGHLIHPPS--		
Aurein 2.2	MAFLKKSFLVFLGLVSLSDICEKEKR-QNEDEDENEAANHEEGSEEKR--GLFDIVK----K--VVG-----ALGSLG----		
Frenatin 1.1	MAFLKKSFLVFLGLVSLSDICEKEKKE-QEDEDENEEKESEEGSEEKR--GLLDTLG-----GILG-----LGR----		
Dermaseptin	MAFLKKSFLVFLFLAIVPLSDICEEEKRE-EENEKQED----DDQSE-KR--GLVSGL-----LNTAGG-----LLGDLLGSLGSLSGGES--		
DRP-AC-1	MAFLKKSLLLVFLGLVSLSDICEEEKRE-NEDEEKQED----DDQSENKR--GLLSGI-----LNTAGG-----LLGNLIG---SLSNGES--		
PBN2	MAFLKKSFLVFLFLALVPLSDICEE-KK-SEEENEKQED---D-QSEEKR--GLVTSL-----IK--GAG-----K---LLGGLFG---SVTGGQS--		
Dermatoxin	MAFLKKSFLVFLGLVPLSDICESEKRE-GENEEQED----D-QSEEKRSLSFLKGVG-TTLA---SVG-----K---VVSDQFG--KLLQAGQG--		
Phylloxin	MVFLKKSLLLVFLVGLVSLSDICEENKRE--EHEEIEEN---KEKAEKR--GWMSKI---AS---GIG-----T---FLSGMQQ-----G----		
Riparin 1.4	MKIIV--VLAVLM--LVSA---QVCLVSAEMGHSSDNELSSRDV--VKR-----FFLPPCAYKGTG--		

**Figure 7.14:** Inferred amino acid sequence alignment of hyloid and ranid preprodermaseptins, fallaxidin 3.1 and riparin 1.4. Gaps (-) have been introduced to maximise sequence similarities. See Table 7.1 for references.

## 7.4 Experimental

The specimens of adult hybrid females were bred from a captive population of Magnificent Tree Frog (*L. splendida*) and Common Green Tree Frog (*L. caerulea*) and provided by M. and H. Vaux (Brama Lodge, South Australia, Australia) [372]. Specimens of Streambank Froglet (*C. riparia*) were obtained in the Flinders Ranges, several hundred kilometres north of Adelaide in South Australia [44, 47]. The froglets were kept in captivity for the duration of the study. Eastern Dwarf Tree Frog (*L. fallax*) specimens were provided from a private captive population by Stuart Blackburn (Modbury, South Australia, Australia).

### 7.4.1 Secretion Harvesting

The skin secretions were obtained from the dorsal skin glands by gentle transdermal electrical stimulation by the technique of Tyler *et al.* [83]. In brief, the animals were held by their hind legs and the skin was washed with sterile MQ water twice. The dorsal skin glands were stimulated using a bipolar electrode of 21 G platinum connected to a Palmer Student Model electrical stimulator (C.F. Palmer, London, Ltd. Myographic Works, London, UK). The electrodes were rubbed on the skin in a circular motion, using 10 V at a 5 ms pulse rate for a duration of 20 – 30 s (hybrid). For *C. riparia* and *L. fallax* specimens, 10 V at a pulse rate of 3 ms and 2 V at a pulse rate of 4 ms were used respectively. The induced secretions were washed using sterile MQ water and immediately snap-frozen in liquid nitrogen and lyophilised. The samples were stored at –80 °C prior to analysis.

Secretions were obtained with the assistance of Assoc. Prof. Michael J. Tyler (School of Environment and Earth Sciences, The University of Adelaide, Australia). The procedure was approved by the University of Adelaide Animal Ethics Committee. No animals were harmed during the procedure.

### 7.4.2 Cloning of Precursor cDNA from Lyophilised Skin Secretion

All of the chemical preparations used in these studies that were not supplied in the kits, were prepared by the Central Services Unit, Molecular and Biomedical Sciences, the University of Adelaide, unless indicated otherwise.

### 7.4.2.1 mRNA Extraction

3 mg of the lyophilised skin secretion was dissolved in 550  $\mu$ L of lysis/RNA stabilisation buffer (DynaL Biotech, UK). The polyadenylated (polyA<sup>+</sup>) mRNA was isolated using magnetic Oligo-dT beads as described by the manufacturer (DynaL Biotech, UK) and subject to reverse transcription procedures (see Section 7.4.2.2).

### 7.4.2.2 cDNA synthesis

The cDNA was synthesised by reverse transcription PCR (BD SMART RACE cDNA Amplification Kit, BD Bioscience Clontech, USA). The 3'-RACE Ready product used a 5' primer (5'-(T)<sub>25</sub>VN-3')<sup>6</sup>, whilst the 5'-RACE Ready product used a modified lock-docking 3' primer (5'-AAGCAGTGGTATCAACGCAGAGTAC(T)<sub>30</sub>VN-3')<sup>6</sup> and BD SMART IIA oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3') with two degenerate nucleotide positions at the 3' end to position the primer at the start of the polyA<sup>+</sup> tail and eliminate 3' heterogeneity [569]. The reaction employed the manufacturers conditions.

### 7.4.2.3 Polymerase Chain Reaction

The resultant cDNA was subjected to 3'-RACE Ready procedures to acquire full-length precursor nucleic acid sequence data using a SMART-RACE kit (BD Biosciences Clontech, USA). The 3'-RACE Ready reactions used a UPM primer (supplied in the kit) (long: 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3' and short: 5'-CTAATACGACTCACTATAGGGC-3') and a sense primer (Geneworks, South Australia, Australia) (see Table 7.4 for sense primers for the individual species). The sense primer for the hybrid and *L. fallax* was designed from the 5'-untranslated region of cDNAs previously cloned from *L. caerulea* skin (EMBL Accession: AY218778-AY218782) [25]. Since the sequences of the 5'-untranslated region of cDNAs of similar species within the *Crinia* genus were not known, a reverse primer designed from the partial sequence of riparin 1.2, 1.4 and 1.5 (PCAXKGTC) was used. The sense primer was then

---

<sup>6</sup>Mixed Base codes: N = A,C,G or T; V = A, G or C

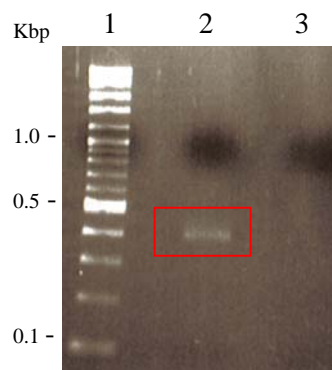
designed complementary to a segment of the 5'-untranslated region as determined from the reverse primer.

**Table 7.4:** Primers used for the amphibian cDNA amplification using 3'-RACE Ready reactions.

Amphibian	Primer*
Hybrid	S1: 5'-GVCTTGTAAGACCAAVCATG-3'
<i>Crina riparia</i>	S2: 5'-GGTTCTTTTGAGAAAAAGGARTCATGAAAATCATTG-3' R1: 3'-RCAGGTTCCYTTTRTRAGCRCAYGG-5'
<i>Litoria fallax</i>	S6: 5'-ATGGCTTCYYTRAARAARTCT-3'

\*Mixed base codes: R (AG), Y (CT), M (AC), K (GT), S (GC), W (AT), H (ACT), B (GCT), V (AGC), D (AGT) and N (AGCT).

