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Peptides 25 (2004) 1035–1054
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Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance

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Received 11 December 2003; received in revised form 10 March 2004; accepted 11 March 2004

Available online 10 May 2004

Abstract

Host-defence peptides secreted from the skin glands of Australian frogs and toads, are, with a few notable exceptions, different from those produced by anurans elsewhere. This review summarizes the current knowledge of the following classes of peptide isolated and characterized from Australian anurans: neuropeptides (including smooth muscle active peptides, and peptides that inhibit the production of nitric oxide from neuronal nitric oxide synthase), antimicrobial and anticancer active peptides, antifungal peptides and antimalarial peptides. Other topics covered include sex pheromones of anurans, and the application of peptide profiling to (i) recognize particular populations of anurans of the same species and to differentiate between species, and (ii) investigate evolutionary aspects of peptide formation.

Keywords: Australian amphibians; Host-defence peptides; Bioactive peptides; Pheromones; Skin secretions; NMR; Mass spectrometry; Evolution

1. Introduction

Amphibians have rich chemical arsenals that form an integral part of their defence systems, and also assist with the regulation of dermal physiological action. In response to a variety of stimuli, host-defence compounds are secreted from specialized glands onto the dorsal surface and into the gut of the amphibian. Many of these peptides exhibit either potent vasodilator or antimicrobial activity [6,8,34,45]. Such peptides are secreted from the skin glands of metamorph and adult animals [25] but in at least one species (*Litoria splendida*) it has been shown that tadpoles contain the same host-defence peptides as the adult [103].

During the past decade we have isolated and identified peptides from the secretions of skin glands of 35 species of Australian frogs and toads from the genera *Litoria, Uperoleia*, *Limnodynastes*, *Cyclorana* and *Crinia*. The dorsal glands are best illustrated by *L. splendida* (Fig. 1: large parotoid and rostral glands on the head) [5], and *Litoria caerulea* (Fig. 2: granular glands over the whole dorsal surface) [5]. We obtain the secretions by electrical stimulation of the glands on the dorsal skin. This process, illustrated in Fig. 3, may be repeated at monthly intervals and does not harm the amphibians [95]. It is not unusual to be able to identify all of the major bioactive peptides using just one secretion from one animal. We have identified neuropeptides, membrane active peptides which exhibit antimicrobial, anticancer and sometimes antifungal activity, antimalarial peptides, and peptides which inhibit the formation of nitric oxide (NO) from neuronal nitric oxide synthase. Many of these peptides show multifaceted activity. The best examples of this are the caerin 1 peptides of Australian tree frogs of the genus *Litoria*, of which caerin 1.1 is an example (sequence shown below).

Caerin 1.1 GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2

A major peptide of the glandular secretions of the tree frogs *L. splendida* and *L. caerulea*, has two amphipathic helices separated by a central flexible hinge region [109]. This peptide is a wide spectrum antibiotic (mainly against

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doi:10.1016/j.peptides.2004.03.006

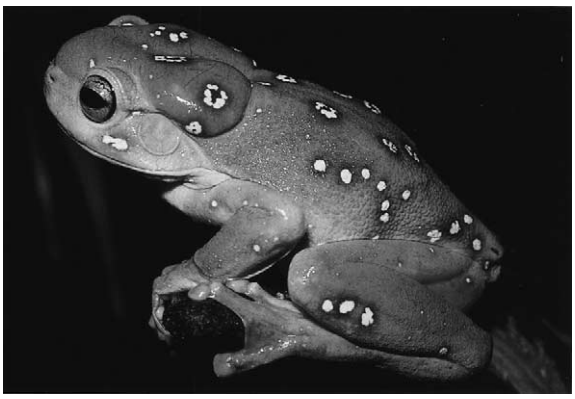


Fig. 1. *Litoria splendida*.

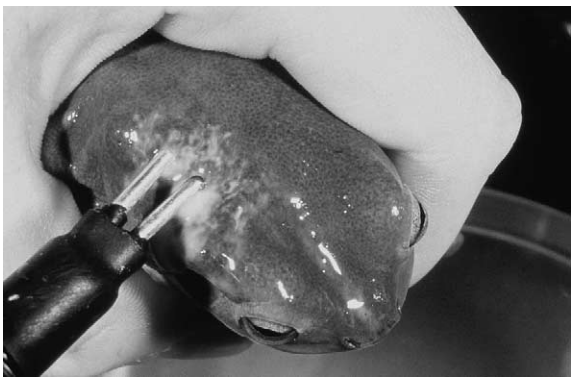


Fig. 3. Milking *Litoria caerulea*.

gram positive organisms but also against some gram negative organisms (MIC 1–100 μg/ml range)), shows IC_{50} in the 10^{-6} M range against all the major human cancer types, is active against some viruses (HIV and Herpes simplex 1 (MIC 20 and 24 μg/ml, respectively)), kills nematodes (at concentrations of 10^{-6} M), is active against the malaria parasite *Plasmodium falciparum* (MIC 10 μg/ml) and inhibits the formation of NO from neuronal nitric oxide synthase at an IC_{50} concentration of 37 μM. Caerin 1.1 lyses red blood cells at >250 μg/ml, a concentration greater than that required for the activities listed above. Some of these bio-activities are probably in excess of requirement as far as anurans are concerned, but tree frogs certainly use caerin 1 peptides as antimicrobials, and they may well use them to control the number of nematodes in the gut. Although there is currently little malaria in Australia, some anurans (e.g. species of the genus *Rana* [76]), are prone to infestation by *Plasmodium*, and caerin 1.1 can certainly deal with these parasites (for *L. caerulea* and *L. splendida*) if required to do so.

An earlier review [15] concentrated on antimicrobially active peptides from Australian anurans; this review will deal with all of the various types of peptide, and their activities, summarized above. In addition, the review will deal with (a) peptide sex pheromones of amphibians, and (b) the use of peptide profiling to assist with (i) the identification of different species of anuran, (ii) the identification of different populations of the same species, and (iii) the investigation of evolutionary trends of amphibians. The review will concentrate principally on the unique peptides of Australian anurans: bioactive peptides from anurans elsewhere have been dealt with by others [6,8,34,45]. The sequences of all peptides discussed in this article are listed (in alphabetical order of trivial names) in Table 1.

Fig. 2. *Litoria caerulea*.

2. Structure determination

2.1. Primary structure determination

Several methods have been utilized to determine the primary structure of the various host-defence peptides isolated from these secretions. These methods involve complementary use of mass spectrometry and automated Edman sequencing.

2.2. Positive ion mass spectrometry

The sequencing of peptides using positive ion mass spectrometry (MS) has been standard for some time and has been described extensively [9]. We have predominately used the B and Y + 2 fragmentations to elucidate the primary structure of peptides. The information provided by these fragmentations is summarized in Scheme 1. Briefly, B fragmentations provide sequencing information from the C-terminal end of the peptide while Y + 2 fragmentations provide sequencing information from the N-terminal end. In addition, the first B cleavage ion can be used to identify whether the peptide is a free carboxylic acid (loss of 18 Da from the MH^+ species) or has been post-translationally modified to the amide (loss of 17 Da from the MH^+ species). For other positive ion fragmentations of peptides, see Ref. [9].

MS coupled with enzymic digestion of the peptide can provide sequence information. For example, isobaric Lys and Gln are distinguished using Lys-C digestion. This enzyme cleaves at the C-terminal end of Lys.

Table 1
Alphabetical listing of selected amphibian peptides

Name	Sequence	MW	Species	Activity*
Aurein 1.1	GLFDIIKKIAESI-NH_2	1444	a	1, 2
Aurein 1.2	GLFDIIKKIAESF-NH_2	1478	a	1, 2
Aurein 2.1	GLLDIVKKVVGAFGSL-NH_2	1613	a	1, 2
Aurein 2.2	GLFDIVKKVVGALGSL-NH_2	1613	a	1, 2, 4
Aurein 2.3	GLFDIVKKVVGAIGSL-NH_2	1613	a	1, 2, 4
Aurein 2.4	GLFDIVKKVVGTLAGL-NH_2	1630	a	1, 2, 4
Aurein 2.5	GLFDIVKKVVGAFGSL-NH_2	1647	a	1, 2
Aurein 3.2	GLFDIVKKIAGHIASSI-NH_2	1766	a	1, 2
Aurein 4.1	GLIQTIKEKLKELAGGLVTGIQS-OH	2394	a	
Caeridin 1.1	GLLαDGLLGTGL-NH_2	1140	b, c, d, e, f	
Caeridin 1.2	GLLβDGLLGTGL-NH_2	1140	d	
Caeridin 1.4	GLLαDGLLGGLGL-NH_2	1096	e, f	
Caeridin 1.5	GLLβDGLLGGLGL-NH_2	1096	e, f	
Caeridin 2	GLLDVVGNLLGGLGL-NH_2	1408	c, d	
Caeridin 3	GLFDAIGNLLGGLGL-NH_2	1428	c, d	
Caeridin 4	GLLDVVGNVLHSGL-NH_2	1504	c	
Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	2582	b, c, d	1, 2, 3, 4
Modification 1	GLLSVLGSVAKHVL**G**HVV**G**VIAEHL-NH_2	2502		1, 2
Modification 2	GLLSVLGSVAKHVL**A**HVV**A**VIAEHL-NH_2	2530		
Caerin 1.1.1	LSVLGSVAKHVLPHVVPVIAEHL-NH_2	2412	d	
Caerin 1.1.2	SVLGSVAKHVLPHVVPVIAEHL-NH_2	2299	d	
Caerin 1.1.3	VLPHVVPVIAEHL-NH_2	1420	b, c, d	
Caerin 1.1.5	GLLSVLGSVAKHVLPH-OH	1625	b, c, d	
Caerin 1.3	GLLSVLGSVAQHVLPHVVPVIAEHL-NH_2	2582	c	1, 2
Caerin 1.4	GLLSSLGSVAKHVLPHVVPVIAEHL-NH_2	2600	c, d	1
Caerin 1.5	GLLSVLGSVVKHVIPHVVPVIAEHL-NH_2	2610	c	1, 2
Caerin 1.6	GLFSVLGAVAKHVLPHVVPVIAEKL-NH_2	2591	e, f	1, 2, 4
Caerin 1.7	GLFKVLGSVAKHLLPHVAPVIAEKL-NH_2	2634	e, f	1, 2
Caerin 1.8	GLFKVLGSVAKHLLPHVVPVIAEKL-NH_2	2662	f	1, 2, 3, 4
Caerin 1.9	GLFGVLGSIAKHVLPHVVPVIAEKL-NH_2	2591	f	1, 2, 3, 4
Caerin 1.10	GLLSVLGSVAKHVLPHVVPVIAEKL-NH_2	2573	b	1, 2, 3, 4
Caerin 1.11	GLLGAMFKVASKVLPHVVPAITEHF-NH_2	2659	g	1
Caerin 2.1	GLVSSIGRALGGLLADVVKSKGQPA-OH	2392	b	1, 4
Caerin 2.2	GLVSSIGRALGGLLADVVKSKEQPA-OH	2464	c	1, 4
Caerin 2.4	GLVSSIGKALGGLLADVVKTKEQPA-OH	2450	c	4
Caerin 2.5	GLVSSIGRALGGLLADVVKSKEQPA-OH	2448	d	1, 4
Caerin 3.1	GLWQKIKDKASELVSGIVEGVK-NH_2	2382	b, c	1
Caerin 3.2	GLWEKIKEKASELVSGIVEGVK-NH_2	2397	c	1
Caerin 3.3	GLWEKIKEKANELVSGIVEGVK-NH_2	2424	c	1
Caerin 3.4	GLEWKIREKANELVSGIVEGVK-NH_2	2452	c	1
Caerin 4.1	GLWQKIKSAAGDLASGIVEGIKS-NH_2	2326	c	1
Caerin 4.2	GLWQKIKSAAGDLASGIVEAIKS-NH_2	2340	c	1
Caerin 4.3	GLWQKIKQAAGDLASGIVEGIKS-NH_2	2353	c	1
Caerulein 1.1	pEQDY(SO_3)TGWMDF-NH_2	1351	h	5
Caerulein 1.2	pEQDY(SO_3)TGWFDF-NH_2	1367	b, i	5
Caerulein 2.1	pEQDY(SO_3)TGAHMDF-NH_2	1373	i	5
Caerulein 2.2	pEQDY(SO_3)TGAHFDF-NH_2	1389	i	5
Caerulein 3.1	pEQDY(SO_3)GTGWMDF-NH_2	1408	i	5
Caerulein 3.2	pEQDY(SO_3)GTGWFDF-NH_2	1424	i	5
Caerulein 4.1	pEQDY(SO_3)TGSHMDF-NH_2	1389	i	5
Caerulein 4.2	pEQDY(SO_3)TGSHFDF-NH_2	1405	i	5
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	1613	i	1, 2, 3, 4
Modification 1	GLF**A**VIKKVASVIGGL-NH_2	1569		1, 2, 3, 4
Modification 2	GLFDVI**A**KVASVIGGL-NH_2	1556		1, 2, 3, 4
Citropin 1.2	GLFDIIKKVASVVGGL-NH_2	1613	i	1, 2, 3, 4
Citropin 1.3	GLFDIIKKVASVIGGL-NH2	1627	i	1, 2, 3, 4
Dahlein 1.1	GLFDIIKNIVSTL-NH_2	1430	j	1
Dahlein 1.2	GLFDIIKNIFSGL-NH_2	1434	j	1
Dahlein 4.1	GLWQLIKDKIKDAATGLVTGIQS-NH_2	2486	j	
Dahlein 5.1	GLLGSIGNAIGAFIANKLKP-OH	1952	j	4

