Host-defence peptides from the glandular secretions of amphibians: structure and activity

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This review covers the literature on the subject of biologically active peptides from the glands of amphibians. These include neuropeptides, antimicrobial and anticancer active peptides, antiviral agents, fungicides and peptides which complex with Ca^{2+} calmodulin. Other topics covered include sex pheromones from amphibians, and the use of peptide profiling to differentiate between species and different populations of the same species.

1 Introduction

Amphibians have 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 specialised glands on the dorsal surface and into the gut of the amphibian. There are many different types of compounds in these secretions; these include amines, alkaloids and peptides. This review is concerned with the structures and activities of the host-defence peptides of amphibians. Among these active peptides are neuropeptides, those with antimicrobial, anticancer, antiviral and fungicide activities,[1–5] those which complex with the regulatory protein Ca^{2+} calmodulin,[6] and finally, sex pheromones.[6]

In early research carried out with anurans, many hundreds of dried skins of a particular species were extracted to obtain active peptides,[7] a method which, today, is environmentally unacceptable. This method is also chemically unsound because the active peptides are stored in the inactive propeptide form in the glands.[6] Modern methods utilise techniques which do not involve killing the animal; for example, injection with noradrenaline,[8] or the non-invasive electrical stimulation method to effect release of the secretion onto the skin.[9] Using these methods, active peptides may be isolated and identified from the skin secretion of just one animal.[6] The active peptides are contained in the skin glands of metamorph and adult animals,[10] and in at least one species (*Litoria splendida*) it has been shown that tadpoles produce the same active peptides as the adult.[11] Active peptides are purified by either column chromatography, electrophoresis or (more usually) by high performance liquid chromatography. Sequence determination of peptides is carried out using mass spectrometric and/or automated Edman degradation methods, with the secondary structure obtained by 2D NMR or (less likely for peptides) by X-ray diffraction methods.[6] mRNA/cDNA encoding of the peptides provide the structures of the initially formed prepropeptides.[12]

One of the most fascinating aspects arising from studies of active peptides from amphibians is that major peptides in secretions often have multi-faceted activities. Three much-studied examples are cited:

(i) The potent neuropeptide caerulein [pEQDY(SO_3)-TGWMDF-NH_2] was first isolated from the Australian green tree frog *Litoria caerulea*,[3,13] and is also produced by other species of the genus *Litoria*,[6] together with *Xenopus laevis*[3]

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and *Leptodactylus labyrinthicus.*[3] Caerulein both contracts and relaxes smooth muscle (depending on the origin of the smooth muscle) and affects blood pressure at better than nanomolar concentrations. Caerulein is also an analgesic some 2000 times more active than morphine.[3]

(ii) The most studied of all anuran membrane-active peptides, magainins 1 and 2 [*e.g.* magainin 2 (GIGKFLHSAKKFGKAFVGEIMNS)], were isolated independently by Williams[14,15] and Zasloff[16,17] from the African clawed frog *Xenopus laevis.* Both magainin 1 and 2 are wide-spectrum amphipathic helical peptides that are antimicrobially active, anticancer agents and fungicides at μM concentrations. The natural magainins and some synthetic modifications also lyse protozoa,[18] and magainin 2 amide and analogues have shown promise as spermicides[19] and contraceptives.[20,21]

(iii) The caerin 1 membrane-active peptides from species of the genus *Litoria*[6,22] [*e.g.* caerin 1.1 (GLLSVLGSVAKHVLPHVLPHVVPVIAEHL-NH_2), which has two helices separated by a flexible hinge region[23,24]] are wide-spectrum antibiotics, anticancer agents active at μM concentrations against all human tumours tested by the National Cancer Institute (NCI; in their routine screening program), and antiviral agents against viruses with envelopes [*e.g.* HIV and *Herpes simplex* 1 (MIC 7.8 and 11.3 μM respectively for caerin 1.1[6,25]]. They also kill nematodes[6] and inhibit the formation of NO from neuronal nitric oxide synthase (nNOS) at μM concentrations.[6,26,27]

In this review, generally, only those amphibian peptides whose activities have been studied will be mentioned. Different groups of researchers often use different test organisms and record activities in different ways, which makes the consolidation of such data somewhat difficult. In the case of antimicrobial and neuropeptide activities, the activities will be generalised in tables with selected examples described in the text. Peptides whose activities have not been determined will not be included, unless there is a particular reason for such inclusion. Recently, mRNA and/or cDNA methods have been used extensively to uncover the DNA coding for the precursors of active peptides and also to identify new active peptides. Peptides which have been identified from DNA sequences will only be described if they have been isolated as native (active) peptides from the amphibian in question. Unexpressed peptides identified by these methods will not be included.

2 Antibacterial and anticancer active peptides

2.1 Introduction

Many frog and toad species have glandular secretions which contain at least one wide-spectrum antibiotic peptide together with a number of other peptides which show narrow-spectrum activity against one or several bacteria. Such a cocktail of antibiotic peptides provides enhanced protection against a range of bacteria.[1–6] Many amphibian wide-spectrum antibiotic peptides also exhibit anticancer activity,[28] for example when investigated by the National Cancer Institute (Washington DC) using *in vitro* testing of their chemosensitivity towards 60 human tumour cell lines.[29] This joint antibiotic/anticancer activity suggests the

likelihood of a similar mechanism of action against bacterial and cancer cells.

The antibiotic peptide is synthesised as a signal–spacer peptide precursor, in which the signal portion of the precursor peptide directs the peptide to the appropriate place in the gland before being cleaved by a protease releasing the inactive spacer peptide. When the animal is attacked, stimulated or sick, a second protease removes the spacer and the active peptide is secreted onto the skin or into the gut as required.[30] It is not unusual for the active antibiotic peptide to be cytotoxic to the frog or toad, the consequence of which is that a third protease deactivates the active peptide after some period of time on the skin (normally 5–30 minutes depending on the species). This degradation either involves cleavage of the peptide in the centre (*e.g.* the magainins[31]) or removal of several amino acid residues from the N-terminal end of the peptide.[6]

The antibiotic and anticancer activity is a result of the active peptide inducing alterations in the hydrophobic–hydrophilic seal of the cell membrane, effecting lysis of the bacterial or cancer cell. This often occurs at a concentration lower than that necessary to lyse normal eukaryotic cells. A number of different scenarios have been proposed to explain membrane permeation or lysis, but essentially there are two major mechanisms. The first is the barrel-stave or pore-forming mechanism, where α-helical amphipathic peptides bind initially to the outside of the lipid bilayer, and then penetrate the bilayer to produce defined pores which are oriented perpendicular to the plane of the bilayer.[32–38] A minimum of 20 amino acid residues is required to span the bilayer, but there are examples where smaller peptides can dimerise to effect full penetration of the barrier.[39] The second process is called the carpet mechanism, where peptides remain bound to the membrane interface and disrupt the bilayer by a detergent-like or carpet-like effect. Above a critical concentration, holes are formed due to strain on the bilayer, and the membrane degrades into micelle-like complexes.[39–42] Both of these mechanisms cause disruption of membrane function, resulting in an excessive flux of ions and small molecules across the cytoplasmic membrane bilayer, ultimately leading to cell lysis.

Both the primary and secondary structures of an antibiotic/anticancer peptide have a direct influence on the activity. Features including the degree of helicity, the charge state, amphipathicity and hydrophobicity are also significant.[43–45]

Tables 1, 3 and 5 summarise the sequences and general activities of over three hundred peptides (isolated from frogs and toads) whose antimicrobial activities have been determined. Peptides are listed alphabetically as trivial names. It has also been reported that salamanders contain antibiotic peptides in their glandular secretions but the sequences of these have not, as yet, been reported.[62] The antimicrobial/anticancer active peptides from the *Litoria*, *Uperoleia* and *Crinia* genera are dealt with first because we have more detailed data concerning the active peptides from these anuran genera.

2.2 Antibacterial and anticancer active peptides from the genera *Litoria*, *Uperoleia* and *Crinia*

There are three major types of antimicrobial peptides listed in Table 1. These are (i) wide-spectrum antibiotics based on the citropin 1.1 structure (aureins 1–3, citropins 1 and 2, signiferins 2 and uperins 2–4), (ii) wide-spectrum antibiotics based on the caerin 1.1 structure (caerins 1 and maculatins 1), and (iii) narrow-spectrum antibiotics (*e.g.* caerins 2–4 and maculatin 3). The antibiotic activities of selected peptides from these three groups are listed in Table 2. The wide-spectrum antibiotics of classes (i) and (ii) are also anticancer agents, all active against the major human cancer cell lines (leukaemia, lung, colon, CNS, melanoma, ovarian, renal, prostate and breast cancers) tested by NCI (Washington DC). These peptides generally show EC_{50} values at concentrations of 10^{-6} or 10^{-5} M.[6] The most active of these anticancer peptides are citropin 1.1 and caerin 1.1, which show activities against all the tested human tumour lines at 10^{-6} M, a concentration at which these two peptides do not lyse red blood cells.[6] Narrow-spectrum antibiotics of type (iii) show no anticancer activity at concentrations $\leq 10^{-4}$ M.

Most of the wide-spectrum antibiotic/anticancer peptides listed in Table 1 have post-translationally modified $CONH_2$ groups at the C-terminal end of the peptide. This has the effect of increasing the positive charge of the peptide and is generally essential for the activity of these peptides. Not all amphibian wide-spectrum antibiotic peptides have C-terminal $CONH_2$ groups. For example, the disulfide-containing antibiotics from the genus *Rana* have C-terminal CO_2H groups (see Table 3).

2.2.1 Citropin 1 type antibiotic peptides. The peptides aureins 1–3, citropins 1 and 2, signiferins 2 and uperins 2–4 have been shown by 2D NMR experiments in model phospholipids to be amphipathic, *i.e.* α-helices with well-defined hydrophobic and hydrophilic regions. The peptides studied by this technique are aurein 1.2,[46] citropin 1.1,[55] and uperin 3.6.[63]

The structure of citropin 1.1, shown in Fig. 1, is typical of these structures. The activities of aurein 2.4 and citropin 1.1 against a number of bacteria are listed in Table 2. Both show significant activity against Gram-positive organisms, but less activity against Gram-negative bacteria. The spectrum of activities of natural L-citropin 1.1 is very similar to that of synthetic D-citropin 1.1 (see Table 2), ruling out the possibility that citropin 1.1 interacts with specific chiral receptors.[6] A number of synthetic citropin 1.1 analogues have been prepared. Replacing Lys7 and Lys8 or Leu16 with Ala destroys both antibiotic and anticancer activity. However, increasing the positive charge of citropin 1.1 within the amphipathic framework increases the activity against both Gram-positive and Gram-negative organisms [see Ci1.1m (Table 2)].[64] Most of the antibiotic peptides considered in this section contain a Gly residue at the N-terminal end of the peptide. The signiferin 2 antibiotics are exceptions, since the sequences of both signiferin 2.1 and 2.2 commence with Ile. The N-terminal Ile is not essential

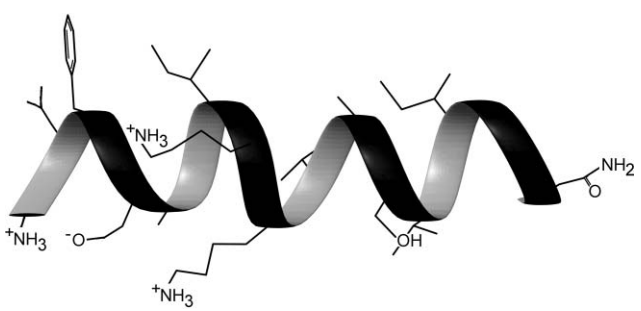

Fig. 1 Citropin 1.1. Structure determined by 2D NMR study in micelles.

Table 1 Antibiotic peptides from the genera *Litoria* (*L.*), *Uperoleia* (*U.*) and *Crinia* (*C.*)

Name	Sequence	M.W.	Activity		Species
Aurein 1.1	GLFDIIKKIAESI-NH_2	1444	+	w	*L.raniformis*[46]
Aurein 1.2	GLFDIIKKIAESF-NH_2	1478	+/-	w	*L.raniformis*[46]
Aurein 2.1	GLLDIVKKVVGAFGSL-NH_2	1613	+	w	*L.aurea, L.raniformis*[46]
Aurein 2.2	GLFDIVKKVVGALGSL-NH_2	1613	+	w	*L.aurea*[46]
Aurein 2.3	GLFDIVKKVVGAIGSL-NH_2	1613	+	w	*L.aurea*[46]
Aurein 2.4	GLFDIVKKVVGTLAGL-NH_2	1627	+	w	*L.aurea*[46]
Aurein 2.5	GLFDIVKKVVGAFGSL-NH_2	1647	+	w	*L.aurea, L.raniformis*[46]
Aurein 2.6	GLFDIAKKVIGVIGSL-NH_2	1627	+	w	*L.raniformis*[46]
Aurein 3.1	GLFDIVKKIAGHIAGSI-NH_2	1736	+	w	*L.aurea, L.raniformis*[46]
Aurein 3.2	GLFDIVKKIAGHIASSI-NH_2	1766	+	w	*L.aurea, L.raniformis*[46]
Aurein 3.3	GLFDIVKKIAGHIVSSI-NH_2	1794	+	w	*L.raniformis*[46]
Aurein 5.2	GLMSSIGKALGGLIVDVLKPKTPAS-OH	2450	+	n	*L.aurea, L.raniformis*[46]
Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	2582	+/-	w	*L.splendida, L.caerulea, L.gilleni*[47-49]
Caerin 1.2	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	2552	+	w	*L.caerulea*[48]
Caerin 1.3	GLLSVLGSVAQHVLPHVVPVIAEHL-NH_2	2582	+	w	*L.caerulea*[48]
Caerin 1.4	GLLSSLGSVAKHVLPHVVPVIAEHL-NH_2	2600	+/-	w	*L.caerulea, L.gilleni*[48,49]
Caerin 1.5	GLLSVLGSVVKHVOPHVVPVIAEHL-NH_2	2610	+/-	w	*L.caerulea*[48]
Caerin 1.6	GLFSVLGAVAKHVLPHVVPVIAEKL-NH_2	2591	+/-	w	*L.splendida, L.xanthomera, L.chloris*[47,50,51]
Caerin 1.7	GLFKVLGSVAKHLLPHVAPVIAEKL-NH_2	2634	+/-	w	*L.xanthomera, L.chloris*[50,51]
Caerin 1.8	GLFKVLGSVAKHLLPHVVPVIAEKL-NH_2	2662	+/-	w	*L.chloris*[51]
Caerin 1.9	GLFGVLGSIAKHVLPHVVPVIAEKL-NH_2	2591	+/-	w	*L.chloris*[51]
Caerin 1.10	GLLSVLGSVAKHVLPHVVPVIAEKL-NH_2	2573	+/-	w	*L.splendida*[47]
Caerin 1.11	GLLGAMFKVASKVLPHVVPAITEHF-NH_2	2659	+	w	*L.eucnemis*[52]
Caerin 1.17	GLFSVLGSVAKHLLPHVAPIIAEKL-NH_2	2606	+	w	*L.gracilenta*[53]
Caerin 1.18	GLFSVLGSVAKHLLPHVVPVIAEKL-NH_2	2620	+	w	*L.gracilenta*[53]
Caerin 1.19	GLFKVLGSVAKHLLPHVAPIIAEKL-NH_2	2600	+	w	*L.gracilenta*[53]
Caerin 1.20	GLFGILGSVAKHVLPHVIPVVAEHL-NH_2	2600	+	w	*L.caerulea/L.splendida* hybrid[54]
Caerin 2.1	GLVSSIGRALGGLLADVVKSKGQPA-OH	2392	-	n	*L.splendida*[47]
Caerin 2.2	GLVSSIGRALGGLLADVVKSKEQPA-OH	2464	+/-	n	*L.caerulea*[48]
Caerin 2.5	GLVASIGRALGGLLADVVKSKEQPA-OH	2448	+	n	*L.gilleni*[49]
Caerin 2.6	GLVSSIGKVLGGLLADVVKSKGQPA-OH	2392	+	n	*L.caerulea/L.splendida* hybrid[54]
Caerin 2.7	GLVSSIGKALGGLLVDVVKSKGQPA-OH	2392	+	n	*L.caerulea/L.splendida* hybrid[54]
Caerin 3.1	GLWQKIKDKASELVSGIVEGVK-NH_2	2382	+	n	*L.splendida, L.caerulea*[47,48]
Caerin 3.2	GLWEKIKEKASELVSGIVEGVK-NH_2	2397	+	n	*L.caerulea*[48]
Caerin 3.3	GLWEKIKEKANELVSGIVEGVK-NH_2	2424	+/-	n	*L.caerulea*[48]
Caerin 3.4	GLWEKIREKANELVSGIVEGVK-NH_2	2452	+/-	n	*L.caerulea*[48]
Caerin 3.5	GLWEKVKEKANELVSGIVEGVK-NH_2	2392	+	n	*L.gracilenta*[53]
Caerin 4.1	GLWQKIKSAAGDLASGIVEGIKS-NH_2	2326	+/-	n	*L.caerulea*[48]
Caerin 4.2	GLWQKIKSAAGDLASGIVEAIKS-NH_2	2340	+/-	n	*L.caerulea*[48]
Caerin 4.3	GLWKIKQAAGDLASGIVEGIKS-NH_2	2353	+/-	n	*L.caerulea*[48]
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	1613	+	w	*L.citropa*[55]
Citropin 1.1.3	GLFDVIKKVASVIGLASP-NH_2	1813	+	n	*L.citropa*[55]
Citropin 1.2	GLFDIIKKVASVVGGL-NH_2	1613	+	w	*L.citropa, L.subglandulosa*[55,56]
Citropin 1.3	GLFDIIKKVASVIGGL-NH_2	1627	+	w	*L.citropa*[55]
Citropin 2.1	GLIGSIGKALGGLLVDVLKPKL-NH_2	2160	+	n	*L.citropa*[55]
Citropin 2.1.3	GLIGSIGKALGGLLVDVLKPKLQAAS-OH	2517	+	n	*L.citropa*[55]
Dahlein 1.1	GLFDIIKNIVSTL-NH_2	1430	+	w	*L.dahlii*[57]
Dahlein 1.2	GLVFDIIKNIFSGL-NH_2	1434	+	w	*L.dahlii*[57]
Maculatin 1.1	GLFGVLAKVAAHVVPAIAEHF-NH_2	2145	+/-	w	*L.genimaculata*[58]
Maculatin 1.2	GLFGVLAKVASHVVAAIAEHFQA-NH_2	2360	+	n	*L.genimaculata*[58]
Maculatin 1.3	GLLGLLGSVVSHVVPAIVGHF-NH_2	2068	+	w	*L.eucnemis*[52]
Maculatin 1.4	GLLGLLGSVVSHVLPAITQHL-NH_2	2121	+	w	*L.eucnemis*[52]
Maculatin 2.1	GFVDFLKKVAGTIANVVT-NH_2	1878	+	w	*L.genimaculata*[58]
Maculatin 3.1	GLLQTIKEKLESLESLAKGIVSGIQA-NH_2	2395	+	n	*L.genimaculata*[58]
Signiferin 2.1	IGHLIKTALGMLGL-NH_2	1547	+	w	*C.signifera*[59]
Signiferin 2.2	IGHLIKTALGFLGL-NH_2	1563	+	w	*C.signifera*[59]
Uperin 2.1	GIVDFAKKVVGGIRNALGI-NH_2	1925	+	n	*U.inundata*[60]
Uperin 2.3	GFFDLAKKVVGGIRNALGI-NH_2	1973	+	n	*U.inundata*[60]
Uperin 2.5	GIVDFAKGVLGKIKNVLGI-NH_2	1939	+	n	*U.inundata*[60]
Uperin 2.8	GILDVAKTLVGKLRNVLGI-NH_2	1977	+	w	*U.mjobergii*[61]
Uperin 3.1	GVLDAFRKIATVVKNVV-NH_2	1826	+	n	*U.inundata*[60]
Uperin 3.5	GVGDLIRKAVSVIKNIV-NH_2	1778	+	w	*U.mjobergii*[61]
Uperin 3.6	GVIDAAKKVVNVLKNLP-NH_2	1826	+	w	*U.mjobergii*[61]
Uperin 4.1	GVGSFIHKVVSAIKNVA-NH_2	1723	+	n	*U.inundata*[60]

