Peptides from Australian Frogs. Structures of the Caerins and Caeridin 1 from *Litoria splendida*

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Seven peptides have been isolated from the parotoid and rostral glands of the Australian green tree frog *Litoria splendida*. These include caerulein (a known hypotensive toxin), and six new compounds named caerin 1.1 (*M*, 2582), 1.1.1 (2412), 1.1.2 (2299), 2.1 (2392), 3.1 (2382) and caeridin 1 (1139). The primary structures of the peptides have been determined by FAB mass spectrometric methods, and confirmed by automated sequencing. The major peptides have been synthesised using *t*-BOC (*N*-*tert*-butoxycarbonyl) *N*-protection and a *p*-methylbenzhydrylamine support.

Amphibians live in environments where there are numerous micro-organisms and animal predators, and their dermal secretions contain a variety of host defence peptides including toxins and antibiotics (for reviews see refs. 1–13). These peptides are located in granular glands distributed throughout the dorsal surface and occasionally aggregated in hypertrophied areas, a feature peculiar to amphibians. During the last three decades, a number of families of amphibian peptides have been identified including the angiotensins, bombesins, bradykinins, caeruleins, dermorphins, tachykinins, magainins and bombinins.[1–13] Many of these studies have involved killing large numbers of animals in order to isolate peptides from their dried skins, but recently, noradrenaline injections have been used to stimulate release of glandular secretions from certain species.[14,15] Most amphibian peptides have molecular weights below 2000 Da. However, some, like the magainins[14,16] and bombinins[15,17] have molecular weights between 2000 and 3000 Da; these particular peptides show potent antimicrobial activity and are thought to exist as single amphipathic α-helices which associate with lipid membranes and disrupt normal membrane functions (see *e.g.* refs. 8, 13, 15).

Testing for pharmacological activity in the skin secretions of Australian frogs commenced in the 1960s;[3,18] this led to the isolation of the hypotensive peptides caerulein (from *Litoria caerulea*)[19] and uperolein (from *Uperoleia* species).[20] Caerulein is possibly a hormone,[13] and it has been used clinically.[21] There are more than 200 species of frog now known in Australia,[22] and during the past seven years we have isolated and characterised over 50 peptides from just ten of these species. We now report the primary structures of the first series of these peptides, the caerins ($M > 2000$ Da)† and caeridin 1 (molecular weights of the caeridins are less than 2000 Da)† from *Litoria splendida*.

Results and Discussion

The 'magnificent green tree frog', *Litoria splendida*, was first reported by Tyler, Davies and Martin in 1977.[24] It is one of the largest frogs in Australia, measuring some twelve cm in length, and is confined to the Kimberley area of Western Australia. The peptide material is contained principally in the large parotoid and rostral glands, which are situated at the rear and the front of the head respectively. Frog populations in many countries, including Australia, are in decline,[25] and it is essential that we collect the glandular secretions without harming the animal. We have developed a benign method in which mild electrical stimulation of the parotoid glands results in release of the glandular secretion, without any apparent adverse effect on the animal.[26] This process may be used on average every month to produce a regular supply of glandular secretion. On average, each 'milking' produces about 70 mg of peptide material, an impressive yield considering the size of the frog.

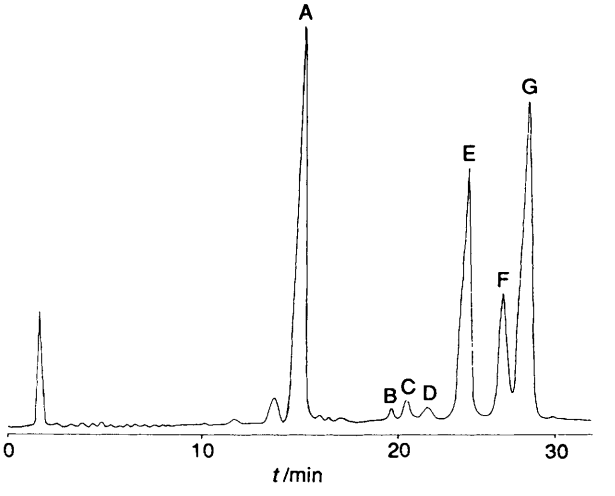

Fig. 1 Preparative HPLC trace of the glandular secretion from *Litoria splendida*. For experimental details see Experimental section. **A** [caerulein (**1**)], **B** [caerin 1.1.2 (**4**)], **C** [caerin 1.1.1 (**3**)], **D** [caeridin 1 (**7**)], **E** [caerin 3.1 (**6**)], **F** [caerin 2.1 (**5**)] and **G** [caerin 1.1 (**2**)].

