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# The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, *Ascaphus truei*

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**Abstract**

The tailed frog *Ascaphus truei* occupies a unique position in phylogeny as the most primitive extant anuran and is regarded as the sister taxon to the clade of all other living frogs. Eight structurally related peptides, termed ascaphins 1–8, were isolated from norepinephrine-stimulated skin secretions of *A. truei* and were shown to possess differential growth inhibitory activity against *Escherichia coli* and *Staphylococcus aureus*. Ascaphins 2–7 may be represented by the consensus amino acid sequence $GX_2DX_2KGAAKX_3KTVAX_2IANX{\cdot}COOH$ whereas ascaphin-1 ($GFRDVLKGAAKAFVKTVAGHIAN{\cdot}NH_2$) and ascaphin-8 ($GFKDLLKGAAKALVKTVLF{\cdot}NH_2$) contain a C-terminally α-amidated residue. The ascaphins show no appreciable structural similarity with other families of antimicrobial peptides from frog skin but display limited sequence identity with the cationic, amphipathic α-helical peptides pandinin 1 and opistoporin 1, isolated from the venoms of African scorpions. Ascaphin-8 shows the highest potency against a range of pathogenic microorganisms but has the greatest haemolytic activity. The data indicate that the host defence strategy of using antimicrobial peptides in skin secretions arose early in the evolution of anurans.
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The emergence of strains of pathogenic bacteria and fungi with resistance to commonly used antibiotics has necessitated a search for novel types of antimicrobial agents to which the microorganisms have not been exposed. The synthesis of peptides with antimicrobial activity in granular glands located in the skin is a feature of several anuran (frog and toad) species, particularly those belonging to the families Bombinatoridae, Hylidae, Hyperoliidae, Myobatrachidae, Pipidae, and Ranidae (reviewed in [1–3]). These antimicrobial peptides comprise between 12 and 48 amino acid residues and are characterized by a remarkable degree of molecular heterogeneity that is considered to be important in protecting the organism against invasion by a wide range of pathogenic microorganisms [4]. The peptides can be grouped on the basis of limited structural similarity into families, many of which are believed to be related evolutionarily having arisen as a result of multiple gene duplication events [5]. The antimicrobial peptides lack any consensus amino acid sequences that are associated with biological activity but, with few exceptions, they are cationic, relatively hydrophobic and have the propensity to form an amphipathic α-helix in a membrane-mimetic solvent such as trifluoroethanol [6].

The frog skin antimicrobial peptides have potential as therapeutic agents. On the positive side, because of their relatively non-specific mechanism of action, the development of resistance to the peptides occurs at rates that are orders of magnitude lower than those observed for

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conventional antibiotics [7]. The major obstacles to their development as useful anti-infectives are their toxicities, particularly if they are to be administered systemically, and their short half-lives in the circulation. Thus, future therapeutic applications are more likely to involve topical rather than systemic administration.

The tailed frog *Ascaphus truei* (family Ascaphidae) occupies a uniquely important position in phylogeny as the most primitive extant anuran and is considered to be the sister-group to the clade of all other living frogs [8]. *A. truei* is generally considered to be the only member of the family Ascaphidae but Rocky Mountain populations of tailed frog have been regarded by some authors as the separate species, *Ascaphus montanus* [9]. The species retains a number of ancestral morphological characteristics found in no other frogs [10]. The 46-chromosome karyotype of *A. truei* is remarkably similar to that of the coelacanth, *Latimeria chalumnae* [11]. The tailed frog has a very limited distribution inhabiting cold high-gradient, rocky streams in the Cascades and coastal ranges from southern British Columbia to Northwestern California. Adults are primarily nocturnal, foraging in the vicinity of streams for arthropods and a variety of other prey. Little is known about tailed frog's defence strategy against either microorganisms or predators. Garter snakes (*Thamnophis* spp.) have often been observed to reject adult specimens after initially capturing them [12] and this may be due to distasteful or toxic skin secretions.