+ Gram-positive; - Gram-negative; w wide spectrum; n narrow spectrum

Table 2 Antibiotic activities of selected peptides from species of the *Litoria*, *Uperoleia* and *Crinia* genera[a,b,c]

Bacterium[d]	A2.4	C1.1	C1.1D	C1.1m	C1.19	Ci1.1	Ci1.1D	Ci1.1m	M1.1	S2.1	S2.1m	U3.6	C2.5	C3.3	C4.1
Bacillus cereus	25	50	50	—	100	50	50	25	50	25	25	25	—	—	—
Enterococcus faecalis	—	25	25	—	25	—	—	—	—	100	50	—	—	—	—
Leuconostoc lactis	12	1.5	3	50	3	6	3	3	3	12	25	3	—	—	—
Listeria innocua	100	25	50	100	25	25	25	12	100	50	50	50	—	—	—
Micrococcus luteus	25	12	6	100	12	12	25	6	12	25	25	50	<0.4	3	12
Staphylococcus aureus	12	3	3	100	3	25	25	12	6	12	25	25	—	—	—
Staphylococcus epidermidis	25	12	12	25	12	12	12	6	12	25	12	12	—	—	—
Streptococcus uberis	25	12	25	12	12	25	12	12	3	25	25	12	—	—	—
Enterobacter clocae	—	—	—	100	—	—	—	—	—	—	—	—	—	—	—
Escherichia coli	—	100	100	6	—	—	—	50	—	—	—	25	25	—	—
Pasteurella multocida	25	25	25	3	25	—	—	100	50	—	—	25	—	6	<0.4
Pseudomonas aeruginosa	—	—	—	50	—	—	—	—	—	—	—	—	—	—	—

[a] Minimum inhibitory concentration (MIC) values (μg mL^{-1}). [b] When no value is indicated, there is no activity ≤100 μg mL^{-1}. [c] Peptide sequences are listed in Table 1 unless indicated to the contrary. A2.4 is aurein 2.4; C1.1 is caerin 1.1; C1.1D is the all-D form of C1.1 (GllsvlGsvakhvphvvpviaehl-NH_2); C1.1m is a synthetic modification of caerin 1.1 [sequence of modification is GLLKKLK**K**VAK**K**VLP**K**VVPVIAE**K**L-NH_2 (changed residues bold)]; C1.19 is caerin 1.19; Ci1.1 is citropin 1.1; Ci1.1D is the all-D form of citropin 1.1 (GlfdvikkvasviGGl-NH_2); Ci1.1m is a synthetic modification of citropin 1.1 [sequence of modification GLFAVI**K**K**K**VASVI**K**GL-NH_2 (changed residues bold)]; M1.1 is maculatin 1.1; S2.1 is signiferin 2.1; S2.1m is a synthetic modification of signiferin 2.1 [sequence of modification is **G**IGHL**I**KTALGM**L**GL-NH_2 (changed residue bold)]; U3.6 is uperin 3.6; C2.5 is caerin 2.5; C3.3 is caerin 3.3; and C4.1 is caerin 4.1. [d] Pathogens listed in the first group are Gram-positive organisms, while those in the second group are Gram-negative organisms.

for activity, since the synthetic modification Gly1 signiferin 2.1 has a very similar spectrum of antibiotic activities to that of signiferin 2.1 (see Table 2).

Solid-state NMR experiments in micelles show that aurein 1.2 and citropin 1.1 penetrate model bilayers at an angle of about 50° to the plane of the membrane (see Fig. 2).[65,66] These peptides are not long enough to span the whole of a bacterial membrane; a peptide with a minimum of 20 residues is required for this. It is therefore likely that the citropin 1.1 type peptides disrupt bacterial membranes by the carpet mechanism. This has been confirmed by confocal fluorescence spectroscopy.[38]

Fig. 2 Representation of a small peptide (aurein 1.2) penetrating the bacterial lipid bilayer (from solid-state NMR investigation).

The structures of some aurein precursors have been determined by cDNA methods.[67]

2.2.2 Caerin 1 and maculatin 1 peptides. 3′-RACE analysis of mRNA from *Litoria caerulea* has revealed a number of cDNAs encoding caerin 1 peptides. A comparison of the amino acid sequences of the caerin 1 precursors indicate that both the signal and spacer portions are highly conserved. The structure of the precursor to caerin 1.1 is shown below.[68] The C-terminal $CONH_2$ group of caerin 1.1 is a post-translational modification effected from Gly (see sequence below).

```
MASLKKSLFLVIFLGLVSLSIC                    Signal (pre)
EEEKRQEDEDEHEEEGESQEEGSEEKR    Acidic spacer (pro)
GLLSVLGSVAKHVLPHVVPVIAEHL(G)               Caerin 1.1
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The caerin 1 and maculatin 1 antibiotic peptides have helical regions at each end of the peptide connected by a flexible hinge region, as shown by 2D NMR experiments in model lipids for caerin 1.1,[23] caerin 1.1 modifications,[69] caerin 1.4[70] and maculatin 1.1.[71] The structure of caerin 1.1 is shown in Fig. 3A. There have been 20 natural caerin 1 peptides isolated so far from species of the genus *Litoria* and these, together with the related maculatin 1 peptides, show significant activity as antibiotics (see Table 2) and anticancer agents. As an illustration, Fig. 4 shows electron microscope pictures of the action of maculatin 1.1 against *Staphylococcus aureus.* The caerins 1 are also fungicides and antiviral agents for viruses with envelopes. These activities will be described in later sections.

The antibiotic activities of natural L-caerin 1.1 are very similar to those of synthetic D-caerin 1.1 (see Table 2), ruling out the possibility that caerin 1.1 interacts with specific chiral receptors.[6] The presence of the central hinge is essential for the activities of the caerins 1 and maculatins 1. For example, if the two central Pro residues of caerin 1.1 are replaced by Ala, the hinge of caerin 1.1 disappears and the hydrophobic and hydrophilic zones become less defined than those of caerin 1.1 (see Fig. 3B for the structure of Ala 15,19 caerin 1.1). Ala 15,19 caerin 1.1 shows only minimal

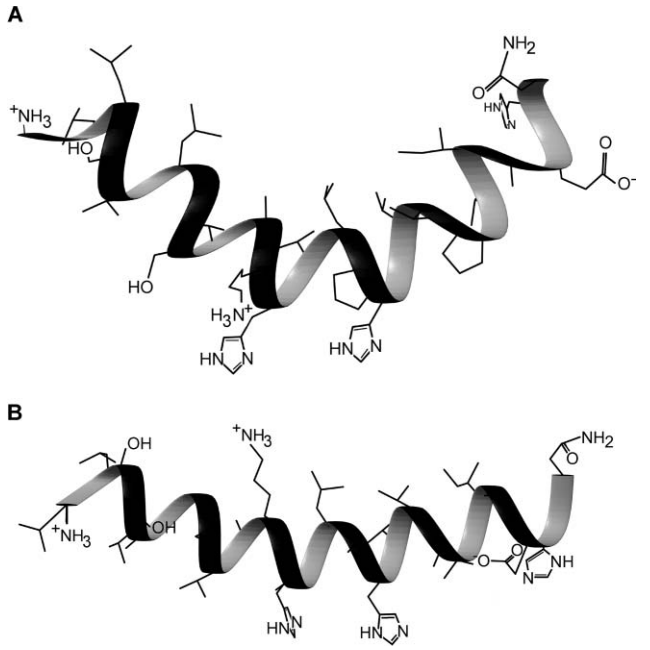

Fig. 3 (A) Caerin 1.1. (B) Ala15 ala19 caerin 1.1. Structures determined by 2D NMR study in micelles.

antibiotic and anticancer activity.[69] Synthetic caerins 1.1 in which the cationic charge is significantly increased demonstrate lower activity towards Gram-positive organisms but increased activity towards Gram-negative bacteria (compare the activities of caerin 1.1 and C1.1m listed in Table 2).

Solid-state NMR experiments[65,66] and Langmuir monolayer experiments[72] indicate that both caerin 1.1 and maculatin 1.1 penetrate model bilayers, demonstrating that these are membrane-active antibiotics. In addition, ^{31}P NMR experiments demonstrate directly that these two peptides interact with the membrane lipids of live bacterial cells.[73] Both maculatin 1 and caerin 1 peptides are, in theory, long enough to span a bacterial bilayer. Both FT/IR[74] and confocal fluorescence spectroscopy[38] techniques suggest that maculatin 1.1 penetrates the bacterial bilayer by a pore-forming mechanism. However, it is not yet clear whether the caerin 1 peptides operate by the carpet or pore mechanisms.

2.2.3 Narrow-spectrum antibiotics. The caerins 2, 3 and 4 isolated from a number of species of the genus *Litoria* show narrow-spectrum antibiotic activity. This is illustrated in Table 2 for caerins 2.5, 3.3 and 4.1. These three compounds show activity against some Gram-negative organisms. Narrow-spectrum antibiotic peptides normally show no anticancer activity, but may have some other role in the amphibian skin. For example, the caerins 2 inhibit the production of nitric oxide by neuronal nitric oxide synthase (see later). The caerins 2 are unusual amongst antimicrobial peptides from the genus *Litoria* in that they contain a C-terminal CO_2H group. The cDNA method has been used to sequence the precursor of caerin 2.1 from *Litoria splendida*.[75] The sequence of the caerin 2.1 precursor is shown below with the signal and spacer regions showing similarity to those of caerin 1.1 (see above).

```
MAFLKKSIFLVLFLGLVSLSIC                Signal (pre)
EQEKREEENEEEYNEIEEGSEEKR       Acidic spacer (pro)
GLVSSIGRALGGLLADVVKSKGQPA              Caerin 2.1
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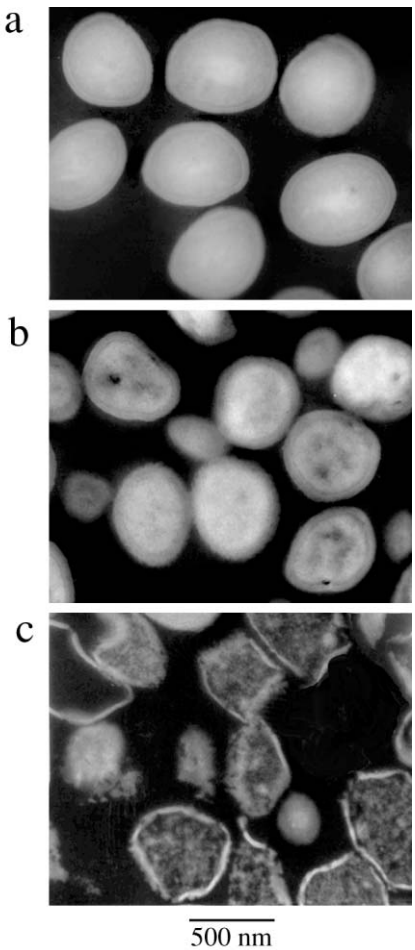

Fig. 4 Electron micrographs of (a) *Staphylococcus aureus*, (b) *S. aureus* plus 8 μg mL^{-1} of maculatin 1.1, and (c) *S. aureus* plus 16 μg mL^{-1} of maculatin. Pictures in b and c were taken after exposure for 30 min.

The structure of caerin 4.1 has been determined by NMR experiments using micelles.[76] Caerin 4.1 is an amphipathic α-helix with a higher degree of hydrophilicity than the wide-spectrum caerin 1 antibiotics.