The HPLC trace resulting from the separation of 2 mg of the peptide mixture is shown in Fig. 1. Five major, and two minor peptides were isolated. One of the major peptides was identified as caerulein **1**, the hypotensive toxin already isolated from the closely related species *Litoria caerulea*.[19] Fast atom bombardment mass spectrometry (FAB MS) indicated that the other major peptides have molecular weights of 2582, 2392, 2382 and 1139: these have been named caerin 1.1, caerin 2.1, caerin 3.1 and caeridin 1 respectively. We estimate that each 'milking' produces, on average, 16 mg of caerulein, 25 mg of caerin 1.1, 5 mg of caerin 2.1, 12 mg of caerin 3.1 and 2 mg of caeridin 1.

† We were unable to call these peptides 'splendidins' since this name has been used before for a naturally occurring terpene.[23] The major peptides described in this paper are also the parent compounds of a number of families of peptides isolated from the related species *Litoria caerulea*; thus we have derived their trivial names from this species.

Pyr Gln Asp Tyr(SO_3H) Thr Gly Trp Met Asp Phe(NH_2)
1

Table 1 The primary structures of the caerins and caeridin 1 from *Litoria splendida*

Caerin 1.1 (2582) **2**
Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu(NH_2)
Caerin 1.1.1 (2412) **3**
Leu Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu(NH_2)
Caerin 1.1.2 (2299) **4**
Ser Val Leu Gly Ser Val Ala Lys His Val Leu Pro His Val Val Pro Val Ile Ala Glu His Leu(NH_2)
Caerin 2.1 (2392) **5**
Gly Leu Val Ser Ser Ile Gly Arg Ala Leu Gly Gly Leu Leu Ala Asp Val Val Lys Ser Lys Gly Gln Pro Ala(OH)
Caerin 3.1 (2382) **6**
Gly Leu Trp Gln Lys Ile Lys Asp Lys Ala Ser Glu Leu Val Ser Gly Ile Val Glu Gly Val Lys(NH_2)
Caeridin 1 (1139) **7**
Gly Leu Leu Asp Gly Leu Leu Gly Thr Leu Gly Leu(NH_2)

Table 2 MS/MS Data for peptides formed by tryptic digest of caerins 1.1, 1.1.1 and 2.1[a]

Caerin 1.1		
m/z 777	('b' ions)	*m/z* 647, 510, 381, 310 and 197 [Leu(21) Ala Glu His Leu(NH_2)(25)]
	('Y + 2' ions)	*m/z* 680, 581, 468, 397 and 268 [Pro(19) Val Leu Ala Glu(23)]
Caerin 1.1.1		
m/z 873	('b' ions)	*m/z* 727, 656, 557, 470, 413 and 300 [Leu(4) Gly Ser Val Lys(9)]
	('Y + 2' ions)	*m/z* 760, 673, 574, 461, 404 and 317 [Leu(1) Ser Val Leu Gly Ser(6)]
Caerin 2.1		
m/z 788	('b' ions)	*m/z* 614, 557, 444, 357 and 270 [Ser(4) Ser Leu Gly Arg(8)]
	('Y + 2' ions)	*m/z* 731, 618, 519, 432, 345 and 232 [Gly(1) Leu Val Ser Ser Leu(6)]
m/z 1055	('b' ions)	*m/z* 909, 810, 711, 596, 525, 412 and 299 [Leu(13) Leu Ala Asp Val Val Lys(19)]
	('Y + 2' ions)	*m/z* 871, 757, 644, 531, 460 and 345 [Gly(11) Gly Leu Leu Ala Asp(16)]

[a] For a recent review of peptide fragmentations see ref. 36. 'b' Cleavages are those which form R–C≡O^+ ions (*e.g.* $RCO\overset{+}{N}H_2CH_2CO_2H \rightarrow RCO^+ + NH_2CH_2CO_2H$); 'Y + 2' cleavages form $R\overset{+}{N}H_3$ ions (*e.g.* $NH_2CH_2CONH_2R \rightarrow R\overset{+}{N}H_3 + NHCH_2CO$).