The vast majority of the frog skin antimicrobial peptides purified to date are from anurans classified in the superfamily Neobatrachia, or "higher" frogs, which comprise approximately 95% of known species (reviewed in [13]). This study provides insight into the evolution of amphibian defence strategies by describing the purification and the structural and biological characterization of a family of structurally related antimicrobial peptides from norepinephrine-stimulated skin secretions from the most primitive living anuran, *A. truei.*

## Materials and methods

*Collection of skin secretions.* Adult and sub-adult specimens of *A. truei* ($n = 32$, body weight range 3–11 g) were collected in the neighbourhood of Korbel, Humbolt County, Northern California, USA (California Department of Fish and Game Permit No. 801134-5). Skin secretions were collected as previously described [14,15]. In brief, the animals were injected bilaterally with norepinephrine (20 nmol/g body wt.) and placed in a solution (50 ml) of composition 25 mM sodium chloride/25 mM ammonium acetate, pH 7.0, for 15 min. The combined secretions and washings were acidified by addition of trifluoroacetic acid (1 ml) and passed at a flow rate of 2 ml $min^{-1}$ through 2 Sep-Pak C-18 cartridges (Waters Associates) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9: 0.1, v/v/v) and freeze-dried. All animals were released unharmed at the exact sites of collection within 24 h.

*Antimicrobial and haemolytic assays.* Standard strains were purchased from the American Type Culture Collection (Rockville, MD) and from the Hungarian National Type Culture Collection (Budapest, Hungary). Antimicrobial activities of the peptides were monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller–Hinton broth (50 μl) with an inoculum (50 μl of $10^6$ colony forming U $ml^{-1}$) from a log-phase culture of *Staphylococcus aureus* (NCTC 8325) and *Escherichia coli* (ATCC 25922) in 96-well microtitre cell-culture plates for 18 h at 37 °C in a humidified atmosphere in air. After incubation, the absorbance at 630 nm of each well was determined using a microtitre plate reader. Minimum inhibitory concentrations (MIC) for *E. coli*, *S. aureus*, and *Staphylococcus epidermidis* (RP62A), *Streptococcus* Group B (HNTCC 80130), *Enterococcus faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *Enterobacter cloacae* (NHTCC 53001), *Klebsiella pneumoniae* (KK3 9904), *Proteus mirabilis* (ATCC 25933), and *Pseudomonas aeruginosa* (ATCC 27853) were measured by a standard microdilution method [16] and were taken as the lowest concentration of peptide where no visible growth was observed. Incubations with *Candida albicans* (ATCC 90028) were carried out in RPMI 1640 medium for 48 h at 35 °C. In order to monitor the validity of the assays, incubations with bacteria were carried out in parallel with increasing concentrations of the broad-spectrum antibiotic, bacitracin, and incubations with *C. albicans* in parallel with amphotericin B.

Peptides in the concentration range 1–200 μM were incubated with washed human erythrocytes ($2 \times 10^7$ cells) from healthy donors in Dulbecco's phosphate-buffered saline, pH 7.4 (100 μl), for 1 h at 37 °C. After centrifugation (12,000*g* for 15 s), the absorbance at 450 nm of the supernatant was measured. A parallel incubation in the presence of 1% v/v Tween 20 was carried out to determine the absorbance associated with 100% haemolysis. The $HC_{50}$ value was taken as the mean concentration of peptide producing 50% haemolysis.

*Purification of the peptides.* The skin secretions, after concentration and partial purification on Sep-Pak cartridges, were redissolved in 0.1% (v/v) trifluoroacetic acid/water (2 ml). The sample was injected onto a ($1 \times 25$ cm) Vydac 218TP510 (C-18) reverse-phase HPLC column (Separations Group) equilibrated with 0.1% trifluoroacetic acid/water at a flow rate of 2 ml $min^{-1}$. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min and to 49% (v/v) over 60 min using linear gradients. Absorbance was monitored at 214 and 280 nm and fractions (1 min) were collected. The fractions containing antimicrobial activity were sequentially rechromatographed on ($0.46 \times 25$ cm) Vydac 214TP54 (C-4) and ($0.46 \times 25$ cm) Vydac 219TP54 (phenyl) columns. The concentration of acetonitrile in the eluting solvent was raised from 21% to 49% over 50 min and the flow rate was 1.5 ml $min^{-1}$.