The polymerase chain reaction (PCR) cycling procedure was employed using a polymerase mix that contained *Taq* DNA polymerase under the following conditions: initial denaturation step at 94 °C for 60 s, 30 cycles of denaturation at 94 °C for 30 s, primer annealing for 30 s, and extension at 72 °C for 180 s and a finish extension at 72 °C for 10 min. Primer annealing temperature of 50 °C was used for all amphibian species with the exception of the hybrid, where an annealing temperature of 56 °C was used. The PCR products were identified by gel electrophoresis (2 % agarose gel) (eg. Figure 7.15), followed by gel purification of any identified bands.



**Figure 7.15:** Gel electropherogram of PCR product from 3'-RACE reaction of the hybrid DNA using a sense primer S1. Lane 1 contains 100 bp increment nucleotide calibration ladder, 2-log ladder; Lane 2 contains 400 bp cDNA fragment (boxed in red); and Lane 3 contains non-template control. For the full scale of marker, refer to Figure C1.

#### 7.4.2.4 Gel Electrophoresis

The DNA integrity of the PCR products was determined by separation on agarose gel using TBE running buffer (0.09 M Tris base, 0.09 M boric acid, 2.5 mM EDTA, pH 8.3). The agarose gel concentration (in TBE buffer) varied from 1 - 3 % depending on the size of the DNA. The PCR products and DNA loading dye (10x, 50 % (v/v) glycerol, 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF) were loaded into the gel and electrophoresed at 100 V for 30 min. The DNA was visualised by incubating the gel in GelRed (nucleic acid gel stain) (Biotium Inc. USA) for 10 min and viewing the gel using short wave UV. Photographs were taken.

#### 7.4.2.5 Purification of PCR Products

The PCR products were gel purified by separation of products using gel electrophoresis. The PCR products and DNA loading dye were loaded into a 1.5 % agarose gel and electrophoresed at 80 V for 40 min. The gel was stained in GelRed for 10 min and viewed using long wave UV. The desired band of DNA was cut out of the gel using a sterile scalpel. The DNA was then purified from the agarose gel using a QIAquick Gel Extraction kti (Qiagen) as per manufacturers instructions.

#### 7.4.2.6 Cloning of PCR Fragments

The purified PCR products were cloned using the pGEMT-Easy vector system (Promega) (Figure 7.16) and transformed into competent DH5 $\alpha$  *E. coli* cells and grown on agar containing ampicillin. The ligase reaction was set up using T4 DNA ligase and pGEMT-Easy vector and the standard protocol described by the manufacturer (Promega). The ligation reaction (10  $\mu$ L) was added to 100  $\mu$ L of competent DH5 $\alpha$  cells and incubated on ice for 5 min. The cell mixture was then heat shocked at 42 °C for 2 min and placed on ice for 5 min. The cells were plated onto *Luria-Bertani* (LB) agar plates containing ampicillin and incubated overnight at 37 °C.

## NOTE:

This figure is included on page 249 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 7.16:** Bacterial plasmid pGEMT Easy Vector circle map and sequence reference points. Amp<sup>r</sup> represents the ampicillin resistance gene. The position of the restriction enzyme sites is indicated in the boxes on the right [570].

After overnight incubation, several colonies were selected and grown overnight in LB media (per L: 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.5) containing ampicillin (100 µg.mL<sup>-1</sup>). The plasma was extracted using a QIAprep Spin MiniPrep kit (Qiagen). The purified plasmids were digested with EcoR1 (New England BioLabs Inc.) to determine the presence of cloned DNA inserts of the expected sizes (400 – 600 bp). The positive plasmids were sequenced using BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems, USA), a RPS primer (5`-CACACAGGAAACAGCTATGACCATC-3`) and the 30 cycles of the following: 96 °C for 30 s, 50 °C for 15 s and 60 °C for 240 s. Precipitation of the resultant sequencing products was achieved by use of the ethanol/EDTA/sodium acetate method (BigDye Terminator V3.1 Cycle Sequencing kit, Applied Biosystems, USA) and sequenced using an ABI 3700 DNA Analyzer automated sequencer (Microbiology Department of the Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia).

The nucleotide chromatogram of the cDNA sequences was visualised using Chromas 1.45. The cDNA sequences were translated into amino acid sequences using the ExPASy (Expert Protein Analysis System) proteomics server (Swiss Institute of Bioinformatics, Switzerland) translation tools. The signal spacer connectivities were determined using the PSORT II prediction program (<http://psort.nibb.ac.jp/>). Sequences were aligned using the multiple sequence alignment tool ClustalW2 (EMBL-EBI tools, European Molecular Biology Laboratory) [571].

### 7.4.3 Preparation of DH5 $\alpha$ Competent Cells

An overnight culture of DH5 $\alpha$  cells was prepared in 2 mL of LB media. A subculture of 330  $\mu$ L was placed in 10 mL of LB media and incubated for 1.5 to 2 hrs at 37 °C until the  $A_{600\text{nm}}$  reached approximately 0.6. A subculture of 5 mL was placed into pre-warmed LB media and incubated at 37 °C for 1.5 hrs. The culture was placed on ice for 5 min and centrifuged (4000 rpm, 4 °C, 5 min). The supernatant was discarded, the cells resuspended in 40 mL of transformation buffer I (30 nM potassium acetate, 100 mM RbCl, 10 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 50 mM  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 15 % glycerol; pH 5.8, filtered sterile) and placed on ice for 5 min. The cells were then centrifuged (4000 rpm, 4 °C, 5 min), the supernatant discarded and resuspended in 4 mL of transformation buffer II (10 mM MOPS, 10 mM RbCl, 75 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 15 % glycerol; pH 6.5, filtered sterile). This solution was placed on ice for 5 min, aliquoted and stored at  $-80$  °C.



## Chapter 8 Summary

### 8.1 *Litoria fallax*

The glandular skin secretion of the Eastern Dwarf Tree Frog *Litoria fallax* contains ten peptides named fallaxidins. cDNA cloning identified these peptides together with four novel peptides that were not detected in the peptide profile. Among the peptides from the skin secretion are: (i) several small peptides, fallaxidins 1 and 4.1. The activities of these peptides are unknown, but it has been shown that they are not smooth muscle active, opioids or antimicrobially active, nor do they effect proliferation of lymphocytes; (ii) two weakly active antimicrobial agents, fallaxidins 2; and (iii) a moderate antimicrobial agent, fallaxidin 3.1.

Fallaxidin 3.1 has an unusual sequence for an amphibian antimicrobial agent, containing three Pro residues together with a C-terminal free acid. The modified fallaxidin 3.1(NH<sub>2</sub>) shows increased antimicrobial activity against Gram-positive organisms, in addition to demonstrating inhibition of nNOS with an IC<sub>50</sub> value of 15.4 μM. The improved activity is attributed to an increase in positive charge, as no difference was observed in the overall secondary structure of the acid and amide in aqueous TFE. The 3D structure of fallaxidin 3.1(NH<sub>2</sub>) was determined in DPC micelles. The peptide adopts a helical like structure about its length with a disruption in the central region due to the presence of Pro and Gly residues periodically 3 to 4 residues apart. It is possible that the disruption in the central region imparts some flexibility to the peptide, which may contribute to its ability to interact with the bacterial membrane.