The minor peptides are present in submilligram amounts: they have the molecular weights 2412 and 2299. Since their respective structures are directly related to that of caerin 1.1, we have called them caerin 1.1.1 and caerin 1.1.2. Certain of the caerin peptides show potent antimicrobial and antiviral activity: for example caerin 1.1 is active against *Staphylococcus aureus* and the *Herpes simplex* virus.[27]

The Primary Structures of the Peptides.—The structures of the caerins and caeridin 1 were determined primarily by mass spectrometry using the technique of fast atom bombardment (FAB). The majority of the peptides have amide functions at the *C*-terminal position: this was confirmed by conversion of the peptide into the methyl ester, followed by determination of the appropriate molecular weight by FAB MS. Manual Edman[28]/FAB MS was used to determine as many amino acid residues from the *N*-terminal end of the peptide as possible. In the case of caerin 2.1, which has a free carboxy group at the *C*-terminal end of the peptide, manual *C*-terminal degradation/FAB MS[29] (see Experimental section for details) was used to determine portion of the sequence from the *C*-terminal end of the peptide. Enzymic digest (typically trypsin and/or chymotrypsin) cleaved each peptide except caeridin 1. Each such hydrolysis usually resulted in two peptide fractions which were subjected to Edman/FAB MS analysis, and where appropriate, *C*-terminal degradation/FAB MS sequencing. In certain cases, sequence information was also provided by the MS/MS technique. Combination of all the above data usually provides the primary sequence of the peptide, with the exception that isomeric Leu and Ile cannot always be differentiated by these mass spectrometric techniques.* This problem was resolved using an automated sequencer,[31] which also confirmed the overall structure of each peptide. All peptides, with the exception of the minor species caerin 1.1.1 and 1.1.2, have been synthesised using the *t*-BOC (*N-tert*-butoxycarbonyl) method.[32] The structure determination of each peptide is considered, in summary, below.†

Caerin 1.1. Caerin 1.1 [structure **2** (Table 1)] has a molecular weight of 2582 Da, and contains 25 amino acid residues.[27] Its structure has been reported in a preliminary communication.[35] Although structurally quite different, it may nevertheless be placed in the same general category of amphibian peptides as the magainins[14,16] (from *Xenopus laevis*), and the bombinins[15,17] (from *Bombina* species). Manual Edman degradation/FAB MS identified the first ten amino acid residues from the *N*-terminal end of the peptide as Gly Leu Leu Ser Val Leu Gly Ser Val Ala. Tryptic digest of caerin 1.1 cleaves the peptide between Lys(11) and His(12), yielding two peptides of molecular weights 1043 and 1558. The peptide of molecular weight 1043 contains the first 11 amino acid residues of caerin 1.1. Manual Edman/FAB MS determined residues 1–5 to be Gly Leu ‡ Leu Ser Val, while *C*-terminal FAB MS analysis identified residues 6–11 as Gly Ser Val Ala Lys. Thus the structure of peptide 1043 is Gly Leu Leu Ser Val Leu Gly Ser Val Ala Lys. The second peptide of molecular weight 1558, contains residues 12–25 of caerin 1.1. Manual Edman/FAB MS identified residues 12–18 as His Val Leu Pro His Val Val. The peptide remaining after removal of these seven residues has an $(MH)^+$ ion at *m/z* 777: the amino acid sequence of this species is Pro Val Leu Ala Glu His Leu(NH_2) from the MS/MS data shown in Table 2 (in this case we cannot use *C*-terminal degradation since the *C*-terminal residue contains a $CONH_2$ group). Thus peptide 1558 has the structure His Val Leu Pro His Val Val Pro Val Leu Ala Glu His Leu(NH_2). This information collectively, allows the formulation of the

* MS/MS data can, on occasions, differentiate between Leu and Ile by losses of C_3H_6 from the former.[30] These fragmentations are not always observed in our spectra, consequently we rely on automated sequencing to distinguish between Leu and Ile.

