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Peptidomic approach identifies cruzioseptins, a new family of potent antimicrobial peptides in the splendid leaf frog, *Cruziohyla calcarifer*

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ABSTRACT

Phyllomedusine frogs are an extraordinary source of biologically active peptides. At least 8 families of antimicrobial peptides have been reported in this frog clade, the dermaseptins being the most diverse. By a peptidomic approach, integrating molecular cloning, Edman degradation sequencing and tandem mass spectrometry, a new family of antimicrobial peptides has been identified in *Cruziohyla calcarifer*. These 15 novel antimicrobial peptides of 20–32 residues in length are named cruzioseptins. They are characterized by having a unique shared N-terminal sequence GFLD– and the sequence motifs –VALGAVSK– or –GKAAL(N/G/S) (V/A)V– in the middle of the peptide. Cruzioseptins have a broad spectrum of antimicrobial activity and low haemolytic effect. The most potent cruzioseptin was CZS-1 that had a MIC of 3.77 μM against the Gram positive bacterium, *Staphylococcus aureus* and the yeast *Candida albicans*. In contrast, CZS-1 was 3–fold less potent against the Gram negative bacterium, *Escherichia coli* (MIC 15.11 μM). CZS-1 reached 100% haemolysis at 120.87 μM. Skin secretions from unexplored species such as *C. calcarifer* continue to demonstrate the enormous molecular diversity hidden in the amphibian skin. Some of these novel peptides may provide lead structures for the development of a new class of antibiotics and antifungals of therapeutic use.

Biological significance: Through the combination of molecular cloning, Edman degradation sequencing, tandem mass spectrometry and MALDI-TOF MS we have identified a new family of 15 antimicrobial peptides in the skin secretion of *Cruziohyla calcarifer*. The novel family is named "Cruzioseptins" and contains cationic amphipathic peptides of 20–32 residues. They have a broad range of antimicrobial activity that also includes effective antifungals with low haemolytic activity. Therefore, *C. calcarifer* has proven to be a rich source of novel peptides, which could become leading structures for the development of novel antibiotics and antifungals of clinical application.

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1. Introduction

Antimicrobial peptides (AMPs) are a diverse group of oligopeptides that constitute the effector molecules of the innate immune response. They occur in all domains in nature, including bacteria, protozoa, fungi, molluscs, arthropods, vertebrates, and plants. AMPs have a broad spectrum of antimicrobial activity and provide protection against bacteria, fungi, parasites and viruses; however, recent research has provided evidence of additional roles in inflammation, immunity and wound healing [1].

AMPs are extremely diverse in primary structure. There is no clear correlation between structure, potency and selectivity. However, size, charge, hydrophobicity, and amphipathicity are crucial physicochemical properties for their biological activity [1,2]. Most antimicrobial peptides contain between 8 and 45 amino acids and a positive net charge of +2 to +6 at pH 7 [3]. In addition, AMPs are usually amphipathic, with a hydrophobic face containing approximately 50% of hydrophobic amino acids. The main mechanism of action involves electrostatic contact of cationic peptides with the anionic membrane of the target microorganisms followed by insertion into the membrane interior. The hydrophobic face interacts with the lipid core while the hydrophilic face interacts with the phospholipids of the cell membrane, and various models have been described, including: carpet-like, toroidal pore, and barrel-stave [1,2]. In addition, some natural AMPs undergo post translational modifications (PTMs) that are required for their antimicrobial function. Common PTMs include: phosphorylation, replacement of L-amino acids with their D-isomers, methylation, amidation, glycosylation, and disulphide bridges [4].

Amphibian skin is one of the richest sources of antimicrobial peptides. Until 2015, around 1600 AMPs had been reported from 165 species and 26 genera [5]. These peptides have been arranged into at least

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100 peptide families based on sequence similarities. Remarkably, >165 antimicrobial peptides have been reported in the dermaseptin superfamily which occurs in the skins of Central and South American frogs that belong to the Phyllomedusinae subfamily including the genera: *Phyllomedusa* (12 spp.), *Agalychnis* (5 spp.), and *Phasmahyla* (1 sp.) [5–8].

An important characteristic of the members of the dermaseptin superfamily is the highly conserved amino acid sequence in their precursor N-terminal region that correspond to the signal peptide and acidic spacer peptide. This conservation usually extends to the non-translated regions at the 5′ side of the precursor nucleotide sequence. Indeed, the extremely conserved sequences have allowed the design of primers able to target this region and have been instrumental in the discovery of a large number of related peptides. These peptides have been classified in the following families: dermaseptins sensu stricto, dermatoxins, phylloxins, phylloseptins, plasticins, medusins, caerin-related peptides and orphan peptides [8–18].

Most studies have been focused on *Phyllomedusa* and *Agalychnis*, while other genera such as *Cruziohyla* remain unexplored. *Cruziohyla* includes two species: *Cruziohyla calcarifer* that occurs in the Caribbean lowlands from eastern Honduras to the Pacific lowlands of northwestern Ecuador, and *Cruziohyla craspedopus* that occurs in the Amazon lowlands from Colombia to Peru [19]. *Cruziohyla calcarifer* was recently relocated from the genus *Agalychnis* to the new genus *Cruziohyla*[20] and, considering their taxonomic proximity to *Agalychnis*, it was presumed that this taxon also produce bioactive peptides in their skin.