The skin peptide profile of *L. fallax* is unique in comparison with other *Litoria* species studied thus far. *L. fallax* does not secrete any peptides that display strong wide spectrum antimicrobial activity, or potent inhibition of nNOS. Additionally, there are several small peptides with unknown biological function present as major components of the secretion. However, the peptides from *L. fallax* do display some sequence similarities to the peptides of two Groupings of the genus *Litoria*, namely the *Litoria citropa* Group and the *Litoria rubella* Group [14, 27]. cDNA cloning has suggested that the fallaxidin peptides originated

from the same ancestor gene as the caerins from *L. caerulea* and *L. splendida* (Section 8.4).

## 8.2 Dahlein 5 Peptides and the Binding of Calmodulin

Numerous peptides that inhibit the formation of NO by inhibiting nNOS have been isolated from amphibian skin secretions. It is believed that these peptides bind to the enzyme regulatory cofactor CaM, resulting in a conformational change in CaM that subsequently prevents it from associating with nNOS, thus inhibiting the enzyme. The most active of the dahlein 5 peptides, dahlein 5.6 isolated from *L. dahlii* inhibits nNOS at concentrations of 1.6  $\mu\text{M}$  with a Hill slope of 2.1. As CaM-binding peptides have been shown to adopt amphipathic  $\alpha$  helices upon binding, the solution structure of dahlein 5.6 was investigated to determine whether this peptide fits this model. The solution structure of dahlein 5.6 was determined in membrane mimicking solvents, aqueous TFE and DPC micelles and shown to adopt a bent amphipathic  $\alpha$  helix, thus fitting the model.

To confirm that the inhibitory effect of dahlein 5.6 was due to the enzyme cofactor CaM, MS studies were completed. ESI-MS confirmed the presence of a dahlein 5.6-CaM complex in the gas-phase. A non-covalent assembly was formed with a 1:1:4 CaM/dahlein 5.6/ $\text{Ca}^{2+}$  stoichiometry. NMR investigations were used to explore this complex further.  $^{15}\text{N}$ - $^1\text{H}$  HSQC spectra of titrations were performed by subsequent additions of increasing concentrations of unlabelled dahlein 5.6 to  $^{15}\text{N}$ -labelled  $\text{Ca}^{2+}$  CaM. It was found that the complex formed with a slow exchange binding regime and a 1:1 stoichiometry. Significant chemical shift changes were observed for a large proportion of residues throughout CaM, suggesting that the protein undergoes considerable conformational change upon binding dahlein 5.6. Furthermore, dahlein 5.6 was labelled with  $^{15}\text{N}$  at amide positions of residues Leu3, Gly7, Phe10, Ala15 and Leu18 and shown to adopt random structures in aqueous solutions. The interaction of  $^{15}\text{N}$ -labelled dahlein 5.6 and  $\text{Ca}^{2+}$  CaM was monitored by  $^{15}\text{N}$ - $^1\text{H}$  HSQC titration. Distinct spectral differences were seen for all of the labelled residues, suggesting that the entire length of the peptide is directly involved in formation of the complex with  $\text{Ca}^{2+}$  CaM. In addition, the chemical shift changes for the labelled residues suggest that upon binding to the protein, dahlein 5.6 undergoes a conformational transformation from a random structure to an  $\alpha$  helix.

### 8.3 Amphibian Neuropeptides

The granular secretion of amphibians contains numerous peptides that exert activities in the CNS, termed neuropeptides. The biological activities, in particular the smooth muscle activity, proliferation of lymphocytes and opioid activities were investigated to provide some insight into the role of these peptides in the host defence of the animal. Several host defence peptides from *Crinia* species were shown to act as neuropeptides; signiferin 1 was found to contract smooth muscle from  $10^{-9}$  M and effect proliferation of lymphocytes at  $10^{-6}$  M, while riparins 1.1 and 1.2 were also found to effect proliferation of lymphocytes, but did not contract smooth muscle. The activity of the disulfide peptides was mediated through CCK<sub>2</sub> receptors.

A major component of the skin secretion of *L. rothii* in the summer months, rothein 1 was found to exert proliferation of lymphocytes, but was inactive in smooth muscle assays. Another major secretion component of *L. rothii* is caerulein 1.2, which was shown to contract smooth muscle with similar potency to CCK-8-NS and effect proliferation of lymphocytes, exclusively through CCK<sub>2</sub> receptors. These two peptides together, provide defence for the animal in the summer against both predators and pathogens.

Caerulein 1.2 was also isolated from *L. citropa*, along with a series of related peptides, including the ubiquitous amphibian neuropeptide caerulein. The series of related caerulein peptides, showed a variety of potencies in the contraction of smooth muscle, providing a structure activity relationship indicating that: (i) the length of the chain from the sulfated Tyr residue to the C-terminal amide; and (ii) the presence of Trp, fourth residue from the C-terminal (WXDF-NH<sub>2</sub>) were both important for receptor recognition. Furthermore, substitution of the Met (third residue X from C-terminal) with Phe resulted in a decrease in activity.

The tryptophyllin peptides are among one of the greatest mysteries surrounding the components of amphibian skin secretions. The biological activity of many tryptophyllins is unknown, however here, two tryptophyllin peptides, tryptophyllin L 1.2 and [Arg<sup>4</sup>]tryptophyllin L 3.1, were found to inhibit electrically stimulated GPI through a naloxone-sensitive pathway (opioid receptors) at modest potencies in the range of  $10^{-6}$  M. It was shown that a long aliphatic side chain on the C-terminal residue was essential for

opioid receptor recognition and binding. The activation of opioid receptors by these tryptophyllins would result in numerous activities in the CNS to regulate physiological conditions and defend against predators.

## 8.4 Evolutionary Significance of Amphibian Prepropeptides

Host defence peptides from the granular secretions of anurans are synthesized within and released from larger precursor molecules. The cDNA sequences of the genes that encode for the skin peptides of several *Litoria* species and *Crinia riparia* were isolated and identified to provide information about the relationships existing among these species with other anuran species and to trace the evolution of the amphibian peptides.

The cDNA sequence for the caerin 1.1 precursor was isolated from an individual hybrid animal formed as a result of interspecies breeding of *L. splendida* and *L. caerulea*. Clones with two different sequences for preprocaerin 1.1 were identified in the hybrid, whilst only one sequence has been previously identified among clones for each of the parent species [25, 372], a feature that is consistent with interspecies breeding. The occurrence of the two distinct precursor clones for caerin 1.1 in the hybrid offspring can be explained as a result of the presence in each parent species of either a single locus containing two or more alleles that encode for caerin 1.1 or more than one locus containing alleles for caerin 1.1. However, at present there is insufficient data to allow rigorous distinction between the two explanations.

Nine distinct precursor sequences that encoded for fourteen fallaxidin peptides were isolated from *L. fallax*. These precursors encode for peptides with a variety of biological functions, yet the N-terminal signal and acidic spacer regions are highly conserved. A comparison of the inferred amino acid sequence of these conserved regions of the precursors with the corresponding regions of previously isolated antimicrobial peptide cDNAs from other amphibian hylid and ranid species has indicated a high degree of sequence homology. This suggests that these precursors have arisen from a common ancestral origin in the early stages of amphibian evolution.