2.3 Antibiotic peptides from the genus *Rana*

Species of the genus *Rana* contain an extraordinary number of antibiotic peptides in their skin secretions. Over 400 peptides have been isolated to date. The majority contain a disulfide linkage at the C-terminal end of the peptide, and these cationic peptides normally contain a C-terminal CO_2H group (rather than the typical $CONH_2$ group of the many antibiotic peptides listed in Table 1). Some 200 *Rana* peptides have been tested for antibiotic activity, and these are listed in Table 3. The majority of the listed peptides have been tested only against one Gram-positive bacterium (usually *Staphylococcus aureus*), and one Gram-negative organism (usually *Escherichia coli*). These peptides, designated 'o' in Table 3, generally show antibiotic activity at MIC 10^{-6}–10^{-5} M. Those peptides that have been tested against a number of pathogens are all wide-spectrum antibiotics, and are designated 'w' in Table 3. Different research groups tend to test routinely against

Table 3 Antibiotic peptides from the genus *Rana* (*R.*)

Name	Sequence	M.W.	Activity	Species
bPcAP	GVVKVSRLKGESLRRRL-OH	1865	+/- w	*R.catesbeiana*[77]
bPaAP	IIKVPLKKFKSMREVMRADHGIKAPVVDPATKY-OH	3961	+/- w	*R.catesbeiana*[77]
Brevinin 1	FLPVLAGIAAKVVPALFCKITKKC-OH	2529	+/- w	*R.brevipoda*[78,79]
Brevinin 1ARa	FLPLVRVAAKILPSVFCAISKRC-OH	2530	+/- o	*R.areolata*[80]
Brevinin 1AUa	FLPILAGLAAKLVPKVFCSITKKC-OH	2559	+/- w	*R.aurora aurora*[81]
Brevinin 1AUb	FLPILAGLAANILPKVFCSITKKC-OH	2559	+/- w	*R.aurora aurora*[81]
Brevinin 1Ba	FLPFIAGMAAKFLPKIFCAISKKC-OH	2643	+ o	*R.berlandieri*[82]
Brevinin 1Bb	FLPAIGMAAKFLPKIFCAISKKC-OH	2567	+/- o	*R.berlandieri*[82]
Brevinin 1Bc	FLPFIAGVAAKFLPKIFCAISKKC-OH	2611	+ o	*R.berlandieri*[82]
Brevinin 1Bd	FLPAIAGVAAKFLPKIFCAISKKC-OH	2535	+/- o	*R.berlandieri*[82]
Brevinin 1Be	FLPAIVGAAAKFLPKIFCVISKKC-OH	2563	+/- o	*R.berlandieri*[82]
Brevinin 1Bf	FLPFIAGMAANFLPKIFCAISKKC-OH	2629	+/- o	*R.berlandieri*[82]
Brevinin 1BYa	FLPILASLAAKFGPKLFCLVTKKC-OH	2607	+/- o	*R.boylii*[83]
Brevinin 1BYb	FLPILASLAAKLGPKLFCLVTKKC-OH	2573	+/- o	*R.boylii*[83]
Brevinin 1BYc	FLPILASLAATLGPKLLCLITKKC-OH	2526	+ o	*R.boylii*[83]
Brevinin 1Da	ILPLLLGKVVCAITKKC-OH	1811	+/- o	*R.dalmatina*[84]
Brevinin 1E	FLPLLAGLAANFLPKIFCKITRKC-OH	2676	+/- w	*R.esculenta*[78,85]
Brevinin 1Ea	FLPAIFRMAAKVVPTIICSITKKC-OH	2649	+/- o	*R.esculenta*[78,85]
Brevinin 1Eb	VIPFVASVAAEMMQHVYCAASRKC-OH	2610	+/- o	*R.esculenta*[78,85]
Brevinin 1Lb	FLPMLAGLAASMVPKFVCLITKKC-OH	2580	+/- o	*R.luteiventis*[82]
Brevinin 1OKa	FFGSMIGALAKGLPSLISLIKK-NH_2	2290	+/- o	*R.okinavana*[86]
Brevinin 1OKc	FFGSIIGALAKGLPSLISLIKK-NH_2	2272	+/- o	*R.okinavana*[86]
Brevinin 1Pa	FLPIIAGVAAKVFPKIFCAISKKC-OH	2563	+/- o	*R.pipiens*[82]
Brevinin 1Pb	FLPIIAGIAAKVFPKIFCAISKKC-OH	2577	+/- o	*R.pipiens*[82]
Brevinin 1Pc	FLPIIASVAAKVFSKIFCAISKKC-OH	2583	+/- o	*R.pipiens*[82]
Brevinin 1Pd	FLPIIASVAANVFSKIFCAISKKC-OH	2569	+/- o	*R.pipiens*[82]
Brevinin 1PLa	FFPNVASVPGQVLKKIFCAISKKC-OH	2623	+/- o	*R.palustris*[87]
Brevinin 1PLb	FLPLIAGLAANFLPKIFCAITKKC-OH	2591	+/- o	*R.palustris*[87]
Brevinin 1PLc	FLPVIAGVAAKFLPKIFCAITKKC-OH	2577	+/- o	*R.palustris*[87]
Brevinin 1PRa	FLSLALAALPKLFCLIFKKC-OH	2238	+ o	*R.pirica*[88]
Brevinin 1Sa	FLPAIVGAAGQFLPKIFCAISKKC-OH	2521	- o	*R.sphenocephala*[89]
Brevinin 1Sb	FLPAIVGAAGKFLPKIFCAISKKC-OH	2535	- o	*R.sphenocephala*[89]
Brevinin 1Sc	FFPIVAGVAGQVLKKIYCTISKKC-OH	2612	- o	*R.sphenocephala*[89]
Brevinin 1SPa	FFPIIAGMAAKLIPSLFCKITKKC-OH	2637	+/- o	*R.septentrionalis*[90]
Brevinin 1SPb	FLPIIAGMAAKVICAITKKC-OH	2088	+/- o	*R.septentrionalis*[90]
Brevinin 1SPd	FFPIIAGMAAKVICAITKKC-OH	2122	+/- o	*R.septentrionalis*[90]
Brevinin 1SY	FLPVVAGLAAKVLPSIICAVTKKC-OH	2440	+/- o	*R.sylvatica*[91]
Brevinin 1T	VNPIILGVLPKFVCLITKKC-OH	2197	+ w	*R.temporaria*[78]
Brevinin 1Ta	FITLLLRKFICSITKKC-OH	2026	+ w	*R.temporaria*[78]
Brevinin 2	GLLDSLKGFAATAGKGVLQSLLSTASCKLAKTC-OH	3251	+/- w	*R.brevipoda*[78,79]
Brevinin 2E	GIMDTLKNLAKTAGKGALQSLLNKASCKLSGQC-OH	3361	+/- w	*R.esculenta*[78,85]
Brevinin 2Ea	GILDTLKNLAISAAKGAAQGLVNKASCKLSGQC-OH	3242	+ o	*R.esculenta*[78,85]
Brevinin 2Eb	GILDTLKNLAKTAGKGALQCLVKMASCKLSGQC-OH	3316	+ o	*R.esculenta*[78,85]
Brevinin 2Ec	GILLDKLKNFAKTAGKGVLQSLLNTASCKLSGQC-OH	3519	+ o	*R.esculenta*[78,85]
Brevinin 2Ed	GILDSLKNLAKNAGQILLNKASCKLSGQC-OH	2999	+ o	*R.esculenta*[78,85]
Brevinin 2Ef	GIMDTLKNLAKTAGKGALQSLVKMASCKLSGQC-OH	3365	- o	*R.esculenta*[92]
Brevinin 2Eg	GIMDTLKNLAKTAGKGALQSLLNHASCKLSGQC-OH	3371	- o	*R.esculenta*[92]
Brevinin 2Eh	GIMDTLKNLAKTAGKGALQSLLNHASCKLSKQC-OH	3442	- o	*R.esculenta*[92]
Brevinin 2Ei	GILSTIKDFAIKAGKGAAKGLLEMASCKLSGQC-OH	3309	- o	*R.esculenta*[93]
Brevinin 2Ej	GIFLDKLKNFAKGVAQSLLNKASCKLSGQC-OH	3181	- o	*R.esculenta*[93]
Brevinin 2Oa	GLFNVFKGALKTAGKHVAGSLLNQLKCKVSGGC-OH	3346	+/- o	*R.omativentris*[94]
Brevinin 2Ob	GIFNVFKGALKTAGKHVAGSLLNQLKCKVSGEC-OH	3417	+/- o	*R.omativentris*[94]
Brevinin 2PRa	GLMSLFKGVLKTAGKHIFKNVGGSLLDQAKCKITGEC-OH	3892	+/- w	*R.pirica*[88]
Brevinin 2PRb	GLMSLFRGVLKTAGKHIFKNVGGSLLDQAKCKITGEC-OH	3919	+/- w	*R.pirica*[88]
Brevinin 2PRc	GLMSVLKGVLKTAGKHIFKNVGGSLLDQAKCKISGQC-OH	3829	+/- w	*R.pirica*[88]
Brevinin 2PRd	GLMSVLKGVLKTAGKHOFKNVGGSLLDQAKCKITGQC-OH	3843	+/- w	*R.pirica*[88]
Brevinin 2PRe	GLLSVLKGVLKTAGKHIFKNVGGSLLDQAKCKISGQC-OH	3810	+/- w	*R.pirica*[88]
Brevinin 2Va	GIWDTLKNVGKAVLGKVLENV-NH_2	2251	+/- o	*R.virgatipes*[95]
Brevinin 2Rel	GIWDTIKSMGKVFAGKILQNL-NH_2	2371	+/- o	*R.septentrionalis*[90]
Bullfrog buforin 1	SGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLRKGNY-OH	4260	+/- w	*R.catesbeiana*[77]
CPRF-Ea	GLGSILGKILNVAGKVGKTIGKVADAVGNKE-OH	3055	- o	*R.esculenta*[93]
CPRF-Eb	GLGSFLKNAIKIAGKVGSTIGKVADAIGNKE-OH	3055	- o	*R.esculenta*[93]
CPRF-Ec	GLGSFFKNAIKIAGKVGSTIGKVADAIGNKE-OH	3089	- o	*R.esculenta*[93]
Esculentin 1	GIFSKLGRKKIKNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC-OH	4884	+/- w	*R.esculenta*[85]
Esculentin 1a	GIFSKLAGKKIKNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC-OH	4799	+/- o	*R.esculenta*[85]
Esculentin 1c	GIFSKLAGKKIKNLLISGLKNIGKEVGMDVVRTGIDIAGCKIKGEC-OH	4813	+/- w	*R.esculenta*[85,96]
Esculentin 1ARa	GIFSKINKKKAKTGLFNIIKTVGKEAGMDVIRAGIDTISCKIKGEC-OH	4924	+/- o	*R.areolata*[80]
Esculentin 1ARb	GLFPKFNKKKVKTGIFDIIKTVGKEAGMDVLRTGIDVIGCKIKGEC-OH	4995	+/- o	*R.areolata*[80]
Esculentin 1PLa	GLFPKINKKKAKTGVFNIIKTVGKEAGMDLIRTGIDTIGCKIKGEC-OH	4948	+/- o	*R.palustris*[87]
Esculentin 1PLb	GIFTKINKKKAKTGVFNIIKTIGKEAGMDVIRAGIDTISCKIKGEC-OH	4938	+/- o	*R.palustris*[87]
Esculentin 2a	GILSLVKGVAKLAGKGLAKEGGKFGLELIACKIAKQC-OH	3711	+/- o	*R.esculenta*[85]
Esculentin 2b	GIFSLVKGAAKLAGKGLAKEGGKFGLELIACKIAKQC-OH	3717	+/- o	*R.esculenta*[85]
Esculentin 2B	GLFSILRGAAKFASKGLGKDLTKLGVDLVACKISKQC-OH	3835	+/- o	*R.berlandieri*[82]
Esculentin 2L	GILSLFTGGIKALGKTLFKMAGKAGAEHLACKATNQC-OH	3737	+/- o	*R.luteiventris*[82]

Table 3 (*Contd.*)

Name	Sequence	M.W.	Activity		Species
Esculentin 2P	GFSSIFRGVAKFASKGLGKDLARLGVNLVACKISKQC-OH	3968	-	o	*R.pipiens*[82]
Esculentin 2Pla	GLFSILKGVGKIALKGLAKNMGKMGLDLVSCKISKEC-OH	3849	+/-	o	*R.palustris*[87]
Gaegurin 1	SLFSLIKAGAKFLGKNLLKQGACYAACKASKQC-OH	3459	+/-	w	*R.rugosa*[97]
Gaegurin 2	GIMSIVKDVAKNAAKEAAKGALSTLSCKLAKTC-OH	3319	+/-	w	*R.rugosa*[97]
Gaegurin 3	GIMSIVKDVAKTAAKEAAKGALSTLSCKLAKTC-OH	3306	+/-	w	*R.rugosa*[97]
Gaegurin 4	GILDTLKQFAKGVGKDLVKGAAQGVLSTVSCKLAKTC-OH	3747	+/-	w	*R.rugosa*[97]
Gaegurin 5	FLGALFKVASKVLPSVFCAITKKC-OH	2567	+/-	w	*R.rugosa*[97]
Gaegurin 6	FLPLLAGLAANFLPTIICKISYKC-OH	2608	+/-	w	*R.rugosa*[97]
Japonicin 1	FFPIGVFCKIFKTC-OH	1648	+/-	o	*R.japonica*[98]
Japonicin 2	FGLPMMSILPKALCILLKRKC-OH	2356	+/-	o	*R.japonica*[98]
MRP 1	FIGSALKVLAGVLPSVISWVKQ-NH_2	2310	+/-	w	*R.temporaria*[99]
MRP 2	AIGSILGALAKGLPTLISWIKNR-NH_2	2390	+/-	w	*R.tagoi*[100]
Nigrocin 1	GLLDSIKGMAISAGKGALQNLLKVASCKLDKTC-OH	3345	+/-	w	*R.nigromaculata*[101]
Nigrocin 2	GLLSKVLGVGKKVLCGVSGLC-OH	2029	+/-	w	*R.nigromaculata*[101]
Palustrin 1b	ALFSILRGLKKLGNMGQAFVNCKIYKKC-OH	3143	-	o	*R.palustris*[87]
Palustrin 1c	ALSILRGLEKLAKMGIALTNCKATKKC-OH	2873	-	o	*R.palustris*[87]
Palustrin 1d	ALSILKGLEKLAKMGIALTNCKATKKC-OH	2845	-	o	*R.palustris*[87]
Palustrin 2AR	GFISTVKNLATNVAGTVIDTIKCKVTGGC-OH	2909	-	o	*R.areolate*[80]
Palustrin 2b	GFFSTVKNLATNVAGTVIDTLKCKVTGGCRS-OH	3186	-	o	*R.palustris*[87]
Palustrin 2c	GFLSTVKNLATNVAGTVIDTLKCKVTGGCRS-OH	3152	-	o	*R.palustris*[87]
Palustrin 3a	GIFPKIIGKGIKTGIVNGIKSLVKGVGMKVFKAGLNNIGNTGCNEDEC-OH	4932	-	o	*R.palustris*[87]
Palustrin 3b	GIFPKIIGKGIKTGIVNGIKSLVKGVGMKVFKAGLSNOGNTGCNEDEC-OH	4902	-	o	*R.palustris*[87]
Palustrin 3AR	GIFPKIIGKGIVNGIKSLAKGVGMKVFKAGLNNIGNTGCNNRDEC-OH	4645	-	o	*R.areolata*[80]
Ranacyclin E	SAPRGCWTKSYPPKPCK-OH	1904	+/-	w	*R.esculenta*[102]
Ranalexin	FLGGLIKIVPAMICAVTKKC-OH	2104	+/-	w	*R.catesbeiana*[103]
Ranalexin 1Ca	FLGGLMKAFPALICAVTKKC-OH	2109	+/-	w	*R.clamitans*[104]
Ranalexin 1Cb	FLGGLMKAFPAIICAVTKKC-OH	2109	+/-	o	*R.clamitans*[104]
Ranalexin 1G	FLGGLMKIIPAAFCAVTKKC-OH	2109	+/-	o	*R.grylio*[105]
Ranalexin 1Vb	FLGGLFKLVPSVICAVTKKC-OH	2120	+/-	o	*R.virgatipes*[95]
Ranatuerin 1	SMLSVLKNLGKVGLGFVACKINKQC-OH	2649	+/-	o	*R.catesbeiana*[103]
Ranatuerin 1C	SMLSVLKNLGKVGLGLVACKINKQC-OH	2615	+/-	o	*R.clamitans*[103,104]
Ranatuerin 1Ga	SMISVLKNLGKVGLGFVACKVNKQC-OH	2635	+/-	o	*R.grylio*[105]
Ranatuerin 2	GLFLDTLKGAAKDVAGKLEGLKCKITGCKLP-OH	3186	+	o	*R.catesbeiana*[103]
Ranatuerin 2ARa	GLMDTVKNAAKNLAGQLLDTIKCKMTGC-OH	2937	+	o	*R.areolata*[80]
Ranatuerin 2ARb	GILDTIKNAAKTVAVGLLEKIKCKMTGC-OH	2918	-	o	*R.areolata*[80]
Ranatuerin 2AUa	GILSSFKGVAKGVAKNLAGKLLDELKCKITGC-OH	3260	+/-	w	*R.aurora aurora*[81]
Ranatuerin 2B	GLLDTIKGVAKTVAASMLDKLKCKISGC-OH	2862	+/-	o	*R.berlandieri*[82]
Ranatuerin 2BYa	GIMDSVKGLAKNLAGKLLDSLKCKITGC-OH	2875	+/-	o	*R.boylii*[83]
Ranatuerin 2BYb	GILSTFKGLAKGVAKDLAGNLLDKFKCKITGC-OH	3308	-	o	*R.boylii*[83]
Ranatuerin 2Ca	GLFLDTLKGAAKDVAGKLLEGLKCKIAGCKP-OH	3156	+/-	o	*R.catesbeiana*, *R.clamitans*[103,104]
Ranatuerin 2Cb	GLFLDTLKGLAGKLLQGLKCIKAGCKP-OH	2784	+/-	o	*R.catesbeiana*, *R.clamitans*[103,104]
Ranatuerin 2G	GLLLDTLKGAAKDIAGIALEKLKCKITGCKP-OH	3180	+/-	o	*R.grylio*[105]
Ranatuerin 2La	GILDSFKGVAKGVAKDLAGKLLDKLKCKITGC-OH	3288	+/-	o	*R.luteiventris*[82]
Ranatuerin 2Lb	GILSSIKGVAKGVAKNVAAQLLDTLKCKITGC-OH	3198	+/-	o	*R.luteiventris*[82]
Ranatuerin 2Ma	GLLSSFKGVAKGVAKDLAGKLLEKLKCKITGC-OH	3272	-	o	*R.muscosa*[106]
Ranatuerin 2Mb	GIMDSVKGVAKNLAAKLLEKLKCKLITGC-OH	2928	-	o	*R.muscosa*[106]
Ranatuerin 2Ok	SFLNFFKGAAKNLLAAGLDKLKCKISGTQC-OH	3183	+/-	o	*R.okinavana*[86]
Ranatuerin 2P	LMDTVKNVAKNLAGHMLDKLKCKITGC-OH	3000	+/-	o	*R.pipiens*[82]
Ranatuerin 2PLa	GIMDTVKNVAKNLAGQLLDKLKCKITAC-OH	2987	-	o	*R.palustris*[87]
Ranatuerin 2PLb	GIMDTVKNAAKDLAGQLLDKLKCRITGC-OH	2974	-	o	*R.palustris*[87]
Ranatuerin 2PLc	GLLDTIKNTAKNLAVGLLDKIKCKMTGC-OH	2960	-	o	*R.palustris*[87]
Ranatuerin 2PLd	GIMDSVKNVAKNIAGQLLDKLKCKITGC-OH	2959	-	o	*R.palustris*[87]
Ranatuerin 2PLe	GIMDSVKNAAKNLAGQLLDTIKCKITAC-OH	2917	-	o	*R.palustris*[87]
Ranatuerin 2PLf	GIMDTVKNAAKDLAGQLDKLKCRITGC-OH	2862	-	o	*R.palustris*[87]
Ranatuerin 2PRa	GLMDVFKGAAKNLLASALDKIRCKVTKC-OH	2992	-	o	*R.pirici*[88]
Ranatuerin 2Va	GVFLDTLKGVGKDAAVKLLEALQCKFGVCKN-OH	3261	-	o	*R.virgatipes*[95]
Ranatuerin 2Vb	GVFLDALKGVGKGVAVSLLNGLKCKLGVC-OH	2855	-	o	*R.virgatipes*[95]
Ranatuerin 2Vc	GVFLNTIKEVGKDAAVKLLEALQCKFGVCKT-OH	3320	-	o	*R.virgatipes*[95]
Ranatuerin 3	GFLDIINKLGKTFAGHMLDKIKCTIGTCPPSPC-OH	3414	+	o	*R.catesbeiana*[103]
Ranatuerin 4	FLPFIARLAAKVFPSIICSVTKKC-OH	2651	+	o	*R.catesbeiana*[103]
Ranatuerin 6	FISAIASMLGKFL-OH	1396	+	o	*R.catesbeiana*[103]
Ranatuerin 7	FLSAIASMLGKFL-OH	1396	+	o	*R.catesbeiana*[103]
Ranatuerin 8	FISAIASFLGKFL-OH	1412	+	o	*R.catesbeiana*[103]
Ranatuerin 9	FLFPLITSFLSKVL-OH	1623	+	o	*R.catesbeiana*[103]
Rugosin A	GLLNTFKDWAISIAKGAGKGVLTTLSCKLDKSC-OH	3437	+	w	*R.rugosa*[107]
Rugosin B	SLFSLIKAGAKFLGKNLLKQGAQYAACKVSKEC-OH	3513	+/-	w	*R.rugosa*[107]
Rugosin C	GILDSFKQFAKGVGKDLIKGAAQGVLSTMSCKLAKTC-OH	3813	+	w	*R.rugosa*[107]