Several studies have demonstrated the robustness of complementing data from shotgun molecular cloning, Edman N-terminal sequencing and tandem mass sequencing for peptidomic studies on frog skin secretions [10,21–23]. In the current study, a new family of 15 antimicrobial peptides is reported in the splendid leaf frog, *Cruziohyla calcarifer* and is named cruzioseptins. These contain an N-terminal sequence motif, GFLD– and the sequences –VALGAVSK– or –GKAAL(N/G/S) (V/A)V– in the mid-regions of their mature peptides. Cruzioseptins showed a broad spectrum of antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with low haemolytic effects.

2. Methods

2.1. Skin secretion extraction

Two adult specimens were collected in northwestern Ecuador (Esmeraldas Province, Durango) in November 2013. Four captive reared sub-adult specimens (from Esmeraldas Province, Reserve Otokiki) were provided in 2015 by Centro Jambatu for Research and Conservation of Amphibians in Ecuador. Skin secretions were obtained after gently massaging the dorsal side of the animals. Secretions were washed off the animals with distilled water. Samples were immediately frozen and stored at −20 °C. The frogs collected in the field were returned to their habitat after the extraction. Samples were freeze-dried for analysis in Queen's University Belfast.

Twelve additional samples were taken from a group of 13-month-old captive bred frogs, whose parental line came from a Costa Rican population.Specimens were housed in terraria as pets in Belgium and Austria. Samples were extracted in the same way as described above, but instead of freeze-dried they were acidified with TFA and were transported at room temperature to the laboratory facilities in Queen's University Belfast.

2.2. Molecular cloning

Lyophilized skin secretions were dissolved in buffer A (99.95% water; 0.05% trifluoroacetic acid), pooled, and aliquoted into two tubes. One was employed for molecular cloning and the other for HPLC fractionation.

One aliquot, equivalent to skin secretion of 2.5 frogs of the Ecuadorian sample, or 1.3 mg of the Costa Rican sample, was dissolved in 1 ml of cell lysis/ binding buffer, and polyadenylated mRNA was isolated using magnetic Dynabeads Oligo (dTs) as described by the manufacturer (Dynal Biotec, UK). Isolated mRNA was subjected to 3′-rapid amplification of cDNA by using the SMART-RACE kit (Clontech, UK). In brief, three sets of 3′-RACE reactions were employed. Firstly, 3′RACE used a nested universal primer (NUP) provided with the kit and the sense primer 1 (S1: 5′-CAGCACTTTCTGAATTACAAGACCAA-3′) that was complementary to

Table 1
Antimicrobial peptides of *Cruziohyla calcarifer* identified by molecular cloning.

Peptide	Signal peptide / Acidic spacer
1	* * - * * * - * - * - * * - * * * * * *
CZS-1 (14)	M A F L K K S L F L V L F L G L V S L S I C E E E K R E E - N E E E Q D D D E Q S E E K R
CZS-2 (2)	M A F L K K S L F L V L F L G L V S L S I C E E E K R E E E N E E V Q E D D D Q S E E K R
CZS-3 (1)	- K R
CZS-4 (1)	- K R
CZS-5 (1)	- K R
CZS-6 (2)	M A Y L K K S L F L V L F L G L V S L S I C E E E K R E E E N E E E Q E D D D Q S E E K R
CZS-7 (4)	M A K L K K S L F L V L F L G L V S L S I C E E E K R E E E N E E V Q E D D D Q S E E K R
CZS-8 (6)	M A F L K K C L F L V L F L G L V S L S I C E E E K R E E E N E E V Q E D D D Q S E E K R
CZS-9 (1)	- K R
CZS-11 (6)	M V K L K K S L F L V L F L G L V S L S I C E E E K R E E E N E E V Q E D D D Q S E E K R
CZS-12(10)	M A F L K K S L F L V L F L G L V S L S I C E E E K R E E E N E E V Q E D D D Q S E E K R
	Mature peptide
46	* * * * - - - * * * * - - * * - * * - - * - - - - - - - *
CZS-1 (14)	G F L D I V - - - - K G V G K V A L G A V S K L F G Q E E R * -
CZS-2 (2)	G F L D V I - - - - K H V G K A A L G V V T H L I N Q G E Q * -
CZS-3 (1)	G F L D V V - - - - K H I G K A A L G A V T H L I N Q G E Q * -
CZS-4 (1)	G F L D V I - - - - K H V G K A A L S V V S H L I N E G E H * -
CZS-5 (1)	G F L D V I K H V G K A V G K A A L N A V N D M V N K P E Q Q S
CZS-6 (2)	G F L D V I T H V G K A V G K A A L N A V T E M V N Q A E Q * -
CZS-7 (4)	G F L D V V K H V G K A V G K A A L N A V T E M V N Q A E Q * -
CZS-8 (6)	G F L D V I K H V G K A A G K A A L N A V T E M V N Q G E Q * -
CZS-9 (1)	G F L D V I T H V G K A V G K A A L N A V N E M V N Q G E Q * -
CZS-11 (6)	G F L D I V K H V G K A A G K A A L N A V T E M V N Q G E Q *
CZS-12(10)	G F L D V V K H V G K A V G K A A L N A V N D L V N Q G E Q *

*Conserved sites, (x) number of clones with the same sequence. Accession numbers: KX065078-KX065088, COHK07-COHK-12.

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