*Structural characterization.* The primary structures of the peptides were determined by automated Edman degradation using a Procise 494 sequenator (Applied Biosystems). MALDI mass spectrometry was carried out using a Voyager RP MALDI-TOF instrument (Perspective Biosystems) equipped with a nitrogen laser (337 nm). The instrument was operated in reflector mode with delayed extraction and the accelerating voltage in the ion source was 25 kV. The accuracy of mass determinations was within 0.05%.

*Peptide synthesis.* Ascaphin-1 (C-terminally α-amidated and free acid forms), ascaphin-5 and ascaphin-8 (C-terminally α-amidated and free acid forms) were synthesized by Sigma–Genosys USA and purified to near homogeneity (>96% purity) by reverse-phase HPLC on a ($2.5 \times 25$ cm) Vydac 218TP1022 (C-18) preparative column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0/78.9/0.1) at a flow rate of 6 ml $min^{-1}$. The concentration of acetonitrile was raised to 56% over 60 min. The identity of the peptides was confirmed by mass spectrometry.

## Results

### *Purification of the peptides*

The elution profile on a Vydac C-18 semi-preparative column of norepinephrine-stimulated skin secretions

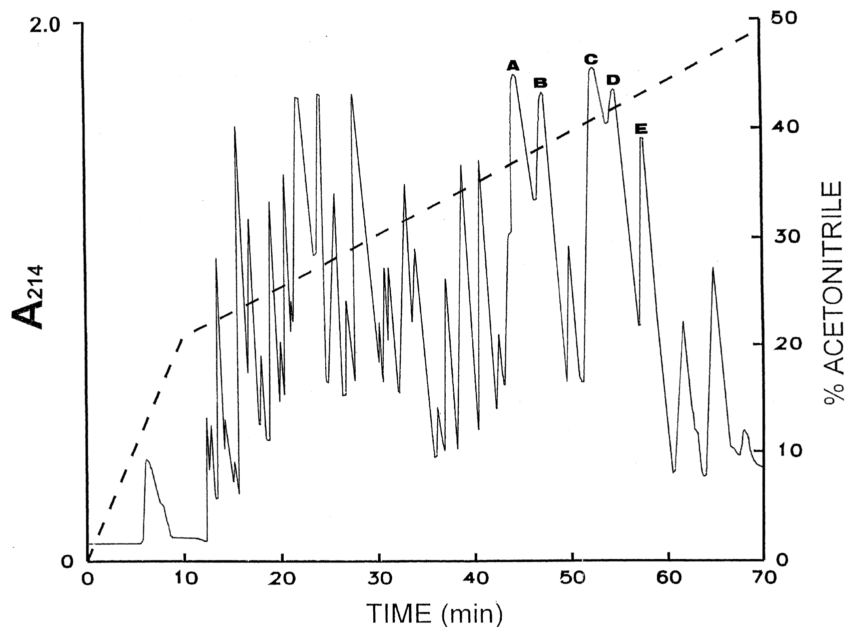


Fig. 1. Reverse-phase HPLC on a semipreparative Vydac C-18 column of pooled samples of *A. truei* skin secretions after partial purification on Sep-Pak cartridges. The peaks designated A–E were associated with antimicrobial activity and contained: A, ascaphin-1; B, ascaphin-2 and -3; C, ascaphin-4 and -5; D, ascaphin-6 and -7; and E, ascaphin-8. The dashed line shows the concentration of acetonitrile in the eluting solvent.

from *A. truei*, after partial purification on Sep-Pak cartridges, is shown in Fig. 1. The prominent peaks designated A–E contained components that strongly inhibited the growth of *E. coli* whereas, under the assay conditions used, only peaks C–E inhibited the growth of *S. aureus*. Peak A was subsequently shown to contain ascaphin-1; peak B, ascaphin-2 and -3; peak C, ascaphin-4 and -5; peak D, ascaphin-6 and -7; and peak E, ascaphin-8. The active peptides were purified to near homogeneity, as assessed by peak symmetry and mass spectrometry, by further chromatography on Vydac C-4 and phenyl columns. The same conditions were used to purify all peptides. The final yields (nmol) of purified peptides were ascaphin-1 320, ascaphin-2 25, ascaphin-3 155, ascaphin-4 30, ascaphin-5 65, ascaphin-6 30, ascaphin-7 125, and ascaphin-8 115.