Table 3 (*Contd.*)

Name	Sequence	M.W.	Activity	Species
RV23	RIGVLLARLPKLFSLFKLMGKKV-OH	2626	+/- o	*R.aurora draytonii*[108]
Temporin A	FLPLIGRVLSGIL-NH_2	1395	+/- w	*R.temporaria*[109]
Temporin B	LLPILGNLLNGLL-NH_2	1390	+/- w	*R.temporaria*[109]
Temporin C	LLPILGNLLNGLL-NH_2	1360	+ o	*R.temporaria*[109]
Temporin D	LLPIVGNLLNSLL-NH_2	1377	+ o	*R.temporaria*[109]
Temporin E	VLPIIGNLLNSLL-NH_2	1377	+ o	*R.temporaria*[109]
Temporin F	FLPLIGKVLSGIL-NH_2	1368	+/- o	*R.temporaria*[109]
Temporin G	FFPVIGRILNGIL-NH_2	1457	+/- o	*R.temporaria*[109]
Temporin H	LSPNLLKSLL-NH_2	1095	+ o	*R.temporaria*[109]
Temporin K	LLPNLLKSLL-NH_2	1121	+/- o	*R.temporaria*[109]
Temporin L	FVQWFSKFLGRIL-NH_2	1639	+/- o	*R.temporaria*[109]
Temporin 1ARa	FLPIVGRLISGLL-NH_2	1397	+/- o	*R.areolate*[80]
Temporin 1AUa	FLPIIGQLLSGLL-NH_2	1381	+ w	*R.aurora aurora*[81]
Temporin 1BYa	FLPIIAKVLSGLL-NH_2	1381	+ o	*R.boylii*[83]
Temporin 1Cb	FLPLFASLIGKLL-NH_2	1429	+ o	*R.catesbeiana*, *R.clamitans*[103,104]
Temporin 1Cc	FLPFLASLLTKVL-NH_2	1460	+ o	*R.catesbeiana*, *R.clamitans*[103,104]
Temporin 1Cd	FLPFLASLLSKVL-NH_2	1446	+ o	*R.catesbeiana*, *R.clamitans*[103,104]
Temporin 1Ce	FLPFLATLLSKVL-NH_2	1460	+ o	*R.catesbeiana*, *R.clamitans*[103,104]
Temporin 1Da	NFLGTLVNLAKKIL-NH_2	1541	+/- o	*R.aurora draytonii*[108]
Temporin 1Db	HFLGTLVNLAKKIL-NH_2	1565	+/- o	*R.aurora draytonii*[108]
Temporin 1Ec	FLPVIAGLLSKLF-NH_2	1417	+ o	*R.esculenta*[93]
Temporin 1Gb	SILPTIVSFLSKFL-NH_2	1563	+ o	*R.grylio*[105]
Temporin 1Gc	SILPTIVSFLTKFL-NH_2	1578	+ o	*R.grylio*[105]
Temporin 1Gd	FILPLIASFLSKFL-NH_2	1608	+ o	*R.grylio*[105]
Temporin 1La	VLPLISMALGKLL-NH_2	1366	+ o	*R.luteiventis*[82]
Temporin 1Lb	NFLGTLINLAKKIM-NH_2	1575	+/- o	*R.luteiventis*[82]
Temporin 1Lc	FLPILINLIHKGLL-NH_2	1603	+/- o	*R.luteiventis*[82]
Temporin 1M	FLPIVGKLLSGLL-NH_2	1367	+ o	*R.mucosa*[106]
Temporin 1Oa	FLPLLASLFSRLL-NH_2	1487	+ o	*R.ornativentris*[94]
Temporin 1Ob	FLPLIGKILGTIL-NH_2	1395	+ o	*R.ornativentris*[94]
Temporin 1Oc	FLPLLASLFSRLF-NH_2	1521	+ o	*R.ornativentris*[94]
Temporin 1Od	FLPLLASLFSGLF-NH_2	1422	+ o	*R.ornativentris*[94]
Temporin 1P	FLPIVGKLLSGLL-NH_2	1368	+ o	*R.pipiens*[82]
Temporin 1PLa	FLPLVGKILSGLI-NH_2	1368	+ o	*R.palustris*[87]
Temporin 1PRa	ILPILGNLLNGLL-NH_2	1360	+/- o	*R.pirica*[88]
Temporin 1PRb	ILPILGNLLNSLL-NH_2	1390	+/- o	*R.pirica*[88]
Temporin 1SPb	FLSAITSLLGKLL-NH_2	1373	+ o	*R.septentrionalis*[90]
Temporin 1Tga	FLPILGKLLSGIL-NH_2	1381	+ o	*R.tagoi*[100]
Temporin 1Va	FLSSIGKILGNLL-NH_2	1372	+/- w	*R.virgatipes*[95]
Temporin 1Vb	FLSIIAKVLGSLF-NH_2	1405	+ w	*R.virgatipes*[95]
Temporin 1Vc	FLPLVTMLLGKLF-NH_2	1489	+/- w	*R.virgatipes*[95]
Tigerin 1	F<u>CTMIPIPRC</u>Y-NH_2	1341	+/- w	*R.tigerina*[110]
Tigerin 2	RV<u>CFAIPLPIC</u>H-NH_2	1366	+/- w	*R.tigerina*[110]
Tigerin 3	RV<u>CYAIPLPIC</u>Y-NH_2	1408	+/- w	*R.tigerina*[110]
Tigerin 4	RV<u>CYAIPLPIC</u>-NH_2	1245	+/- w	*R.tigerina*[110]

+ Gram-positive; - Gram-negative; w wide spectrum; n narrow spectrum; o means that only one Gram-positive (usually *Staphylococcus aureus*) and one Gram-negative organism (usually *Escherichia coli*) has been tested. Underlined sequences indicate a disulfide linkage.

different bacteria, so a consolidated table of relative activities is difficult to construct. Further, when the same peptide is tested against the same bacterial strain by several groups, the MIC values are often different. Even so, typical antibiotic activities of some *Rana* disulfide peptides are listed in Table 4. These activities are significant; the interesting feature is that the peptides illustrated in Table 4 are routinely active against both Gram-positive and Gram-negative organisms.

A number of *Rana* antibiotic disulfide-containing peptides have been sequenced by cDNA cloning techniques. Examples are shown below for brevinin 1E,[85] esculatin 1 and ranacyclin T.[102] The signal and anionic spacer portions of each precursor show some similarity.

```
MFTLKKSNLLLPFLGTIMLSLC                Signal (Pre)
EEERDADEEERRDNFDESEVEVEKR       Acidic spacer (Pro)
FLPLLAGLAAMFLPKIRCKITRKC              Brevinin 1E

MFTLKKPLLLIVLLGMISLSLC                Signal (Pre)
EQERNADEEEGSEIKR                Acidic spacer (Pro)
GIFSKLAGKKLKNLLISGLKNVGKEVSMDVVRTGID
IAGCKIKGEC                            Esculatin 1

MFTWKKTLLVLFFLGVVSLSLC                Signal (Pre)
VEERDADEEDGGEVMEEEVKR           Acidic spacer (Pro)
GALRGCWTKSYPPKPCK(G)                 Ranacyclin T
```

Table 4 Antibiotic and antifungal activities of some peptides from the genus *Rana*[a,b,c]

Bacterium[d]	B1E	B1Aua	B1Aub	B2E	E1c	bPaAP	bPcAP	TA
Bacillus subtilis	6	—	—	6	3	6	6	—
Bacillus megaterium	6	—	—	6	3	—	—	1
Micrococcus luteus	6	—	—	—	—	—	—	—
Staphylococcus aureus	12	20	3	25	12	—	—	12
Staphylococcus epidermidis	—	20	6	—	—	—	—	12
Streptococcus mutans	—	—	—	—	—	6	6	—
Enterobacter clocae	—	5	13	—	—	—	—	—
Escherichia coli	12	5	13	25	25	10	10	4
Pseudomonas aeruginosa	30	5	25	25	12	10	6	>100
RBC	5	>100	5	>100	>100	—	—	>100
Candida albicans	100	40	3	100	6	10	10	3

[a] Minimum inhibitory concentration (MIC) values (μg mL^{-1}). [b] A dash (—) means not tested. [c] Peptide sequences are listed in Table 3: B1E is brevinin 1E; B1Aua is brevinin 1Aua; B1Aub is brevinin 1Aub; B2E is brevinin 2E; E1c is esculatin 1c; and TA is temporin A. [d] The first group of organisms are Gram-positive bacteria, the second group Gram-negative bacteria. RBC indicates red blood cells. *Candida albicans* is a fungus.

Table 5 Antibiotic peptides from the genera *Ascaphus* (*A.*), *Bombina* (*Bo.*), *Bufo* (*Bu.*), *Hyla* (*H.*), *Kassina* (*K.*), *Leptodactylus* (*L.*), *Phyllomedusa* (*P.*) and *Xenopus* (*X.*)

Name	Sequence	M.W.	Activity		Species
Ascaphin 1	GFRDVLKGAAKAFVKTVAGHIAN-NH_2	2368	+/-	w	*A.truei*[126]
Ascaphin 3	GFRDVLKGAAKAFVKTVAGIIANI-OH	2482	+/-	o	*A.truei*[126]
Ascaphin 5	GIKDWIKGAAKKLIKTVASHIANQ-OH	2589	+/-	w	*A.truei*[126]
Ascaphin 7	GFKDWIKGAAKKLIKTVASSIANQ-OH	2573	+/-	o	*A.truei*[126]
Ascaphin 8	GFKDLLKGAAKALVKTVLF-NH_2	2071	+/-	w	*A.truei*[126]
BLP 1	GIGASILSAGKSALKGLAKGLAEHFAN-NH_2	2579	-	o	*Bo.orientalis*[127]
BLP 2	GIGSAILSAGKSALKGLAKGLAEHFAN-NH_2	2579	-	o	*Bo.orientalis*[127]
BLP 3	GIGAAILSAGKSALKGLAKGLAEHF-NH_2	2378	-	o	*Bo.orientalis*[127]
Bombinin	GIGALSAKGALKGLAKGLAEHFAN-NH_2	2292	+/-	o	*Bo.vaerigata*[78,128]
Bombinin H1	IIGPVLGMVGSALGGLLKKI-NH_2	1934	+/-	o	*Bo.vaerigata*[129]
Bombinin H3	HiGPVLGMVGSALGGLLKKI-NH_2	1934	+/-	o	*Bo.vaerigata*[129]
Bombinin H4	LiGPVLGLVGSALGGLLKKI-NH2	1916	+/-	o	*Bo.vaerigata*[129]
Buforin 1	AGRGKQGGKVRAKAKTRSSRAGLQFPVGRVHRLLR KGNY-OH	4309	+/-	w	*Bu.bufo gargarizans*[130,131]
Buforin 2	TRSSRAGLQFPVGRVHRLLRK-OH	2432	+/-	w	*Bu.bufo gargarizans*[130,131]
Dermadistinctin K	GLWSKIKAAGKEAAKAAAKAAGKAALNAVSEAV-OH	3150	+/-	w	*P.distincta*[132]
Dermadistinctin L	ALWKTLLKNVGKAAGKAALNAVTDMVNQ-OH	2924	+/-	w	*P.distincta*[132]
Dermadistinctin M	ALWKTMLKKLGTMALHAGKAAFGAAADTISQ-OH	3200	+/-	w	*P.distincta*[132]
Dermadistinctin Q1	ALWKNMLKGIGKLAGQAALGAVKTLVGAES-OH	2994	+/-	w	*P.distincta*[132]
Dermadistibctin Q2	GLWSKIKEAAKTAGLMAMGFVNDMV-OH	2667	+/-	w	*P.distincta*[132]
Dermaseptin B2	GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV-NH_2	3179	+/-	o	*P.bicolor*[12]
Dermaseptin B3	ALWKNMLKGIGKLAGQAALGAVKTLVGA-OH	2778	+/-	w	*P.bicolor*[133]
Dermaseptin B4	ALWKDILKNVGKAAGKAVLNTVTDMVNQ-NH_2	2995	+/-	w	*P.bicolor*[133]
Dermaseptin O1	GLWSTIKQKGKEAAIAAAKAAGQAALGAL-OH	2793	+/-	w	*P.oreades*[134]
Dermaseptin S1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGRQ-OH	3452	+/-	w	*P.sauvagei*[135]
Dermaseptin S2	ALWFTMLKKLGTMALHAGKAALGAAANTISQGTQ-OH	3470	+/-	w	*P.sauvagei*[78,136]
Dermaseptin S3	ALWKNMLKGIGKLAGKAALGAVKKLVGAES-OH	3021	+/-	w	*P.sauvagei*[78,136]
Dermaseptin S4	ALWMTLLKKVLKAAAKALNAVLVGANA-OH	2777	+/-	w	*P.sauvagei*[78,136]
Dermaseptin S5	GLWSKIKTAGKSVAKAAAKAAVKAVTNAV-OH	2838	+/-	w	*P.sauvagei*[78,136]
Dermatoxin	SLGSFLKGVGTTLASVGKVVSDQFGKLLQAGQ-OH	3191	+/-	w	*P.bicolor*[137]
Distinctin	ENREVPPGFTALIKTLRKCKII-OH | NLVSGLIEARKYLEQLHRKLKNCKV-OH	5478	+/-	w	*P.distincta*[138]
Hylaseptin P1	GILDAIKAIAKAAG-OH	1310	+/-	w	*H.punctata*[139]
Kassinatuerin 1	GFMKYIGPLIPHAVKAISDLI-NH_2	2281	+/-	o	*K.senegalensis*[140]
Magainin 1	GIGKFLHSAGKFGKAFVGWIMNS-OH	2394	+/-	w	*X.laevis*[14-17]
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS-OH	2465	+/-	w	*X.laevis*[14-17]

Table 5 (*Contd.*)

