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The Maculatin Peptides from the Skin Glands of the Tree Frog *Litoria genimaculata*: A Comparison of the Structures and Antibacterial Activities of Maculatin 1.1 and Caerin 1.1

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Abstract: Six peptides have been isolated and characterized from the dorsal glands of the tree frog *Litoria genimaculata*. One of these is the known hypotensive peptide caerulein; the others have been named maculatins. The amino acid sequences of the maculatin peptides have been determined using a combination of fast atom bombardment mass spectrometry and automated Edman sequencing. Four of the maculatin peptides show antibiotic activity, with maculatin 1.1 [GLFGVLAKVAAHVVPAIAEHF(NH_2)] showing the most pronounced activity, particularly against Gram-positive organisms. Maculatin 1.1 resembles the known caerin 1 antibiotic peptides, except that four of the central amino acid residues (of the caerin 1 system) are missing in maculatin 1.1. A comparison of the antibiotic activity of maculatin 1.1 with those of caerin 1.1 is reported. ©1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *Litoria genimaculata*; skin glands; glandular secretions; peptides; antibiotic activity; maculatins 1; caerin 1.1

INTRODUCTION

Our work on the characterization of host defence peptides from the skin glands of tree frogs of the genus *Litoria* [1–5] has recently been extended to include *L. genimaculata* [6]. *Litoria genimaculata* is a medium sized tree frog measuring up to 5 cm in length, which is found in rainforests in both New Guinea and northern Australia [7]. In Australia, *L. genimaculata* is found within a 700 km transect of the Queensland coastline from Cape Melville to Cairns. The single specimen used in this study was obtained from near Cairns. *Litoria genimaculata* has a dorsal coloration which is rich brown, interspersed with patches of metallic green and copper shading.

We report the structures and antibiotic activities of the major peptides contained in skin secretions of *L genimaculata*, and compare the activity of the major antibiotic agent maculatin 1.1 with those of the related caerin 1.1.

MATERIALS AND METHODS

Preparation of skin secretions, HPLC separation of the glandular secretion and structure etermination of the peptides using FAB MS with a VG ZAB 2HF

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mass spectrometer and automated Edman sequencing were carried out as described previously [4].

Five of the maculatin peptides were synthesized by Chiron Mimotopes (Clayton, Victoria, Australia) using L-amino acids via the standard *N*-α-Fmoc method [8]. Each synthetic peptide was shown to be identical with the natural maculatin by FAB mass spectroscopy, Edman sequencing and co-elution of the synthetic and natural peptides by HPLC.

Antibiotic testing of the maculatin and caerin peptides was carried out by Dr B. Winter of the Microbiology Department, Institute of Medical and Veterinary Science, Adelaide, Australia. The microorganisms used are listed in Table 1. The procedures are standard [9] and involve the measurement of inhibition zones produced by the applied peptide on a thin algarose plate containing the microorganisms under study. Activities are recorded as MIC values, i.e. the minimum inhibitory concentration of peptide per ml required to inhibit the growth of the named microorganism totally.

RESULTS AND DISCUSSION

Mild electrical stimulation of the skin of *L. genimaculata* effects release of the skin secretion, a procedure that can be performed monthly without harming the animal [10]. The HPLC separation (Figure 1) shows a variety of components and, from these, six peptides (designated **a** to **f** on Figure 1 in order of retention time) have been isolated and characterized. Each 'milking' provides some 4 mg of solid following lyophilization, and HPLC data indicate that some 2.5 mg of this solid is peptide material, of which **c** and **f** (each 0.4 mg) are the major components.

FAB mass spectrometry gave the following MH^+ values for the peptide components: **a**, 1354; **b**, 1975; **c**, 1878; **d**, 2395; **e**, 2360 and **f**, 2145 Da. The number of CO_2H and $CONH_2$ groups in the new peptides is determined by converting each peptide to its methyl ester and determining the difference in molecular weights of the peptide and the methyl ester. The mass increments ($CO_2H \rightarrow CO_2Me$) and $CONH_2 \rightarrow CO_2Me$) are 14 and 15 Da respectively. The MH^+ values of the esters of the various components are **b**, 2004; **c**, 1922; **d**, 2482; **e**, 2404 and **f**, 2174. Thus **b** and **f** have one CO_2H and one $CONH_2$ group, **c** and **e** one CO_2H and two $CONH_2$ groups, and **d** three CO_2H and three $CONH_2$ groups.

A combination of FAB MS and Edman sequencing data provides the amino acid sequences of the peptides. Names and structures of the six peptides are listed in Table 2. Component **a** is the hypotensive peptide caerulein identical with the same peptide isolated from other tree frogs of the genus *Litoria* [1–5]. Other peptides are all new.