The four distinct precursor clones isolated from *C. riparia* showed a high degree of sequence similarity among the clones. In contrast, the sequences of the skin peptide

precursors from *C. riparia* are significantly diverse in comparison to other precursor cDNAs isolated from various hylid and ranid species. This suggests that these preproriparins either originated from the same common ancestral but have undergone substantial divergent evolution relative to the hylid and ranid frogs or that they have originated from distinct ancestral genes.

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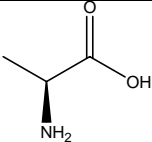
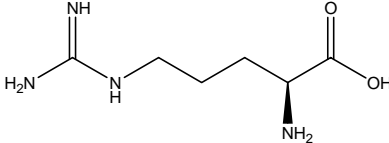
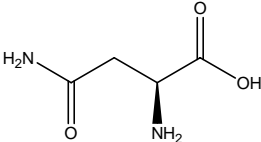
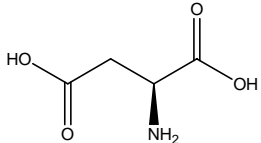
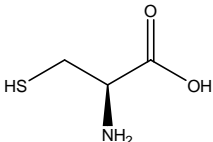
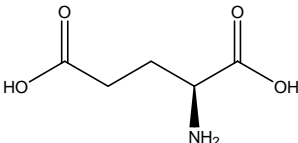
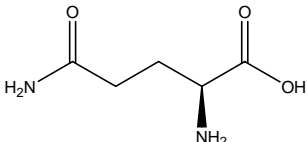
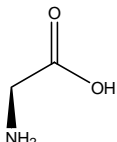
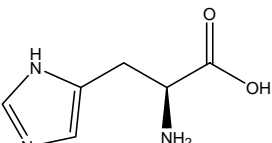
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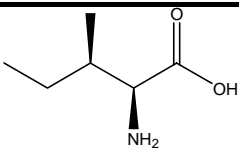
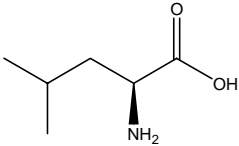
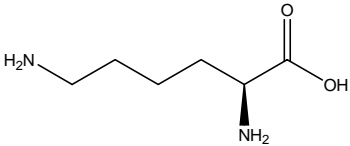
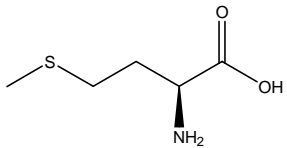
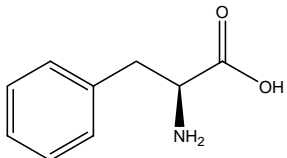
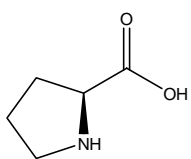
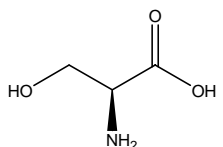
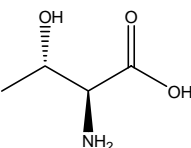
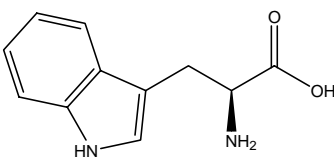
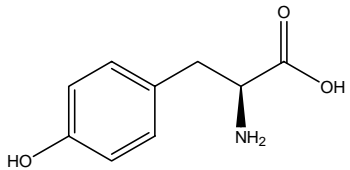
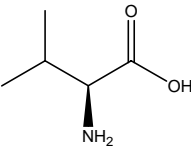
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# Appendix A

## Twenty Common Amino Acids

**Table A1:** The structure and nominal mass of the twenty common amino acids.

Amino Acid	Structure	Nominal Mass
Alanine Ala A		71
Arginine Arg R		156
Asparagine Asn N		114
Aspartic Acid Asp D		115
Cysteine Cys C		103
Glutamic Acid Glu E		129
Glutamine Gln Q		128
Glycine Gly G		57
Histidine His H		137

Amino Acid	Structure	Nominal Mass
Isoleucine Ile I		113
Leucine Leu L		113
Lysine Lys K		128
Methionine Met M		131
Phenylalanine Phe F		147
Proline Pro P		97
Serine Ser S		87
Threonine Thr T		101
Tryptophan Trp W		186
Tyrosine Tyr Y		163
Valine Val V		99

**Table A2:** Characteristic negative ion fragmentations within side chains of amino acid residues from the  $[M-H]^-$  ions in small peptides.

<b>Amino Acid</b>	<b>Loss (or formation)</b>	<b>Mass</b>
Ala	Me $\cdot$	15
Val	Pr $\cdot$	43
Leu (Ile)	Bu $\cdot$	57
Phe	PhCH $_2^-$	91
Tyr	<i>p</i> -HOC $_6$ H $_4$ CH $_2^-$	107
	O=C $_6$ H $_4$ =CH $_2$	106
Trp	C $_9$ H $_7$ N	129
<b>Ser</b>	<b>CH<math>_2</math>O</b>	<b>30</b>
<b>Thr</b>	<b>MeCHO</b>	<b>44</b>
Cys	H $_2$ S	34
Met	MeSH	48
	MeSMe	62
	CH $_2$ CH $_2$ SMe	75
<b>Asp</b>	<b>H<math>_2</math>O</b>	<b>18</b>
<b>Asn</b>	<b>NH<math>_3</math></b>	<b>17</b>
<b>Glu</b>	<b>H<math>_2</math>O</b>	<b>18</b>
<b>Gln</b>	<b>NH<math>_3</math></b>	<b>17</b>
Arg	NH=C=NH	42

## Appendix B

### Secondary Structure Determination of Fallaxidin 3.1

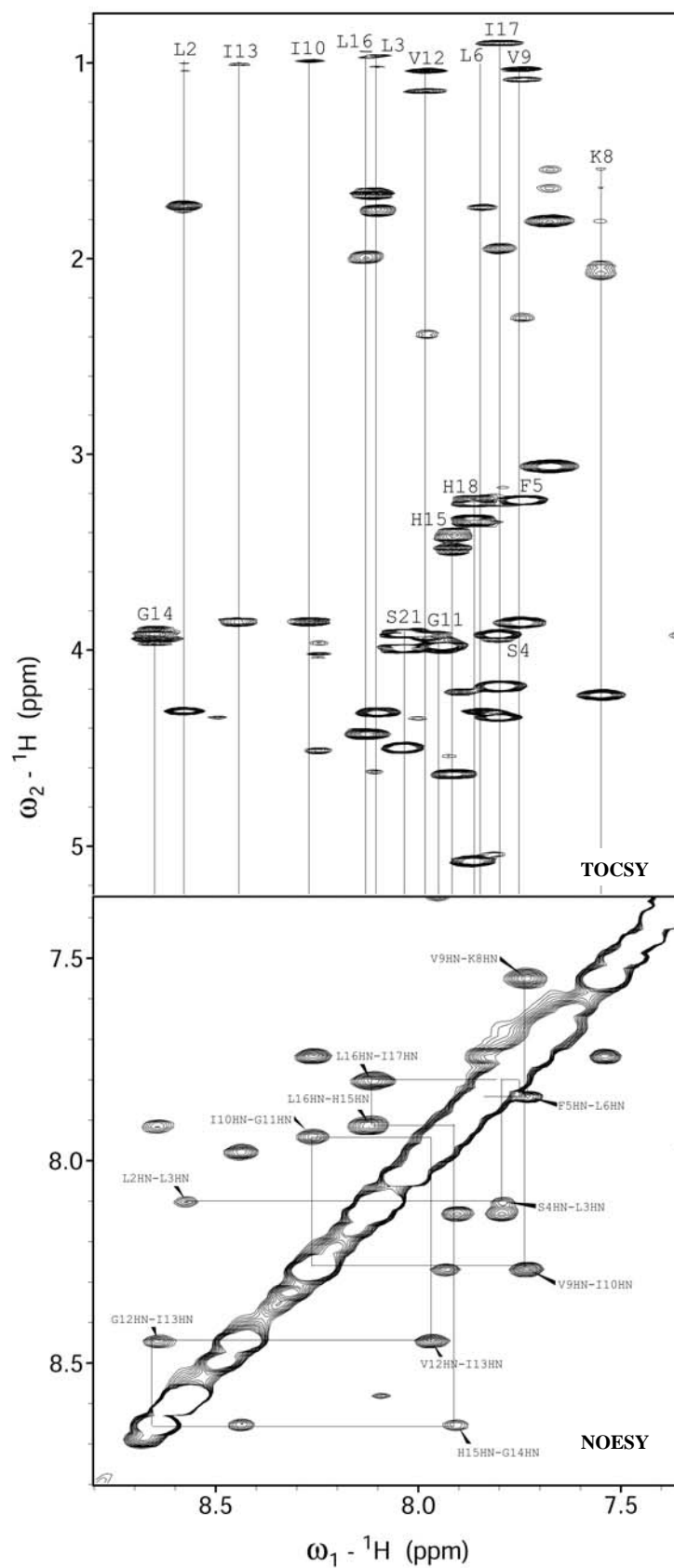
**Table B1:**  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts for fallaxidin 3.1 in TFE/H<sub>2</sub>O (1:1 v/v), pH 2.10, 25 °C. n.o. indicates that the resonance was not observed.

