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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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**KEYWORDS**

Cruzioseptins; antimicrobial peptides; peptidomic; molecular cloning; skin secretions; tandem mass spectrometry.
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 have 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 120.87µM. 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.

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 contain 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.

**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 8–45 amino acids and a positive net charge of +2–+6 at pH7 [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 100 peptide families based on sequence similarities. Remarkably, more than 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 are the highly conserved amino acid sequences in their precursor N-terminal regions 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: *C. calcarifer* that occurs in the Caribbean lowlands from eastern Honduras to the Pacific lowlands of northwestern Ecuador, and *C. 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 are
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
eaureus*, *Escherichia coli*, and *Candida albicans* with low haemolytic effects.

**METHODS**

**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 -20ºC. 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.

**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 1ml 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’-
CAGCCTTTCTGAATTACAAGACCA-3’) that was complementary to the signal sequence of the phylloseptin-S5 precursor of *Phyllomedusa sauvagii*. Secondly, 3’RACE employed an NUP primer and the sense primer 2 (S2: 5’-TAGACCAAACATGGCTTTCCTGA) designed to target the signal sequence of the first antimicrobial peptide of *Cruziohyla calcarifer* (CZS-1), which was first identified with the primer sense 1 described above. The third 3’RACE included an NUP primer and the sense primer 3 (S3: 5’-AAGAGAGGCTTCCTGGAT-3’), which was also designed based on the sequence of CZS-1 but this time targeting the sequence corresponding to the first 4 amino acids of the mature sequence of the CZS-1 peptide. These primers were designed employing Primer3 and Primer-BLAST online softwares. The 3’-RACE reactions were purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequence.

Reverse-phase HPLC fractionation and Edman degradation
The second aliquot of freeze-dried skin secretion (corresponding to 2.5 frogs) was dissolved in 1.2 ml of buffer A (99.95% H₂O, 0.05% trifluoroacetic acid) and clarified by centrifugation. 1 ml supernatant was subjected to reverse phase HPLC employing Waters Binary pump HPLC system fitted with an analytical column Phenomenex Jupiter C18 (4.6 x 250 mm). Peptides were eluted with a linear gradient formed from 100% buffer A (99.95% H₂O, 0.05% trifluoroacetic acid) to 100% buffer B (80.00% Acetonitrile, 19.95% H₂O, 0.05% trifluoroacetic acid) in 240 min at a flow rate 1 ml/min. Fractions (1 ml) were collected every minute. Detection at 214 and 280 nm was continuous.

Skin secretion of two specimens of *C. calcarifer* from a Costa Rican population was subjected to reverse-phase HPLC using a Diphenyl column C18. Peptides were eluted in a gradient from 1% buffer A (99.95:0.05% H₂O/trifluoroacetic acid) to 80% buffer B (80.00:19.95:0.05% acetonitrile/H₂O/trifluoroacetic acid) in 80 min and fractions were collected every minute. Those fractions were tested for antimicrobial activity and the active fractions 47-53, 59 were re-chromatographed on a VydaC18 column until clear peaks were obtained. Those samples were sequenced by automated Edman degradation. These analyses were performed in Chris Shaw lab 15 years ago (unpublished data).

**MALDI-TOF MS**

The molecular masses of peptides and proteins in each chromatographic fraction were analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE mass spectrometer (Perceptive Biosystems, MA, USA) in positive detection mode employing α-cyano-4-hydroxycinnamic acid matrix. Two microliters of sample plus 1 µl of matrix (10 mg/ml) were allowed to dry and were later analysed in the range of 500–5000 Da.
Tandem mass spectrometry sequencing

20 µl of the remaining skin secretion fraction were diluted in buffer A and was pumped directly onto an analytical HPLC column (Phenomenex C-18; 4.6x150 mm) connected to an LCQ Fleet ESI ion-trap mass spectrometer (Thermo Fisher, San Jose, CA, USA). The linear elution gradient was formed from 100% buffer A (99.90% H₂O, 0.1% formic acid) to 100% buffer B (19.9% H₂O, 80% acetonitrile, 0.1% formic acid) in 135 min at a flow rate 20 µl/min. Mass analysis was performed in a positive ion mode with acquired spectra in the range of m/z 500–2000 with >50% relative intensity during HPLC-MS. Parameters for electrospray ionization ion-trap mass spectrometry (ESI/MS) were: spray voltage +4.5kV, drying gas temperature 320ºC, drying gas flow 200 µl/min, and maximum accumulation time –for the ion trap– 350 ms. The first mass analysis was performed in full scan mode, then peptide ions with >50% relative intensity were selected for fragmentation by collision induced dissociation (CID), to generate b and y ions that were detected in a second mass analysis. The instrument was controlled by Xcalibur software (Thermo, USA) and data analysis was performed using Proteome Discover 1.0 (Thermo, USA). Sequest™ algorithm was employed to compare the acquired fragment ion profiles with the theoretical fragment ions generated from a FASTA database specific for this species built by molecular cloning (as described above) to confirm the amino acid sequences of individual peptides.