### *Structural characterization*

The amino acid sequences of the ascaphins were determined by automated Edman degradation and are shown in Fig. 2. In all cases, the molecular masses of the peptides determined by MALDI mass spectrometry were consistent with their proposed structures assuming that ascaphin-1 and ascaphin-8 contain a C-terminally α-amidated residue Fig. 2. The primary structures of ascaphin-1 and ascaphin-8 were confirmed by chemical synthesis of both the amidated and free acid forms of the peptide. The retention times on reverse-phase HPLC of the synthetic C-terminally α-amidated peptides and endogenous peptides were identical. The retention time of endogenous ascaphin-5 was identical to that of the synthetic free acid form of the peptide.

### *Antimicrobial activities*

MIC values against reference strains of *E. coli* and *S. aureus* and $HC_{50}$ values against human erythrocytes of the endogenous ascaphins present in major abundance in the skin secretions are shown in Table 1. MIC values against reference strains of a range of Gram-positive and Gram-negative bacteria and the yeast opportunistic pathogen, *C. albicans*, and $HC_{50}$ values of synthetic replicates of ascaphins-1, -5, and -8 are shown in Table 2.

## Discussion

The study has led to the identification of the ascaphin family of structurally related antimicrobial peptides present in relatively high concentration in the skin secretions of *A. truei*. As shown in Fig. 3, ascaphin-6, containing only the substitution $Gln^{24} \rightarrow Glu$, may represent an artifactually modified form of ascaphin-7 arising from hydrolysis during the purification procedure. Otherwise, the identification of multiple forms of the ascaphins suggests that the peptides are probably the product of expression of at least seven structurally related genes that may have arisen as a result of a series of gene duplication events [5]. The production of antimicrobial peptides in the skin of the most primitive extant

| Peptide | Primary structure | $M_r$ (obs) | $M_r$(calc) |
|---|---|---|---|
| Ascaphin-1 | GFRDVLKGAAKAFVKTVAGHIAN.$NH_2$ | 2368.3 | 2368.4 |
| Ascaphin-2 | -----------Q----------I | 2539.6 | 2539.4 |
| Ascaphin-3 | ----------------------I | 2482.5 | 2482.4 |
| Ascaphin-4 | --K-WI-----KLI----AN---Q | 2584.6 | 2584.5 |
| Ascaphin-5 | -IK-WI-----KLI----S----Q | 2589.6 | 2589.5 |
| Ascaphin-6 | --K-WI-----KLI----SS---E | 2574.3 | 2574.4 |
| Ascaphin-7 | --K-WI-----KLI----SS---Q | 2573.5 | 2573.4 |
| Ascaphin-8 | --K-L-------L----LF.$NH_2$ | 2017.3 | 2017.3 |

Fig. 2. Primary structures and molecular masses of the ascaphins. (–) Denotes residue identity. $M_r$ (obs) is the observed molecular mass and $M_r$ (calc) is the calculated mass based upon the proposed structures. $M_r$ (calc) for ascaphin-1 and ascaphin-8 refers to the C-terminally α-amidated form of the peptides.

Table 1
Antimicrobial and haemolytic activities of the endogenous peptides isolated from the skin secretions of *A. truei*

| Peptide | MIC (μM) | | $HC_{50}$ (μM) |
|---|---|---|---|
| | *E. coli* | *S . aureus* | |
| Ascaphin-1 | 6 | >50 | >200 |
| Ascaphin-3 | 6 | >50 | >200 |
| Ascaphin-7 | 3 | 25 | >200 |
| Ascaphin-8 | 6 | 6 | 50 |

frog demonstrates that this defence strategy arose very early in evolution of the anurans.

A BLAST search of the peptides/proteins in the National Center for Biotechnology Information (Bethesda, MD) database indicates that the ascaphins showed little structural similarity to previously described peptides isolated from the skins of frogs of other species but identifies a region of sequence identity with a domain at the N-termini of pandinin 1 [17] and opistoporin 1 [18] Fig. 3. These antibacterial peptides were isolated from the venoms of the African scorpions *Pandinus imperator* and *Opistophtalmus carinatus*, respectively. Secondary structure predictions and circular dichroism measurements demonstrate that both scorpion toxins adopt α-helical conformations in a membrane-mimetic solvent such as trifluoroethanol. Prediction of the secondary structure by the method of Kellner et al. [19] indicates that the central regions [residues (4–17)] of all the ascaphins also have a strong propensity to adopt an α-helical conformation. Helical wheel projections illustrate the amphipathic nature of the helix with the conserved $Lys/Arg^3$, $Lys^7$, $Lys^{11}$, and $Lys^{15}$ residues segregating on one face of the helix and the hydrophobic residues at positions 2, 5, 6, 13, and 17 segregating on the opposite face. Thus, the mechanism of action of the ascaphins in producing bacterial cell lysis is likely to be the same as that of other frog skin cationic, amphipathic α-helical peptides [20].