Residue	Chemical Shift				
	HN	$\alpha\text{H}$	$\beta\text{H}$	Other H	$\alpha^{13}\text{C}$
Gly1	n.o.	4.04, 3.93	-	-	n.o.
Leu2	8.58	4.32	2.01, 1.87	$\gamma\text{-CH}$ 1.74 $\delta\text{-CH}_3$ 1.05, 1.01	55.76
Leu3	8.10	4.33	1.76	$\gamma\text{-CH}$ 1.68 $\delta\text{-CH}_3$ 1.02, 0.97	55.27
Ser4	7.80	4.35	3.93	-	58.97
Phe5	7.74	4.80	3.24	H 2,6 7.30 H 3,5 7.34 H 4 7.39	58.27
Leu6	7.84	4.32	2.42, 2.20	$\gamma\text{-CH}$ 1.74 $\delta\text{-CH}_3$ 1.02, 0.97	56.73
Pro7	-	4.32	2.45, 2.01	$\gamma\text{-CH}_2$ 2.19, 2.10 $\delta\text{-CH}_2$ 3.77, 3.59	64.73
Lys8	7.56	4.24	2.07	$\gamma\text{-CH}_2$ 1.65, 1.55 $\delta\text{-CH}_2$ 1.81 $\varepsilon\text{-CH}_2$ 3.07 $\varepsilon\text{-NH}_3^+$ 7.67	57.59
Val9	7.74	3.87	2.31	$\gamma\text{-CH}_3$ 1.09, 1.04	64.06
Ile10	8.26	3.86	1.95	$\gamma\text{-CH}_2$ 1.76, 0.87 $\gamma\text{-CH}_3$ 0.99 $\delta\text{-CH}_3$ 0.87	63.46
Gly11	7.94	3.99	-	-	n.o.
Val12	7.97	3.94	2.39	$\gamma\text{-CH}_3$ 1.15, 1.04	65.16
Ile13	8.44	3.86	2.06	$\gamma\text{-CH}_2$ 1.66, 1.35 $\gamma\text{-CH}_3$ 1.01 $\delta\text{-CH}_3$ 0.90	n.o.

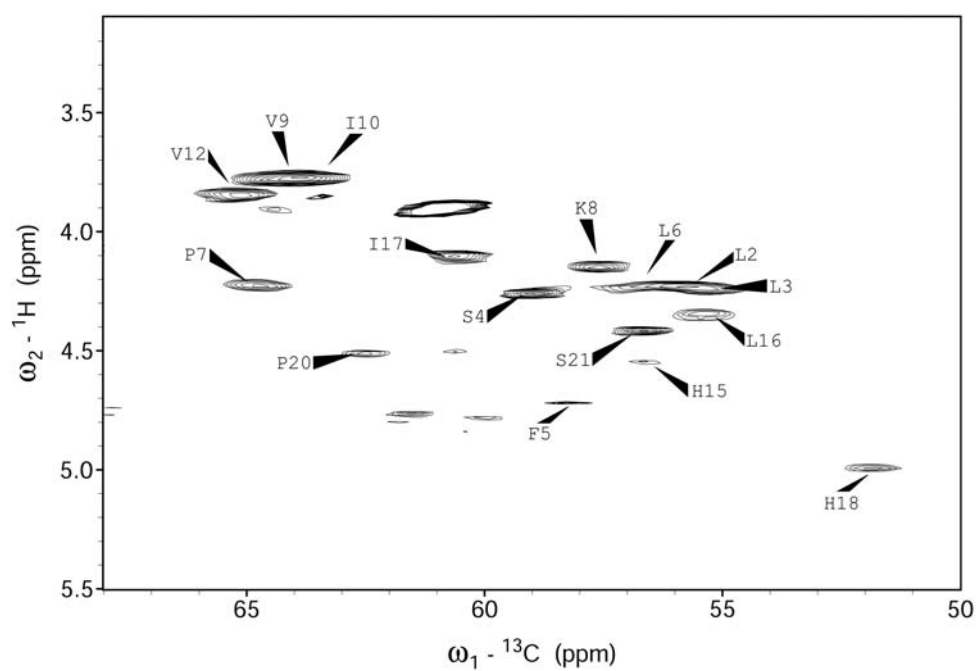
Table B1: continues.

Residue	Chemical Shift				
	HN	$\alpha$ H	$\beta$ H	Other H	$\alpha^{13}\text{C}$
Gly14	8.67	3.95, 3.93	-	-	n.o.
His15	7.91	4.64	3.49, 3.42	H 2 7.45 H 4 8.60	56.65
Leu16	8.12	4.44	2.00, 1.89	$\gamma$ -CH 1.68 $\delta$ -CH <sub>3</sub> 0.94	55.37
Ile17	7.80	4.19	1.96	$\gamma$ -CH <sub>2</sub> 1.48, 1.33 $\gamma$ -CH <sub>3</sub> 0.99 $\delta$ -CH <sub>3</sub> 0.90	60.60
His18	7.86	5.08	3.34, 3.25	H 2 7.36 H 4 8.60	51.86
Pro19	-	4.80	2.45, 2.07	$\gamma$ -CH <sub>2</sub> 2.11 $\delta$ -CH <sub>2</sub> 3.80, 3.61	n.o.
Pro20	-	4.60	2.40	$\gamma$ -CH <sub>2</sub> 2.14 $\delta$ -CH <sub>2</sub> 3.90, 3.74	62.49
Ser21	8.04	4.51	4.00, 3.92	-	56.68