Table 1 (*Continued*)

Name	Sequence	MW	Species	Activity*
Dynastin 1	GLVSNLGI-OH	729	k	
Dynastin 2	GLLSSLGLNL-OH	986	l	
Dynastin 3	GLVPNLLNNLGL-OH	1236	m	
Dynastin 4	GLVSNLGI-OH	772	n	
Dynastin 5	GLISNLGI-OH	786	n	
Dynastin 6	GAVSGLLTNL-OH	944	n	
Dynastin 7	GAVSGLLTNLGL-OH	1144	n	
Electrin 2.1	NEEEKVKWEPDVP-NH_2	1743	o	
Fletcherin	AGPVSKLVSGIGL-OH	1197	p	
Frenatin 1	GLLDALSGILGL-NH_2	1140	q	
Frenatin 2	GLLGTLGNLLNGLGL-NH_2	1423	q	
Frenatin 3	GLMSVLGHAVGNVLGGLFKPKS-OH	2180	q	4
Lesueurin	GLLDILKKVGKVA-NH_2	1352	r	4
Mactulatin 1.1	GLFGVLAKVAAHVVPAIAEHF-NH_2	2145	s	1, 2, 3, 4
Maculatin 1.2	GLFGVLAKVASHVVAAIAEHFQA-NH_2	2360	s	1, 2
Maculatin 1.3	GLLGLLGSVVSHVVPAIVGHF-NH_2	2068	g	1, 2
Maculatin 1.4	GLLGLLGSVVSHVLPAITQHL-NH_2	2121	g	1, 2
Maculatin 2.1	GFVDFLKKVAGTIANVVT-NH_2	1878	s	1, 2
Maculatin 3.1	GLLQTIKEKLESLAKGIVSGIQA-NH_2	2395	s	
Rubellidin 4.1	GLGDILGLLGL-NH_2	1039	t	
Rubellidin 4.2	AGLLDILGL-NH_2	883	t	
Rothein 2.1	AGGLDDLLEPVLNSADNLVHGL-NH_2	2230	u	
Rothein 3.1	ASAAGAVRAGGLDDLLEPVLNSADNLVHGL-NH_2	2964	u	
Signiferin 1	RLC*IPYIIPC*-OH (*indicates disulfide bridge)	1187	v	5
Splendipherin	GLVSSIGKALGGLLADVVKSKGQPA-OH	2364	b, c	4, 6
Tryptophyllin L 1.1	PWL-NH_2	414	t	
Tryptophyllin L 1.2	FPWL-NH_2	561	o, t	
Tryptophyllin L 1.3	pEFPWL-NH_2	672	t	
Tryptophyllin L 1.4	FPFPWL-NH_2	805	t	5
Tryptophyllin L 2.1	IPWL-NH_2	527	t	
Tryptophyllin L 3.1	FPWP-NH_2	545	o, t	
Tryptophyllin L 3.2	FPWP-OH	546	t	
Tryptophyllin L 3.3	pEFPWF-NH_2	706	t	
Tryptophyllin L 4.1	LPWY-NH_2	577	t	
Tryptophyllin L 4.2	FLPWY-NH_2	724	t	
Tryptophyllin L 5.1	pEIPWFHR-NH_2	965	t	
Uperin 1.1	pEADPNAFYGLM-NH_2	1208	w	5
Uperolein	pEPDPNAFYGLM-NH_2	1232	x	5

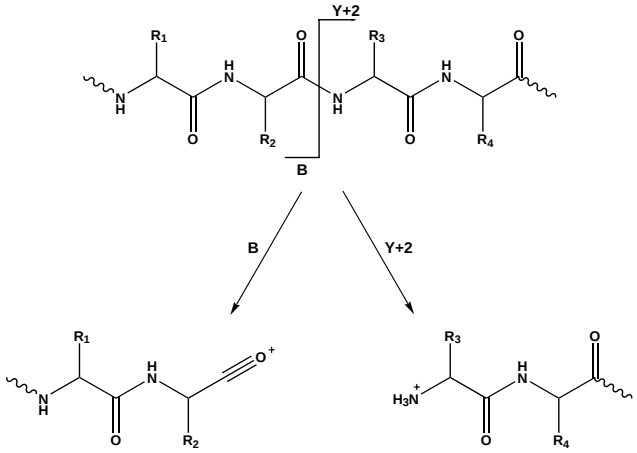
*Activity nomenclature: (1) antibiotic activity; (2) anticancer activity; (3) fungicide activity; (4) nNOS inhibitor; (5) neuropeptide, smooth muscle active; (6) aquatic sex pheromone.

Species: (a) *Litoria aurea*, *Litoria raniformis* [70]; (b) *Litoria splendida* [99]; (c) *Litoria caerulea* [89]; (d) *Litoria gilleni* [105]; (e) *Litoria xanthomera* [84,85]; (f) *Litoria chloris* [81]; (g) *Litoria eucnemis* [19]; (h) various species of the genus *Litoria* [34]; (i) *Litoria citropa* [107]; (j) *Litoria dahlii* [106]; (k) *Limnodynastes interioris* [60]; (l) *Limnodynastes dumerilii* [60]; (m) *Limnodynastes terraereginae* [60]; (n) *Limnodynastes salmini* [18]; (o) *Litoria electrica* [101]; (p) *Limnodynastes fletcheri* [18]; (q) *Litoria infrafrenata* [104]; (r) *Litoria lesueuri* [32]; (s) *Litoria genimaculata* [69]; (t) *Litoria rubella*[81,83]; (u) *Litoria rothii* [106]; (v) *Crinia signifera* [51] (w) *Uperoleia inundata* [1]; (x) many species of the genus *Uperoleia* [34].

2.2.1. Negative ion mass spectrometry

We also use negative ion mass spectrometry to assist in the primary sequencing of peptides. There are several cleavage processes that provide analogous information to that provided by the B and Y + 2 fragmentations in the positive ion mode. These cleavages are summarized in Scheme 2. The α cleavage process provides sequence information from the N-terminal end of the peptide while the β cleavage process provides information from the C-terminal end [13]. Generally, α fragmentation is more pronounced than β fragmentation [13].

Several other cleavages have been discovered that provide additional sequence information. These cleavages identify specific residues and/or the position of these residues in the peptide. The first set of cleavages identify the presence of specific residues by a characteristic loss of a neutral from the $(M–H)^-$ species, eg. CH_2O (Ser) [13], MeCHO (Thr) [13], H_2S (Cys) [11], H_2O (Asp, Glu) [13], NH_3 (Asn, Gln) [13].

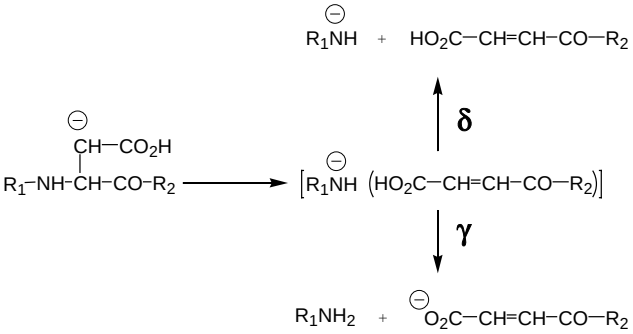


Scheme 1.

The second set of fragmentations involves backbone cleavages initiated from specific residues. The fragmentations result from cleavage of the bond between the NH and αC generating two possible ions depending on where the charge resides, namely δ (charge resides on the N-terminal fragment) and γ (charge resides on the C-terminal fragment) cleavage ions. Amino acid residues that undergo this type of fragmentation are Ser [13], Thr [13], Glu [13], Cys [11], Gln [13], Asp [13], Asn [13], and Phe [20]. A particular example is shown for Asp in Scheme 3. γ-Cleavage ions are generally more abundant than δ ions. For details of the other backbone cleavages see review [13].

Neither positive nor negative ion backbone fragmentations distinguish Leu and Ile. This is done by automated Edman degradation [13], which also confirms the total sequence.

The positive and negative ion spectra of a 16-residue peptide (1) are shown below for comparison. The collision induced mass spectrum of the MH^+ ion of (1) is shown in Fig. 4a. The B ions are drawn schematically above the spectrum while the Y + 2 ions are drawn below the spectrum. There are a total thirteen B ions and eleven Y + 2 ions, identifying the entire peptide sequence with the exceptions



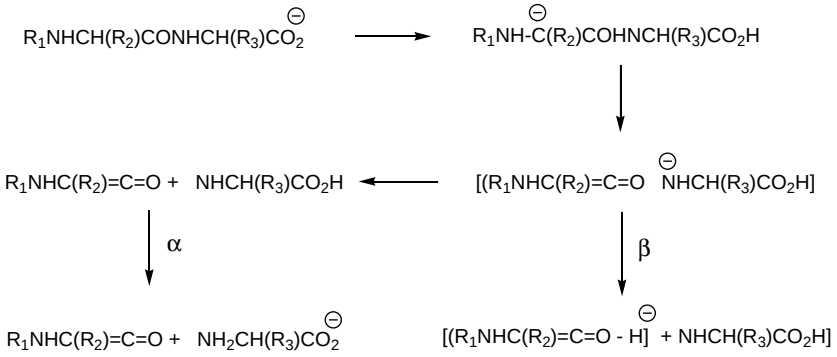
Scheme 3.

of the relative orientation of the first two residues (Gly Leu) and differentiation between isomeric Ile and Leu and isobaric Gln and Lys. The collision induced MS/MS data for the $(M–H)^-$ species of (1) is shown in Fig. 4b. The base peak in the spectrum is the $[(M–H)^–CH_2O]^-$ peak at *m/z* 1609 (loss of CH_2O is the side-chain cleavage of Ser) from which the majority of the remaining fragmentation results. Thirteen α ions and five β ions originate from this ion, identifying the peptide sequence with the exception of the relative orientation of the first two and last two residues. The lack of any γ fragmentation arising from residues 7 and 8 suggests that these residues are Lys rather than the isobaric Gln. Also the peaks at *m/z* 596 and 578 are backbone cleavage ions of Ser11 [13], identifying the position of this residue. The two spectra together identify the entire sequence of peptide (1) except for isomeric Ile and Leu and the relative orientation of the first two residues (Gly Leu).

(1) GLFAVIKKVASVIKGL(NH_2)

2.3. Three-dimensional structure determination

The elucidation of the three-dimensional (3D) structure of the various amphibian peptides is accomplished using a combination of two-dimensional (2D) nuclear magnetic resonance (NMR) experiments and computer modeling.



Scheme 2.

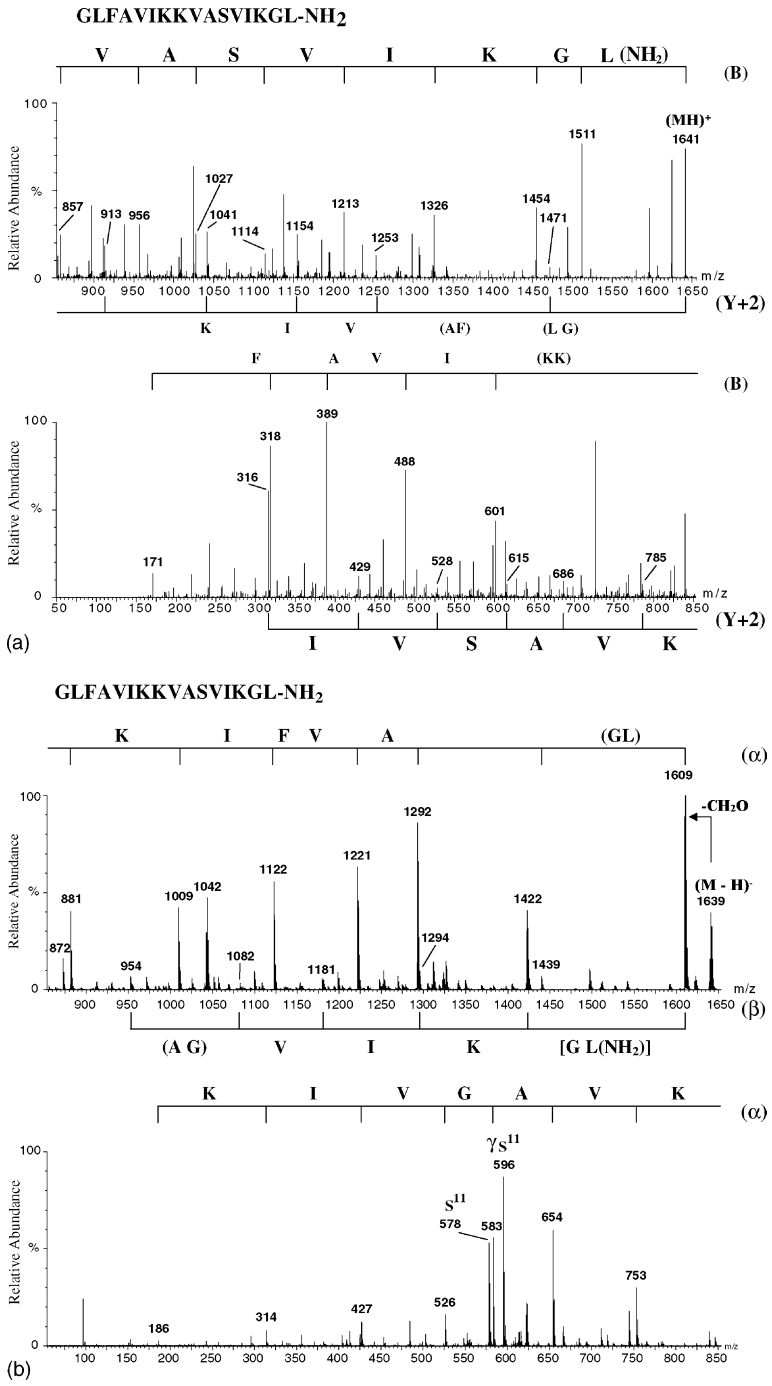


Fig. 4. (a) Collision induced MS/MS mass spectrum of the $(MH)^+$ ion of (1). B fragmentations are drawn schematically above the spectrum while Y + 2 fragmentations are shown below the spectrum. Magnification ranges: 495–1654 (2×). (b) Collision induced MS/MS mass spectrum of the $(M–H)^-$ ion of (1). The α and β fragmentation originating from the $[(M–H)^–CH_2O]^-$ ion are drawn above and below the spectrum, respectively. All other fragmentation is annotated on the spectrum. Magnification ranges: 58–597 (36×), 597–1432 (24×).

