Journal of Peptide Science, Vol. 3, 181–185 (1997)

New Caerin Antibacterial Peptides From the Skin Glands of the Australian Tree Frog *Litoria xanthomera*

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Received 25 April 1996
Accepted 6 August 1996

Abstract: The secretion of the skin glands of the 'orange-thighed frog' *Litoria xanthomera* contains seven peptides. One of these is the known hypotensive peptide caerulein. Two new peptides, caerin 1.6 [GLFSVLGAVAKHVLPHVVPVIAEKL(NH_2)], and caerin 1.7 [GLFKVLGSVAKHLLPHVAPVIAEKL(NH_2)] show antibacterial properties. Two other peptides lack the first two amino acid residues of caerins 1.6 and 1.7 and show no antibacterial activity. The identification of the peptides in *Litoria xanthomera* confirms that this species is related to *Litoria caerula, Litoria gilleni* and *Litoria splendida* but not as closely as those three species are related to each other. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

J. Pep. Sci. 3: 181–185, 1997

(No. of Figures: 3. No. of Tables: 2. No. of Refs: 18.)

Keywords: *Litoria xanthomera; skin glands; glandular secretion; antibacterial peptides; caerins 1*

INTRODUCTION

Our work on the isolation and characterization of host defence peptides from the *Litoria* genus of amphibians [1–5] has recently concentrated on such products from the 'orange-thighed frog' *Litoria xanthomera*. *Litoria xanthomera* [6] (Figure 1) is a medium sized tree frog, measuring up to 5.5 cm in length. It is found only in tropical rainforest along a 600 km transect of the northern Queensland coastline (from Cape Melville to Ingham). It may be visually identified by its very characteristic orange thighs and orange eyes. It is related to the other tree frogs *L. splendida*. *L. caerulea* and *L. gilleni* in that they all have a haploid chromosome number of 13 [7], but it differs from them in that its glandular secretion is contained in granular glands which are distributed over the whole dorsal surface rather than being located in specific rostral and/or parotoid glands.

In this paper we report the structures and biological activities of the peptides contained in the skin secretions of *L. xanthomera* and confirm that this frog is distantly related to the green frogs *L. splendida*, *L. caerula* and *L. gilleni* based on the peptide compositions found.

MATERIALS AND METHODS

Preparation of skin secretions, HPLC separation of the glandular secretion and structure determination of the peptides using FAB MS with a VG ZAB 2HF mass spectrometer and automated Edman sequencing were carried out as described previously [5].

Preparation of Synthetic Peptides

Four peptides (caerins 1.6, 1.6.1, 1.7 and 1.7.1) were synthesized commercially, by Chiron Mimotopes, Clayton, Victoria, Australia, using L-amino

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CCC 1075-2617/97/030181-05

Figure 1 *Litoria xanthomera*.

acids via the standard *N*-α-Fmoc method. Full details including protecting groups and deprotection have been reported recently [8]. Each synthetic caerin was shown to be identical with the natural caerin by FAB mass spectrometry, Edman sequencing and co-elution of the synthetic and natural peptides on HPLC.

Antimicrobial testing on synthetic peptides was carried out by Dr Bruce Winter of the Microbiology Department of the Institute of Medical and Veterinary Science, Adelaide, Australia. The method involved the measurement of inhibition zones produced by the applied peptide on a thin agarose plate containing the micro-organisms under study. The procedures are standard and have been fully documented [9]. The micro-organisms used are listed in Table 2. Activities are recorded as MIC values, i.e. the minimum inhibitory concentration of peptide per ml required to inhibit the growth of the named micro-organism totally.

RESULTS AND DISCUSSION

Several specimens of *L. xanthomera* were collected from near Cairns (Queensland) and kept in captivity. Mild electrical stimulation of the skin effects the release of skin secretions, and this can be done on a monthly basis without harming the animals [10]. The HPLC separation (Figure 2) indicates that there are seven major components (**a** to **g**), all of which have been subsequently shown to be peptides. The HPLC data indicate that of the 5 mg (on average) of solid material produced per frog following each 'milking', ca. 3 mg, is peptide material, with components **f** (0.6 mg) and **g** (1.25 mg) being the major components.