Sequences and Bioactivity Relationships of the Maculatin Peptides

All of the maculatin peptides listed in Table 2 are post-translationally modified in that they contain C-terminal $CONH_2$ groups. The nomenclature and

Table 1 Antibiotic Activities of the Maculatins from *Litoria genimaculata* and of Caerin 1.1

	MIC (μg/ml)[a] Maculatins					
Organism	1.1	1.1.1	1.2	2.1	3.1	Caerin 1.1[c]
Bacillus cereus	50			100		50
Escherichia coli	100					
Leuconostoc lactis	25					1.5
Listeria innocua	100			100		25
Micrococcus luteus	12.5			100		12.5
Pasteurella multocida	100					25
Staphylococcus aureus	6–12.5[b]		50	100		3–12.5
Staphylococcus epidermidis	12.5			50		12.5
Streptococcus faecalis	25					25
Streptococcus uberis	3		6	25	25	12.5

[a] If there is no figure indicated, the MIC value is >100 μg/ml. [b] Value depends upon strain used. [c] Caerin 1.1: GLLSVLGSVAKHVLPHVVPVIAEHL (NH_2) [1–3]

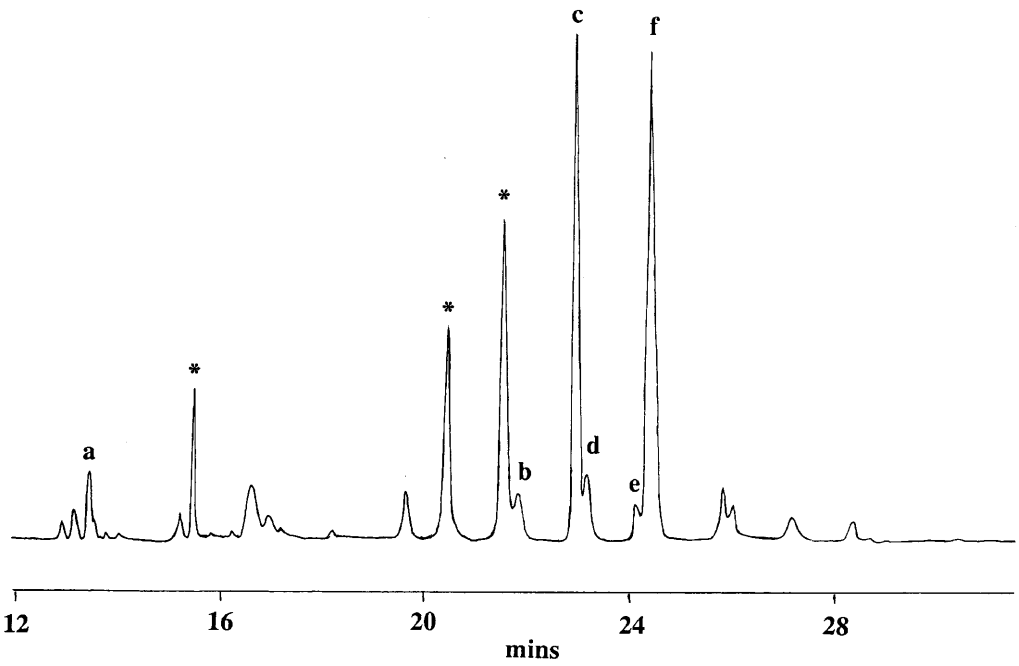

Figure 1 HPLC separation of 0.1 mg of the treated lyophilized glandular secretion of *Litoria genimaculata*. Experimental procedures have been reported [4]. The components are as follows (cf Table 2): **a**, caerulein ($MH^+ = 1354$ Da); **b**, maculatin 1.1.1 ($MH^+ = 1975$); **c**, maculatin 2.1 ($MH^+ = 1878$); **d**, maculatin 3.1 ($MH^+ = 2395$); **e**, maculatin 1.2 ($MH^+ = 2360$); and **f**, maculatin 1.1 ($MH^+ = 2145$). Peaks marked with an asterisk (*) contain only traces of peptide material, and their compositions have not been further investigated.

numbering system is analogous to that used previously for the caerin peptides [1–3, 5]. Maculatins 2.1 and 3.1 show no significant resemblance to any peptides so far isolated from amphibians. In contrast, maculatin 1.1 shows a structural resemblance to the previously reported caerin 1 group of peptides [1–3, 5] (there are nine members of the caerin 1 family; see Table 1 for the structure of caerin 1.1) except that there are four amino acid residues of the caerin 1 structure missing in the maculatin 1.1 structure. Maculatin 1.1.1 corresponds to maculatin 1.1 except that the first two amino acid residues from the *N*-terminal end of maculatin 1.1 are missing. Maculatin 1.2 has a similar structure to maculatin 1.1, except that in maculatin 1.2, Ser (11) replaces Ala (11), and two more residues (Glu-22

Table 2 Amino Acid Sequences of the Skin Peptides from *Litoria genimaculata*

Name (MH^+)	HPLC component	Sequence
Caerulein (1354)	**a**	pEQDY(SO_3H)TGWMDF(NH_2)
Maculatin 1.1 (2145)	**f**	GLFGVLAKVAAHVVPAIAEHF(NH_2)
Maculatin 1.1.1 (1975)	**b**	FGVLAKVAAHVVPAIAEHF(NH_2)
Maculatin 1.2 (2360)	**e**	GLFGVLAKVASHVVPAIAEHFQA (NH_2)
Maculatin 2.1 (1878)	**c**	GFVDFLKKVAGTIANVVT (NH_2)
Maculatin 3.1 (2395)	**d**	GLLQTIKEKLESLESLAKGIVSGIQA (NH_2)

and Ala(NH_2)-23) are present at the C-terminal end of the peptide.