2.3.1. Solvent systems

The solvent system in which the NMR experiments are run is important. Solvent systems are chosen so as to mimic different conditions within the body. Three solvent systems have been used, viz. (i) varying mixtures of water and 2,2,2-trifluoroethanol (TFE) are used as a membrane mimicking solvent. TFE is known to disrupt intermolecular hydrogen bonds between the water and the peptide thus increasing the effectiveness of the intra-molecular bonds in the peptide responsible for the secondary structure [58,61]; (ii) a membrane mimicking solvent can also be generated using micelles. Micelles are spherical aggregates of amphiphilic

lipid molecules that form in water when the concentration of lipids is sufficiently high. For our investigations, the commonly used lipid is zwitterionic dodecylphospatidycholine (DPC). These lipids form stable micelles at concentrations of about 1 mM [53]; and (iii) NMR data were also obtained in water, allowing the structure of the peptides outside the membrane to be elucidated. In pure water, peptides often tend to form inter-molecular hydrogen bonds with the water, thereby disrupting the intra-molecular hydrogen bonding within the peptide responsible for the secondary structure.

2.3.2. ^1H NMR experiments

^{1}H 2D NMR experiments are used to assign the various proton resonances within the peptide under study. The experiments used include:

(i) Correlated spectroscopy experiments (COSY) indicate protons that are spin-spin coupled to each other (i.e. protons that are on adjacent nuclei) [37]. This is particularly useful for assigning proton resonances within specific spin systems (i.e. individual residues).

(ii) Total correlated spectroscopy experiments (TOCSY) provide additional information to that in a COSY spectrum by indicating all proton resonances in the same spin system. With respect to peptides and proteins, each amino acid residue constitutes a separate spin system [37].

(iii) Nuclear overhauser spectroscopy (NOESY) experiments indicate, via dipolar coupling, protons that are within 5 Å of each other in space. A spatial interaction is represented by a cross peak: the volume of the cross peak is inversely proportional to the sixth power of the distance between the two protons in question [37].

Citropin 1.1 GLFDVIKKAVASVIGGL(NH_2)

Sections of NOESY and TOSCY spectra of citropin 1.1 are shown in Fig. 5. The TOCSY spectrum shown on top indicates the through-bond connectivities within the spin systems of each residue. The NH region of the NOESY spectrum is shown below and depicts through-space NOE connectivities between sequential backbone NH protons.

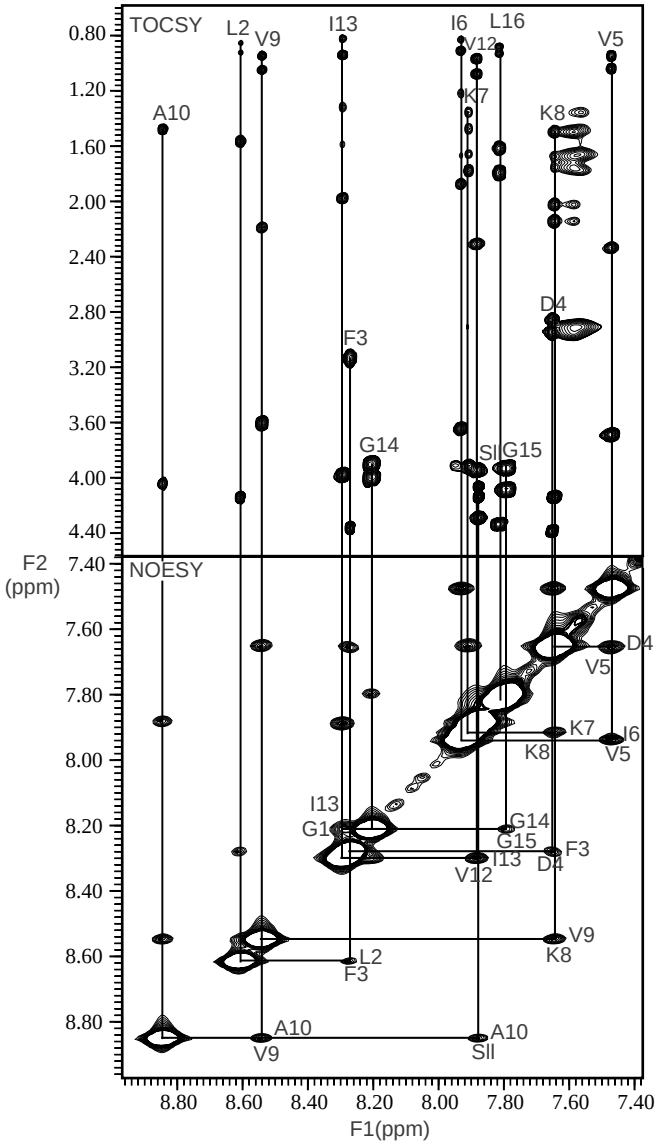


Fig. 5. Partial NOESY and TOCSY spectra of citropin 1.1 in TFE/water. Vertical lines connect the resonances in each spin system. These are labelled with the standard single-letter abbreviations for the residue type and a number indicating the sequential position of the residue. NOEs between sequential NH protons are indicated in the NOESY spectrum.

Having assigned the proton resonances in the peptide, the cross peaks in the NOESY spectrum are assigned accordingly. The best scenario is that each peak is uniquely assigned although this is usually improbable with systems as large as peptides. There are usually at least several peaks that have multiple assignments. The volume of each cross peak is determined and using the inverse sixth power relationship between volume and distance, a series of distance restraints is generated (i.e. a list of the distances between specific protons in the peptide) [110].

A series of dihedral restraints can also be generated from the $J^3_{NH\alpha H}$ spin–spin coupling constants measured from the high-resolution 1D ^{1}H NMR spectrum of the peptide [24]. These two restraint series are used in restrained molecular dynamics and simulated annealing calculations to generate the 3D solution structure of the peptide [110]. Beginning with an initial structure of poorly defined geometry [26], the system is manipulated such that all of the restraints are satisfied with the least number of violations. The most stable conformation (i.e. global minimum) is located by refining the distance restraints by removing the ambiguities in the assignments producing a representation of the peptide in solution [7,91]. The solution structures of six peptides (described in detail in this article) determined by the method described above are shown in Figs. 6–11. As an example, the solution structure of citropin 1.1 (derived in part from the data shown in Fig. 5) is shown in Fig. 7.

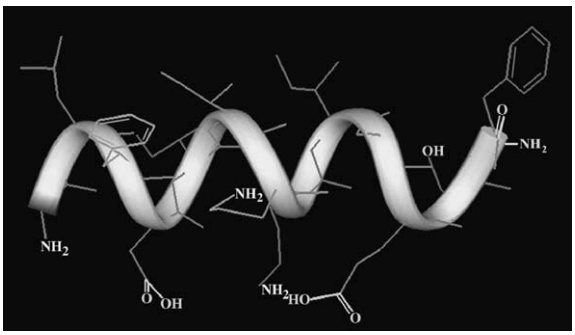


Fig. 6. The solution structure of aurein 1.2 as determined in TFE/water.

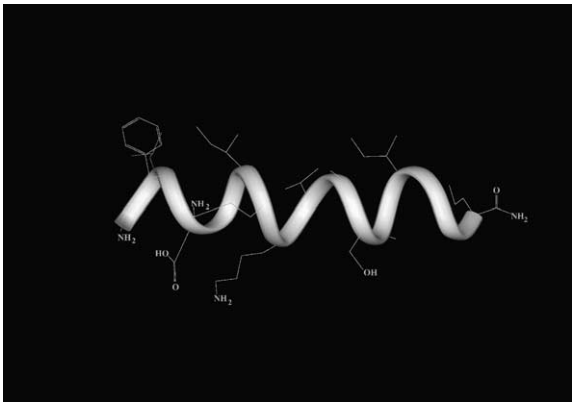


Fig. 7. The solution structure of citropin 1.1 as determined in TFE/water.

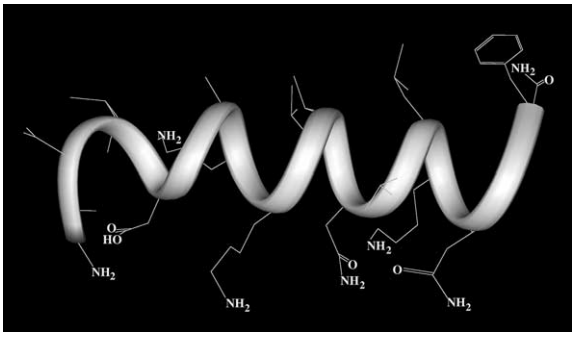


Fig. 8. The solution structure of uperin 3.6 as determined in TFE/water.

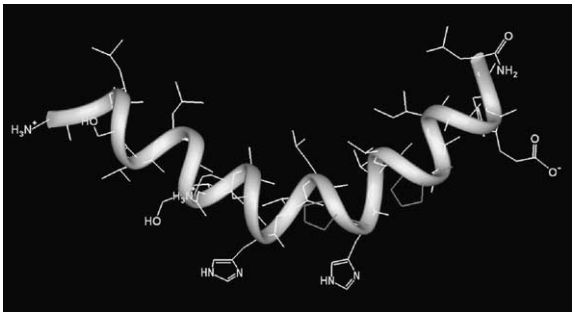


Fig. 9. The solution structure of caerin 1.1 as determined in TFE/water.

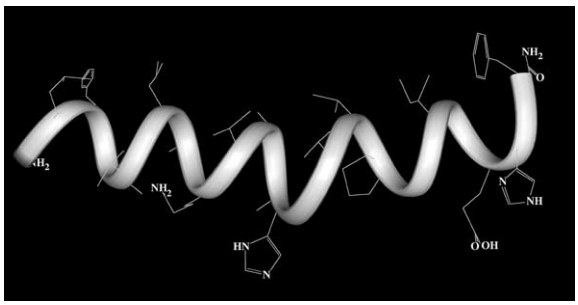


Fig. 10. The solution structure of maculatin 1.1 as determined in TFE/water.

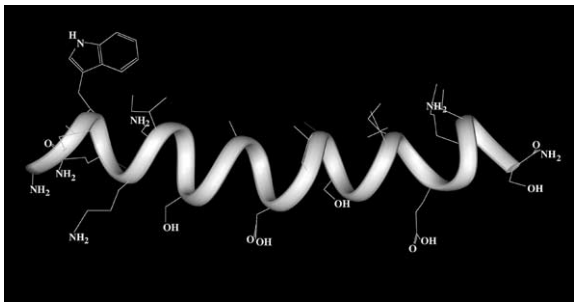


Fig. 11. The solution structure of caerin 4.1 as determined in TFE/water.

3. Antibacterial and anticancer peptides and fungicides

3.1. Antibacterial and anticancer active peptides

The dermal secretions of most Australian frog species contain at least one broad-spectrum antibiotic, and often a number of peptides with varied specificity to allow enhanced protection against a range of bacteria. Biological testing has revealed additional anticancer properties for a number of these peptides, with such coincident activity presumably due to a similar mechanism of action at both bacterial and cancer cells. The peptides are synthesized as a signal-spacer-peptide precursor, in which the signal directs the peptide to the gland before being cleaved by an endoprotease to give the spacer-peptide moiety, which is inactive and as such safe to store. Upon stimulation, the spacer is removed by a second endoprotease and the active peptide delivered onto the skin [38]. In the case of broad-spectrum antibiotics, a third endoprotease degrades and deactivates the peptide after a period of time on the skin (5–30 min depending on the species) [64].