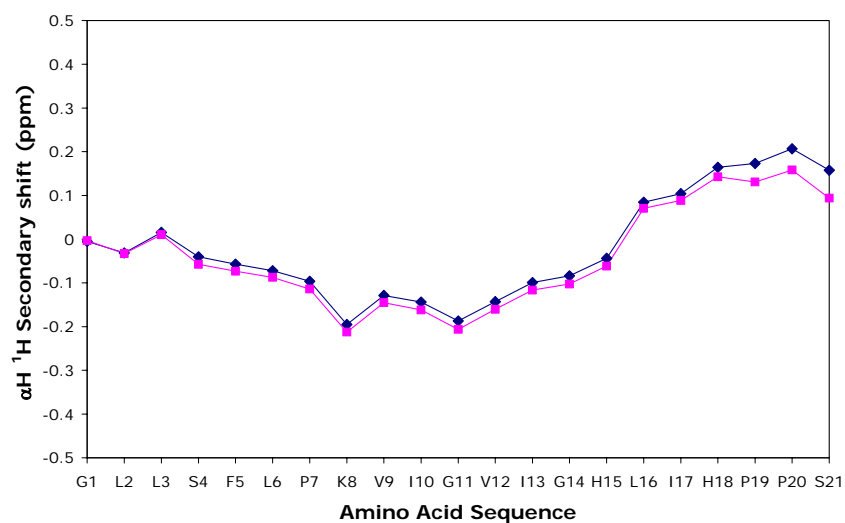


**Figure B1:** Partial TOCSY and NOESY spectra of fallaxidin 3.1 in TFE/H<sub>2</sub>O (1:1 v/v). In the TOCSY spectrum, vertical lines connect resonances in the same spin system. NOEs between sequential amide protons are indicated in the NOESY spectrum.

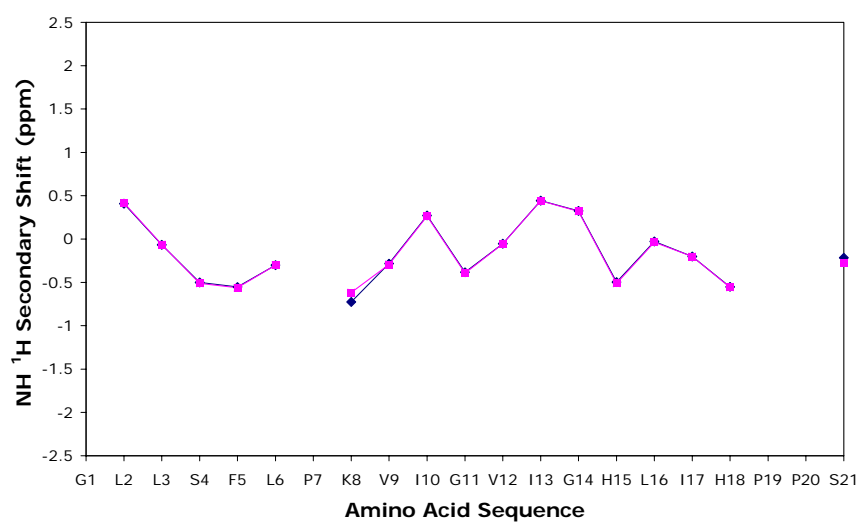


**Figure B2:** Partial  ${}^{13}\text{C}$ - ${}^1\text{H}$  HSQC spectrum of fallaxidin 3.1 in TFE/ $\text{H}_2\text{O}$  (1:1 v/v). The  $\alpha\text{H}$   ${}^1\text{H}/\alpha\text{C}$   ${}^{13}\text{C}$  connectivities for the residues are indicated.

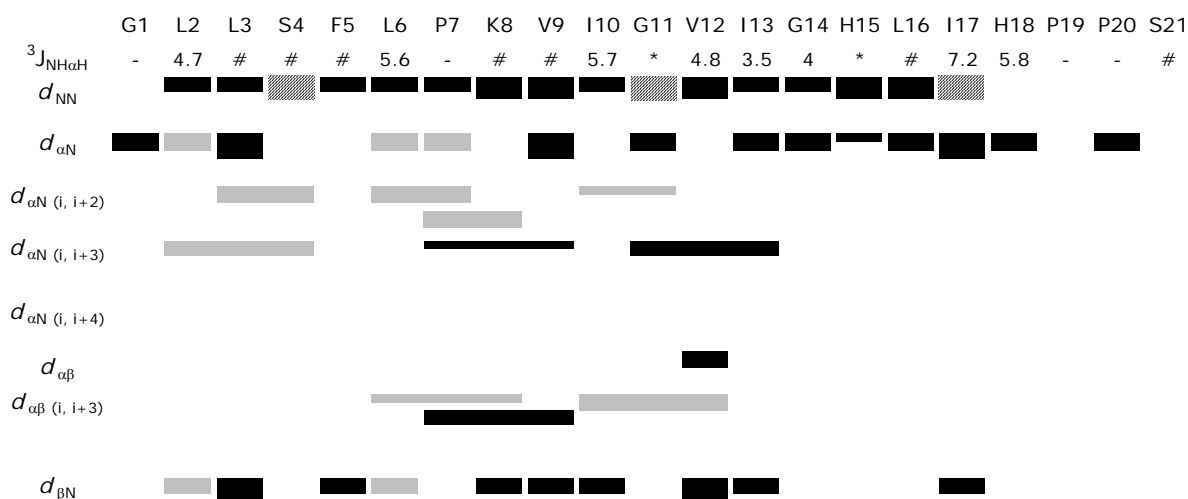
(a)



(b)