Name	Sequence	M.W.	Activity		Species
Maximin 1	GIGTKILGGVKTALKGALKELASTYAN-NH_2	2673	+/-	w	*Bo.maxima*[141]
Maximin 2	GIGTKILGGVKTALKGALKELASTYVN-NH_2	2702	+/-	w	*Bo.maxima*[141]
Maximin 3	GIGGKILSGLKTALKGAAKELASTYLH-OH	2698	+/-	w	*Bo.maxima*[141]
Maximin 4	GIGGVLLSAGKAALKGLAKVLAEKYAN-NH_2	2611	+/-	w	*Bo.maxima*[141]
Maximin 5	SIGAKILGGVKTFFKGALKELASTYLQ-OH	2841	+/-	w	*Bo.maxima*[141]
Maximin H1	ILGPVISTIGGVLGGLLKNL-NH_2	1933	+/-	w	*Bo.maxima*[141]
Maximin H2	ILGPVLSMVGSALGGLIKKI-NH_2	1965	+/-	w	*Bo.maxima*[141]
Maximin H3	ILGPVLGLVGNALGGLIKKI-NH_2	1944	+/-	w	*Bo.maxima*[141]
Maximin H4	ILGPVISKIGGVLGGLLKNL-NH_2	1960	+/-	w	*Bo.maxima*[141]
Maximin S4	RSNKGFNFMVDMIQALSK-NH_2	2085	+/-	o	*Bo.maxima*[141,142]
Ocellatin 1	GVVDILKGAGKDLLAHLVGKISEKV-NH_2	2560	-	o	*L.ocellatus*[143]
Ocellatin 2	GVLDIFKDAAKQILAHAAEQI-NH_2	2251	-	o	*L.ocellatus*[143]
Ocellatin 3	GVLDILKNAAKNILAHAAEQI-NH_2	2202	-	o	*L.ocellatus*[143]
Pentadactylin	GLLDTLKGAAKNVVGSLASKVMEKL-NH_2	2540	+/-	w	*L.pentadactylus*[144]
PGLa	GMASKAGAIAGKIAKVALKAL-NH_2	1967	+/-	w	*X.laevis*[145]
PGQ	GVLSNVIGYLKKLGTGALNAVLKQ-OH	2455	+/-	w	*X.laevis*[145]
Phylloseptin 1	FLSLIPHAINAVSAIAKHN-NH_2	2016	+/-	w	*P.hypochondrialis*[146]
Phylloxin	GWMSKIASGIGTFLSGIQQ-NH_2	1979	+/-	w	*P.bicolor*[147]
Pseudin 1	GLNTLKKVFQGLHEAIKLINNHVQ-OH	2715	-	o	*P.paradoxa*[148]
Pseudin 2	GLNALKKVFQGIHEAIKLINNHVQ-OH	2685	+/-	o	*P.paradoxa*[148]
Pseudin 3	GINTLKKVIQGLHEVIKLVSNHE-OH	2571	-	o	*P.paradoxa*[148]
Pseudin 4	GINTLKKVIQGLHEVIKLVSNHA-OH	2511	-	o	*P.paradoxa*[148]
XPF 1	GWAGKIGQTLGKIKKVGLKELIQPK-NH_2	2660	+/-	w	*X.laevis*[78,145]
XT 1	GFLGPLLKLAAKGVAKVIPHLIPSRQQ-OH	2852	+/-	o	*X.tropicalis*[149]
XT 2	GVWSTVLGGLKKFAKGGLEAIVNPK-OH	2570	-	o	*X.tropicalis*[149]
XT 4	GVFLDALKKFAKGGMNAVLNPK-OH	2318	+/-	o	*X.tropicalis*[149]
XT 6	GFLGSLLKTGLKVGSNLL-NH_2	1816	+/-	o	*X.tropicalis*[149]
XT 7	GLLGPLLKIAAKVGSNLL-NH_2	1776	+/-	o	*X.tropicalis*[149]

+ Gram-positive; - Gram-negative; w wide spectrum; o means that only one Gram-positive (usually *Staphylococcus aureus*) and one Gram-negative organism (normally *Escherichia coli*) have been tested.

2D NMR spectroscopy has been used to determine the secondary structures for a number of *Rana* disulfide antibiotics; for example, brevinin 1E,[111] nigrocin 2,[112] gaegurin 4,[113] gaegurin 5[114] and gaegurin 6.[115] These peptides are mostly unstructured in water, but when the NMR spectra are measured in either trifluoroethanol or model micelles, significant secondary structure is observed. For example, nigrocin 2 (GLLSKVLGVLGVGKKVLCGVSGLC-OH) shows a stable α-helix from Leu3 to Gly18, followed by the disulfide ring,[112] whereas brevinin 1E[111] and gaegurin 4[113] have two helical regions separated by a flexible hinge [*cf.* the caerins 1 (Fig. 3)]. Gaegurin 4 (GILDTLKQFAKGVGKDLVKGAAQGVLSTVSCKLAKTC-OH) shows an α-helix from Ile2 to Ala10, a flexible loop between Lys11 and Lys15, and an α-helix between Asp16 and Lys32 followed by the disulfide ring.

The precise role of the disulfide ring in the antibiotic activity of these membrane-active peptides is not known: for example, oxidised and reduced forms of the brevinins 1 both show significant antibiotic activity.[111,116] However, for esculentin 1, the cyclic form killed bacteria more rapidly than the linear, although MIC values were comparable.[117]

The mechanism of antimicrobial action of *Rana* peptides containing disulfide bonds has been investigated principally for the gaegurins. Gaegurin 4 has been shown to form voltage-dependent pores in lipid bilayers.[118] The C-terminal disulfide does not have an important role in the structure and activity of gaegurins 4 or 5,[118,119] and is not critical in inducing pore formation.[120] The electrostatic interactions of the disulfide region with phospholipids may however play a role in specificity of action.[120]

Finally, there are *Rana* antibiotic peptides which do not have disulfide functionality. These include the bP peptides from *Rana catesbeiana*,[77] and the temporins from a number of *Rana* species.[80–83,87,88,90,94,109,121,122] The temporins are amongst the smallest *Rana* antibiotic peptides, containing only 10 to 13 amino acid residues. The temporins are α-helical, amphipathic, hydrophobic, cationic, contain C-terminal $CONH_2$ groups and are active mainly against Gram-positive bacteria. They show classical membrane bilayer activity, in that the natural (L) and synthetic (D) forms of temporin A show similar ranges of antibiotic activities (see Table 4 for some antibiotic activities of temporin A).[122] The lytic activity of most of the temporins is due principally to hydrophobic interactions with the membrane, suggesting a barrel-stave mechanism of action.[123] In contrast, temporin L increases the permeability of bacterial cell membranes through the formation of pore-like openings causing leakage of small molecules and cell death.[124,125]

2.4 Antimicrobial peptides from the genera *Ascaphus*, *Bombina*, *Bufo*, *Hyla*, *Leptodactylus*, *Phyllomedusa* and *Xenopus*

Although bombinin was the first antibiotic peptide to be isolated from an anuran (*Bombina variegata*),[128] from a historical point of

view the magainin peptide antibiotics isolated from the African clawed frog *Xenopus laevis* have been the most studied.[14–17] A 2D NMR study in trifluoroethanol–water[150] and micelles[151] together with a Fourier transform infrared investigation[152,153] indicates that the magainins adopt stable α-helical conformations (see Fig. 5 for the secondary structure of magainin 2). The magainins are amphipathic, cationic and hydrophobic, and exhibit modest antibiotic activity against both Gram-positive and Gram-negative organisms (see Table 6 for magainin 2). The magainins penetrate bacterial membrane bilayers by a pore mechanism.[154–156] A consideration of the activities of magainins and some synthetic modifications show that they exhibit anticancer,[157] antiviral[158] and antifungal activity,[159] and they also lyse protozoa,[18] and show spermicidal activity.[19–21] It has been proposed that the synthetic modification Ala(8,13,18)magainin 2 may have potential in an anti-implantation strategy for intercepting pregnancy.[160]

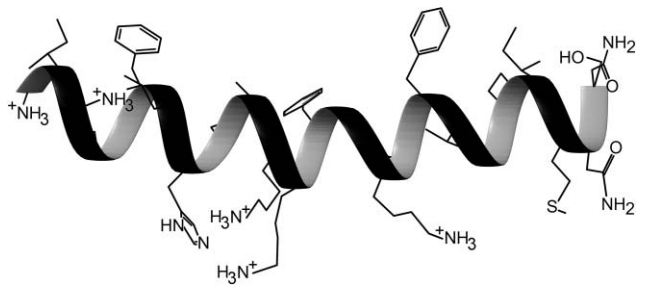

Fig. 5 Magainin 2. Structure determined by 2D NMR study in micelles.

Most of the other peptides listed in Table 5 are conventional membrane-active peptides. For example, 2D NMR studies have shown that hylaseptin P1 (GILDAIKAIAKAAG-OH)[139] and dermaseptin B2 (GLWSKIKEVGKEAAKAAAKAAGKAAL-GAVSEAV-NH_2),[161] which have cationic charges of +1 and +2 respectively, adopt stable α-helical structures in trifluoroethanol–water.

The carpet model is proposed for the action of dermaseptin S and its other natural analogues.[162] The mechanism of action of buforin 2 appears to be different. This peptide crosses lipid bilayers without effecting cell lysis: it has a strong affinity for RNA and DNA, suggesting that the ultimate target may be intracellular components.[163,164]

Distinctin, isolated from *Phyllomedusa distincta*, has strong antibacterial activity, consists of two peptide chains linked by a disulfide bridge, and is the first example of a heterodimeric antibiotic peptide isolated from frog skin.[138] NMR experiments reveal that this peptide adopts a symmetrical full-parallel four-helix bundle after homo-dimerisation in water, forming voltage-dependent pore-forming aggregates (see Fig. 6).[165]

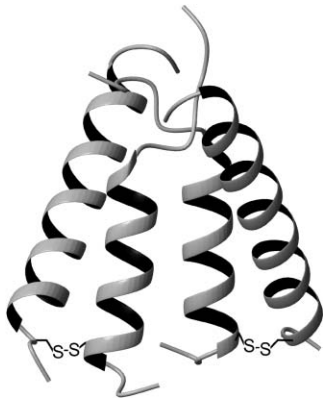

Fig. 6 Distinctin. Structure determined by 2D NMR study in water. Only the peptide backbone is shown for ease of representation. The disulfide bonds which link chains in each monomer are indicated.

Antibacterial activities of selected peptides are recorded in Table 6.

cDNA techniques have been used to sequence the precursors of a number of the peptides listed in Table 5; for example, buforin 1,[123] the magainins,[14,15] the maximins[141] and the dermaseptins and related species.[17,133,166,167]

3 Antiviral peptides

The first report of antiviral activity for the caerins 1 was for caerin 1.1, which showed activity against viruses with envelopes, *e.g.* HIV (MIC 7.7 μM) and *Herpes simplex* 1 (MIC 9.2 μM).[6] A more extensive survey of 14 antimicrobial peptides against HIV has shown that caerin 1.1, caerin 1.9 and maculatin 1.1 (see Table 1 for sequences), all wide-spectrum antibiotics with hinged secondary structures (see Fig. 3A), show MIC values of 7.8, 1.2 and 11.3 μM respectively.[25] Other antimicrobial peptides like dermaseptin,[25] and a number of *Rana* peptides[168] show lesser activity, but at concentrations where the peptide is cytotoxic to the target cells. Magainin 2 is inactive.[25]

Caerin 1.1 and 1.9 and maculatin 1.1 completely inhibit HIV infection of T cells within minutes of exposure to the virus. These membrane-active peptides are not toxic to target cells, and act by

Table 6 Antibiotic and antifungal activities of some antibiotic peptides listed in Table 5[a,b,c]

Bacterium[d]	M2	A1	B1	B2	BH2	Dis	Max3	P1
Bacillus megaterium	50	25	4	2	1	—	1	8
Staphylococcus aureus	50	>100	4	4	5	28	3	8
Staphylococcus epidermidis	50	50	—	—	—	—	—	—
Enterobacter clocae	50	6	—	—	—	—	—	—
Escherichia coli	50	3	8	4	4	15	3	8
Pseudomonas aeruginosa	100	25	—	—	>100	28	—	4
Candida albicans	>100	>100	4	4	>100	—	3	—

[a] Minimum inhibitory concentration (MIC) values (μg mL^{-1}). [b] A dash (—) means not tested. [c] Peptide sequences are listed in Table 5: M2 is magainin 2; A1 is ascaphin 1; B1 is bombinin 1; B2 is bombinin 2; BH2 is bombinin H2; Dis is distinctin; Max3 is maximin 3; and P1 is phylloseptin 1. [d] The first group of organisms are Gram-positive: the second group Gram-negative bacteria. *Candida albicans* is a fungus.

disrupting the virus envelope. In contrast, the three peptides are not active against reovirus, a structurally unrelated nonenveloped virus. The peptides also inhibit the transfer of HIV by dendridic cells to T cells. These data suggest that the amphibian-derived peptides can access dendridic cell-sequestered HIV and destroy the virus before it can be transferred to T cells.[25]

4 Antifungal peptides

Most of the wide-spectrum antibiotics listed in Tables 1, 3 and 5 show fungicidal activity at micromolar concentrations (see Tables 3 and 5 for activity against the fungus *Candida albicans*).

Amphibian populations are declining worldwide; a very serious environmental problem.[169,170] Although habitat destruction is certainly a major factor in this decline, another problem involves the infection of amphibians by viruses and fungi. Ranaviruses have led to destruction of amphibians in localised areas of North America and Europe.[171] In contrast, some fungi are causing widespread decline of anuran populations. In particular, the zoosporic chytrid fungus (*Batrachochytrium dendrobatidis*) is seriously affecting anuran populations throughout Central America and Australia.[172–175] The chytrid fungus also infects terrestrial salamanders in North America, but the mortality rates of these salamanders are less than those reported for anurans.[176] Many wide-spectrum anuran antibiotic peptides are active against the chytrid fungus, *e.g.* the temporins from *Rana* species and the magainins from *Xenopus laevis.*[177] Antimicrobial peptides from Australian anurans are also active against this fungus. For example, caerin 1 and maculatin 1 peptides from various Australian species of the genus *Litoria* are active against the chytrid fungus in the μM concentration range.[178] Australian frogs which do not contain antimicrobial peptides in their skin glands (*e.g.* species of the genus *Limnodynastes*) succumb more readily to the chytrid fungus than those which produce membrane-active antimicrobial peptides. Even so, animals which produce potent antifungal peptides from their skin glands are still infected by the fungus.

The question is why are those anurans, which appear to have adequate protection against fungi, are still killed by the chytrid fungus? Perhaps it is simply that the zoospores of the fungus attach to the underside of the animal, an area not effectively reached by the skin secretion. Perhaps the animal does not realise that the fungus is lethal and does not engage its chemical arsenal. Or maybe the fungus contains an enzyme which effectively cleaves and deactivates antifungal peptides. These are matters which require urgent resolution before such fungi reduce the world population of anurans still further.

5 Neuropeptides

The study of anuran neuropeptides is important not just for our understanding of the ecology and physiology of anurans (frogs and toads), but has given important clues to mammalian and human physiology and may be a source of new therapeutics. Pioneering work on the host-defence chemistry of neuropeptides from anurans commenced with the research of Vittorio Erspamer and members of his research group in the 1960s. Some of the work done in isolation and structure determination of these neuropeptides in these early days is quite exceptional, given the paucity of separatory and analytical techniques that were then available. Erspamer's final review[3] was published in 1994 and contains details of the structures and pharmacology of all anuran neuropeptides published up to that time. Many thousands of papers have been published in this area over the years, and over six hundred of these are referenced in Erspamer's review. The reader is referred to this review if specific data are required concerning the pharmacological spectrum of activities of a particular amphibian peptide. Our treatment of this area provides only a brief summary of the early work, and concentrates on work published after 1994.

This section of the review is summarised (for ease of representation) in tabular form for the following neuropeptide types: bombesins (Table 7), caeruleins, tachykinins, bradykinins and tryptophyllins (Table 8), dermorphins and deltorphins (Table 9) and miscellaneous neuropeptides (Table 10). Neuropeptides are normally an integral part of the host-defence system of the animal and also assist with the regulation of dermal physiological action.[1–6] Many of these peptides have a variety of roles in the amphibian integument and body. They generally bind to G-protein-coupled (seven transmembrane domain) receptors with wide distributions in the central nervous system, on smooth muscle and in other areas.

Some of the neuropeptides initially isolated from skin secretions have subsequently been detected in amphibian gut and brain. The major activity of a peptide is quantified in a table: for example, smooth-muscle activity (Tables 7 and 8), opioid activity (Table 9), while in Table 10, the primary function of each peptide is reported. Highlights of this work will be outlined in the text.