Activity is thought to be mediated by disruption of either cancer or bacterial cell membrane integrity, since the all-D isomers have comparable activity with the natural L-form, ruling out interaction with specific chiral receptors [109]. A number of mechanisms have been proposed to rationalize membrane penetration by the peptide, the simplest of

which include the barrel-stave and carpet mechanisms. In the barrel-stave model, peptides aggregate at the membrane surface in α-helical form, driven by electrostatic attraction between charged residues and ionic sites on the bilayer. Subsequent insertion into the membrane then occurs via formation of a trans-membrane barrel-like pore, in which peptides are oriented perpendicular to the plane of the bilayer [33,72]. A minimum of 20 residues is required to span the membrane entirely although a modified model has been proposed whereby shorter peptides can dimerize end-on to effect complete penetration [2]. In contrast, the carpet mechanism is initiated as peptides assemble in α-helical form with their axis parallel to the membrane, forming a carpet-like monolayer on the surface. Above a critical concentration, transient holes are formed due to strain on the bilayer curvature, and the membrane degrades into micelle-like complexes [73,74]. Regardless of the mode of penetration, ultimately the disruption of normal membrane function results in excessive flux of ions and small molecules across the cytoplasmic membrane bilayer, in turn leading to cell lysis.

Amphibian peptides often have no mammalian counterpart, and display varying degrees of specificity for both bacterial and eukaryotic cells. For example, some exhibit broad-spectrum antibiotic activity while others are active against only selected micro-organisms [35]. In addition, other peptides are lethal to tumorigenic cells at concentrations that are harmless to normal cells [28]. This is thought to be a property of membrane construction, with factors including lipid composition, charge and potential influencing the peptides' binding and permeabilizing ability [52]. The efficacy of amphibian peptides, however, is modulated to a greater extent by structural properties of the peptide itself, with features including degree of helicity, charge state, amphipathicity and hydrophobicity being significant [29,93,108]. It is for this reason that both the primary and secondary structure of the peptides have a direct influence on the observed biological activity.

3.1.1. Short, linear, antibacterial and anticancer bioactive peptides (<20 residues): the aureins citropins, uperins and maculatin 2.1

One group of antibacterial and anticancer peptides that have been identified is a series of short peptides (<20 residues) isolated from various species of the *Litoria* and *Uperoleia* genera. This group contains the aureins 1–3 (from *Litoria aurea* and *Litoria raniformis* [70]), the citropins 1 (from *Litoria citropa* [107]), dahlein 1.2 (from *Litoria dahlii* [106]), maculatin 2.1 (from *Litoria genimaculata* [69]) and uperins 3.5 and 3.6 (from *Uperoleia inundata* [17] and *Uperoleia mjobergii* [16]). The sequences of these antibiotic peptides are listed in alphabetical order in Table 1. Their antibiotic activities together with those of some synthetic modifications of citropin 1.1 are recorded in Table 2 [31]. These peptides have also been tested by the National Cancer Institute (Washington) and have activities (IC_{50}) in the 10^{-5} to 10^{-6} M range against all classes of human cancers tested (viz. leukaemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancers) [14,31,68].

There are several characteristics common to these peptides. First, they are all cationic, possessing at least two basic residues occurring at positions 7 and 8 and a free amine at the N-terminal end. Citropin 1.1 is a typical example, with sequence GLFDVIKKVASVIGGL-NH_2. With the exception of uperin 3.6 (which has an Arg at position 7) all the other peptides have the pattern of Lys7 Lys8. When one of these basic residues is replaced with Ala, the activity of the peptide is reduced remarkably (see citropin synthetic modification 2) but replacement of Asp4 with Ala (citropin synthetic modification 1) does not have any major influence on the activity. Replacement of both basic residues with Ala at positions 7 and 8 results in a lack of observable activity [31]. All the peptides are post-translationally modified to the C-terminal amide and this is vital for their observed activity. The size of the peptides also influences their activities. Sixteen or seventeen residues is the optimal length for these linear peptides (cf. aurein 2.1, citropin 1.1 and 1.2, and uperin 3.5 and 3.6). As the length decreases (cf. aurein

Table 2
The antibiotic activities of selected aurein (A) citropin (Ci), dahlein (D), maculatin (M) and uperin (U) peptides listed in Table 2

Organism	MIC (μg/ml)											
	A1.2	A2.1	A3.2	Ci1.1	Mod1	Mod2	Ci1.2	Ci1.3	D1.2	M2.1	U3.5	U3.6
Bacillus cereus	100	50		50	25	100	25	25	100	100	25	25
Leuconostoc lactis	12	6	6	6	3	25	3	6	25		3	3
Listeria innocua	100	6	100	25	25		100	25			25	50
Micrococcus luteus	100	100	100	12	12	100	12	12		100	12.5	25
Staphylococcus aureus	50		50	25	25	100	25	25	100	100	50	25
Staphylococcus epidermidis	50	50	50	12	12	100	25	25	100	50	12.5	12.5
Streptococcus uberis	50	100	50	25	25	100	12	25	100	25	12.5	12.5
*Escherichia coli**					100							
*Pasteurella multocida**	100											

*Gram-negative organism. Sequences are listed in Table 1. Antibiotic results are listed as MIC values (μg/ml). Where no figure is indicated, MIC is >100 μg/ml.

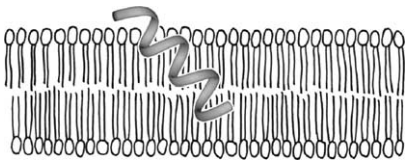


Fig. 12. Model proposed for the insertion of short amphibian peptides in DMPC membranes [49].

1.1, 1.2 and dahlein 1.2) or increases (cf. maculatin 2.1) the activity decreases.

The solution structures of the membrane active aurein 1.2 [70], citropin 1.1 [107] and uperin 3.6 [24] peptides have all been determined and have been shown to adopt well-defined amphipathic α-helical structures. The solution structures as determined in TFE/water are shown in Fig. 6 (aurein 1.2), Fig. 7 (citropin 1.1) and Fig. 8 (uperin 3.6). Solid-state NMR studies using both aurein 1.2 and citropin 1.1 indicate that the peptides first align themselves along the membrane and at higher concentrations tilt into the bilayer at an angle of about 40° (Fig. 12) [49]. This result is consistent with the peptides acting via the carpet mechanism.

3.1.2. Longer, hinged, antibacterial and anticancer peptides: caerins and maculatins

The largest group of antibacterial amphibian peptides isolated to date is that of the caerin peptides, with over 30 identified from more than six Australian frog species of the *Litoria* genus [79,80,84–86,88,89,105]. These can be further divided into four subgroups, with the caerin 1 broad-spectrum peptides the most common. All caerin 1 peptides have similar primary structures based on that of caerin 1.1 (Table 1), and are active mainly against Gram-positive bacteria. Caerins 1.1, 1.3, 1.4, 1.5 and 1.9 are typical of this group, and their activities are shown in Table 3. In addition to their antibiotic activities, these peptides have been tested by the National Cancer Institute (Washington) and have IC_{50} values in the 10^{-5} to 10^{-6} M range against all classes of cancers tested [14].

Until recently, nothing was known of the genes encoding these peptides. However, 3′-RACE analysis of skin mRNA from *L. caerulea* revealed a number of cDNAs encoding for caerin peptides, while also giving an insight into the structure of the pre-pro-peptide precursors [96]. A comparison of the amino acid sequences of the caerin precursors showed the acidic pro-piece is highly conserved, as is the N-terminal signal portion. In addition, these pre-pro-regions of the caerin precursors show significant identity with those from South American hylid frogs [96]. The sequences of the pre-pro-peptide for caerin 1.1 are given below. The C-terminal amide of the native peptide is formed by addition of a glycine residue to the end of the progenitor sequence which is post-translationally modified into an amide group [96].

Signal:	MASLKKSLFLVLLLGFVSVSIC
Spacer:	EEEKRQEDEDEHEEEGESQEEGSEEKR
Native peptide:	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2

Investigation into the solution structures of caerin 1.1 and related peptides suggests they form two amphipathic helices, separated by a more flexible hinge region initiated by Pro15 (Fig. 9) [106,109]. The hinge assists this peptide to interact effectively with the membrane, as optimal orientation of hydrophilic and hydrophobic zones are facilitated. This is supported by solid-state NMR studies in which it appears that at higher concentrations, the N-terminal helix sits on the surface of the membrane while the C-terminal helix penetrates the bilayer at an angle of approximately 40°, consistent with the carpet mechanism of action (Fig. 13) [49]. The molecules do not penetrate deeply into zwitterionic or positively charged membranes, which may explain why these positively charged peptides preferentially lyse bacterial rather than eukaryotic cells [49].

Synthetic modifications show that the antibacterial activity of caerin 1.1 (GLLGVLVSIAKHVLPHVVPVIAEHL-NH_2) is significantly reduced when Pro15 or Pro19 are replaced with Gly (modification 1, Table 3), and to a further

Table 3
The antibiotic activities of selected caerin (C) and maculatin (M) peptides

Organism	MIC (μg/ml)								
	C1.1	Mod 1	Mod 2	C1.3	C1.4	C1.5	C1.9	M1.1	M1.4
Bacillus cereus	50	50		50	50	50	100	25	100
Leuconostoc lactis	1.5	12	25	3	12	3	12	3	6
Listeria innocua	25	50		50	100	50	50	100	100
Micrococcus luteus	12	12		25	0.4	12	50	12	50
Staphylococcus aureus	3	25–50		6–12	100	25	12	6	50
Staphylococcus epidermidis	12	100		12	25	25	25	12	50
Streptococcus uberis	12	12		25	100	50	50	3	50
*Escherichia coli**		50			50				
*Pasteurella multocida**	25	100		50	25	25	50	50	

Sequences of the peptides together with those of the two synthetic modifications of caerin 1.1 are listed in Table 1. *Gram-negative organism. Antibiotic results are listed as MIC values (μg/ml). Where no figure is indicated, MIC is >100 μg/ml.

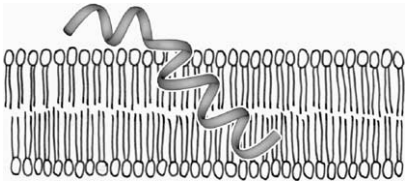


Fig. 13. Model proposed for the insertion of hinged amphibian peptides in DMPC membranes [49].

extent with Ala (modification 2, Table 3). This is thought to be a result of reduced conformational freedom, and demonstrates the importance of the flexible hinge in such peptides [59]. Since the positive charge of Lys11 may be involved in the interaction with anionic phospholipids in the membrane, it is not surprising that modification of this residue would affect the antibiotic activity. Substitution at this site with Gln gives the uncharged, naturally occurring caerin 1.3, which possesses markedly less activity than that of caerin 1.1.

The caerin 1 peptides are generally inactivated by enzymic cleavage of a number of residues from the N-terminus, giving degradation products such as caerin 1.1.1 and 1.1.2 (see Table 1). In addition, caerins 1.1.5 and 1.1.3 (Table 1) are made up of the residues from the first and second helices of caerin 1.1 respectively, and are found to be completely inactive [105]. Thus, it would seem the flexible hinge region and both helices are necessary for antibacterial activity. It was, therefore, surprising to find maculatin 1.1 (GLFGVLAKVAAHVVPAIAEHF-NH_2) from the skin secretion of *Litoria genimaculata* [69]. This molecule shows similar antibiotic activity compared with caerin 1.1 (Table 3), yet lacks four residues including Pro15. The solution structure of maculatin 1.1 has also been investigated, and forms a helix-bend-helix structure similar to that of caerin 1.1 in membrane-like media (Fig. 10) [23]. This molecule also displays similar interaction with model membranes in solid-state NMR studies [49]. In addition to maculatin 1.1, a number of similar peptides (maculatins 1.3 and 1.4 (Table 1)) have subsequently been isolated from *L. eucnemis* [19].

The caerin 2 peptides are the only caerin molecules to contain C-terminal CO_2H groups (Table 1), generally regarded as an indication of poor antibiotic activity. In fact, they show minimal activity against a range of bacteria (Table 4), and essentially no anti-cancer activity against the cell lines tested. However, the caerin 2 peptides inhibit the operation of nNOS (see Section 4.2.). The caerins 3 and 4 are related in that they all contain Trp3 and two or three Lys residues (Table 1). In addition, the caerins 3 and 4 are generally narrow spectrum antibiotics, commonly active only against a few of the species tested. For example, caerin 3.1 shows pronounced activity against *Micrococcus luteus*, while caerin 4.1 is active against *Pasteurella multocida* and *Escherichia coli* (Table 4). The solution structure of caerin 4.1 has been determined and shown to be a linear α-helix with well-defined hydrophobic and hydrophilic domains

Table 4
The antibiotic activities of selected caerin (C) 2–4 peptides

Organism	MIC (μg/ml)					
	C2.1	C2.2	C3.1	C3.2	C4.1	C4.3
Bacillus cereus						
Leuconostoc lactis						
Listeria innocua						
Micrococcus luteus		50	<0.4	3	12	25
Staphylococcus aureus						
Staphylococcus epidermidis						
Streptococcus uberis						
*Escherichia coli**					25	50
*Pasteurella multocida**	25	25				

*Gram-negative organism. Sequences are listed in Table 1. Antibiotic results are listed as MIC values (μg/ml). Where no figure is indicated, MIC is >100 μg/ml.