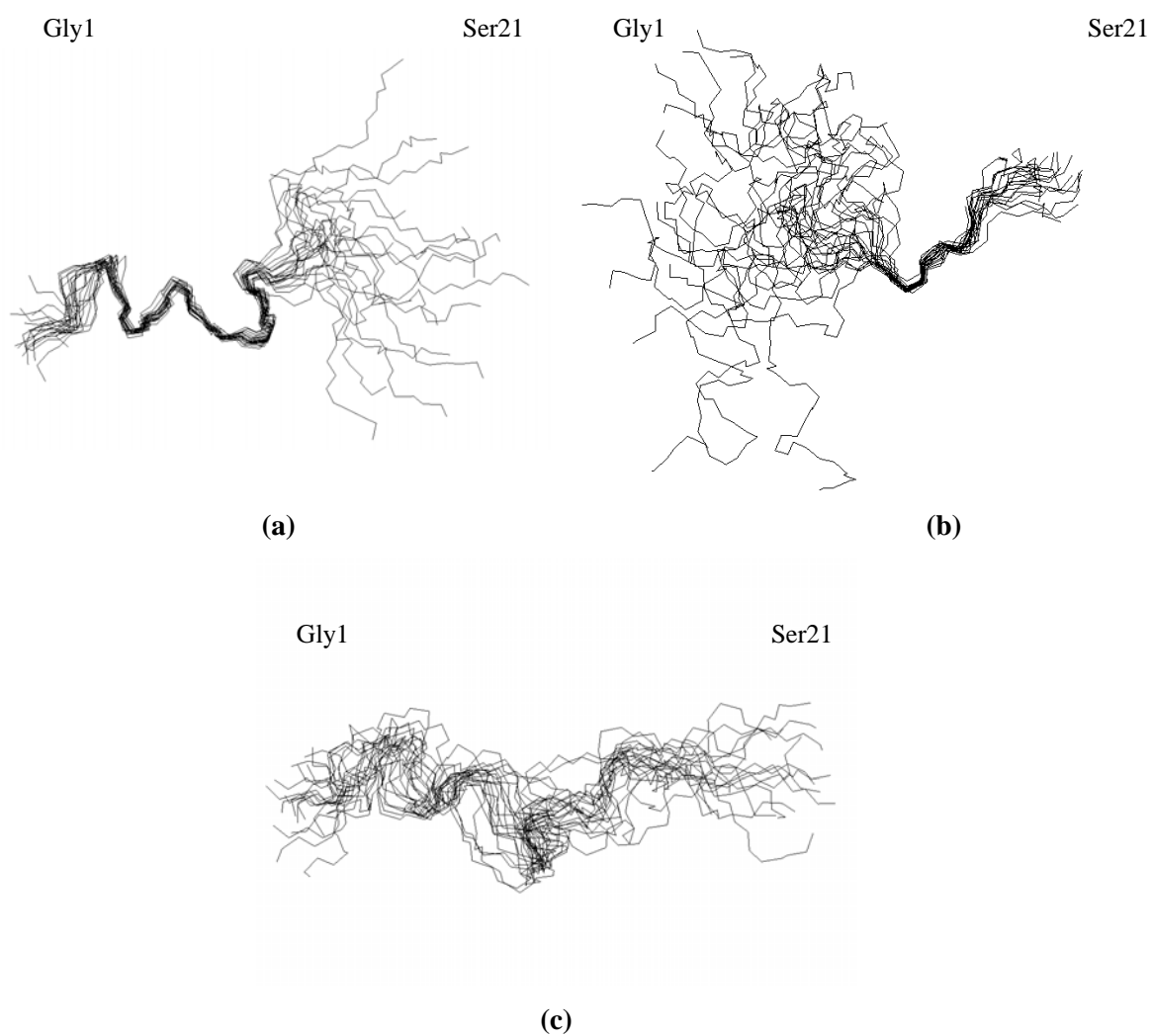
**Figure B3:** (a) The smoothed ( $n = \pm 2$  residues)  $\alpha\text{H } ^1\text{H}$  and (b)  $\text{NH } ^1\text{H}$  secondary shifts of fallaxidin 3.1 (blue) and fallaxidin 3.1(NH<sub>2</sub>) (pink) in TFE/H<sub>2</sub>O (1:1 v/v). Negative values indicate an upfield shift from the random coil values and positive values indicate a downfield shift.



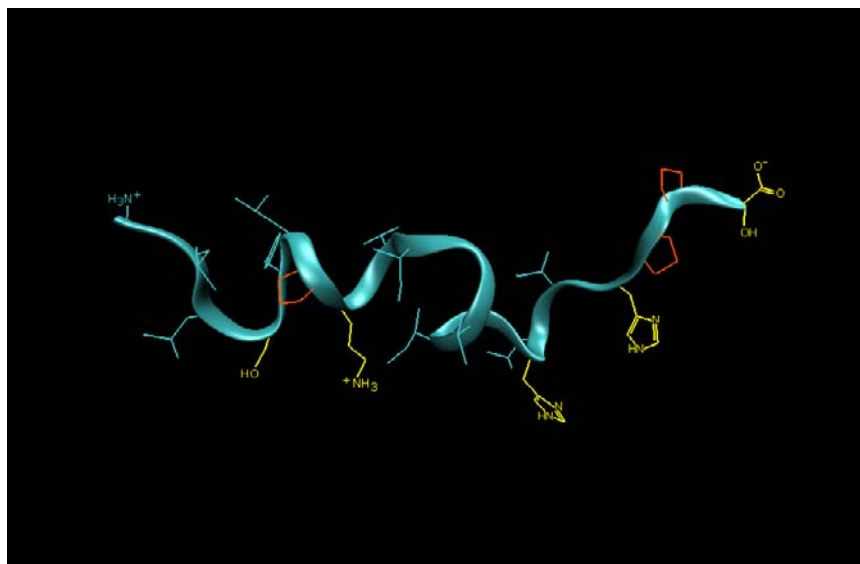
**Figure B4:** A summary of the diagnostic NOEs used in the structure calculations for fallaxidin 3.1 in TFE/H<sub>2</sub>O (1:1 v/v). In Pro where no amide proton is present, NOEs to the δH are shown. The thickness of the bar indicates the relative strength of the NOE (strong < 3.1 Å, medium 3.1 – 3.7 Å, weak > 3.7 Å). Grey shaded bars represent ambiguous NOEs. <sup>3</sup>J<sub>NHαH</sub> values are indicated where applicable. # indicates coupling constants that were not resolved due to signal overlap, \* indicates no coupling constant was detected.

**Table B2:** The experimental distance restraints obtained from the NOESY spectrum of fallaxidin 3.1 in TFE/H<sub>2</sub>O (1:1 v/v).

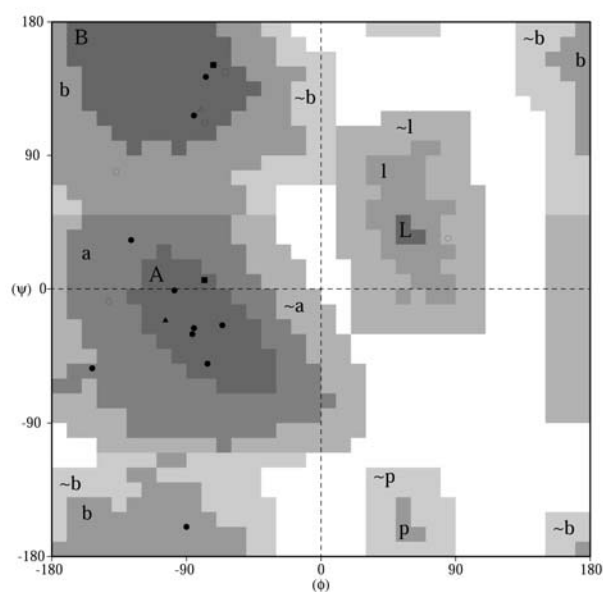
	No. of Experimental Restrains
Sequential NOEs	46
Medium-range NOEs	15
Long-range NOEs	-
Intra-residue NOEs	87
Ambiguous NOEs	37
Total	187



**Figure B5:** The twenty most stable calculated structures of fallaxidin 3.1 in TFE/H<sub>2</sub>O (1:1 v/v). The structures have been superimposed over the backbone atoms of the well-defined (a) residues 5 – 13 and (b) residues 16 – 19 and (c) superimposed over the entire backbone.



**Figure B6:** The lowest calculated potential energy structure of fallaxidin 3.1 in TFE/H<sub>2</sub>O (1:1 v/v). Yellow indicates residues that are hydrophilic in nature. Pro residues are shown in red.



**Figure B7:** Ramachandran plot for the well-defined residues of fallaxidin 3.1 in TFE/H<sub>2</sub>O (1:1 v/v). Favourable regions are labelled A and B for  $\alpha$  helical or  $\beta$  strand structures respectively, while allowable and generous regions are labelled a and b or  $\sim$ a and  $\sim$ b.