Solid phase peptide synthesis (SPPS)

Three peptides CZS-1: GFLDIVKGVGKVALGAVSKLF-amide, CZS-2: GFLDVIKHVGKAALGVTHLINQ-amide, and CZS-3: GFLDVKKHIGHGKAALGAVTHLINQ-amide were chemically synthetized by solid phase Fmoc chemistry using a Tribute peptide synthesizer (Protein technologies, Inc). After
cleavage from resin and de-protection, each peptide was purified by HPLC and their degrees of purity were analysed by MALDI-TOF mass spectrometry.

**Antimicrobial assays**

**Antimicrobial screening**

500 µl of each HPLC fraction were dried in a vacuum concentrator and later diluted in 10 µl of phosphate buffered saline (PBS). Mueller agar plates with *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* in a 10⁶ CFU/ml concentration were prepared and 12 holes were prepared with a sterile Pasteur pipette. 2 µl of each fraction were transferred to one hole of each plate to be tested against the 3 microorganisms. Plates were incubated at 37°C overnight and inhibition zones were recorded as antimicrobial activity.

**Minimal inhibitory concentration MIC and minimal bactericidal concentration MBC assays**

MICs of the synthetic peptides were determined against *E. coli*, *S. aureus* and *C. albicans*. In brief, serial dilutions of each peptide in dimethylsulphoxide (DMSO) were prepared to obtain concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 x10² mg/L. Each microorganism in log phase was diluted to obtain the equivalent of 1x10⁶ colony forming units (CFU)/ml for the bacteria and 1x10⁵ CFU/ml for the yeast. Later, 2 µl of each peptide dilution were transferred to a 96 well sterile plate and 198 µl of the microorganism were added. As controls, 2 µl of DMSO was included instead of peptide and 200 µl of Mueller Hinton Broth in another well. 7 replicates per peptide concentration were performed and the experiment was repeated 3 times in order to confirm the results. Plates were incubated at 37°C for 18–22 h. Growth was monitored at 550 nm in an ELISA plate reader. Later, 10 µl of each concentration without visual growth was sub-cultured on Mueller Hinton agar plates. Plates were incubated at 37°C overnight. MBCs were recorded as the minimal concentration without any growth occurrence.
Haemolysis assay

A suspension of red blood cells (2%) was prepared with defibrinated horse blood (ICS Biosciences) and it was challenged with serial dilutions of the tested peptides resembling the same concentrations employed in the antimicrobial assays previously described. In brief, 200 µl of blood cell suspension were incubated with 200 µl of each diluted peptide and they were incubated at 37ºC for 120 min. Later, samples were centrifuged and supernatants were transferred to a 96 well plate. Lysis of red blood cells was analysed in an ELISA plate reader at λ=550 nm. For negative controls, phosphate buffered saline was added to the cells instead of peptide, and for positive controls, phosphate buffered saline with 2% (v/v) Triton X-100 (Sigma-Aldrich) was employed. The concentrations that produce 100% haemolysis are reported.

Bioinformatic analysis

Nucleotide sequences were analysed by MEGA6.0 and compared by employing the BLAST tool using databases in the National Centre for Biotechnology Information (NCBI) [24,25]. Signal peptides were predicted using the SignalP 4.1 server and theoretical peptide masses were calculated with the peptide mass calculator v3.2 [26,27]. Secondary structure prediction was performed using the GOR4 programme and the physicochemical properties of the peptides were calculated using HeliQuest Computational Parameters and Peptide property calculator Bachem [28-30].