(Fig. 11) [22]. While the solution structures of members of the caerin 2 and 3 families have not yet been investigated, Edmundson projections suggest amphipathicity is not facilitated in an α-helical form, possibly explaining the poor antibacterial efficacy of these molecules. There are a number of peptides related to caerin 3 from other species of the genus *Litoria*; for example aurein 4.1 (Table 1, from *L. aurea* and *L. raniformis* [70]), dahlein 4.1 (Table 1, from *Litoria dahlii* [106]) and maculatin 3.1 (Table 1, from *Litoria genimaculata* [69]). These show no antibiotic activity and their role in the amphibian integument is not known at this time.

3.2. Antifungal peptides

Amphibians are prone to infection by fungi; the worst being the chytrid fungus (*Batrachochytrium dendrobatis*) which is affecting anuran populations worldwide [55,77]. It has already been shown that antibiotic peptides of some frogs from the northern hemisphere are active against the chytrid fungus (e.g. the temporins from *Rana* species and the magainins from *Xenopus laevis* [66]), and it thus seems likely that the membrane-active antimicrobial peptides of Australian anurans should similarly destroy the chytrid fungus. There is some anecdotal evidence for this in that frogs without the protection of skin antimicrobial peptides (e.g. species of the genus *Limnodynastes*) succumb more readily to the chytrid fungus than species from other genera that produce antimicrobial peptides. It has been shown recently that many membrane-active antibiotic peptides from Australian anurans kill the chytrid fungus, generally at concentrations in the micromolar range [65]: this includes the caerins 1, citropins 1, uperins, aureins, dahleins and so on. However, the situation is complex since species which have this apparent antifungal protection still succumb to the chytrid fungus. For example, those *Litoria* species that produce the caerin 1 antimicrobial (and antifungal) peptides may also be killed by the chytrid fungus [77]. The question is why do anurans that, in principle, have (apparently) adequate protection

against the chytrid fungus, still succumb to the fungus? Is the explanation the simple one that the zoospores of the fungus attach mainly to the underneath of the animal and this area is not effectively reached by the skin secretion from the back of the animal? Alternatively, perhaps the animal does not realize that the fungus is lethal, and does not engage its chemical arsenal. Or perhaps the fungus itself has an effective defence against the active peptides; for example a protease that cleaves and deactivates the antifungal peptide. The explanation for this strange phenomenon is not yet known.

4. Neuropeptides

4.1. Caeruleins and uperoleins

The majority of frogs of the genus *Litoria* and toadlets of the *Uperoleia* genus contain at least one neuropeptide of the caerulein and uperolein groups, respectively. The neuropeptide is often the major host-defence peptide in the glandular secretion. Such neuropeptides are both an integral part of the defence system, and also assist with the regulation of dermal physiological action [8,34,45]. The sequences of the caerulein and of uperolein and uperin 1.1 neuropeptides isolated from Australian anurans are listed in Table 1.

Caerulein, pEQDY(SO_3)TGWMDF-NH_2, (which we now call caerulein 1.1 to distinguish it from other caeruleins) is a common neuropeptide found in many frog species worldwide [34]. Caerulein 1.1 exhibits a spectrum of activity similar to that of the mammalian intestinal peptide hormones gastrin and cholecystokin (CCK): it contracts smooth muscle at better than nanomolar concentration, enhances blood circulation, modifies satiety, sedation and thermoregulation, is an analgesic several thousand times more potent than morphine, and has been used clinically during gall bladder operations [34].

The levels of caerulein 1.1, although identical for both male and female *L. splendida*, vary seasonally [102]. These seasonal changes may be involved in thermoregulation. During the spring to autumn period (in the southern hemisphere), which corresponds to the breeding period of *L. splendida*, caerulein 1.1 is the only smooth-muscle active neuropeptide present. During the winter period (June to August) the composition of the glandular skin secretion changes. There is a decrease in the concentration of caerulein 1.1, balanced by the formation of desulfated caerulein 1.1 (pEQDYTGWMDF-NH_2), and caerulein 1.2 [pEQDY(SO_3)TGWFDF-NH_2]. Both caerulein 1.1 and 1.2 show similar smooth muscle activity, but whereas caerulein 1.1 acts at a CCK site directly on smooth muscle, it appears that caerulein 1.2 elicits acetylcholine release, which initiates the smooth muscle activity. The Australian Blue Mountains Tree Frog (*Litoria citropa*) produces a variety of caerulein peptides (see Table 1) [98]: the reason for the presence of so many neuropeptides of the caerulein family in this species is not known at this time.

The hypertensive peptide uperolein [pEPDPNAFYGLM-NH_2] was first isolated from toadlets of the *Uperoleia* genus by Erspamer and colleagues [1]: uperolein is a member of the tackykinin family, exhibiting potent vasodilator and hypertensive action, together with intense spasmogenic activity of smooth muscle [34]. Ala2 uperolein (uperin 1.1, (pEADPNAFYGLM-NH_2) (from *Uperoleia inundata* [1,17])) has similar activity, exhibiting smooth muscle contraction of guinea pig ileum at 0.4 ng/kg (of body weight) and reduction of rabbit blood pressure at 5 ng/kg [17].

4.2. Nitric oxide synthase active peptides

The seemingly ubiquitous involvement of nitric oxide (NO) in biological systems has now resulted in an explosion of interest in the field. At high concentrations, NO behaves as a defensive cytotoxin against tumor cells and pathogens as the immune system utilizes the toxic properties of NO to kill or inhibit the growth of invading organisms. At low concentrations it serves as a cell-to-cell signalling agent, exerting its biological effects by reacting either directly or through other reactive nitrogen intermediates with a variety of targets. The diversity of this potential interaction is reflected in the large number of different systems that utilize NO as a mediator, including regulation of the circulatory and central nervous system, neurotransmission in contractile and sensory tissues, learning, and memory formation [10,62,90].

NO is notable among biological signals for its rapid diffusion, ability to permeate cell membranes and intrinsic instability, properties that eliminate the need for extracellular NO receptors or targeted NO degradation. Therefore, NO differs from most other neurotransmitters and hormones in that it is not regulated by storage, release or degradation, but rather solely by synthesis [62]. Nearly every cell type studied thus far has demonstrated the ability to synthesize NO by one of the three distinct isoforms of the nitric oxide synthase (NOS) enzymes isolated to date [10].

Nitric oxide synthases, expressed as neuronal NOS (nNOS, also called NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) isozymes, oxidize L-arginine (L-Arg) to NO and citrulline, thereby controlling NO distribution and concentration. The names reflect characteristics of the activity or the original tissues in which the enzymes were first described, but it is now known that each of these isoforms is expressed in a variety of tissues and cell types. All three isozymes are homodimers with subunits of 130–160 kDa. They differ in size, amino acid sequence (50–60% identity between any two isozymes), tissue distribution, transcriptional regulation, and activation by intracellular calcium, but they share an overall three-component construction [27], namely:

(i) An N-terminal catalytic oxygenase domain that binds heme (iron protoporphyrin IX), tetrahydrobiopterin (BH_4), and the substrate L-Arg;

(ii) A C-terminal reductase domain that binds flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and NADPH; and
(iii) An intervening calmodulin-binding region that regulates electronic communication between the oxygenase and reductase domains. Occupation of this site facilitates electron transfer from the cofactors in the reductase domain to heme during NO production.

Calmodulin (CaM), a "dumbbell" shaped 148 residue protein, is required for activation of NOS. Calmodulin acts as an electron shuttle and is the cell's main intracellular calcium transporter. It appears that CaM is required to alter the conformation of the reductase domain, increase the rate of electron transfer into the flavins and increase the rate at which the reductase can transfer electrons to acceptors such as the active heme site [90].

Once NOS has been synthesized, its activity can then be regulated by post-translational mechanisms, and it is at this level that NO synthesis by nNOS and eNOS can be tightly controlled. At the normal resting level of Ca^{2+} in the cell, both nNOS and eNOS are inactive. However, when Ca^{2+} levels increase, the binding of calmodulin to these isoforms is triggered, resulting in the stimulation of catalytic activity [46]. The importance of this mechanism is that it allows NO synthesis to be coupled with known physiological stimuli such as neural depolarisation, shear stress, and second messenger systems such as cGMP which lead to rises in Ca^{2+} concentrations. In contrast, calmodulin is tightly bound to iNOS irrespective of the Ca^{2+} concentration, hence the regulation of iNOS occurs generally at the level of transcription.

The majority of frogs of the genus *Litoria* that we have studied contain at least one major peptide in their glandular secretions that inhibits the formation of NO by nNOS. These positively charged peptides fall into one of three categories: (a) peptides of the citropin 1 type, (b) caerin 1 peptides, particularly those with phenylalanine residues at position 3, and (c) the frenatin/splendipherin group of peptides. The most active of these are listed in Table 5 [31,32].

These peptides interfere with communication between Ca^{2+}CaM and nNOS. This is confirmed experimentally since: (i) addition of these peptides to nNOS during in vitro testing results in an inhibition of nNOS and decrease of NO production. Subsequent addition of CaM to these test solutions results in a partial recovery of nNOS activity, (ii) these peptides also inhibit the operation of calcineurin, another enzyme which requires Ca^{2+}CaM as a regulatory protein [32], and (iii) a preliminary 2D NMR study indicates that splendipherin (Table 5) forms a complex with CaM [3], as evidenced by chemical shift changes at significant residues [41].

That these nNOS active peptides interact with Ca^{2+}CaM rather than with the affected enzymes directly leads to the observation that any enzyme requiring Ca^{2+}CaM for function is a potential target of these host-defence peptides. Other examples of enzymes requiring Ca^{2+}CaM include myosin light chain kinase, phosphorylase kinase and adenylate cyclase [44]. Ca^{2+}CaM is also involved in regulation of the eukaryotic cytoskeleton [44] and is required by some protozoa for ciliate movement [57].

Although this means that peptides binding Ca^{2+}CaM probably do not act specifically on any one target enzyme, the probable advantage to the frog is the ability to interfere with many important cellular functions at once, causing maximum disruption to any attacker.

Table 5
nNOS inhibition activities of selected amphibian peptides

Name	Sequence	IC_{50} (μM)	Charge	Species
Inhibitor Group A				
Lesueurin	GLLDILKKVGKVA-NH_2	16.2	+3	a
Aurein 1.1	GLFDIIKKIAESI-NH_2	33.9	+1	b
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	8.2	+2	c
Aurein 2.2	GLFDIVKKVVGALGSL-NH_2	4.3	+2	b
Aurein 2.3	GFLDIVKKVVGIAGSL-NH_2	1.8	+2	b
Aurein 2.4	GLFDIVKKVVGTLAGL-NH_2	2.1	+2	b
Inhibitor Group B				
Dahlein 5.1	GLLGSIGNAIGAFIANKLKP-OH	3.2	+3	d
Frenatin 3	GLMSVLGHAVGNVLGGLFKPKS-OH	6.8	+3	e
Splendipherin	GLVSSIGKALGGLLADVVKSKGQPA-OH	8.5	+3	f
Inhibitor Group C				
Caerin 1.1	GLLGVLVSIAKHVLPHVVPVIAEHL-NH_2	36.6	+1	f
Caerin 1.10	GLLSVLGSVAKHVLPHVVPVIAEKL-NH_2	41	+2	g
Caerin 1.6	GLFSVLGAVAKHVLPHVVPVIAEKL-NH_2	8.5	+2	g
Caerin 1.8	GLFKVLGSVAKHLLPHVVPVIAEKL-NH_2	1.7	+3	g
Caerin 1.9	GLFGVLGSIAKHVLPHVVPVIAEKL-NH_2	6.2	+2	g

(a) *L. lesueuri* [32]; (b) *L. aurea* [70]; (c) *L. citropa*[100]; (d) *L. dahlii* [106]; (e) *L. infrafrenata* [15]; (f) *L. splendida* [99]; (g) *L. chloris* [15].

4.3. Cys containing neuropeptides from the genus Crinia

Although peptides containing Cys residues and disulfide bridges have been isolated from European and Indian frogs of the *Rana* genus [34,45,71,75], Cys containing peptides have only recently been discovered in Australian frogs. *Crinia signifera* has a number of antibiotic and nNOS active peptides in its glandular secretion, but no neuropeptides analogous to the caeruleins or uperoleins. Instead, the major component of the secretion, now called signiferin 1, has the structure shown below [51]. The nomenclature *C is used to indicate the presence of a disulfide bridge.

Signiferin 1	RL*CIPYIIP*C-OH
Tigerinin 2	RV*CFAIPLPI*CH-NH_2
Vasopressin	*CYFQN*CPRG-NH_2

The signiferin 1 sequence has some resemblance to the antibiotic tigerinins [71] (tigerinin 2 has the sequence shown above) and the human pituitary hormones oxytocin and vasopressin (vasopressin sequence shown above). Unlike tigerinin 2, signiferin 1 has no antibiotic activity: in preliminary experiments it has been shown to contract smooth muscle at the 10^{-9} M concentration [50].

4.4. Tryptophyllins

The Red Tree Frog *Litoria rubella* (Fig. 14) [5] is widespread throughout central and northern Australia and has evolved into a number of specific populations within this area [81,83]. It is a remarkable frog that can adapt to a range of climates from desert conditions to those of wet rain forests. There is a related frog called *Litoria electrica* found only in northern Australia in a specific region just below the Gulf of Carpentaria [101]. Both of these frogs produce abundant glandular secretions on the skin, but the secretions contain neither neuropeptides like caerulein nor antibacterial peptides. How then do these animals protect themselves from predators? The granular glands produce large amounts of small peptides related to the tryptophyllins, first discovered in the South American hylid frog *Phyllomedusa rohdei* by Erspamer et al. [36,39,54]. The tryptophyllin peptides from the two Australian frogs are listed together in Table 1.