FAB mass spectrometry gave the following MH^+ values for the seven major peptide components shown in HPLC trace (Figure 2), as follows: **a** (1354 Da), **b** (2464 Da), **c** (2421 Da), **d** (1096 Da), **e** (1140 Da), **f** (2634 Da) and **g** (2591 Da).

Peptides **a** and **e** are respectively the hypotensive peptide caerulein (**1**) and caeridin 1.1 (**3**), identical with those peptides isolated previously from other *Litoria* species [1–4]. The other five peptides are new.

The number of CO_2H and $CONH_2$ groups in each of the new peptides is determined by first determining the molecular weight of its methyl ester derivative [5] using FAB mass spectrometry. The masses of the methyl esters of the five new peptides, listed in the order shown on the HPLC trace (Figure 2), are as follows: **b** (2493 Da), **c** (2450 Da), **d** (1125 Da), **f** (2663 Da) and **g** (2620 Da). The difference between the MH^+ value of the natural peptide and that of its methyl ester gives the required information. In each of the cases listed above, the mass difference is 29 Da. Since the mass increments (CO_2H to CO_2Me) and ($CONH_2$ to CO_2Me) are 14 and 15 respectively, all of the new peptides have one CO_2H and one $CONH_2$ group.

A combination of the FAB MS and Edman sequencing data provides the amino acid sequences of the five peptides. The names and structures of all peptides are listed in Table 1.

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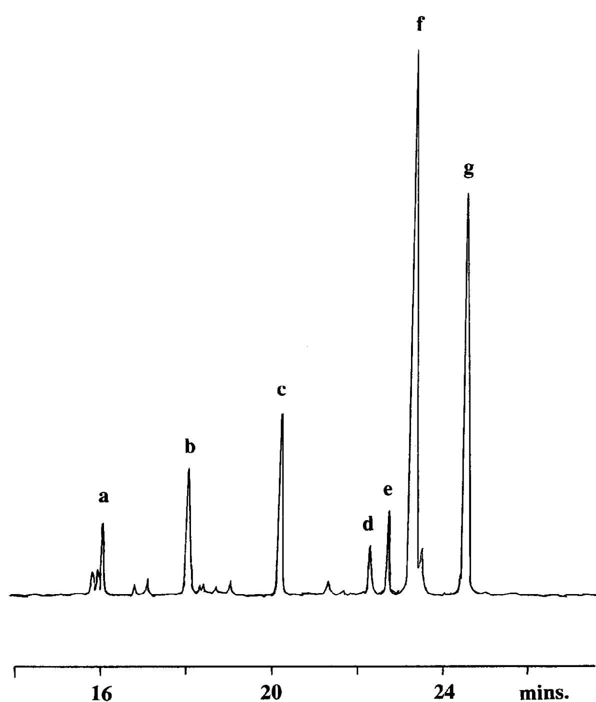
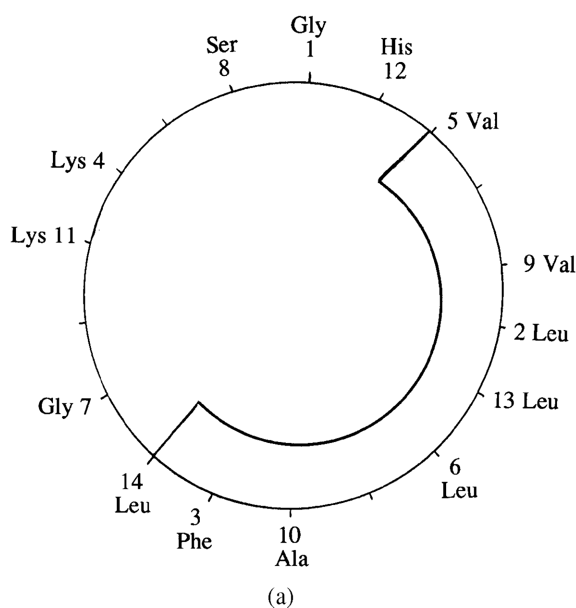


Figure 2 HPLC separation of 0.1 mg of the treated lyophilized glandular secretion of *Litoria xanthomera*. Experimental procedures have been reported [5]. The components are as follows (cf. Table 1): **a** [caerulein ($MH^+ = 1354$ Da)], **b** [Caerin 1.7.1 ($MH^+ = 2464$), **c** [caerin 1.6.1 ($MH^+ = 2421$)], **d** [caeridin 1.4 ($MH^+ = 1096$)], **e** [caeridin 1.1 ($MH^+ = 1140$)]. **f** [caerin 1.7 ($MH^+ = 2634$)] and **g** [caerin 1.6 ($MH^+ = 2591$)].