† A referee has asked whether we have confirmed experimentally whether the caerins and caeridin 1 are derived exclusively from L-amino acids. The short answer is no. However most amphibian peptides only contain L-amino acid residues (exceptions are the opioid peptides, the dermorphins[33] and deltorphins,[34] which contain D-Ala and D-Met respectively at residue 2). Our synthetic peptides are constructed with L-amino acids: each synthetic peptide has the same retention time on the analytical HPLC column as the natural counterpart (such columns can separate deltorphins containing 2-L-Met and 2-D-Met,[34] however we cannot be sure that this would necessarily be the case with our peptides). Both natural and synthetic caerin 1.1 show activity towards *Staphylococcus aureus* within the same range.

‡ As explained above, these analyses do not specifically differentiate between Leu and Ile. For brevity, we specify Leu in the text, with the clear understanding that this residue could be either Leu or Ile. The representations in Table 1 indicate the correct, confirmed structures of the caerins and caeridin 1.

Table 3 Structure determination of caerin 3.1 (**6**) (M = 2382)

(A) *Edman/MS*	Gly Leu [decomposes in third cycle]
(B) *Tryptic digest*	
(*a*) *m/z* 631	('b ions') 485, 357, 171 [Trp(3) Gln Lys]
	('Y + 2' ion) 461 [Gly(1) Leu]
Structure is	Gly(1) Leu Trp Gln Lys
(b) *m/z* 1287	(i) Edman/MS Ala(10) Ser Glu Leu
	(ii) MS/MS
	('b ions' 1141, 1042, 985, 856, 757, 644, 587, 500 and 401
	[Val(14) Ser Gly Leu Val Glu Gly Val Lys[a](22)]
	('Y + 2' ions) 1129, 1000, 887, 788, 701, 644, 531 and 432
	[Glu(12) Leu Val Ser Gly Leu Val(18)]
Structure is	Ala(10) Ser Glu Leu Val Ser Gly Leu Val Glu Gly Val Lys[a]
(C) *Chymotrypsin digest*	Produces peptide *m/z* 2027, confirming Trp(3) [Caerin 3.1 absorbs at 278 nm, characteristic of Trp]
(D) *Lys C digest*	Produces peptides *m/z* 1771 and 1530 confirming Lys(5) and Lys(7)
(E) *C-terminal degradation*	Caerin 3.1 forms *m/z* 1971 confirming Glu(19)

From the collective data above, the remaining residues must be Leu(6) and Asp(8)

[a] Trypsin cleaves the *C*-terminal Lys(NH_2) to Lys.

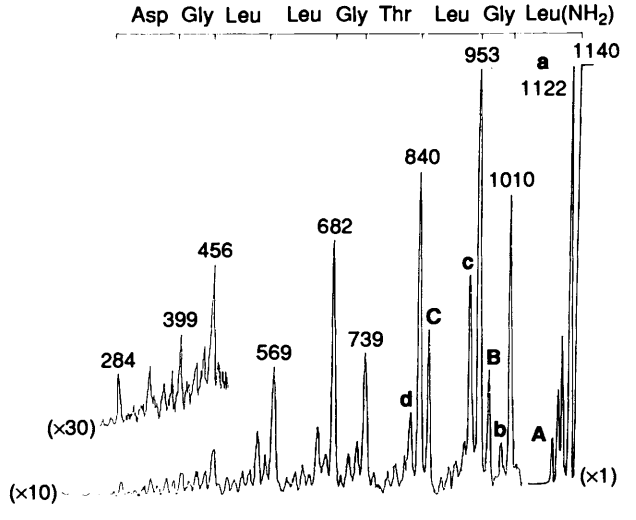

Fig. 2 MS/MS Data for caeridin 1 (**7**). The schematics at the top of the figure indicate the 'b' cleavages (*i.e.* those fragmentations that yield R–C≡O$^+$ product ions). The peaks designed **a** (*m/z* 1122), **b** (992), **c** (935) and **d** (822) are formed by loss of water (from the Thr α side chain) from *m/z* 1140, 1010, 953 and 840 respectively. The peaks designated **A** (*m/z* 1083), **B** (970) and **C** (857) are formed by 'Y + 2' cleavages and indicate the sequence Gly(1) Leu Leu.

primary structure of caerin 1.1 except for the differentiation between Leu and Ile. The final assignment **2** (Table 1), has been made using an automated sequencer.