5.1 Bombesins and litorins

The bombesin peptides (see Table 7) were isolated from the skin and gut of anurans of the genus *Bombina*, while the related litorins (see also Table 7) are produced by species of the genera *Litoria*, *Pseudophyrne* and *Rana.* All of the bombesin/litorin peptides commence with a pyroglutamate residue, the last seven residues are similar, contain a terminal $CONH_2$, and show a similar spectrum of activities.[3] The full sequences of the prepropeptides of bombesin and [Phe13] bombesin have been determined using cDNA cloning.[183]

GRP VPLPAGGGTVLTKMYPRGNH**WA**V**GH**L**M**-NH_2

NMB GNL**WAT**G**H**F**M**-NH_2

Bombesin-like and litorin-like peptides are also found in many vertebrates.[3,188] Bombinin is similar to human gastrin releasing peptide (GRP; for sequence see above) and neuromedin B (NMB).[188] Bombesin can be present as more than one variant in the same animal. For example, bombesin is found in amphibian skin, gut and brain, while [Phe13]bombesin is found exclusively in the brain.[183,189]

Bombesin-like neuropeptides have a wide variety of physiological activity. They produce smooth-muscle contraction (see Table 7), stimulate the growth of both normal and neoplastic tissues, enhance secretion (*e.g.* of gastrin), and have widespread central nervous system effects.[3,188] They also have potent immunological stimulating activity,[190,191] which possibly explains their presence in anuran skin secretions.

Table 7 Smooth-muscle contraction activities of bombesin-type neuropeptides

Name	Sequence [a]	M.W.	Activity of bombesin (%) [b]	Activity of litorin (%) [b]	Species
Bombesins					
Bombesin [d]	pEQRLGNQ**WAVGHLM**-NH_2	1618	100		*Bombina bombina*,[179] *B.orientalis*,[180,181] *Rana pipiens*[182,183]
[pGlu1]bombesin (6-14)	pE**QWAVGHLM**-NH_2	1053	290		*Bombina bombina*[179]
[Phe13] bombesin [d]	pEQRLGNQ**WAVGHFM**-NH_2	1621	[c]		*Bombina orientalis*[180,181]
Alytesin	pEGRLGTQ**WAVGHLM**-NH_2	1536	[c]		*Alytes obstetricians*[179,180]
Ranatensins					
Litorin	pEQ**WAVGHFM**-NH_2	1084	130	100	*Litoria aurea*[179,184]
[Glu(OMe)$_2$]litorin	pEE(OMe)**WAVGHFM**-NH_2	1098		10	*Litoria aurea*[3,184]
PG litorin	pEGGGPGin**WAVGHFM**-NH_2	1352		[c]	*Pseudophyrne guntheri*[3,185]
Rohdei litorin	pEL**WATGHFM**-NH_2	1034		16	*Phyllomedusa rohdei*[181]
Ranatensin	pEVPQ**WAVGHFM**-NH_2	1280		85	*Rana pipiens*[180,181,186]
Phyllolitorins					
Phyllolitorin	pEL**WAVGSFM**-NH_2	1019		2	*Phyllomedusae sauvagei*, *P.burmeisteri*, *P.hypochondrialis*[181,187]
[Leu8]phyllolitorin [d]	pEL**WAVGSLM**-NH_2	985		2	*Phyllomedusae rohdei*, *P.sauvagei*[3,187]
[Thr5,Leu8]phyllolitorin	pEL**WATGSLM**-NH_2	987		3	*Phyllomedusae sauvagei*[3,187]

[a] Core sequence shown in bold, deviations from bombesin core sequence underlined. [b] Threshold concentration of the various peptides in producing Guinea pig colon smooth muscle concentration as a percentage of the threshold concentration of either bombesin (0.06 to 0.3 nM) or litorin. Value shown is the mean of the range of responses for that peptide. [c] Quantitative data not available. [d] cDNA sequencing data for prepropeptide available.

Bombesin- and litorin-type peptides bind to a number of G-protein coupled receptors: the NMB receptor (NMB-R or BB_1), the GRP receptor (GRP-R or BB_2) and the bombesin-like receptor subtypes 3 and 4 (BB_3 and BB_4).[188,189,192] The BB_1 (skin, gut) and BB_2 (brain) receptors are present in many vertebrates, while BB_4 is only found in the brains of anurans.[188,189,192] Bombesin and litorin neuropeptides have nanomolar (nM) affinities for BB_1, BB_2 and BB_4 receptors.[3,188,189,193]

5.2 Caeruleins

Caerulein (see Table 8) is one of the most studied of all amphibian neuropeptides. Caerulein contains pyroglutamate and tyrosine sulfate residues together with a C-terminal $CONH_2$ group. The tyrosine sulfate group is essential for full activity of the peptide. Caerulein is often the major neuropeptide present in the skin secretions of many species of the *Litoria* genus,[3,6,13] together with *Xenopus laevis* and *Leptodactylus labyrinthicus*.[194] The biological activity of caerulein is very similar to those of the mammalian intestinal peptide hormones gastrin and cholecystokinin. Caerulein contracts smooth muscle at nM concentrations. Caerulein, like its mammalian analogue cholecystokinin-8 [CCK-8; DY(SO_3)MGWMDF-NH_2] may act directly on smooth muscle *via* the CCK_1 receptor or indirectly *via* the CCK_2 receptor. The CCK_2 receptor is situated on cholinergic nerves in the myenteric plexus of the gut and stimulates the release of acetylcholine. This then activates muscarinic receptors directly on ileal smooth muscle, producing muscle contraction.[193,224] Caerulein also enhances blood circulation, modifies satiety, sedation and thermoregulation, and is an analgesic several thousand times more potent than morphine.

A number of of cDNA clones have been produced from *Xenopus laevis* that encode preprocaeruleins containing one or more copies of caerulein.[225]

The concentration of caerulein may vary seasonally in the skin secretions of some *Litoria* species. For example, *Litoria splendida* and *L. citropa* produce caerulein in the summer breeding season, while the analogue caerulein 1.2 [(Phe8) caerulein], is the major neuropeptide in the winter.[226–228] Both peptides contract smooth muscle at nM concentrations, but unlike caerulein, caerulein 1.2 only operates indirectly on smooth muscle *via* CCK_2 receptors. The reason for this seasonal change of neuropeptides is not known. *Litoria citropa* also produces a range of other caerulein-type peptides whose activities have not so far been tested.[228]

5.3 Tachykinins

The tachykinin subgroup of peptides occurs widely in various genera of anurans (see Table 8). Most of these peptides are anionic, some contain an N-terminal pyroglutamate and have the C-terminal consensus **FYGLM**-NH_2. Tachykinins have mammalian counterparts, with substance P (SP; RPKPQQFFGLM-NH_2) and the neurokinins (*e.g.* neurokinin A; HKTDSFVGLM-NH_2) being the most familiar.[3,229] The tachykinins may be divided into SP-like, aromatic and aliphatic (see Table 8). The secondary structures of the ranatachykinin peptides have been investigated in micelles using 2D NMR methods.[198] For example, ranatachykinin A (KPSPDRFYGLM-NH_2) from the bullfrog (*Rana catesbeina*) is helical from Pro4 to Leu10, but unstructured elsewhere.

Table 8 Smooth-muscle contraction activities of caerulein, tachykinin, bradykinin and tryptophyllin neuropeptides

Name	Sequence	M.W.	EC_{50}/mol	Species
Caeruleins				
Caerulein	p**EQDY(SO$_3$)TGWMDF**-NH$_2$	1351	10^{-10}	Various *Litoria* species, *Xenopus laevis*, *Leptodactylus labyrinthicus*[3,13,194]
Caerulein 1.2	p**EQDY(SO$_3$)TGW<u>F</u>DF**-NH$_2$	1367	10^{-9}	*Litoria splendida*[47]
Tachykinins				
Substance P like:				
Xenopus SP	KPRPDQ**FYGLM**-NH$_2$	1350	10^{-9}	*Xenopus laevis*[195]
Xenopus NKA	TLTTGKD**F<u>V</u>GLM**-NH$_2$	1281	10^{-9}	*Xenopus laevis*[195]
Ranakinin	KPNPERF**L<u>Y</u>GLM**-NH$_2$	1350	10^{-9}	*Rana ridibunda*[196]
Ranatachykinin A[a]	KPSPDR**FYGLM**-NH$_2$	1309	10^{-9}	*Rana catesbeina*[196-198]
Bufokinin	KPRPDQ**FYGLM**-NH$_2$	1349	10^{-8}	*Bufo marinus, Xenopus laevis, Neoceratodus forsteri*[196,199]
Aromatic tachykinins:				
Physalaemin	pEADPNK**FYGLM**-NH$_2$	1264	10^{-9}	*Physalamus bilogonigerus,*[196,200] *P.fuscumacalatus*[196]
[Lys5,Thr6]physalaemin	pEADPKT**FYGLM**-NH$_2$	1251	10^{-9}	*Uperoleia rugosa*[196]
Uperolein	pEPDPNA**FYGLM**-NH$_2$	1233	10^{-9}	*Uperoleia rugosa, U.marmorata*[196]
Uperin 1.1	pEADPNA**FYGLM**-NH$_2$	1208	10^{-10}	*Uperoleia inundata*[201]
PG-SP1	pEPNPDE**F<u>F</u>GLM**-NH$_2$	1275	10^{-8}	*Pseudophyrne guentheri*[196,202]
Hylambatin	DPPDPDR**FYG<u>M</u>M**-NH$_2$	1438	10^{-9}	*Hylambates maculata*[185,203]
Ranatachykinin B[a]	YKSDSK**FYGL**-NH$_2$	1206	10^{-8}	*Rana catesbeina*[196-198]
Ranatachykinin D	KPNPER**FY<u>A</u>P<u>M</u>**-NH$_2$	1348	10^{-8}	*Rana catesbeina*[196-198]
Ranamargarin	DDASDRAKK**FYG<u>K</u>M**-NH$_2$	1614	10^{-9}	*Rana margaratae*[196,204]
Aliphatic tachykinins:				
Kassinin	DVPKSDQ**FVGLM**-NH$_2$	1333	10^{-9}	*Kassina senegalensis,*[196,205,206] *Neoceratodus forsteri*[196,199]
PG-K3	pEPHPNE**FVGLM**-NH$_2$	1249	10^{-9}	*Pseudophyrne guntheri*[196,202]
Phyllomedusin	pENPNRF**<u>I</u>GLM**-NH$_2$	1170	10^{-9}	*Phyllomedusa bicolor,*[196] *Neoceratodus forsteri*[196,199]
AL-1	GPPDPNKF**<u>I</u>GLM**-NH$_2$	1284	10^{-9}	*Agalychnis callidryas*[205]
AR-1	GPPDPDR**F<u>Y</u><u>P</u><u>G</u>M**-NH$_2$	1347	[b]	*Agalychnis callidryas*[205]
Bradykinins				
Bradykinin[a]	RPP**GFSPFR**-OH	1059	10^{-8}	*Rana temporaria, R. palustris, R.nitromaculata, R.esculenta, Bombina orientalis*[204-213]
[Thr6]bradykinin[a]	RPP**GF<u>T</u>PFR**-OH	1073	10^{-6}	*Rana rugosa, Bombina orientalis*[213]
[Ala3,Thr6]bradykinin[a]	RPA**GF<u>T</u>PFR**-OH	1049	10^{-7}	*Bombina variegata*[214]
[Val1,Thr3,6]bradykinin	VPT**GF<u>T</u>PFR**-OH	1022	10^{-7}	*Bombina variegata, Rana nitromaculata*[214]
[Hyp3]bradykinin	RPHyp**GFSPFR**-OH	1074	10^{-8}	*Heleophyrne purcelli*[205,208]
RD-11	RPP**GFSPFR**VD-OH	1273	10^{-7} [c]	*Ascaphus truei*[215]
AR-10	APVP**G<u>L</u>SPFR**-OH	1039	10^{-6} [c]	*Ascaphus truei*[215]
AV-12	APVP**G<u>L</u>SPFR**VV-OH	1237	10^{-7} [c]	*Ascaphus truei*[215]
Maximakinin[a]	DLPKINRKGPRPP**GFSPFR**-OH	2176	[d]	*Bombina maxima*[216,217]
Phyllokinin	RPP**GFSPFR**IY(SO$_3$)-OH	1417	[e]	*Phyllomedusa rohdei, P.sauvagei*[215,218]
[Hyp3]phyllokinin	RPHyp**GFSPFR**IY(SO$_3$)-OH	1434	[e]	*Agalychnis callidryas*[205]
Kinestatin	pEIP**GL<u>G</u>P<u>L</u>R**-NH$_2$	932	10^{-8}	*Bombina maxima*[219]
Tryptophyllins				
Tryptophyllin L 1.2	**FPWL**-NH$_2$	561	[f]	*Litoria rubella, Litoria electrica*[220,221]
Tryptophyllin L 1.3	pE**FPWL**-NH$_2$	672	10^{-6}	*Litoria rubella*[220]
Tryptophyllin L 1.4	FP**FPWL**-NH$_2$	805	[f]	*Litoria rubella*[220]
	FP**PW<u>M</u>**-NH$_2$	657	[f]	*Phyllomedusa rohdei*[222]
PdT-1	KPHyp**<u>A</u>W<u>V</u>**P-NH$_2$	809	10^{-8}	*Pachymedusa dacnicolor*[223]

[a] cDNA sequence of prepropeptide has been reported. [b] Smooth muscle contraction not quantified. [c] Smooth muscle relaxation. [d] More potent than bradykinin in arterial tissue, much less potent in smooth muscle activity: quantification not provided. [e] Hypotensive more potent than bradykinin, but less potent in smooth muscle contraction: quantification not provided. [f] Not active against smooth muscle.

Table 9 Opioid activities of dermorphin and deltorphin neuropeptides

Name	Sequence [a]	M.W.	EC_{50}/mol [b]	Species
Dermorphins				
Dermorphin [c]	YaFGYPS-NH_2	802	10^{-11}	*Agalychnis callidryas, Phyllomedusa rohdei, P.sauvagei, P.burmeisteri*[3,234-238]
[Hyp6]dermorphin	YaFGYHypS-NH_2	818	10^{-9}	*Agalychnis callidryas, Phyllomedusa rohdei, P.sauvagei*[3,236,237]
[Lys7-OH]dermorphin	YaFGYPK-OH	802	10^{-9}	*Phyllomedusa bicolor*[3,238]
[Trp4,Asn7-OH]dermorphin	YaFWYPN-OH	961	10^{-9}	*Phyllomedusa bicolor*[3,239]
[Trp4,Asn5-OH] dermorphin (1-5)	YaFWN-OH	699	10^{-8}	*Phyllomedusa bicolor*[3,239]
Deltorphins				
Dermenkephalin [c,d]	YmFHLMD-NH_2	954	10^{-6}	*Phyllomedusa bicolor*[3,238-241]
[a2]deltorphin 1 [c]	YaFDVVG-NH_2	768	10^{-6}	*Phyllomedusa bicolor*[3,238-241]
[a2]deltorphin 2 [c]	YaFEVVG-NH_2	782	10^{-6}	*Phyllomedusa bicolor*[3,238-241]
[l2]deltophin	YlFADVASTIGDFFHSI-NH_2	1900	$<10^{-6}$	*Phyllomedusa bicolor*[3,238-241]

[a] a = D-Ala, m = D-Met, l = D-Leu. [b] EC_{50} is reported for inhibition of twitch responses in electrically stimulated mouse vas Deferens, an index of μ opioid receptor activation. [c] cDNA sequence of prepropeptide has been reported. Dermorphin and dermenkephalin are present in the same prepropeptide. Deltorphin 1 and 2 are present in the same prepropeptide. [d] Dermenkephalin is also known as deltorphin A.