Table 1 Amino Acid Sequences of Skin Peptides from *Litoria xanthomera*

Caerulein ($MH^+ = 1354$)	pEQDY(SO_3H)TGWMDF(NH_2)	(**1**)
Caeridin 1.1 ($MH^+ = 1140$)	GLLDGLLGTLGL(NH_2)	(**2**)
Caeridin 1.4	GLLDGLLGGLGL(NH_2)	(**3**)
Caerin 1.6 ($MH^+ = 2591$)	GLFSVLGAVAKHVLPHVVPVIAEKL(NH_2)	(**4**)
Caerin 1.6.1 ($MH^+ = 2421$)	FSVLGAVAKHVLPHVVPVIAEKL(NH_2)	(**5**)
Caerin 1.7 ($MH^+ = 2634$)	GLFKVLGSVAKHLLPHVAPVIAEKL(NH_2)	(**6**)
Caerin 1.7.1	FKVLGSVAKHLLPHVAPVIAEKL(NH_2)	(**7**)

The new peptides are all post-translationally modified and are either related to caerin 1.1 [1] or caeridin 1.1 (**3**). We name them using nomenclature already adopted for those types of peptide. The major components are caerins 1.6 and 1.7, i.e. components **g** and **f** respectively (Figure 2). Amino acid residues different from those of caeridin 1.1 or caerin 1.1 respectively are underlined in Table 1. Caeridin 1.4 differs from caeridin 1.1 at residue 9 (Gly for Thr). Caerin 1.6 differs from caerin 1.1 at



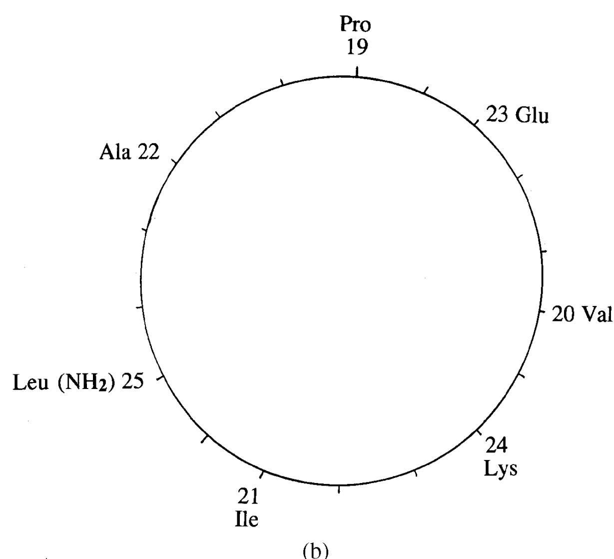


Figure 3 Edmundson projection for caerin 1.7: (a) residues 1–14 (the hydrophobic and hydrophilic regions are indicated) and (b) residues 19–25.

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residues 3 (Phe for Leu) and 24 (Lys for His), while caerin 1.7 is different from caerin 1.1 at six residues, viz 3 (Phe for Leu), 4 (Lys for Ser), 8 (Ser for Ala), 13 (Leu for Val), 18 (Ala for Val) and 24 (Lys for His). Caerins 1.6.1 and 1.7.1 lack the first two residues from the N-terminal end of caerins 1.6 and 1.7 respectively.

The green tree frogs *L. splendida*, *L. caerulea* and *L. gilleni*, which are closely related, have many skin peptides in common [1–4]. Two of the skin peptides of *L. xanthomera* are also present in the glandular secretions of the above-mentioned *Litoria* species. However, although the major skin peptides of *L. xanthomera* are members of the caerin 1 group of peptides, they are nevertheless different in structure from any caerins reported previously. Thus the described chemistry indicates that *L. xanthomera* is related to *L. splendida*, *L. caerulea* and *L. gilleni*, but not as closely as those three species are related to each other.