Caerin 1.1.1. Caerin 1.1.1 has a molecular weight of 2412, 23 amino acid residues, and is based on the caerin 1.1 structure except that the first two residues Gly and Leu (of caerin 1.1) are missing. The structure determination is essentially the same as that for caerin 1.1, except that Edman/FAB MS demonstrates that Gly(1) and Leu(2) are missing, and tryptic digest gives a peptide of molecular weight 872 instead of 1043. The species *m/z* 873 shows the sequence Leu Ser Val Leu by manual Edman/FAB MS, while MS/MS data (Table 2) from *m/z* 873 indicates the sequence 4–9 to be Leu Gly Ser Val Ala Lys. The structure of caerin 1.1.1 is **3** (Table 1).

Caerin 1.1.2. Caerin 1.1.2 has a molecular weight of 2299, 22 amino acid residues and corresponds to caerin 1.1 except that the first three residues Gly Leu Leu are missing. Tryptic digest gave two peptides of molecular weights 759 and 1558. The first peptide corresponds to the residue 1043 (from caerin 1.1), except that residues (1–3), Gly Leu Leu are missing. The second peptide is identical with that of molecular weight 1558 formed from caerin 1.1. Caerin 1.1.2 has structure **4** (Table 1).

Caerin 2.1. Caerin 2.1 [structure **5** (Table 1)] has a molecular weight of 2392, and contains 25 amino acid residues. Manual Edman/FAB MS gave the first six residues Gly Leu Val Ser Ser Leu. Caerin 1.2 has a *C*-terminal carboxy group, and *C*-terminal/FAB sequencing showed the last four residues to be Gly Gln* Pro Ala. The tryptic digest effected cleavage at a number of positions, but two, *viz* Arg(8)/Ala(9) and Lys(19)/Ser(20), gave two isolable peptides of respective molecular weights 787 and 1054. The peptide of molecular weight 787 showed Gly Leu by Edman/FAB MS analysis, and Gly Arg by *C*-terminal degradation. MS/MS data on the $(MH)^+$ ion *m/z* 788 indicated the sequence Gly Leu Val Ser Ser Leu Gly Arg (see Table 2), *i.e.* residues 1–8 of caerin 2.1. The peptide of molecular weight 1054 was sequenced as follows: Edman/FAB MS analysis yielded Ala Leu Gly Gly; *C*-terminal analysis gave Ala Asp Val Val Lys, and MS/MS data from *m/z* 1055 (see Table 2) gave the full sequence Ala Leu Gly Gly Leu Leu Ala Asp Val Val Lys. This leaves overall, a mass of 215 Da unidentified from caerin 2.1. This could correspond to Ser Lys, but the positions and order of these units cannot be determined from existing data. Automated sequencing resolved the issue, confirming the data above, differentiating between Leu and Ile, and identifying Ser and Lys at positions 20 and 21 respectively.

Caerin 3.1. Caerin 3.1, structure **6** (Table 1), has a molecular weight of 2382, and contains 22 amino acid residues. The presence of tryptophan and the three lysine residues in this molecule make combined enzymic/MS structure determination very complex indeed. The collective data are summarised in Table 3. The proposed structure was confirmed by automated sequencing.

Caeridin 1. Caeridin 1, structure **7** (Table 1), has a molecular weight of 1139, and contains 12 amino acid residues. Manual Edman/FAB MS determined that the first six residues are Gly Leu Leu Asp Gly Leu. The molecule is unaffected by either trypsin or chymotrypsin, and *C*-terminal degradation was unsuccessful. The collisional activation MS/MS spectrum of the $(MH)^+$ ion (*m/z* 1140) is shown in Fig.2: the indicated data (*C*-terminal fragmentations, 'b' ions) establish residues (4–12) as Asp Gly Leu Leu Gly Thr Leu Gly Leu (NH_2). This is the only peptide in this series to contain Thr; its presence is demonstrated by the pronounced losses of water observed in the mass spectrum.

Other Structural Features.—Having determined the primary sequences of the peptides, it is now instructive to see which of them can be fitted to a well behaved α-helix using an Edmundson wheel projection. These representations are shown for the major peptides in Fig. 3. The first fourteen residues of caerin 1.1 (**2**) divide nicely into hydrophilic and hydrophobic zones, however the Pro residues (15 and 19) preclude the formation of a perfect α-helix after residue 15. Caerin 2.1 **5** also does not give a perfect Edmundson representation, however,

* The residue Gln(23) caused problems during most degradative procedures including automated sequencing. Its amide side chain appears unusually labile under the mildest of conditions, partially hydrolysing to Glu.