RESULTS

Molecular cloning of novel antimicrobial peptide precursor-encoding cDNAs

Seven full-length and four partial-length cDNAs encoding novel peptides were cloned from the cDNA library that was constructed from the skin secretion of Cruziohyla calcarifer
The novel peptides are named Cruzioseptins (CZS) to represent their origin in *Cruziohyla* – a genus in honour of a Brazilian herpetologist, Carlos Alberto Gonçalves da Cruz, in recognition of his various contributions to knowledge of *Phyllomedusinae* [20]. The open reading frames of these sequences contained 195–231 nucleotides. Translated amino acid sequences revealed that the precursors consisted of: (1) a putative signal peptide of 22 residues; (2) an acidic spacer peptide of 23 residues containing 2 pro-peptide convertase processing sites; and (3) a mature peptide of 20–32 residues (Figure 1). In addition, 6 of the 15 peptides were C-terminally amidated with a Gly (G) residue as the amide donor (Table 2). Nucleotide sequences were submitted to the GenBank (NCBI) under accession numbers: KX065078–KX065088.

Each novel nucleotide sequence was analysed using the NCBI database and they showed 80–91% similarity with dermaseptins from *Phyllomedusa hypochondrialis* (Accession number AM229015.1), *Agalychnis annae* (Accession number AJ005187.1), and *P. bicolor* (Accession number Y16564.1). In addition, the BLAST/p (protein/protein) comparisons using only the translated mature sequences of these peptides, showed a lower similarity (45–90%) with dermaseptins. For example: CZS-4 was 45% similar to dermaseptin-B6 from *P. bicolor* (accession number AFR78287.1), CZS-6 was 65% similar to dermaseptin SVII from *P. sauvagii* (accession number CAD92230.1), and CZS-8 was 90% similar to dermadistinctin-L from *P. distincta* (accession number P83639.1). However, when the translated amino acid sequences of the mature peptides CZS-1 and 15 were subjected to BLAST/p analysis, no significant hits were found, not with any amphibian skin antimicrobial peptide or with antimicrobial peptides from other sources.

**Edman degradation sequencing**
Cruzioseptins 10–15 were found first by antimicrobial activity screening of reverse phase HPLC fractions of *C. calcarifer* skin secretions from the Costa Rican population. Peptides were re-chromatographed for purification and sequenced by Edman degradation. The sequences are shown in Table 2. Later, two of them were cloned from the same population, but none were cloned from the Ecuadorian population to date. The peptide sequences were submitted to the UniProt Knowledgebase under accession numbers: C0HK07- C0HK012.

**Isolation and structural analysis of cruzioseptin**

During functional screening of HPLC fractions of the skin secretion of *C. calcarifer*, antimicrobial activity against *S. aureus* and *C. albicans* was identified in fractions 162, 163, 171 and 172 (Figure 2). Cruzioseptin-1 was identified in HPLC fractions 171 and 172 based on its monoisotopic molecular mass [M+H]**+** m/z of 2117.54 as determined by MALDI-TOF mass spectrometric analysis and confirmed by LCQ ESI MS full scan that revealed ions 2+ = m/z 1059.75 and 3+ m/z = 706.67 (Figure 3). In addition, cruzioseptin-2 was identified in HPLC fractions 162 and 163 due to its monoisotopic molecular mass [M+H]**+** m/z of 2427.42, as found by MALDI-TOF and confirmed by a LCQ ESI MS full scan, where ions 2+ m/z = 1215.08, 3+ m/z = 810.50, and 4+ m/z = 316.25, were identified (Figure 4).

It is remarkable that all cruzioseptins 1 to 15 were 100% identified by LCQ MS/MS fragmentation sequencing employing the whole skin secretion of *C. calcarifer* (Table 2).

**Antimicrobial and haemolytic assays of cruzioseptins**

Once sequences were confirmed, cruzioseptins 1-3 were selected for further analysis. CZS 1 and 2 were chosen because these peptides were identified in HPLC fractions as detailed above, but in order to determine their potency and specificity more pure peptides were
required. CZS-3 was included later due to the sequence similarity with CZS-2, aiming to
determine the effect of the 3 amino acid differences in its antimicrobial activity.