We have described before how tree frogs of the genus *Litoria* are able to deactivate their major antibacterial peptide by removing the first two amino acid residues from the N-terminal end of the peptide [5]. Since a similar structural relationship is observed between maculatins 1.1 and 1.1.1, this suggests that maculatin 1.1 may exhibit antibiotic activity. Thus we have carried out antibacterial testing on the five maculatin peptides. We did not have sufficient of the natural maculatins from *L. genimaculata* to assess their antimicrobial activities properly, so we had all the peptides synthesized for this purpose. The results of the antibacterial tests are recorded in Table 1. They show that maculatin 1.1 shows significant antibacterial activity, while the related maculatin 1.1.1 (lacking the first two residues of maculatin 1.1) shows no activity at MIC values < 100 μg/ml. Interestingly, maculatin 1.2 (a modified maculatin 1.1 but with two extra residues) exhibits minimal antibiotic activity. Maculatin 2.1 shows some activity, but maculatin 3.1 shows no useful activity and its role in the amphibian skin is not known.

The relationship in structure and antibiotic activity between maculatin 1.1 and the natural caerin 1 peptides needs further consideration. The most widespread caerin 1 peptide is caerin 1.1 [1–3]: its structure and antibiotic activities against a number of pathogens are recorded in Table 1.

Amphibian peptides such as the magainins [11, 12] and the bombinins [13, 14] interact with the lipid bilayer of the bacterial cell wall in a sheet-like arrangement, adopting an α-helical configuration which disrupts normal membrane function and leads to lysis of the bacterial cell wall [15, 16]. It is likely that the caerin 1.1 interacts with the bacterial cell wall in the same manner, since natural (all L) caerin 1.1 shows the same antibiotic activity as the synthetic (all D) caerin 1.1 [17 and cf 15]. However, ^{1}H-NMR studies [17] indicate that the secondary structure of caerin 1.1 is more complex than those of either the magainins or the bombinins. In solution, caerin 1.1 adopts two well-defined helices, the first from Gly-1 to Lys-11 and the second from Val-17 to Leu(NH_2)-25, with the two helices separated by a more flexible hinge region. The hinge allows the two helical regions to be oriented such that the side chains form a continuous hydrophobic face on the concave side of the peptide with the hydrophilic face on the convex side [17]. Pro-15 directly contributes to the lack of a rigid helical structure in the central region of caerin 1.1 (central Pro residues also affect the helicity of other membrane-interacting peptides such as alamethicin [18], melittin [19] and cecropin A [20]). The two Pro residues of caerin 1.1 are necessary for activity: replacing one or both with Gly significantly reduces the activity of the peptide [17]. Removing two amino acid residues from either end of a caerin 1 peptide destroys the antibiotic activity [5].

One of the four residues present in caerin 1.1 but lacking in maculatin 1.1 is Pro-15, a residue that is necessary for the antibiotic activity of the caerin 1 peptides [17]. Whether the other three missing residues are from (i) the inner end of the first helix (residues 7–11 of caerin 1.1) or (ii) the central hinge region containing residues 12–14 of caerin 1.1, or some combination of (i) and (ii) is not known. That maculatin 1.1 should exhibit good antibiotic activity can only mean that amino acid residues can be removed from the inner end of the first helical region and/or the hinge region of caerin 1.1 without destroying the antibiotic activity of the peptide. In contrast, the addition of two residues to the C-terminal end of the maculatin 1.1 structure (as in maculatin 1.2, Table 2), results in major loss of activity (see Table 1).

CONCLUSIONS

The tree frog *Litoria genimaculata* produces three host defence peptides in its skin glands, viz. the neuropeptide caerulein and the two antibiotic peptides maculatins 1.1 and 2.1. The frog is able to deactivate maculatin 1.1 by removing its first two amino acid residues.

There is a structural resemblance between maculatin 1.1 and the caerin 1 group of peptides isolated previously from the green tree frogs *L. caerulea*, *L. gilleni*, *L. splendida* and *L. xanthomera* [1–3, 5]. The difference is that maculatin 1.1 lacks four of the amino acid residues of a caerin type 1 peptide. Even though *L. genimaculata* is not grouped directly from a phylogenetic viewpoint with the green frogs mentioned above [7], the structural relationship between the maculatin 1 and caerin 1 peptides suggests that the five named *Litoria* species may have a common ancestor.

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