Table 10 Activities of miscellaneous neuropeptides

Name	Sequence [a]	M.W.	Activity	EC_{50}/mol	Species
Crinia-angiotensin 2	APGDRIYVHPF-OH	1270	[b]		*Crinia georgiana, C.glauerti, C.leai*[3,246]
Signiferin 1	RLCIPYIIPC-OH	1157	[b]	10^{-9}	*Crinia signifera*[247,248]
Riparin 1	RLCIPVIFPC-OH	1187	[b]	10^{-8}	*Crinia riparia*[248,249]
Rothein 1	SVSNIPESIGF-OH	1148	[c]	10^{-7}	*Litoria rothii*[250]
Temporin 1Gb	SILPTIVSFLSKFL-NH_2	1564	[d]	10^{-6}	*Rana grylio*[251]
Temporin 1Gd	FILPLIASFLSKFL-NH_2	1608	[d]	10^{-6}	*Rana grylio*[251]
PLR [e]	LVRGCWTKSYPPKPCFVR-OH	2136	[f]	10^{-6}	*Rana pipiens*[252,253]
Pipinin 1	FLPIIAGVAAKVFPKICAISKKC-OH	2562	[f]		*Rana pipiens*[254]
Pipinin 2	FLPIIAGIAAKVFPKIFCAISKKC-OH	2573	[f]		*Rana pipiens*[255]
Pipinin 3	FLPIIASVAAKVFSKIFCAISKKC-OH	2579	[f]		*Rana pipiens*[255]
Brevinin 1	FLPVLAGIAAKVVPALFCKITKKC-OH	2525	[g]	10^{-6}	*Rana palustris*[256]
Palustrin 1c	ALSILRGLEKLAKMGIALTNCKATKKC-OH	2873	[g]		*Rana palustris*[257]
Granuliberin R	FGFLPIYRRPAS-NH_2	1422	[f]		*Rana rugosa*[256]
FSIP	AVWKDFLKNIGKAAGKAVLNSVTDMVNE-OH	3030	[g]		*Agalychnis litodryas*[255]
BST1	NFVCPPGQTFQTCASSCPKTCETRNKLVLCDKK CNQRCGCISGTVLKSKDSSECVHPSKC-OH	6368	[h]		*Bombina bombina*[258]
BOT1 [i]	NFVCPPGQSFQTCASSCPKTCETRNKVVLCDKK CNQRCPCVSGTVLKSKGSSECVHPSKC-OH	6446	[h]		*Bombina orientalis*[259]
Unnamed	LMCRMHQTYSACKGHCPPTCQFKKGPPLCSKK CVGACICKAPYIARSKTDNRCVLPEDC-OH	6620	[h]		*Rana areolata*[260]
Sauvagine	pEGPPISIDLSLELLRKMIEIEKQEKEKQQAA NNRLLLDTI-NH_2	4600	[b]		*Phyllomedusa sauvagei*[255,261,262]

[a] Underlining under a section contained within two Cys residues indicates disulfide functionality. [b] Contracts smooth muscle. [c] Lymphocyte proliferator. [d] Smooth muscle relaxant. [e] 2D structure (NMR) reported. [f] Histamine release agent. [g] Insulin release agent. [h] Trypsin inhibitor. [i] cDNA sequenced.

Smooth-muscle contraction is a major activity of tachykinin neuropeptides, but they also act as neurotransmitters and neuromodulators in the central nervous system, gastrointestinal tract and cardiovascular systems.[3] In mammals, tachykinins act *via* G-protein coupled neurokinin NK_1, NK_2 and NK_3 receptors.[230] These receptors are widely distributed on nerve terminals and cell bodies, a wide variety of smooth muscle, and endocrine cells such as the adrenal medulla.[230] Tachykinin peptides (like the caeruleins) produce intestinal contraction (i) through receptors located on enteric neurones in the central nervous system, which release

acetylcholine, initiating smooth-muscle contraction, and (ii) in a nerve-independent process, acting through receptors directly situated on smooth muscle. In anurans, tachykinins operate by the latter process, *i.e.* through NK_1 receptors situated on smooth muscle.[230]

5.4 Bradykinins

The bradykinin peptides are unusual (among neuropeptides) in that they contain C-terminal CO_2H residues. They are distributed amongst a number of anuran genera (see Table 8). In some species, bradykinins are the major peptides produced in skin secretions.[229] The bioactivities of the bradykinins are less than those of the bombesins, litorins, caeruleins or tachykinins, but this may be offset by the large quantities of bradykinins formed by anurans.[229] Their biological roles include smooth-muscle contraction or relaxation of intestinal, urogenital and respiratory tracts together with regulation of blood pressure.[3,231] They also have potent immunostimulatory effects, activate nociceptive pathways in mammals, and deter predation.[229] In mammals, smooth-muscle contraction is effected *via* the G-protein coupled B_1 receptors directly on smooth muscle or indirectly *via* B_2 receptors in the central nervous system.[231,232]

```
MFTLKKSLLLLFFLGTINLSLC                Signal (Pre)
KQERDADEDENEREAKVEDVKRAGY
SRMIR                          Acidic spacer (Pro)
RPPGFSPFR                              Bradykinin
```

A number of bradykinins have been sequenced using cDNA cloning methods.[211,213,214,216–219] The preprobradykinin sequence is shown above. The other bradykinin peptides sequenced by this method (see Table 8) show little similarity in the prepro regions of the peptides compared with that shown above for bradykinin. As an example, the cDNA clone of the precursor of kinestatin contains 114 amino acid residues, of which 84 constitute the central pro piece.[219] The full sequence is listed below.

```
MRLWFCLSFFIVLCLEHFPG                  Signal (Pre)
TLADERNNRDYTIRTRLHGHHKPSRNNRYAIKTSIH
GEHIPRNVPESEEKTEQLLRDLPKINRKGPRPPGFS
PFRGKFHSQSLR                   Acidic spacer (Pro)
QIPGLGPLR(G)                           Kinestatin
```

5.5 Tryptophyllins

There have been some forty tryptophyllins isolated from frogs of the *Phyllomedusa* and *Litoria* genera. The role of most of these peptides is quite unknown. In the case of *Litoria rubella* and *L. electrica*, there are no neuropeptides (like caerulein) and no antimicrobial species (like caerin 1.1) present in the skin secretions.[220,221] The tryptophyllin examples shown in Table 8 are major peptides present in the glandular secretion, and must be host-defence peptides. Tryptophyllin L 1.3 (pEFPWL-NH_2) is the only tryptophyllin from *Litoria* to show any smooth-muscle activity (at a modest μM concentration). No tryptophyllin shows antimicrobial or nNOS activity. One of Erspamer's tryptophyllins (FPPWM-NH_2) induces sedation and behavioural sleep in birds, and is also immunoreactive to a set of cells in the rat adenohypophysis.[222] The tryptophyllin peptides show some sequence similarity to the brain endomorphins YPWF-NH_2 and YPWG-NH_2 that have affinity for the γ-receptor.[233]

```
MNFLKKSLFLVLFLGFVSISFC                Signal (Pre)
DEEKRQDDDEGNEREEKKEIQEDGN
QEERRD                         Acidic spacer (Pro)
KP(P)AWVP(G)                              PdT-1
```

The cDNA sequence of the precursor of a tryptophyllin like peptide (PdT-1; KPHypAWVP-NH_2) from *Pachymedusa dacnicolor* has been determined and is listed above. Unlike other tryptophyllins, this peptide contracts smooth muscle at a concentration of 10^{-8} M.[223] The signal part of this peptide has some similarity with that of the bradykinin precursor (see above).

5.6 Dermorphins and deltorphins

Dermorphins and deltorphins (see Table 9) are unusual among amphibian peptides because they have a D-amino acid residue at position 2 (D-Ala, D-Leu or D-Met), and this residue is essential for full biological activity. Extensive pharmacological testing of these peptides has been described by Erspamer[3,238] and others.[239–241] The potent analgesic effect of the dermorphin and deltorphin neuropeptides is due to activation of μ and δ opioid receptors respectively.[3,242] Opioid receptors are widely distributed in the brain, spinal cord and peripheral nervous system on cell bodies and nerve terminals, and are also present in a variety of immune cells.[238] Table 9 shows the effect of dermorphins and deltorphins on the electrically evoked switch response in the mouse vas Deferens; an index of activity at μ opioid receptors. This response is only seen when the peptides are injected into the spinal cord or brain ventricles.[242,243] The most active of these opioid peptides is dermorphin, which shows analgesic effects at an EC_{50} of 10^{-11} mol per mouse.

The cDNA encoding preprodermorphin has been reported.[244] This encodes for a peptide which proteolytically cleaves to produce one molecule of dermenkephalin and three molecules of dermorphin. Similarly, the preprodeltorphin encodes three molecules of deltorphin 1 and one of deltorphin.[245] As an example, the sequence of the dermorphin precursor is shown below.

```
MSFLLKKSLLLILFLGLVSLSVC                Signal (Pre)
KEEKRETEEENENEENHEEEGSEMKR     Acidic spacer (Pro)
YAFGYPS(G)                              Dermorphin
```

5.7 Miscellaneous neuropeptides

A number of amphibian neuropeptides with various activities are listed in Table 10. The disulfide-containing peptides isolated from the *Crinia* genus are of interest. Structural work on *Crinia* disulfides is recent,[247–249] and preliminary pharmacological testing results indicate that signiferin 1 and riparin 1 have quite different roles in the amphibian integument. Signiferin 1 is smooth-muscle active while riparin 1 has no activity on smooth muscle, but acts to proliferate lymphocytes (*i.e.* is an immunomodulator). Both peptides act *via* CCK_2 receptors. Their 2D NMR structures are shown in Fig. 7.[249]

This raises the question as to the activities of the disulfide antibiotic peptides from ranid frogs (for sequences see Table 3).

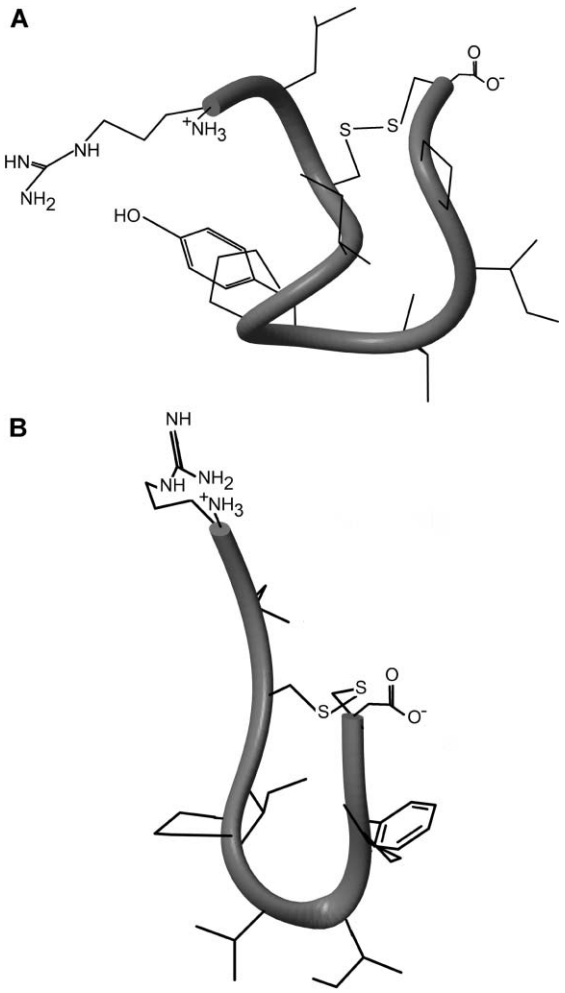

Fig. 7 (A) Signiferin 1, (B) Riparin 1. Structures determined by 2D NMR in trifluoroethanol–water.

The precise role of the disulfide bridge is not fully understood in the antimicrobial context, and it is already known that some of these *Rana* disulfides have roles in addition to microbial activity; *e.g.* the pipinins 1–3 (histamine release agents) and brevinin 1 and palustrin 1c (insulin release agents) (see Table 10). Other *Rana* disulfide peptides show sequence similarity to the signiferin and riparin peptides (from *Crinia* species), and it may be that some of the shorter *Rana* disulfide-containing peptides have some type of neuropeptide activity as well as their antimicrobial activity. These include the tigerins, ranalexins and the shorter gaegurins, japonicins and nigrocins (Table 3). Other peptides, *e.g.* BST1,[258] BOT1[259] and an unnamed peptide from *Rana areolata*[260] (see Table 10) are trypsin inhibitors.

6 Amphibian peptides that complex with Ca^{2+} calmodulin

Most frogs of the genus *Litoria* so far studied produce active peptides which inhibit the formation of nitric oxide (NO) by neuronal nitric oxide synthase (nNOS). Some fifty such peptides have been identified to date. Selected examples and their activities are shown in Table 11.

NO is unique 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. NO differs from other neurotransmitters and hormones in that its synthesis is regulated by three NOS isoforms. At low concentrations, NO serves as a cell-to-cell signalling agent. Nearly every cell type studied thus far has demonstrated the ability to synthesise NO by one of the three isoforms of NOS, namely neuronal NOS (nNOS, also called NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). A large number of different systems utilise NO as a mediator, including regulation of the circulatory and central

Table 11 nNOS inhibition activities of selected amphibian peptides

Name	Sequence	IC_{50}/μM	Charge	Species
Inhibitor Group A				
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2	8.2	+2	*Litoria citropa*
Citropin 1.1 d	Glfdvikkvasvi**D**Gl-NH_2	30.7	+2	
Citropin 1.1 mod	GLFDVIKKVASVIKKL-NH_2	0.9	+4	
Aurein 2.3	GFLDIVKKVVGIAGSL-NH_2	1.8	+2	*L.aurea*
Aurein 2.4	GLFDIVKKVVGTLAGL-NH_2	2.1	+2	*L.aurea*
Inhibitor Group B				
Frenatin 3	GLMSVLGHAVGNVLGGLF**K**P**K**S-OH	6.8	+3	*Litoria infrafrenata*
Frenatin 3 mod	GLMKVLGKAVGNVLGGLF**K**P**K**S-OH	1.4	+5	
Splendipherin	GLVSSIGKALGGLLADVV**K**S**K**GQPA-OH	9.0	+3	*L.splendida*
Caerin 2.6	GLVSSIGKLLGGLLADVV**K**S**K**GQPA-OH	6.6	+3	*L.caerulea/L.splendida* hybrid
Dahlein 5.1	GLLGSIGNAIGAFIAN**K**L**K**P-OH	3.2	+3	*L.dahlii*
Dahlein 5.2	GLLASIGKVLGGYLAE**K**L**K**P-OH	1.2	+2	*L.dahlii*
Dahlein 5.3	GLLASLGKVFGGYLAE**K**L**K**PK-OH	1.4	+3	*L.dahlii*
Dahlein 5.6	GLLASLGKVFGGYLAE**K**L**K**PK-OH	1.6	+3	*L.dahlii*
Inhibitor Group C				
Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	36.6	+1	*Litoria caerulea, L.splendida, L.gilleni*
Caerin 1.6	GLFSVLGAVAKHVLPHVVPVIAEKL-NH_2	8.5	+2	*L.chloris*
Caerin 1.8	GLFKVLGSVAKHLLPHVVPVIAEKL-NH_2	1.7	+3	*L.chloris*
Caerin 1.9	GLFGVLGSIAKHVLPHVVPVIAEKL-NH_2	6.2	+2	*L.chloris*
Caerin 1.19	GLFKVLGSVAKHLLPHVAPIIAEKL-NH_2	4.1	+3	*L.gracilenta*
Caerin 1.19.3	GSVAKHLLPHVAPIIAEKL-NH_2	inactive	+2	*L.gracilenta*

nervous system, neurotransmission in contractile and sensory tissues, together with learning and memory function.[263–265] Any amphibian predator which ingests a peptide that inhibits the formation of NO will almost certainly be adversely affected.

Nitric oxide synthases oxidise L-arginine to NO and citrulline, thereby controlling NO distribution and concentration. All three isoforms are homodimers with subunits of 130–160 kDa, differing in amino acid sequence identity, but sharing an overall three-component construction, namely: (i) An N-terminal catalytic oxygenase domain that binds heme, tetrahydrobiopterin and 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.[266]

Ca^{2+} calmodulin (Ca^{2+} CaM) is a dumbbell-shaped 148-residue protein which is required for the activation of nNOS: it acts as an electron shuttle and calcium transporter. It also alters the conformation of the reductase domain, allowing reactions to proceed at the heme site.[265] The nNOS-active amphibian peptides interfere with communication between Ca^{2+} CaM and nNOS. Addition of these peptides to nNOS during *in vitro* production inhibits the formation of NO at μM concentrations. Subsequent addition of Ca^{2+} CaM results in partial recovery of nNOS activity.[26,27] Complexes between Ca^{2+} CaM and the active peptides shown in Table 1 can be detected using 2D NMR to study the titration of Ca^{2+} CaM with the active peptide, and by electrospray mass spectrometry.[267] A current 3D NMR study of the complex between caerin 1.8 (Table 1) and Ca^{2+} CaM indicates that the CaM changes from a dumbbell to an ovoid shape in order to encapsulate the active peptide;.[267] *cf.* refs. 268 and 269. This change in shape adversely affects binding of the complex at the Ca^{2+} CaM binding domain.

Selected nNOS-active peptides have been shown to also inhibit the operation of the enzyme calcineurin.[26] CaM is not only the regulatory protein for the NOS isoforms but also for calcineurin, other kinase-phosphorylating enzymes and adenylate cyclase.[270] Ca^{2+} CaM is also involved in regulation of the eukaryotic cytoskeleton[270] and is required by some protozoa for ciliate movement.[271] The likelihood is that the active amphibian peptides will therefore interfere with many cellular functions at once, causing maximum inconvenience and deterrence to any attacker.

The nNOS-inhibiting peptides fall into three major groups. Group B comprises peptides which show only nNOS activity. All of these contain KXK or KXKYK residues [X and Y may be Leu, Pro or Ser (see Table 11)], towards the C-terminal end of the peptide, and the activity within this group of peptides increases with increasing positive charge. 2D NMR studies of these peptides indicates that there is an initial α-helical region followed by a more random region (see, for example, Frenatin 3, Fig. 8).[272] The dahleins 5 (from *Litoria dahlii*[6,57]) are amongst the most active nNOS inhibitors so far isolated from amphibians.