Fig. 14. *Litoria rubella*.

Erspamer has found that neither his nor our tryptophyllins show significant smooth muscle activity (no effect below a concentration of 10^{-6} M), and we have shown that neither tryptophyllins L 1.2 (FPWL-NH_2), 1.3 (pEFPWL-NH_2) nor 3.1 (FPWP-NH_2) inhibit neuronal nitric oxide synthase [32]. One of Erspamer's tryptophyllins (FPPWM-NH_2) induces sedation and behavioral sleep in birds, and is also immunoreactive to a set of cells in the rat adenohypophysis [63]. Recently, the precursor cDNA for a novel tryptophyllin (LPHAWVP-NH_2) from the Mexican leaf frog (*Pachymedusa dacnicolor*) has been cloned and shown to contract smooth muscle at nanomolar concentrations [21]. It is also of interest that the tryptophyllin peptides show some sequence similarity to the human brain endomorphins (e.g. YPWF-NH_2 and YPWG-NH_2) that have a very high affinity for the γ-receptor [112]). At this time, the role of the tryptophyllins shown in Table 1 is still undetermined.

5. Amphibian pheromones

Pheromones are substances that are released to cause a behavioral response in a conspecific, and are commonly involved in mating and courtship. Although alarm responses had been characterized in *Bufo bufo* tadpoles upon exposure to crushed tadpole as early as 1949 [12], the first pheromone identified from a vertebrate was only discovered quite recently, in 1995 [43]. This female-attractant sex pheromone came from the Japanese fire-bellied newt, *Cynops pyrrhogaster*. Sodefrin, a 10-residue peptide was isolated in 1995 and was named for the Japanese word "to solicit" [43]. During species specificity testing, it was discovered that another newt species, *C. ensicauda*, also had a female attractant aquatic sex pheromone. This 10-residue peptide differs from sodefrin at positions 3 and 8, was isolated in 2000 and named silefrin [111].

Sodefrin	SIPSKDALLK-OH
Silefrin	SILSKDAQLK-OH

These peptide hormones are secreted from the abdominal glands through the cloacae of the animals and are both species-specific female attractants.

A 22-kDa proteinaceous courtship pheromone has also been discovered in a terrestrial salamander, *Plethodon jordani*. This protein hormone is deposited directly onto the skin of the female by the male from his mental glands, located under the chin. This pheromone is thought to shorten the courtship process [67].

The first anuran sex pheromone was isolated from *L. splendida* in 1997. *L. splendida*, also known as the Magnificent Tree Frog, was first identified in 1977 by Tyler et al. [94]. Monthly secretions were collected from male and female specimens over a period of three years using the surface electrical stimulation method [97]. The chromatograms of these secretions indicated a small component present in the male secretions only. Comparison of the

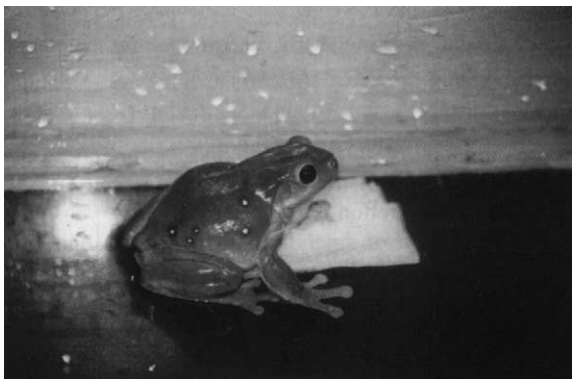


Fig. 15. Female *Litoria splendida* sitting on a pheromone sample during behavioral testing.

chromatograms from the three year period show that this compound, a 25-residue peptide, now named splendipherin, is produced in the highest levels during the mating season. Splendipherin GLVSSIGKALGGLLADVVKSKGQPA-OH

The peptide levels peak in February/March, at this point it constitutes up to 1% of the total secretion material, dropping to as low as 0.1% from June through to November, and was therefore investigated for a possible role in the breeding cycle of this species.

Behavioral tests were conducted in a 2-m glass tank containing a 2-cm depth of water. Females of the species exposed to the hormone at a concentration of ~10 pM, were attracted to the source with remarkably rapid response times [99]. Recognition of the peptide was apparent within twenty seconds of its introduction into the tank, and within an average of 6 min, female frogs would find and sit on the source of the pheromone (Fig. 15) [99] until physically removed. The tests were repeated with male *L. splendida* specimens and with *L. caerulea*, a related species of frog and showed that the pheromone is a species-specific female attractant with no effect on males or on other species.

As the peptide was not being moved toward the female by agitation of the water during the behavioral tests, as is the case with the newt hormones sodefrin and silefrin [43,111], nor being directly applied, as with the terrestrial salamander [67], we are interested in how the peptide moves through the aquatic environment. The structure of splendipherin has been determined using NMR and simulated annealing calculations; this structure is currently being employed in studies to determine the mode of action of splendipherin at the water surface [4].

6. Miscellaneous peptides

6.1. Antimalarial peptides

Anurans breed in aquatic environments that abound with insects, including mosquitoes. Even though malaria is rare in Australia, it is known that some European ranid frogs are prone to infestation by malaria parasites [76]. Perhaps anurans have evolved chemical protection against insects? Gas chromatographic separation of those components of the glandular secretion of *L. caerulea*, which are soluble in organic solvents, with mosquitoes enclosed in a container through which the effluent of the gas chromatogram passed, showed the presence of several volatile mosquito repellants. It was not possible to quantitatively reproduce the results of this experiment; the volatile components were present in trace amounts only, and some components were variable, differing from day to day. One of the insecticides was shown by GC/MS to be a methyl acetophenone (probably the *ortho*-isomer) [78].

Certain amphibian peptides kill the malaria parasite (*P. falciparum*). For example, the caerin 1 peptides are active at micromolar concentrations; caerin 1.8 (see Table 1 for the sequence) is the most potent. We are currently investigating the mode of action of the caerins 1 and other antimalarial peptides [92].

6.2. Inactive peptides

Marsh frogs of the genus *Limnodynastes* produce copious secretions from their dorsal granular glands, and in some cases from tibial glands on their legs. Only minute quantities of peptides are found in these secretions. We have named these peptides dynastins, and their sequences are listed in Table 1. An example is dynastin 1 (GLVSNLGI-NH_2, from *Limnodynastes interioris* [60]). The dynastins are all small anionic peptides, contain C-terminal CO_2H residues, and they exhibit no bio-activity in any of the test regimes that we now use. It is clear that this genus of frog has a very different defence mechanism to that of other animals that we have studied, and we do not know what that is.

Most of the tree frogs of the genus *Litoria* that we have studied produce inactive caeridin (e.g. caeridin 1, (GLLDGLLGTGL-NH_2) from various *Litoria* species [1]), frenatin (e.g. frenatin 1, (GLLDALSGILGL-NH_2) from *Litoria infrafrenata* [104]), or rubellidin (e.g. rubellidin 4.1 (GLGDILGLLGL-NH_2) from *L. rubella* [81,83]) peptides together with active peptides in their glandular secretions. These inactive peptides (see above and Table 1) show some structural resemblance to the dynastin peptides, but unlike the dynastins, they are all post translationally modified (C-terminal $CONH_2$). Their role in the amphibian integument is unknown.

Finally, there are peptides isolated from *L. electrica* and *Litoria rothii* that are unlike any other peptides obtained from Australian anurans. These include the anionic peptides electrin 2.1 (NEEEKVKWEPDVP-NH_2) from *L. electrica* [101] and the rotheins 2 and 3 (Table 1; e.g. rothein 2.1 (AGGLDDLLEPVLNSADNLVHGL-NH_2) from *L. rothii* [106]), which have shown no activity in any of our standard testing programs. They bear some resemblance to spacer peptides (e.g. the spacer peptides of the pre-pro-caerins

1 (see Section 3.3)) but most of these peptides from *L. electrica* and *L. rothii* are post translationally modified, containing C-terminal $CONH_2$ functionality.

7. Evolutionary implications

The recognition of taxa such as genera, species and subspecies was originally based entirely upon morphological characteristics. In the 1950s behavioral characteristics such as advertisement calls and other attributes were included in the definitions of taxa. With the development of biochemical techniques a new dimension was added [40].

A variety of tissue secretions has been used to determine the evolutionary relationships of frogs: e.g. *Bufo* parotoid gland toxins [47], skin alkaloids [56] and globin polypeptides [30].

With the development of a non-invasive technique to 'milk' dermal secretions from frog skin [95] it has become possible to study variations in individuals within species in Australia that have a broad geographic range, so demonstrating a divergence not suspected previously. An example is the Red Tree Frog, *L. rubella* (see Fig. 14), which occupies much of the northern half of the Australian continent as shown in Fig. 16. Specimens of *L. rubella* were examined from 15 localities. The peptide profiles differed in the majority of localities (see e.g. Fig. 17), the exceptions being from three adjacent localities in South Australia [81,83]. There is every indication that at least six populations of '*L. rubella*' can be recognized. What is required is a comparable study of pre-mating isolating mechanisms of *L. rubella* at these localities, to determine whether the populations have evolved isolating mechanisms separating them into distinct species. Preliminary data [48] of *L. rubella* taken at localities along the Stuart Highway from Marree (in South Australia) to Darwin (in the Northern Territory) (see Fig. 16), a distance of 2200 km, suggest

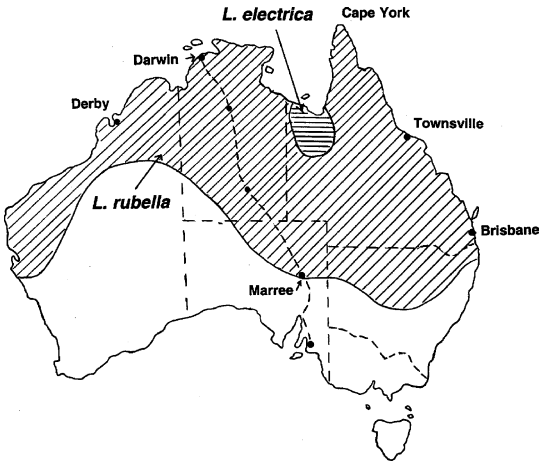


Fig. 16. Geographical distribution of *Litoria rubella* and *Litoria electrica* in Australia: (---) State boundaries; (---·---) Stuart Highway.

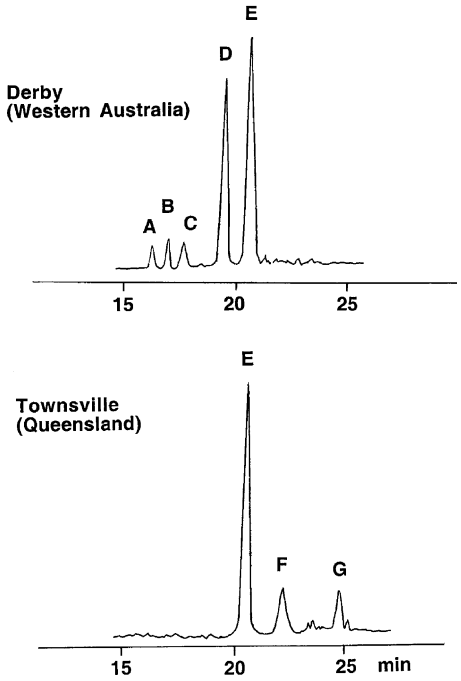


Fig. 17. HPLC peptide profiles of *Litoria rubella* [81,83] from Derby (Western Australia), and Townsville (Queensland). Peptides are as follows; (A) IEFFA-OH; (B) IEFFT-NH_2; (C) VDFFA-OH; (D) pEIPWFHR-NH_2; (E) FPWL-NH_2; (F) FPWP-NH_2; (G) FPFPWL-NH_2. For further information, including nomenclature of tryptophyllins, see Section 4.

that pre-mating data are consistent with peptide-profiling information.

To date, the only authenticated separation of a population from *L. rubella* is the recognition of *L. electrica* for individuals at localities south of the Gulf of Carpentaria in Queensland [42] (see Fig. 16). Peptide studies confirm the distinctness of this population as a separate species [101]. The peptide profile of *L. electrica* shows two tryptophyllins as the major components (the same as components E and F in Fig. 17), but it also contains six other peptides not produced by *L. rubella*. These data support the classification of *L. electrica* as a species separate from *L. rubella*, but also indicate the close relationship between the two species.

Of particular interest are the variations in the peptide profiles of *L. rubella* from the south (Brisbane) to the north (Cape York) of the Queensland eastern seaboard, a distance of 2300 km [82]. Fraction F (see Fig. 17) is minor compared with E in the south, but increases regularly to constitute the largest fraction in the north. Clinal variations in peptide profiles of this type can be interpreted as a progressive stage in evolution. Essentially, peptide studies provide an indication of genetic change.