Cruzioseptins 1, 2 and 3 were synthesized by solid phase Fmoc chemistry, purified by HPLC,
and the sequences were confirmed by LCQ MS/MS sequencing (Figure 5 and 6). Physico-
chemical properties of CZS1–3 are summarized in Table 3. Synthetic pure peptides were
employed in antimicrobial and haemolytic assays. Cruzioseptin-1 displayed potent broad-
spectrum antimicrobial activity against all three microorganisms tested with MICs of 15.11
µM against *E. coli* and 3.77 µM against *S. aureus* and *C. albicans*. In addition, the MBC was
below 15.11 µM for the three microorganisms. At the antimicrobial concentration of 3.77
µM, this peptide showed only 1% haemolytic activity while reaching 20% haemolysis at
15.11 µM. CZS-1 reached 100% haemolysis at 120.87 µM. In addition, cruzioseptin-2
showed moderate broad-spectrum antimicrobial activity against *E. coli* (MIC of 26.35 µM),
*S. aureus* (6.59 µM), and *C. albicans* (13.18 µM). The MBC concentrations were below
52.69 µM. for the three microorganism. Nevertheless, haemolytic activity at 13.18 µM was
only 26% reaching 100% haemolysis at 210.96 µM. In contrast, synthetic cruzioseptin-3 was
less potent that CZS-1 and CZS-2 showing MICs of 13.32 µM against the three
microorganisms tested. Moreover, the MBC was similar to CZS-2 (53.31 µM). However,
haemolysis at this concentration was only 6%. CZS-3 produced 100% haemolysis at 213.33
µM. Results of these tests are summarized in Table 4 and Figure 8.

**DISCUSSION**

Antimicrobial peptides secreted by phyllomedusine frog skins are extremely diverse. At least
eight families of antimicrobial peptides have been reported so far. These peptides have been
classified based on similarities of their primary structures and/or structural motifs. The most
diverse family is the dermaseptins *sensu stricto*, which contains more than 75 peptides described from 15 species [8].

Through a combination of molecular cloning, Edman degradation sequencing, and LCQ tandem MS/MS, a new family named ‘cruzioseptins’ of 15 antimicrobial peptides were found in the splendid leaf frog, *Cruziohyla calcarifer*. All these novel peptides share these unique structural sequences: (1) the N-terminal motif GFLD–; and (2) the motif –GKAAL(N/G/S)(V/A)V– or –VALGAVSK–. In fact, 13 of the cruzioseptins (CZS-2 to CZS-14) present the motif –GKAAL(G/N/S)(V/A)V– and 2 cruzioseptins (CZS-1 and 15) present the motif –VALGAVSK– (Table 2). Their precursor sequences are extremely conserved, sharing high similarity in the signal and acidic spacer sequences at the N-terminal ends but showing important variation in the mature sequences at their C-terminal ends. A BLAST/n search in the NCBI database identified the precursor sequences of these peptides as members of the dermaseptin superfamily. In addition, the BLAST/p comparisons with the translated mature sequences of these peptides, showed 45–90% similarity to dermaseptins. However, CZS-1 and CZS-15 did not produce any significant hits when compared with BLAST/p, suggesting that these were a well differentiated group of peptides that we recognize as a new family based on having a set of unique shared structural motifs and sequences. With a closer analysis of CZS-8, 11 and 14 sequences, it was found that the similarities with dermaseptins were concentrated in the centre of the mature peptides where these cruzioseptins share the dermaseptin motif –AAGKAALNV–. However, all cruzioseptins lack the characteristic Trp (W) in position 3 of dermaseptins. For that reason, and for having the motif GFLD– at their N-terminals, and the motifs –GKAAL(N/G/S) (V/A)V– or –VALGAVSK– at the mid-region, these novel antimicrobials were not classified as dermaseptins; instead, they were assigned to a new family of antimicrobial peptides – the cruzioseptins.
The GFLD– N-terminal motif is also found in other four amphibian skin antimicrobial peptides, including: ranatuerin-3 from *Rana catesbeiana* (accession number P82780.1), brevinins 2PTd and 2Pte from *Pulchrana picturata* (accession numbers POC8T6.1 and POC8T7.1, respectively), and frenatin-4 from *Litoria infrafrenata* (accession number P82023.1). These species belong to the families Ranidae and Hylidae. However, neither ranatuerin, brevinin or frenatin families contain GFLD– as a specific motif, so their appearance in these families is most likely a result of convergent evolution. On the other hand, the strongly-conserved nucleotide precursor sequences of cruzioseptins at their N-terminals in common with other members of the dermaseptin superfamily, such as litorins and caerin of the Australian frogs of the Pelodryinae subfamily, supports the view that the genetic origin of the ancestral gene precursor of cruzioseptins was present in the common ancestor which originated prior to the fragmentation of Gondwana. In addition, the extraordinary diversity of cruzioseptins found in a single species provides evidence, once again, that evolutionary mechanisms such as hypermutability of the C-terminal domain, gene duplication, and diversifying selection can provide a wide range of antimicrobial protection [9,31].