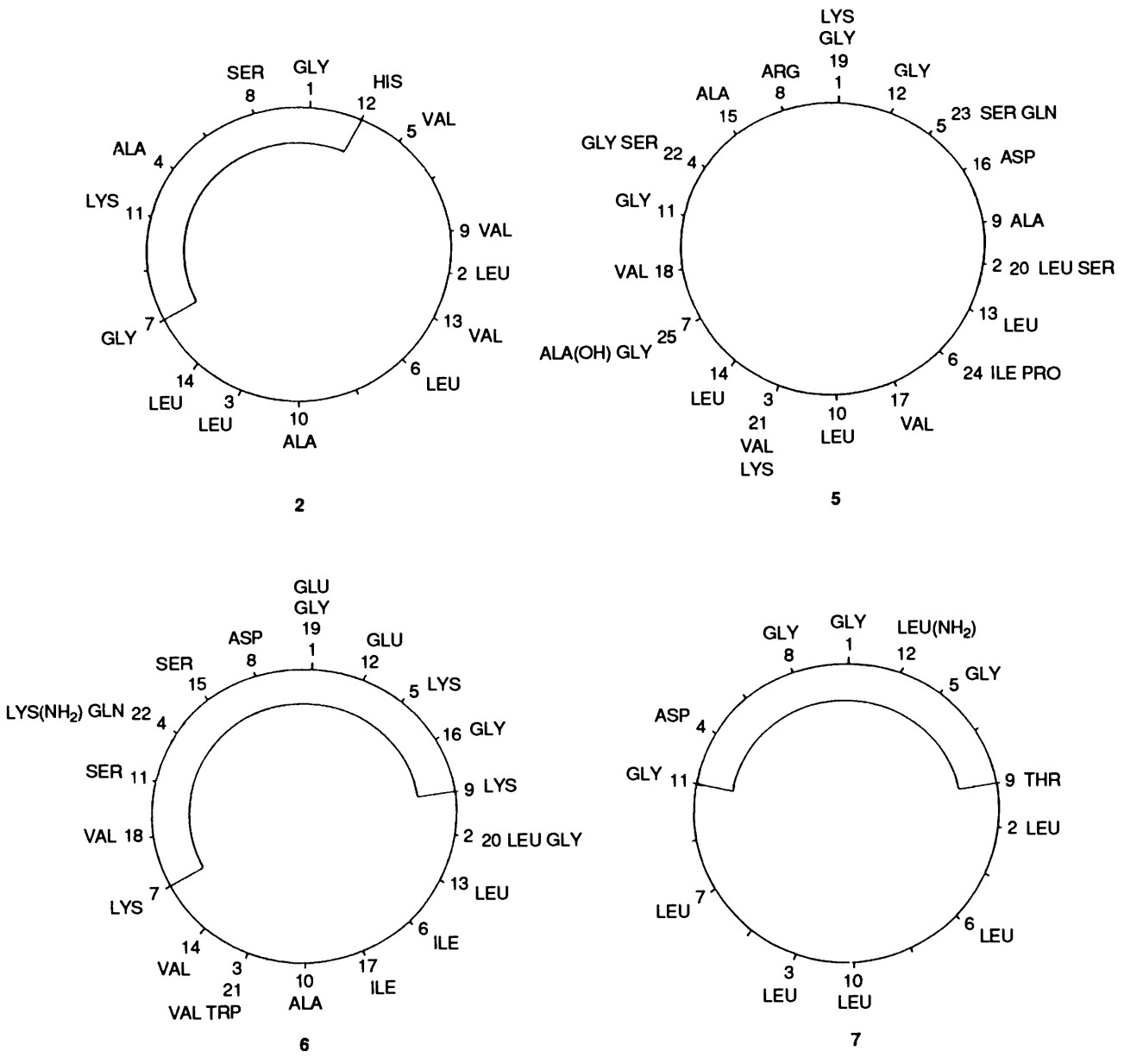

Fig. 3 Edmundson wheel representations for caerin 1.1 (**2**) (the first fifteen residues), caerin 2.1 (**5**), caerin 3.1 (**6**) and caeridin 1 (**7**)

it shows a mainly hydrophilic zone from Gly(11) to Asp(16), and a mainly hydrophobic zone from Ala(9) to Val(18). Caerin 3.1 (**6**) and caeridin 1 (**7**), both show classical behaviour, with two reasonably consistent zones. Caeridin 1 (**7**) is interesting, in that all of its hydrophobic residues correspond to Leu.