The time scale of evolutionary change in peptide profiles may be short. Studies on the Green Tree Frog, *L. caerulea* [87] indicate significant differences in the peptide profiles of animals collected from different parts of Australia. There appear to be two major populations of *L. caerulea*, one in

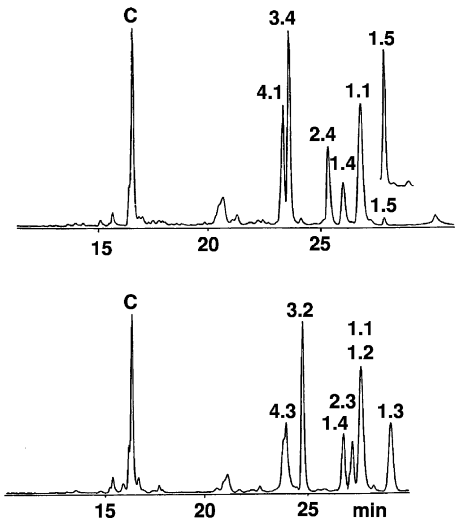


Fig. 18. HPLC peptide profiles of *Litoria caerulea* from (A) Proserpine (Queensland) and (B) Borroloola (Northern Territory). Peaks identified by numbers are caerin peptides: these numbers correspond to the sequences listed in Table 1. The peak designated by C is the neuropeptide caerulein 1.1 [pEQDY(SO_3)TGWMDF-NH_2].

the Northern Territory, the second along the Queensland eastern coastal region. The HPLC peptide profiles of animals obtained from these two areas are shown in Fig. 18. Whether these changes are predator or climate driven are not known at this time. Of particular interest are the differences in peptide profiles that have been noted between individuals at Darwin in the Northern Territory from those at Melville Island (off the coast from Darwin) [87]. These populations have been separated by the ocean for only 10,000 years.

We conclude that dermal peptide profiles of frogs provide a labile index of relationships that should be included in any evaluation of evolution of closely related species. The only proviso here is that the neuropeptide content of the peptide profile of an anuran may vary seasonally and this must be taken into account, e.g. the caerulein 1.1/1.2 variation of *L. splendida* (see Sections 4 and 5and [99]).

8. Conclusions

Australian amphibians have some of the most diverse yet simple host-defence peptides to be reported in the Animal Kingdom, ranging from neuropeptides with a wide range of activities to antibiotic, antiviral, antifungal, anticancer and antimalarial peptides. The range of 3D structures for the amphipathic antibiotic peptides (from simple amphipathic α helices to α helices with a central hinge) is spectacular, taking into account that most of these peptides have much the same spectrum of antibacterial activities. The modes of action of some of these active peptides are known (e.g. the smooth muscle activity of the caeruleins, and the membrane destroying power of the antimicrobial peptides), while others are currently under investigation (e.g. how structurally diverse peptides such as the frenatins 3, caerin 1 and citropin 1 peptides all inhibit the formation of NO from nNOS, and how the caerins 1 destroy the malaria parasite). The research that we carry out is primarily curiosity driven: we are fascinated by the host-defence chemistry of these primitive creatures. The impressive range of bio-activity of these peptides indicates that consideration of certain anuran peptides for pharmaceutical, clinical and/or agricultural use should be explored.

Acknowledgments

We thank the Australian Research Council, the South Australian Anticancer Foundation, and The University of Adelaide for providing the funding for this research.

References

[1] Anastasi A, Erspamer V, Endean R. Isolation and amino acid sequence of caerulein, the active decapeptide of the skin of *Hyla caerulea*. Arch Biochem Biophys 1968;125:57–68.
[2] Andreu D, Ubach J, Boman A, Wahlin B, Wade D, Merrifield RB, et al. Shortened cecropin A-melittin hybrids—significant size reduction retains potent antibiotic activity. FEBS Lett 1992; 296:190–4.
[3] Apponyi MA, Booker GW, Bowie JH. Unpublished observations.
[4] Apponyi MA, Bowie JH. Unpublished observations.
[5] Barker J, Grigg GC, Tyler MJ. A field guide to Australian frogs. NSW: Surrey Beatty and Sons; 1995.
[6] Barra D, Simmaco M. Amphibian skin: a promising resource for antimicrobial peptides. TIBTECH 1995;13:205–9.
[7] Barsukov IL, Lian L. Structure determination from NMR data. I. Analysis of NMR data. In: Roberts, GCK, editor. NMR of macromolecules: a practical approach. New York: Oxford University Press; 1993. p. 315–57.
[8] Bevins CL, Zasloff M. Peptides from frog skin. Annu Rev Biochem 1990;59:395–414.
[9] Biemann K, Martin SA. Mass spectrometric determination of the amino acid sequence of peptides and proteins. Mass Spectrom Rev 1987;6:1–76.
[10] Billiar TR. Nitric oxide, novel biology with clinical relevance. Ann Surg 1995;221:339–49.
[11] Bilusich D, Brinkworth CS, McAnoy AM, Bowie JH. The fragmentation of (M-H)-anions derived from underivatized peptides. The side chain loss of H2S from Cys. A joint experimental and theoretical study. Rapid Commun Mass Spectrom 2003;17:2488–94.
[12] Birch MC. Pheromones 32. Amsterdam: North-Holland; 1974.
[13] Bowie JH, Brinkworth CS, Dua S. Collision-induced fragmentations of the (M-H)-parent anions of underivatized peptides: an aid to structure determination and some unusual negative ion cleavages. Mass Spectrom Rev 2002;21:87–107.
[14] Bowie JH, Chia BCS, Tyler MJ. Host defence peptides from the skin glands of Australian amphibians: a powerful chemical arsenal. Pharmacol News 1998;5:16–21.
[15] Bowie JH, Wegener KL, Chia BCS, Wabnitz PA, Carver JA, Tyler MJ, et al. Host defence antibacterial peptides from skin secretions of Australian amphibians. The relationship between structure and activity. Protein Peptide Lett 1999;6:259–70.
[16] Bradford AM, Bowie JH, Tyler MJ, Wallace JC. New antibiotic uperin peptides from the dorsal glands of the Australian toadlet *Uperoleia mjobergii*. Aust J Chem 1996;49:1325–31.

[17] Bradford AM, Raftery MJ, Bowie JH, Tyler MJ, Wallace JC, Adams GW, et al. Novel uperin peptides from the dorsal glands of the Australian Flood Plain Toadlet *Uperoleia inundata*. Aust J Chem 1996;49:475–84.
[18] Bradford AM, Raftery MJ, Bowie JH, Wallace JC, Tyler MJ. Peptides from Australian frogs—the structures of the dynastins from *Limnodynastes salmini* and fletcherin from *Limnodynastes fletcheri*. Aust J Chem 1993;46:1235–44.
[19] Brinkworth CS, Bowie JH, Tyler MJ, Wallace JC. A comparison of the host defence skin peptides of the New Guinea Tree frog (*Litoria genimaculata*) and the Fringed Tree frog (*Litoria eucnemis*). The link between the caerin and the maculatin antimicrobial peptides. Aust J Chem 2002;55:605–10.
[20] Brinkworth CS, Dua S, McAnoy AM, Bowie JH. Negative ion fragmentations of deprotonated peptides: backbone cleavages directed through both Asp and Glu. Rapid Commun Mass Spectrom 2001;15:1965–73.
[21] Chen T, Orr DF, O'Rourke M, McLynn C, Biourson AJ, McLean S, et al. *Pachymedusa dacnocolor* tryptophyllin-1; structural characterization, pharmacological activity and cloning of precursor cDNA. Regulatory peptides, in press.
[22] Chia BCS, Carver JA, Lindner RA, Bowie JH, Wong H, Lie W. Caerin 4.1, an antibiotic peptide from the Australian tree frog *Litoria caerulea*. The NMR-derived solution structure. Aust J Chem 2000;53:257–65.
[23] Chia BCS, Carver JA, Mulhern TD, Bowie JH. Maculatin 1.1, an antimicrobial peptide from the Australian tree frog, *Litoria genimaculata*. Solution structure and biological activity. Eur J Biochem 2000;267:1894–908.
[24] Chia BCS, Carver JA, Mulhern TD, Bowie JH. The solution structure of uperin 3.6, an antibiotic peptide from the granular dorsal glands of the Australian toadlet, *Uperoleia mjobergii*. J Peptide Res 1999;54:137–45.
[25] Clark DP, Durell S, Maloy WL, Zasloff M. Ranalexin—a novel antimicrobial peptide from bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymyxin. J Biol Chem 1994;269:10849–55.
[26] Clore GM, Gronenborn AM. Determination of three-dimensional structures of proteins and nucleic acids in solution by nuclear magnetic resonance spectroscopy. Crit Rev Biochem Mol Biol 1989;24:479–564.
[27] Crane BR, Arvai AS, Gachhui R, Wu C, Ghosh DK, Getzoff ED, et al. The structure of nitric oxide synthase oxygenase domain and inhibitor complexes. Science 1997;278:425–31.
[28] Cruciani RA, Barker JL, Zasloff M, Chen H, Colamonici O. Antibiotic magainins exert cytolytic activity against transformed cell lines through channel formation. Proc Natl Acad Sci USA 1991;88:3792–6.
[29] Dathe M, Wieprecht T. Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. Biochim Biophys Acta Biomembr 1999;1462:71–87.
[30] Dessauer HC, Nevo E. Geographic variation of blood and liver proteins in cricket frogs. Biochem Genet 1969;3:171–88.
[31] Doyle J, Brinkworth CS, Wegener KL, Carver JA, Llewellyn LE, Olver IN, et al. nNOS inhibition antimicrobial and anticancer activity of the amphibian skin peptide, citropin 1.1 and synthetic modifications: the solution structure of a modified citropin 1.1. Eur J Biochem 2003;270:1141–53.
[32] Doyle J, Llewellyn LE, Brinkworth CS, Bowie JH, Wegener KL, Rozek T, et al. Amphibian peptides that inhibit neuronal nitric oxide synthase: the isolation of lesueurin from the skin secretion of the Australian Stony Creek Frog *Litoria lesueuri*. Eur J Biochem 2002;269:100–9.
[33] Epand RM, Shai YC, Segrest JP, Anantharamaiah GM. Mechanisms for the modulation of membrane bilayer properties by amphipathic helical peptides. Biopolymers 1995;37:319–38.
[34] Erspamer V. Bioactive secretions of the amphibian integument. In: Heatwole H, editor. Amphibian biology: the integument. vol. 1. Norton, NSW: Surrey, Beatty and Sons; 1994. p. 178–350.
[35] Erspamer V, Erspamer GF, Mazzanti G, Endean R. Active peptides in the skins of one hundred amphibian species from Australia and Papua New Guinea. Comp Biochem Physiol 1984;77C:99–108.
[36] Erspamer V, Melchiorri P, Erspamer GF, Montecucchi PC, de Castiglione R. Phyllomedusa skin: a huge factory and store-house of a variety of active peptides. Peptides 1985;6:7–12.
[37] Evans JNS. Biomolecular NMR spectroscopy. Oxford: Oxford University Press; 1995. p. 55–77.
[38] Ganz T. Biosynthesis of defensins and other antimicrobial peptides. In: Marsh J, Goode J, editors. Antimicrobial peptides. Ciba Foundation Symposium 186. London: John Wiley and Sons; 1994.
[39] Gozzini L, Montecucchi PC, Erspamer V, Melchiorri P. Tryptophyllins from extracts of *Phyllomedusa rhodei* skin: new tetra-, penta- and heptapeptides. Int J Peptide Protein Res 1985; 25:323–9.
[40] Guttman SI. Biochemical techniques and problems in anuran evolution. In: JL, V, editors. Evolutionary biology of the anurans. Contemporary research on major problems. Columbia: University of Missouri Press; 1973. p. 183–203.
[41] Hensmann M, Booker GW, Panayotou G, Boyd J, Linacre J, Waterfield M, et al. Phosphopeptide binding to the N-terminal SH2 domain of the p85-alpha subunit of PI 3′-kinase: a heteronuclear NMR study. Protein Sci 1994;3:1020–30.
[42] Ingram G, Corben C. *Litoria electrica*: a new tree frog from western Queensland. Mem Qld Mus 1993;28:475–8.
[43] Kikuyama S, Toyoda F, Ohmiya Y, Matsuda K, Tanaka S, Hayashi H. Sodefrin: a female-attracting peptide pheromone in newt cloacal glands. Science 1995;267:1643–5.
[44] Klee CB, Vanaman TC. Calmodulin. Adv Protein Chem 1982;35:213–321.
[45] Lazarus LH, Attila M. The toad, ugly and venomous, wears yet a precious jewel in his skin. Prog Neurobiol 1993;41:473–507.
[46] Lincoln J, Hoyle CHV, Burnstock G. Nitric oxide in health and disease. Cambridge: Cambridge University Press; 1997
[47] Low BS. Evidence from parotoid—gland secretions. In: Blair WF, editor. Evolution in the genus Bufo. Austin: University of Texas Press; 1972. p. 244–64.
[48] Low BS. Unpublished observations.
[49] Marcotte I, Wegener KL, Lam Y, Chia BCS, de Planque MRR, Bowie JH, et al. Interaction of antimicrobial peptides from Australian amphibians with lipid membranes. Chem Phys Lipids 2003;122:107–20.
[50] Maselli V, Musgrave I, Bowie JH, Tyler MJ. Unpublished observations.
[51] Maselli V, Pukala TL, Bowie JH, Tyler MJ. Unpublished observations.
[52] Matsuzaki K, Sugishita K, Fujii N, Miyajima K. Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. Biochemistry 1995;34:3423–9.
[53] McDonnell PA, Opella SJ. Effect of detergent concentration on multidimensional solution NMR spectra of membrane proteins in micelles. J Magn Res B 1993;102:120–5.
[54] Montecucchi PC, Gozzini L, Erspamer V, Melchiorri P. The primary structure of tryptophan containing peptides from skin extracts of *Phyllomedusa rhodei* (tryptophyllins). Int J Peptide Protein Res 1984;24:276–85.
[55] Mutschmann F, Berger L, Zwart P, Gaedicke C. Chytridiomycosis in amphibians—first report in Europe http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_11084755&dopt=Abstract.
[56] Myers CW, Daly JW, Garroffo HM, Wisnieski A, Cover JFJ. Discovery of the Costa Rican poison frog *Dendrobates granuliferus* in sympatry with *Dendrobates pumilio*, and comments on taxonomic use of skin alkaloids. Am Mus Novit 1995;3144:1–21.