The antimicrobial properties of the most abundant naturally occuring ascaphins and synthetic replicates of ascaphins -1, -5, and -8 are shown in Tables 1 and 2. The appreciably reduced potency of the free acid forms of ascaphin-1 and -8 indicates that a C-terminally

Table 2
Minimum inhibitory concentrations (μM) and haemolytic activities (μM) of synthetic replicates of peptides isolated from the skin secretions of *A. truei*

| | Ascaphin-1 | | Ascaphin-5 | Ascaphin-8 | |
|---|---|---|---|---|---|
| | Free acid | Amidated | | Free acid | Amidated |
| *E. coli* | 25 | 3 | 6 | 6 | 6 |
| *P. aeruginosa* | >100 | 25 | 13 | 50 | 13 |
| *E. cloacae* | 50 | 6 | 6 | 13 | 6 |
| *K. pneumoniae* | 50 | 6 | 13 | 25 | 6 |
| *P. mirabilis* | >100 | >100 | >100 | >100 | >100 |
| *S. aureus* | >100 | >100 | 50 | 50 | 3 |
| *S. epidermidis* | >100 | 50 | 13 | 25 | 6 |
| *E. faecalis* | >100 | >100 | >100 | >100 | 50 |
| *Streptococcus* Group B | 100 | 13 | 6 | 13 | 6 |
| *C. albicans* | >100 | >100 | 100 | 100 | 25 |
| $HC_{50}$ | >200 | >200 | >200 | >200 | 55 |

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Pandinin 1      GKVWDWIKSAAKKIWSSEPVSQLKGQVLNAAKNYVAEKIGATPT

Opistoporin 1   ---------T---L-N----KE--NTA------L---------S

Ascaphin-4      -FK ----G----LIKTVAANIANQ

Ascaphin-5      -IK ----G----LIKTVASHIANQ

Ascaphin-6      -FK ----G----LIKTVASSIANE

Ascaphin-7      -FK ----G----LIKTVASSIANQ
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Fig. 3. A comparison of the primary structures of ascaphins 4–7 with those of the antimicrobial peptides pandinin 1 and opistoporin 1, isolated from the venoms of the African scorpions *P. imperator* and *O. carinatus*.

α-amidated residue is an important, but not an essential, determinant of activity. With the exception of ascaphin-8, the ascaphins tested showed higher potency against Gram-negative bacilli (*E. coli*, *E. cloacae*, *K. pneumoniae*, and *P. aeruginosa*) than against Gram-positive cocci (*S. aureus*, *S. epidermidis*, and *E . faecalis*). In this respect, the ascaphins resemble more the properties of opistoporin 1 which is also more active against Gram-negative bacteria [18] rather than pandinin 1 which is more active against Gram-positive bacteria [17]. Overall, ascaphin-8 was the most active peptide tested showing the highest potency against *P. aeruginosa*, *S. aureus*, and *S. epidermidis* and significant activity against *E. faecalis* and *C. albicans*. In common with other frog skin peptides that have been investigated in the investigator's laboratory [14,21], the ascaphins were not active against *P. mirabilis*.

The therapeutic potential of ascaphin-8 as an anti-infective agent is limited by its moderately high haemolytic activity ($HC_{50} = 55\,\mu M$) In contrast, ascaphin-1 and ascaphin-5, although displaying high potencies only against Gram-negative bacteria, have very low haemolytic activities ($HC_{50} > 200\,\mu M$) compared with many other frog skin peptides. By way of comparison, the $HC_{50}$ values of brevinin-1E from *Rana esculenta* [22] and temporin L from *Rana temporaria* [23] are less than $1\,\mu M$.

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