Bevins and Zasloff[13] argue that every amphibian peptide should have a mammalian analogue. Using the FASTA search protocol[37] [with GenBank (release #72.0, EMBL (release #31.0), SWISS-PROT (release #22.0) and GenPept (release #72.0)], we find the caerins to share only between 26 and 47% identity with a heterogeneous group of proteins of known sequences. None reached a statistically significant similarity to the caerins as indicated by z scores[38] between 4 and 7. In general, the common feature of these proteins was their association with lipids either as membrane components or in lipid metabolism. However, none of the proteins has a reported three-dimensional structure.[39] Caeridin 1 shares as high as 73% identity with a leucine-rich 11 residue segment of rabbit apolipoprotein B,[40] and a slightly lower identity with another heterogeneous group of lipid associated proteins.[41,42] For example, there is a case where the homologous peptide segment occurs in a putative membrane spanning region of the rat neuronal nicotine acetylcholine receptor.[41] However z scores of less than 4 suggest that these similarities to caeridin 1 are probably not significant.

Experimental

Preparation of Skin Secretions.—Litoria splendida was held by the back legs, the skin moistened with de-ionised water, and stimulated by means of a bipolar electrode of 21G platinum attached to a C. F. Palmer 'Student Model' electrical stimulator. The electrode was rubbed gently in a circular manner on the skin of the parotoid glands of the animal, using 10 volts and a pulse duration of 3 ms.[26] The resulting secretion was washed from the frog with de-ionised water (approximately 50 cm^3), the mixture diluted with an equal volume of methanol containing acetic acid (1 cm^3), sonicated for 5 min to disrupt vesicles, centrifuged, filtered through glass wool, and reduced to 10 cm^3 (rotary evaporator, 25 °C, 14 mmHg). This extract can be

stored for up to 12 months at −10 °C without any noticeable change in the composition of the peptide mixture. [Lyophilisation of this extract gives on average, 70 mg of peptides. Lyophilisation should be avoided however, because HPLC results from lyophilised material (when compared with extracts which were not lyophilised) give reduced recovery of pure material, poorer separation, together with the formation of artefacts (of lower molecular weight) resulting from decomposition of the major peptides.]

HPLC Separation of the Glandular Secretion.—Analytical separation was achieved using a Brownlee aquapore RP-300 analytical HPLC column (4.6 × 75 mm) equilibrated with acetonitrile–aqueous trifluoroacetic acid (0.1% TFA) (1:10). Preparative separation was effected with a Brownlee preparative column [aquapore octyl, 20 μm spherical (100 × 10 mm)]. For preparative separations, each aqueous injection contained *ca.* 2 mg of the peptide mixture: ten individual injections (and separations) were (on average) required to produce workable amounts of purified major peptides, whereas some fifty separations were necessary for the separation and isolation of the minor peptides. Components were eluted using a non-linear gradient generated by a Waters gradient controller and dual pump system, increasing from 10 to 50% acetonitrile over a period of 30 min at a flow rate of 2 cm^3 min^{-1}. The eluent was monitored by UV absorbance at 215 nm using a Waters 481 variable wavelength detector. Fractions were collected, concentrated and dried under reduced pressure for subsequent analysis. A typical preparative separation is shown in Fig. 1.

Mass Spectrometric Investigation.—Fast atom bombardment mass spectra were recorded with a Vacuum Generators ZAB 2HF reverse sector mass spectrometer equipped with an Ion Tech FAB gun operating with argon at 6–8 kV with a current of 1 mA. Routine spectra (for molecular weight data, including those from Edman and *C*-terminal degradations) were obtained by scanning the magnet over a mass range from *m/z* 200–3400 using 2000 mass resolution. Collisional activation MS/MS (ion kinetic energy) data were obtained by setting the magnet to focus the particular ion under study, adding helium to the second collision cell to a pressure of 2×10^{-6} Torr (to produce a reduction in the main beam of some 30%), and scanning the electric sector. Because of the kinetic shift, the peaks in these ion kinetic energy spectra may be up to 1 Da lower than the expected theoretical mass. However it is the mass difference between peaks that provide the sequencing information, and these differences may be reliably determined to 1 Da.