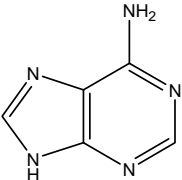
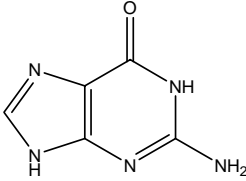
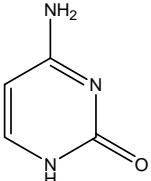
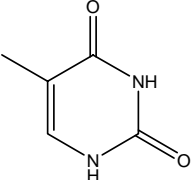
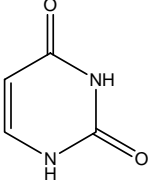
**Table B3:** The structural statistics for the twenty lowest energy structures of fallaxidin 3.1 in TFE/H<sub>2</sub>O (1:1 v/v) following RMD/SA calculations.

<b>Energies (kcal mol<sup>-1</sup>)</b>	<b>TFE/H<sub>2</sub>O</b>
E <sub>total</sub>	19.873 ± 0.659
E <sub>bond</sub>	0.592 ± 0.059
E <sub>angle</sub>	8.437 ± 0.334
E <sub>improper</sub>	0.254 ± 0.038
E <sub>vdm</sub>	10.424 ± 0.376
E <sub>NOE</sub>	0.161 ± 0.098
E <sub>cdih</sub>	0.004 ± 0.007
<b>Well defined residues</b>	5-13, 16-19
<b>RMSD from mean geometry (Å)</b>	
Heavy atoms of well-defined residues (5-13)	0.839 ± 0.269
Backbone atoms of well-defined residues (5-13)	0.335 ± 0.127
Heavy atoms of well-defined residues (16-19)	1.183 ± 0.353
Backbone atoms of well-defined residues (16-19)	0.327 ± 0.169
All heavy atoms	3.339 ± 1.262
All backbone atoms	2.839 ± 1.193
<b>Number of violations &gt; 0.3 Å</b>	4
Maximum violation (Å)	0.523

# Appendix C

## Nucleic Acids

**Table C1:** The structure of the nucleic acids.

Nucleic Acid <sup>1</sup>	Structure
<b>Purines</b>	
Adenine A	
Guanine G	
<b>Pyrimidines</b>	
Cytosine C	
Thymine <sup>2</sup> T	
Uracil <sup>3</sup> U	

**<sup>1</sup>Mixed Base Codes:**

**R** = A or G; **Y** = C or T; **M** = A or C; **K** = G or T; **S** = G or C; **W** = A or T;  
**H** = A, C or T; **B** = G, C or T; **V** = A, G or C; **D** = A, G or T; **N** = A, G, C or T

<sup>2</sup> Present in DNA

<sup>3</sup> Present in RNA



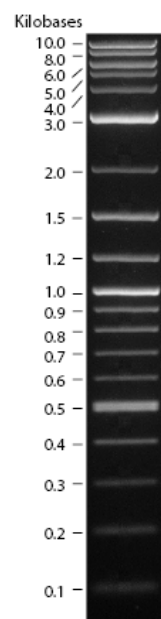
**Table C2:** The Genetic Code. The table shows the 64 codons and the corresponding amino acids for each. The direction of the mRNA is 5' to 3'.

	<b>T</b>			<b>C</b>			<b>A</b>			<b>G</b>		
<b>T</b>	TTT	F	Phe	TCT	S	Ser	TAT	Y	Tyr	TGT	C	Cys
	TTC	F	Phe	TCC	S	Ser	TAC	Y	Tyr	TGC	C	Cys
	TTA	L	Leu	TCA	S	Ser	TAA	*	Stop	TGA	*	Stop
	TTG	L	Leu	TCG	S	Ser	TAG	*	Stop	TGG	W	Trp
<b>C</b>	CTT	L	Leu	CCT	P	Pro	CAT	H	His	CGT	R	Arg
	CTC	L	Leu	CCC	P	Pro	CAC	H	His	CGC	R	Arg
	CTA	L	Leu	CCA	P	Pro	CAA	Q	Gln	CGA	R	Arg
	CTG	L	Leu	CCG	P	Pro	CAG	Q	Gln	CGG	R	Arg
<b>A</b>	ATT	I	Ile	ACT	T	Thr	AAT	N	Asn	AGT	S	Ser
	ATC	I	Ile	ACC	T	Thr	AAC	N	Asn	AGC	S	Ser
	ATA	I	Ile	ACA	T	Thr	AAA	K	Lys	AGA	R	Arg
	ATG	M	Met <sup>#</sup>	ACG	T	Thr	AAG	K	Lys	AGG	R	Arg
<b>G</b>	GTT	V	Val	GCT	A	Ala	GAT	D	Asp	GGT	G	Gly
	GTC	V	Val	GCC	A	Ala	GAC	D	Asp	GGC	G	Gly
	GTA	V	Val	GCA	A	Ala	GAA	E	Glu	GGA	G	Gly
	GTG	V	Val	GCG	A	Ala	GAG	E	Glu	GGG	G	Gly

<sup>#</sup> Codon ATG both codes for methionine and serves as an initiator (start codon)

\* Stop codon – serves as a terminator

**Figure C1:** The 2-log ladder DNA marker for gel electrophoresis. The size of the DNA band is indicated in kilobases.



# Publications

## Publications Related To Ph.D. Thesis

Pukala, T.L., Bertozzi, T., Donnellan, S.C., Bowie, J.H., Surinya-Johnson, K.H., Lui, Y., Jackway, R.J., Doyle, J.R., Llewellyn, L.E. and Tyler, M.J. (2006) Host-defence peptide profiles of the skin secretions of interspecific hybrid tree frogs and their parents, female *Litoria splendida* and male *Litoria caerulea*, *FEBS J.* 273, 3511-3519

Jackway, R.J., Pukala, T.L., Maselli, V.M., Musgrave, I.F., Bowie, J.H., Liu, Y., Surinya-Johnson, K.H., Donnellan, S.C., Doyle, J.R., Llewellyn, L.E. and Tyler, M.J. (2008) Disulfide-containing peptides from the glandular skin secretions of froglets of the genus *Crinia*: Structure, activity and evolutionary trends, *Regul. Pept. in press.*

Doyle, J.R., Llewellyn, L.E., Pukala, T.L., Jackway, R.J. and Bowie, J.H. (2008) Anuran host-defence peptides which inhibit the synthesis of nitric oxide by neuronal nitric oxide synthase. In: *Bioactive Peptides* (Howl, J., ed) CRC Press, *in press.*

Bowie, J.H., Jackway, R.J., Separovic, F., Carver, J.A. and Tyler, M.J. (2008) Host-defence peptides from the secretion of the skin glands of frogs and toads: Membrane-active peptides from the genera *Litoria*, *Uperoleia* and *Crinia*. In: *Bioactive Peptides* (Howl, J., ed) CRC Press, *in press.*

Pukala, T.L., Urathamakul, T., Watt, S.J., Beck, J.L., Jackway, R.J. and Bowie, J.H. (2008) Binding studies of nNOS-active amphibian peptides and Ca<sup>2+</sup> calmodulin, using negative ion ESI mass spectrometry, *Rapid Commun. Mass Spectrom.* *submitted for publication.*

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Jackway, R.J., Pukala, T.L., Maselli, V.M., Musgrave, I.F., Bowie, J.H., Liu, Y., Surinya-Johnson, K.H., Donnellan, S.C., Doyle, J.R., Llewellyn, L.E. and Tyler, M.J. (2008) Disulfide-containing peptides from the glandular skin secretions of froglets of the genus *Crinia*: Structure, activity and evolutionary trends, *Regul. Pept. in press*.

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