Members of the other groups of nNOS-inhibiting peptides have multifaceted activities. Group A peptides include the citropins 1 and aurein peptides. These are α-helical amphipathic peptides (*cf.* Fig. 1) which show major antimicrobial, anticancer and fungicidal activity as well as significant nNOS activities. Some twenty synthetic modifications of citropin 1.1 have been tested for nNOS activity: two are shown in Table 1. It is of interest that the nNOS activities of the L and D isomers of citropin 1.1 are quite different: Ca^{2+} CaM complexes more efficiently with the natural (L) form of citropin 1.1. The most active synthetic modification of citropin 1.1 has an IC_{50} of 9×10^{-7} M with a charge of +5.[27]

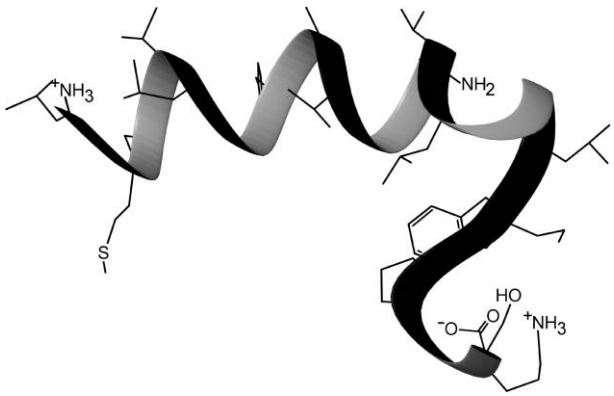

Fig. 8 Frenatin 3. Structure determined by 2D NMR in trifluoroethanol–water.

The caerin 1 peptides comprise the final group of nNOS inhibitors.[6] These hinged peptides (see, for example, Fig. 3A) are amongst the most cytotoxic (to predators) of all *Litoria* peptides, showing wide-spectrum antibiotic, anticancer, fungicidal, antiviral (including HIV) and nNOS activities. The trend of increasing activity with increasing positive charge is again apparent from the data in Table 11, while the importance of hydrophobic groups is shown by the different activities of the natural caerins 1 with Leu3 changed to Phe3.

7 Amphibian pheromones

Amphibians evolved from freshwater fish several hundreds of million years ago. It might be expected that such amphibians could inherit the water-soluble pheromones of the fish ancestor, and also develop volatile pheromones for use on land. Fish have two types of aquatic sex pheromones. They have water-soluble sex pheromones (structures unknown but possibly peptides), which attract males and females of a particular species, together with other pheromones which are transferred from male to female (and sometimes female to male) to initiate the reproductive cycle.[273,274] The most studied fish in this regard are the goldfish,[274–276] in which the pheromones are steroidal compounds, *e.g.* 17α,20β-dihydroxy-4-pregnen-3-one. It has also been shown that 11-ketotestosterone induces male-type sexual behaviour in crucian carp.[277] Finally, an unusual variation on the above: the sea lamprey, which is one of the oldest living relics of vertebrate evolution, spends most of its time in freshwater streams as a non-parasitic form before metamorphosing into a parasitic adult, which inhabits oceans or lakes. The stream-dwelling larval form releases a mixture of two sulfated steroids and a bile acid which lead adults to spawning streams. This migratory pheromone mixture is active at sub-picogram concentrations.[278]

The first aquatic sex pheromone of an amphibian was isolated from the cloacal (tail) gland of the aquatic male salamander *Cynops pyrrogaster* in 1995.[279,280] This female-attracting peptide was named sodefrin and is species-specific. A cDNA investigation indicated that the sodefrin precursor protein contains 189 amino acid residues.[281] A related sex pheromone, silefrin, was isolated

from the cloacal gland of the male aquatic salamander *Cynops ensicauda.*[282] Movement of these pheromones through water is effected by the male lashing his tail: the pheromones attract females within a concentration range of 0.1–1.0 pM.[280,283]

Sodefrin	SIPSKDALLK-OH
Silefrin	SILSKDAQLK-OH

A quite different scenario occurs for the terrestrial salamander *Plethodon jardani.* During the mating display on land, four isoforms of a 22 kDa protein from the male mental glands (beneath the head) are placed directly onto the skin of the female to accelerate the mating process.[284] Whether these proteins are male sex pheromones, or whether one is a carrier for a smaller pheromone, is not known.[285]

The first anuran sex pheromone was isolated from the male of the Magnificent tree frog (*Litoria splendida*).[47,286] Secretions were collected monthly (using the electrical stimulation method[9]) over a three year period from both male and female and then analysed by HPLC and electrospray mass spectrometry. The HPLC profiles indicated a small component present only in male secretions during the reproductive (summer) period. This 25-residue peptide (GLVSSIGKALGGLLADVVKSKGQPA-OH) was named splendipherin, and behavioural tests showed that the pheromone attracted female *L. splendida* at a minimum concentration of 10 pM.[47,286] Splendipherin moves across the surface of water by surface tension gradient. The pheromone is species-specific, having no effect on females of other species. It also has no effect on males of *Litoria splendida* or *L. caerulea.* The tree frog *Litoria splendida* is terrestrial, only coming to the water to breed, and as such, normally has no need of an aquatic sex pheromone. The presence of this pheromone is almost certainly an evolutionary overkill, since these frogs can see each other and readily communicate on land. This is in complete contrast with the aquatic salamanders which spend their lives in water: for these creatures, the aquatic pheromones are essential for their survival.

There are several interesting evolutionary riders to this investigation. Firstly, splendipherin is a trace component of the peptide secretion of male *Litoria splendida*, but a major component of the skin secretions of both male and female of the closely related Common green tree frog *Litoria caerulea.*[48] Splendipherin has no pheromone activity towards the female of *L. caerulea.* Instead, it is used as a host-defence peptide; the major nNOS inhibitor of *L. caerulea* (see Table 11). Both frog species originated from a common ancestor; one uses splendipherin as a sex pheromone, the other as an nNOS inhibitor. The secondary structure of splendipherin, as shown by 2D NMR studies, is shown below in Fig. 9.

Recently, we have had access to a female hybrid produced from a male *Litoria caerulea* and a female *Litoria splendida.* This animal has physical likenesses to each parent. Interestingly, the female hydrid does not recognise the sex pheromone splendipherin of *Litoria splendida.*[287]

8 Evolutionary trends – peptide profiling

The evolutionary relationships of many anurans remain, at least in part, an issue of contention. At one time, all physical characters were considered of equal significance to determine relationships. Currently, a distinction is made between 'ancestral'

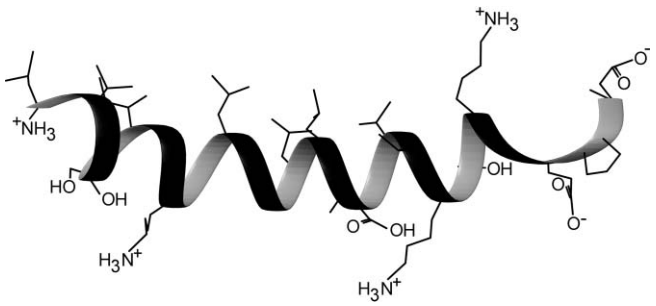

Fig. 9 Splendipherin. Structure determined by 2D NMR study in trifluoroethanol–water.

characters shared by all early frogs, and 'derived' characters which are considered more meaningful in an evolutionary sense. For example, the North American genus *Ascaphus* was at one time united in the same family as the New Zealand genus *Leiopelma* on the basis of solely ancestral features. The two are now considered different representatives of separate families.

How can the skin peptides of anurans be viewed in an evolutionary context? Take the examples of hylid and ranid frogs. The current biogeographic distribution of these families is associated with tectonic events which occurred during the fragmentation of Gondwanaland.[288] The structural diversity of bioactive peptides among hylids and ranids is extraordinary. Such peptides are synthesised in precursor form (prepropeptide) in the multinucleated cells lining the inner walls of the dermal glands, and stored as inactive propeptides. The glands release the active peptides onto the skin as required. Nicolas *et al.*[12] have shown that in spite of the wide variation in the sequences of active peptides from American and Australian hylids and also from ranids, there is some conservation of the signal (pre) sections of the precursor peptides, and has concluded that they all originated from an ancestral gene approximately 150 million years old. This is illustrated by a consideration of the sequences of the signal (pre) portions of the precursor peptides listed in this review: namely, caerin 1.1, caerin 2.1, brevinin 1E, esculatin 1, ranacyclin 1, bradykinin, PdT-1 and dermorphin (the only precursor sequence apparently out of step with this correlation is kinestatin from *Bombina maxima*[116]). Within this context, the molecular phylogeny of the precursors of the dermaseptins,[12] caerins[12] and certain *Rana* antimicrobials[12,116] has recently been proposed. Recent work on ranid frogs should be compared with an earlier study based on electrophoresis patterns of enzymes from skeletal muscles and livers of pond frogs. This suggested that differentiation of species occurred at the same time in Europe and Asia.[289]

Bioactive peptides from anuran skin are able to diverge more rapidly than the physical and biological aspects of the animals. Thus two individuals may be indistinguishable in morphological and advertisement call (a premating isolating mechanism) but have different skin peptide profiles. If the geographical sources become isolated for a sufficient period, genetic divergence could be anticipated to create distinct species (allopatric speciation). Such divergence could be considered an incipient step in the process of speciation. An obvious example of this is the case of the common edible frog *Rana esculenta* Linneaus 1758 which is a hybrid (best regarded as a complex rather than a discrete species) arising from

the marsh frog *Rana ridibunda* Pallas 1771 and the pool frog *Rana lessonae* Camerano 1882.[116]

The genus *Litoria* in continental Australia and the surrounding islands is useful in illustrating the application of peptide profiling in differentiating between species and between different populations of the same species. It is necessary to stress that these comparisons must be carried out at the same time of the year, because there are some Australian *Litoria* species of frog that vary the relative peptide concentrations[47] (or indeed the peptides themselves[47,250]) in the reproductive and inactive seasons of the year.

The skin peptide profile can be used to differentiate all studied species of the genus *Litoria*, even species which are very closely related, *e.g.* (i) *L. splendida*[47] and *L. gilleni*,[49] and (ii) *L. chloris*[51] and *L. xanthomera.*[50] However, studies of the Green Tree Frog *Litoria caerulea* (which is found across the central, northern and eastern areas of Australia), indicate major differences in the peptide profiles of animals collected from different geographic locations. Physically, these animals are identical.[48] There appear to be two major populations,[290] one in the northern periphery of Northern Territory and Western Australia, the second along the Queensland and New South Wales coast. The HPLC peptide profiles of animals collected from these areas are shown in Fig. 10. Of particular interest are the differences in peptide profiles of *L. caerulea* collected in Darwin and from Melville Island (60 km off the coast from Darwin). These populations have been separated by the ocean for only 10 000 years.[6,48]

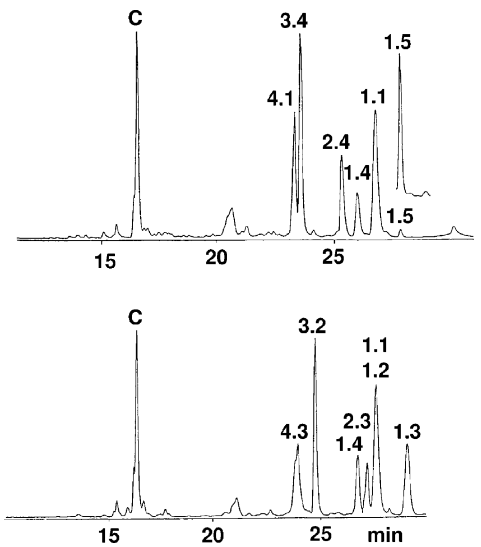

Fig. 10 HPLC peptide profiles of skin glandular secretion of *Litoria caerulea* from (A) Proserpine (Queensland) and (B) Borroloola (Northern Territory). Peaks identified by numbers are caerin peptides: these numbers correspond to the sequences given in Table 1. The peak designated C is the neuropeptide caerulein [pEQDY(SO_3)TGWMDF-NH_2].

A more complex scenario pertains for the Australian Red Tree Frog *Litoria rubella.* This animal is distributed widely throughout Australia, as indicated in Fig. 11. There is a closely related species (*Litoria electrica*) situated near the Gulf of Carpentaria (see Fig. 11): the separation of these two different species has been confirmed by peptide profiling.[221] This indicates that at least six populations (some may be new species) of *Litoria rubella* occur on

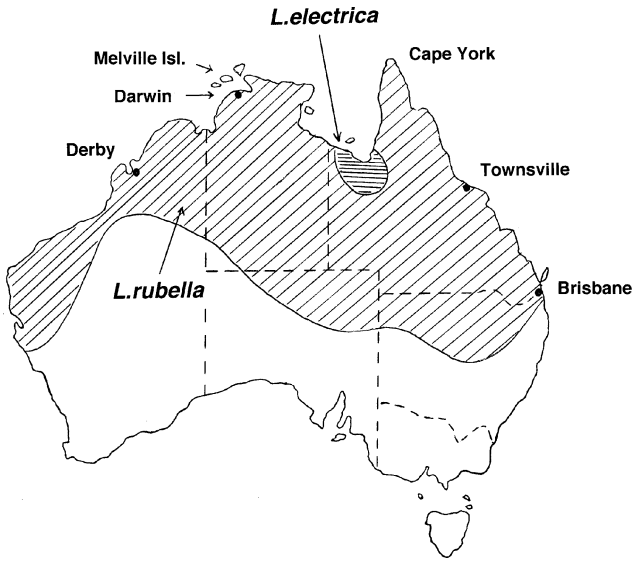

Fig. 11 Geographic distribution of *Litoria rubella* and *Litoria electrica* in Australia. Dashed lines (---) are state boundaries.

the Australian mainland. Examples of the HPLC peptide profiles from animals collected near Derby (Western Australia) and Townsville (Queensland) are shown in Fig. 12.[220] The variations in peptide profiles of *L.rubella* along the coastal strip of Queensland are of particular interest. In the south (Brisbane), fraction F (see Fig. 12) is a minor component compared with fraction E, but F increases steadily as the geographic location moves

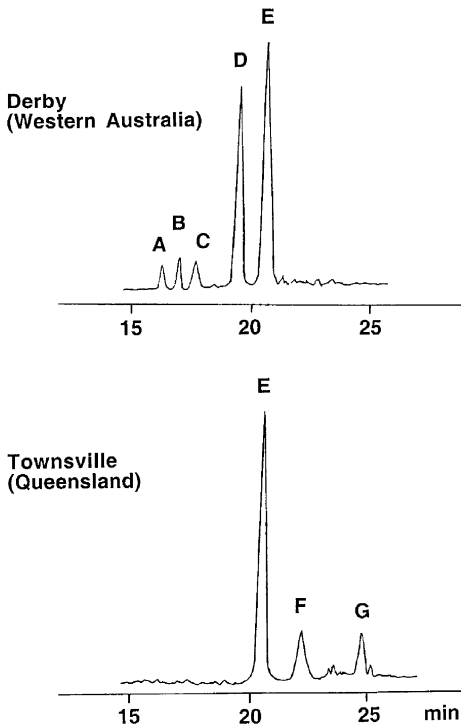

Fig. 12 HPLC peptide profiles of skin glandular secretion of *Litoria rubella* from Derby (Western Australia) and Townsville (Queensland). Tryptophyllin peptide sequences are as follows: (A) IEFFA-OH; (B) IEFFT-NH_2; (C) VDFFA-OH; (D) pEIPWFHR-NH_2; (E) FPWL-NH_2; (F) FPWP-NH_2; (G) FPFPWL-NH_2.

northward to Cape York (a distance of 2300 km), where it is the major component. Clinal changes like this can be considered a progressive stage of evolution, with peptide studies of this type providing a clear indication of genetic change.

9 Summary

Work on amphibian peptides commenced in the mid 20th century when separative techniques were primitive and modern spectroscopic techniques in their infancy. Often, thousands of frogs had to be sacrificed in order to identify one peptide. With the sophisticated analytical techniques available today, the components of the skin secretions can be determined from a single (benign) 'milking' of one animal. Although X-ray techniques are not routine structural methods for peptides (as opposed to proteins), 2D and 3D NMR methods for determining the secondary structure of peptides, although time consuming, are standard procedures. In addition, DNA cloning techniques have advanced to a stage where their application to determining the sequences of precursor peptides is a routine and simple procedure.

Amphibians evolved from freshwater fish in the Devonian period. Their peptide arsenals probably originated from ancestor genes in the same period, evolving to provide defence systems which have protected them over hundreds of millions of years. This was the case until recently, when *Homo sapiens* began the destruction of the amphibian environment and a chytrid fungus began decimating amphibians worldwide.

The extraordinary range of peptides, including antimicrobials, neuropeptides and nNOS-inhibiting peptides, produced by any one species is presumably an evolutionary device to ensure that predators are not able to simply effect resistance to each component of a varied cocktail of active peptides.

10 Acknowledgements

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