Samples (normally 2–5 mm^3) for FAB MS analysis were dissolved in aqueous acetic acid (1%) and dried on the FAB probe tip *in vacuo*. A eutectic mixture of 1,4-dithiothreitol/1,4-dithioerythritol [(5:1), 2 mm^3] was added to the probe tip, followed by aqueous trichloroacetic acid (saturated, 1 mm^3). The mixture on the probe tip was mixed thoroughly before insertion into the ion source.

Manual Edman Degradation.[28]—To the peptide (typically 20–100 mmol) in aqueous pyridine (50%, 200 mm^3), was added a solution of phenyl isothiocyanate in hexane (1%, 100 mm^3) in a 3 cm^3 screw cap glass vial. The solution was purged with oxygen-free nitrogen for 1 min, the vial capped and incubated at 40 °C for 2 h, the mixture extracted with hexane–ethyl acetate (4:1, 2 × 250 mm^3), blown dry under nitrogen, trifluoroacetic acid (200 mm^3) was added, the solution purged with oxygen free nitrogen, the vial capped, incubated at 40 °C for 20 min, and blown dry using nitrogen. The resultant solid was suspended in distilled water (200 mm^3) and extracted with butyl acetate (250 mm^3). An aliquot of the aqueous layer was then removed for FAB MS analysis, and the remaining solution was blown dry and subjected to a further cycle of the degradation process. We have determined the identity of up to ten residues with 20 mmol of peptide using this technique (see e.g. caerin 1.1).

Manual Carboxy C-Terminal Degradation (Modification of Standard Method).[29]—To the peptide (typically 5 mm^3), in a 3 cm^3 screw cap glass vial, was added acetic acid (25 mm^3) and acetic anhydride (75 mm^3) at 25 °C. High-purity nitrogen was bubbled through the solution for 5 min, a saturated solution of thiocyanic acid in acetone (20 mm^3) was added, the vial was capped, and the solution allowed to stir at 55 °C for 40 min. Water (150 mm^3) was added, the resulting solution heated at 55 °C for 5 min., lyophilised, triethylamine (100 mm^3) was added, and the solution was heated at 55 °C for 5 min. The volume of the solution was reduced to 20 mm^3 using a stream of dry nitrogen gas. A portion of the residue (*ca.* 2 mm^3) was removed for FAB MS analysis, while the remainder was lyophilised in preparation for the next cycle of degradation.

Enzymic Hydrolysis.—All peptides were subject to hydrolysis using one or more enzymes, typically trypsin and chymotrypsin. The peptides so produced were subject to Edman/FAB MS, *C*-terminal/FAB MS, and MS/MS (as appropriate) as outlined above. Enzyme–substrate ratios of 1:10 were used [in Tris buffer (0.1 mol dm^{-3}) at pH = 7]; the mixture was incubated at 38 °C for 3 h.

Peptide Sequencing using a Protein Sequencer.[31]—Peptide sequencing was performed on an applied Biosystem 470 A sequencer, equipped with a 900A data analysis module. Initial attempts with the sequencing of the caerin and caeridin peptides gave no more than four successful sequence cycles before all the peptide was washed from the glass fibre filter. Good results were obtained using a disc of immobilon film treated with bioprene in ethanol, onto which the peptide was absorbed from aqueous acetonitrile (90%). The disc was pierced several times with a razor blade in order to aid the flow of solvent.

Peptide Synthesis.—The peptides were synthesised by the *t*-BOC polyamide method[32] using an Applied Biosystems Model 430A solid phase peptide synthesiser. The supports used were *p*-methylbenzhydrylamine for peptides with *C*-terminal $CONH_2$ groups, and 2% divinyl cross linked styrene for peptides with *C*-terminal CO_2H groups. Side chain protecting groups were as follows: benzyl (Tyr Ser Thr Asp Glu), formyl (Trp) and chlorobenzyloxycarbonyl (Lys). Single coupling *via* the symmetrical anhydrides was used for all amino acids except Gln which was double coupled as its hydroxybenzotriazole active ester. Peptides were cleaved from the resin using trifluoromethanesulfonic acid (10%) in trifluoroacetic acid at 0 °C for 1 h with thiocresol acting as the radical scavenger. The synthetic peptide was purified using the HPLC method outlined above (see HPLC separation of glandular secretion).

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