[57] Nakaoka Y, Tanaka H, Oosawa F. Ca^{2+}-dependent regulation of beat frequency of cilia in *Paramecium*. J Cell Sci 1984;65:223–31.
[58] Nelson JW, Kallenbach NR. Stabilization of the ribonuclease S-peptide a-helix by trifluoroethanol. Proteins Struct Funct Genet 1986;1:211–7.
[59] Pukala TL, Brinkworth CS, Carver JA, Bowie JH. Investigating the importance of the flexible hinge in caerin 1.1: the solution structures and activity of two synthetically modified caerin peptides. Biochemistry 2004;43:937–44.
[60] Raftery MJ, Bradford AM, Bowie JH, Wallace JC, Tyler MJ. Peptides from Australian frogs—the structures of the dynastins from the Banjo frogs *Limnodynastes interioris*, *Limnodynastes dumerilii* and *Limnodynastes terraereginae*. Aust J Chem 1993;46:833–42.
[61] Rajan R, Balaram P. A model for the interaction of trifluoroethanol with peptides and proteins. Int J Peptide Protein Res 1996;48:328–36.
[62] Rang HP, Dale MM, Ritter JM. Nitric oxide, editors. In: Pharmacology. Edinburgh: Churchill Livingstone; 1999. p. 188–97.
[63] Renda T, D'Este L, Buffa R, Usellini L, Capella C, Vaccaro R, et al. Tryptophyllin-like immunoreactivity in rat adenohypophysis. Peptides 1985;6:197–202.
[64] Resnick NM, Maloy WL, Guy HR, Zasloff M. A novel endopeptidase from *Xenopus* that recognizes a-helical secondary structure. Cell 1991;66:541–54.
[65] Rollins-Smith LA, Bowie JH, Tyler MJ. Unpublished observations.
[66] Rollins-Smith LA, Carey C, Conlon JM, Reinert LK, Doersam JK, Bergman T, et al. Antimicrobial activity of Temporin family peptides against the Chytrid fungus (*Batrachochytrium dendrobatidis*) associated with global amphibian declines. Agent Chemother 2003;47:1157–60.
[67] Rollman SM, Houck LD, Feldhoff RC. Proteinaceous pheromone affecting female receptivity in a terrestrial salamander. Science 1999;285:1907–9.
[68] Rozek T, Bowie JH, Wallace JC, Tyler MJ. The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs *Litoria aurea* and *Litoria raniformis*. Part 2. Sequence determination using electrospray mass spectrometry. Rapid Commun Mass Spectrom 2000;14:2002–11.
[69] Rozek T, Waugh RJ, Steinborner ST, Bowie JH, Tyler MJ, Wallace JC. The maculatin peptides from the skin glands of the tree frog *Litoria genimaculata*—a comparison of the structures and antibacterial activities of maculatin 1.1 and caerin 1.1. J Peptide Sci 1998;4:111–5.
[70] Rozek T, Wegener KL, Bowie JH, Olver IN, Carver JA, Wallace JC, et al. The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs *Litoria aurea* and *Litoria raniformis*. The solution structure of aurein 1.2. Eur J Biochem 2000;267:5330–41.
[71] Sai KP, Jagannadham MV, Vairaman M, Rajii NP, Devi AS, Nagaraj R. Tigerinins: novel antimicrobial peptides from the Indian frog *Rana tigerina*. J Biol Chem 2001;276:2701–7.
[72] Sansom MSP. The biophysics of peptide models of ion channels. Prog Biophys Mol Biol 1991;55:139–235.
[73] Shai Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by a-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta 1999;1462:55–70.
[74] Shai Y, Oren Z. From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. Peptides 2001;22:1629–41.
[75] Simmaco M, Mignogna G, Barra D. Antimicrobial peptides from amphibian skin: what do they tell us? Biopolymers 1998;47:435–50.
[76] Smith TG, Kim B, Hong H, Desser SS. Intraerythrocytic development of species of Hepatazoon infecting Ranid frogs: evidence for convergence of life cycle characteristics among Apicomplexans. J Parasitol 2000;86:451–8.
[77] Speare R, Berger L. Chytridiomycosis in amphibians in Australia http://www.jcu.edu.au/school/phtm/PHTM/frogs/chyspec.htm
[78] Steinborner ST, Bowie JH, Tyler MJ. Unpublished observations.
[79] Steinborner ST, Bowie JH, Tyler MJ, Wallace JC. An unusual combination of peptides from the skin glands of Ewings tree frog, *Litoria ewingi*—sequence determination and antimicrobial activity. Aust J Chem 1997;50:889–94.
[80] Steinborner ST, Currie GJ, Bowie JH, Wallace JC, Tyler MJ. New antibiotic caerin 1 peptides from the skin secretion of the Australian tree frog *Litoria chloris*—comparison of the activities of the caerin 1 peptides from the genus *Litoria*. Int J Peptide Protein Res 1998;51:121–6.
[81] Steinborner ST, Gao CW, Raftery MJ, Waugh RJ, Blumenthal T, Bowie JH, et al. The structures of four tryptophyllin and three rubellidin peptides from the Australian red tree frog *Litoria rubella*. Aust J Chem 1994;47:2099–108.
[82] Steinborner ST, Wabnitz PA, Bowie JH, Tyler MJ. The application of mass spectrometry to the study of evolutionary trends in amphibians. Rapid Commun Mass Spectrom 1996;10:92–5.
[83] Steinborner ST, Wabnitz PA, Waugh RJ, Bowie JH, Gao CW, Tyler MJ, et al. The structures of new peptides from the Australian Red Tree Frog *Litoria rubella*—the skin peptide profile as a probe for the study of evolutionary trends of amphibians. Aust J Chem 1996;49:955–63.
[84] Steinborner ST, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ, Ramsay SL. New caerin antibacterial peptides from the skin glands of the Australian tree frog *Litoria xanthomera*. J Peptide Sci 1997;3:181–5.
[85] Steinborner ST, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ, Ramsey SL. New caerin antibiotic peptides from the skin glands of the Australian tree frog *Litoria xanthomera*. Sequence determination by mass spectrometry. Rapid Commun Mass Spectrom 1997;11:997–1101.
[86] Stone DJM, Bowie JH, Tyler MJ, Wallace JC. The structure of caerin 1.1, a novel antibiotic peptide from Australian tree frogs. J Chem Soc Chem Commun 1992;72:1224–5.
[87] Stone DJM, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ. Peptides from Australian frogs. The structures of the caerins from *Litoria caerulea*. J. Chem. Res. (S) 1993;138–9; (M) 1993;910–36.
[88] Stone DJM, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ. Peptides from Australian frogs. Structures of the caerins and caeridin 1 from *Litoria splendida*. J. Chem. Soc. Perkin Trans. I 1992;3173–78.
[89] Stone DJM, Waugh RJ, Bowie JH, Wallace JC, Tyler MJ. Peptides from Australian frogs. The structures of the caerins from *Litoria caerulea*. J Chem Res (S) (M) 1993;138:910–36.
[90] Stuehr DJ, Ghosh S. Enzymology of nitric oxide synthases. In: Mayer B, editor. Nitric oxide. Handbook of experimental pharmacology. Berlin: Springer-Verlag; 2000:33–70.
[91] Sutcliffe MJ. Structure determination from NMR data II. Computational approaches. In: Roberts GCK, editor. NMR of macromolecules: a practical approach. New York: Oxford University Press; 1993:359–90.
[92] Tilley L, Maselli V, Bowie JH. Unpublished observations.
[93] Tossi A, Tarantino C, Romeo D. Design of synthetic antimicrobial peptides based on sequence analogy and amphipathicity. Eur J Biochem 1997;250:549–58.
[94] Tyler MJ, Davies M, Martin AA. A new species of large, green tree frog from Northern Western Australia. Trans R Soc S Aust 1977;101:133–8.
[95] Tyler MJ, Stone DJM, Bowie JH. A novel method for the release and collection of dermal, glandular secretions from the skin of frogs. J Pharm Toxicol Methods 1992;28:199–200.
[96] Vanhoye D, Brustion F, Nicolas P, Amiche M. Antimicrobial peptides from hylid and ranid frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but hypermutable antimicrobial domain. Eur J Biochem 2003;270:2068–81.
[97] Wabnitz PA. PhD thesis. Department of Chemistry, University of Adelaide, Adelaide; 1999.

[98] Wabnitz PA, Bowie JH, Tyler MJ. Caerulein-like peptides from the skin glands of the Australian Blue Mountains Tree Frog *Litoria citropa*. Part 1. Sequence determination using electrospray mass spectrometry. R Commun Mass Spectrom 1999;13:2498–502.

[99] Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP. Differences in the skin peptides of the male and female Australian tree frog *Litoria splendida*—the discovery of the aquatic male sex pheromone splendipherin, together with Phe8 caerulein and a new antibiotic peptide caerin 1.10. Eur J Biochem 2000;267:269–75.

[100] Wabnitz PA, Bowie JH, Wallace JC, Tyler MJ. The citropin peptides from the skin glands of the Australian Blue Mountains Tree Frog *Litoria citropa*. Part 2: sequence determination using electrospray mass spectrometry. Rapid Commun Mass Spectrom 1999;13:1724–32.

[101] Wabnitz PA, Bowie JH, Wallace JC, Tyler MJ. Peptides from the skin glands of the Australian Buzzing Tree Frog *Litoria electrica*. Comparison with the skin peptides of the Red Tree Frog *Litoria rubella*. Aust J Chem 1999;52:639–45.

[102] Wabnitz PA, Bowie JH, Tyler MJ, Wallace JC, Smith BP. Differences in the skin peptides in male and female Australian tree frog *Litoria splendida*. Eur J Biochem 2000;267:269–75.

[103] Wabnitz PA, Walters H, Tyler MJ, Wallace JC, Bowie JH. First record of host defence peptides in tadpoles. The magnificent tree frog *Litoria splendida*. J Peptide Res 1998;52:477–81.

[104] Waugh RJ, Raftery MJ, Bowie JH, Wallace JC, Tyler MJ. The structures of the frenatin peptides from the skin secretions of the giant tree frog *Litoria infrafrenata*. J Peptide Sci 1996;2:117–24.

[105] Waugh RJ, Stone DJM, Bowie JH, Wallace JC, Tyler MJ. Peptides from Australian frogs. The structures of the caerins and caeridins from *Litoria gilleni*. J Chem Res (S) (M) 1993;139:937–61.

[106] Wegener KL, Brinkworth CS, Bowie JH, Wallace JC, Tyler MJ. Bioactive dahlein peptides from the skin secretions of the Australian aquatic frog *Litoria dahlii*: sequence determination by electrospray mass spectrometry. Rapid Commun Mass Spectrom 2001;15:1726–34.

[107] Wegener KL, Wabnitz PA, Carver JA, Bowie JH, Chia BCS, Wallace JC, et al. Host defence peptides from the skin glands of the Australian Blue Mountains Tree Frog *Litoria citropa*. Solution structure of the antibacterial peptide citropin 1.1. Eur J Biochem 1999;265:627–37.

[108] Wieprecht T, Dathe M, Schumann M, Krause E, Beyermann M, Bienert M. Conformational and functional study of magainin 2 in model membrane environments using the new approach of systematic double-D-amino acid replacement. Biochemistry 1996;35:10844–53.

[109] Wong H, Bowie JH, Carver JA. The solution structure and activity of caerin 1.1, an antimicrobial peptide from the Australian green tree frog, *Litoria splendida*. Eur J Biochem 1997;247:545–57.

[110] Xu RX, Word JM, Davis DG, Rink MJ, Willard DH, Gampe RT. Solution structure of the human pp60c-src SH2 domain complexed with a phosphorylated tyrosine pentapeptide. Biochemistry 1995;34:2107–21.

[111] Yamamoto K, Kawai Y, Hayashi T, Ohe Y, Hayashi H, Toyoda F, et al. Silefrin, a sodefrin-like pheromone in the abdominal gland of the sword-tailed newt, *Cynops ensicauda*. FEBS Lett 2000;472:267–70.

[112] Zadina JE, Hackler L, Ge L, Kastin AJ. A potent and selective endogenous agonist for the μ-opiate receptor. Nature 1997;386:499–502.