Structure and Bio-activity relationships of Caerin 1 Peptides

A number of amphibian peptides such as the caerins 1 [1–4], the magainins [11,12], the bombinins [13,14] and the gaegurins [15] show wide-spectrum antimicrobial activity. It has been shown [16] that following liberation of the magainins in random conformations from the prepromagainin precursor, the magainin peptides bind electrostatically via the cationic charges on the peptide to the anionic centres of phospholipids on the outer membrane of the bacterial cell. For a review of the biosynthesis of antimicrobial peptides see [17]. The antibacterial peptide then becomes an amphipathic α-helical structure with well-defined hydrophilic and hydrophobic zones. These α-helices form a monolayer which disrupts the bacterial membrane, forms ion channels and ultimately causes cell death. Other amphipathic amphibian peptides may act in similar fashion [16].

The caerin 1 peptides are unusual among antibiotic peptides from amphibians in that they cannot form an ideal α-helix (like for example the magainins) since they contain Pro at residues 15 and 19. Edmundson projections (Figure 3) of caerin 1.7 (**6**) indicate that an α-helical structure encompassing residues 1 to 15 has well-defined hydrophilic and hydrophobic zones. The two Pro residues distort the α-helix after residue 14 (cf. [18]), and an α-helical structure following residue 20 does not have well-ordered hydrophilic and hydrophobic zones. The same scenario pertains to caerin 1.6 (**4**).

We did not have sufficient of the natural caerin peptides from *Litoria xanthomera* to assess their antibacterial activities properly, so we have caerins 1.6, 1.6.1, 1.7 and 1.7.1 synthesized for this purpose. The results of the antibacterial testing are recorded in Table 2 and show that caerins 1.6 (**4**) and 1.7 (**6**) exhibit significant antibiotic activity, a mixture of caerins 1.6 and 1.7 is, overall, not significantly more active than the individual peptides, and caerins 1.6.1 (**5**) and 1.7.1 (**7**), which have the first two residues of caerins 1.6 and 1.7 missing, are inactive. The last point is of particular interest because analogous relationships are also observed for *L. splendida*, *L. caerulea* and *L. gilleni*. In these cases, the major antibiotic agent caerin 1.1 [1] is always accompanied by the inactive caerin 1.1.1

Table 2 Antibiotic Activities of the Caerins from *Litoria xanthomera*

Organism	MIC (μg/ml)[a] for caerins				
	1.6	1.6.1	1.7	1.7.1	[1.6 + 1.7 (1 : 1)]
Bacillus cereus	100		100		25
Escherichia coli					
Leuconostoc lactis	3		3		3
Listerial innocua	50		50		12
Micrococcus luteus	25		12.5		12.5
Pasteurella multoci	25		25		50
Staphylococcus aureus	6–12.5[b]		12.5–50[b]		50
Staphylococcus epidermis	12.5		50		12.5
Streptococcus uberis	25		50		12.5

[a]If no figure is indicated, the MIC value is >100 μg/ml.
[b]Value depends upon strain used.

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(caerin 1.1 minus the first two residues) [1–4]. We have investigated this point further for *L. xanthomera* as follows. When the skin secretion is first produced, it is immediately washed from the skin with water, and an equal volume of methanol is added to deactivate enzyme material. After filtration and lyophilization, the HPLC separation of the peptide components is that shown in Figure 2. However, if the aqueous skin secretion is not treated with methanol, and then lyophilized, caerins 1.6.1 and 1.7.1 are major components. These results suggest that *L. xanthomera*, as well as being able to release the active caerins 1.6 and 1.7 on demand, also has a mechanism to deactivate those peptides by removing their first two amino acid residues.

CONCLUSIONS

The 'orange-thighed frog' *Litoria xanthomera* produces three powerful host-defence peptides in its skin glands, viz. the neuropeptide caerulein and the two antibiotic peptides caerin 1.6 and 1.7.

The frog is able to deactivate its antibiotic peptides by removing their first two amino acid residues.

The observation of caeridin and caerin peptides in the glandular secretion of *L. xanthomera* confirms this species to be related to the green tree frogs *L. splendida*, *L. caerulea* and *L. gilleni*.

Acknowledgements

We thank the Australian Research Council for providing funding for this project, the Queensland Department of Environment and Heritage for a permit to collect *Litoria xanthomera*, and Ms D. Turner for automated peptide sequencing.

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