In addition, three cruzioseptins were chemically synthesized and their antimicrobial profiles were analysed, showing that all three cruzioseptins (CZS1–3) have broad spectra of antimicrobial activity and relatively low haemolytic activity (Table 4). Firstly, CZS-3 showed potent activity (MIC) against the Gram negative bacterium *E. coli* at 13.32 µM, followed by CZS-1 at 15.11 µM and CZS-2 at 23.35 µM. In addition, at these concentrations, the peptides presented relatively little haemolysis (6%, 9%, and 26%, respectively) (Table 4 and Figure 8). However, in comparison with other antimicrobial peptides of similar sequences (50–70% similarity) such as dermaseptin-B4 from *P. bicolor* (accession number P81486) and dermadistinctin-L from *P. distincta* (accession number P83639), cruzioseptins are less potent...
than dermaseptins, whose MICs are 5 and 2.5 µM, respectively (Table 4). Secondly, CZS-1 was the most potent of the three cruzioseptins, being able to inhibit the growth (MIC) of the Gram positive bacterium *S. aureus* at 3.77 µM; to achieve the same goal, CZS-2 is 2-fold less potent and CZS-3 is 3-fold less potent. However, dermaseptin-B4 and dermadistinctin-L are still more potent (MICs 3.0 µM and 1.3 µM, respectively) [32,33]. Finally, CZS-1 was also able to inhibit the growth (MIC) of the yeast *C. albicans* at 3.77 µM while CZS-2 and CZS-3 needed 3-fold this concentration to achieve the same goal (Table 4). Cruzioseptins 1-2 were bactericidal against *E. coli* having the same MIC and MBC concentrations. However, cruzioseptins 1-3 have a bacteriostatic effect against *S. aureus* and *C. albicans*, requiring a two or three folds concentration increase to reach the bactericidal effect (Table 4). This is an important result because there are relatively few peptides that exhibit antifungal activity and the need to develop new antifungal agents is always growing. The differences in activity found between CZS-2 and CZS-3 are very interesting because these peptides are very similar in their primary structures (87%) and both have a charge of +2. They differ only in 3 amino acids: I/V in position 6, V/I in position 9 and V/A in position 16 (Table 3).

The predicted secondary structures and physico-chemical properties of the three cruzioseptins (CZS-1, CZS-2, and CZS-3) are shown in Table 3. All three cruzioseptins have a similar hydrophobicity (H value range 0.523–0.581) and hydrophobic moment (0.441–0.472 µH), although the primary structure of CZS-1 compared to CZS-2 and CZS-3 is different sharing only 12 conserved amino acids (57%). In addition, CZS-1 has a predicted helical domain containing 19.05 % of the peptide that increases to 30.43% for CZS-3 and decreases to 0% for CZS-2. Moreover, CZS-1 possesses a higher net positive charge than CZS-2 and CZS-3 (+3 versus +2). Helical wheel plots showed that all three cruzioseptins are amphipathic having 11–13 amino acids placed in the hydrophobic face (V/ I/ A/ F/ L/ G) and 8–12 amino acids hydrophilic residues placed at the opposite side (Table 3 and Figure 7).
These 3 variations in sequence change the potency of CZS-2 making it weaker than CZS-3 against *E. coli* (13.33 vs 26.35 µM) but more potent against *C. albicans* (13.33 vs 6.59 µM).

In summary, the antimicrobial potency observed for CZS-1 could be due to its +3 charge, in contrast to the +2 of CZS-2 and CZS-3. However, in comparison with other antimicrobial peptides such as dermaseptin-B4 and dermadistinctin-L, CZS-2 is weaker against *E. coli*, but potent against *S. aureus*. Moreover, CZS showed potency against *S. aureus* and *C. albicans* with only 1% haemolysis at those concentrations, which makes CZS-1 an interesting peptide and warrants further study into its potential antibiotic and antifungal functions.

In conclusion, cruzioseptins, a novel antimicrobial peptide family, is reported in *Cruziohyla calcarifer*. Three synthetic cruzioseptins displayed broad-spectrum antimicrobial activity against *S. aureus*, *C. albicans* and less potently against *E. coli* with minor haemolytic activity. These data show once again, the phenomenal peptide diversity produced in the skin of phyllomedusine frogs such as the previously unstudied *C. calcarifer*. Interplay between molecular cloning and tandem mass spectrometry sequencing, together with functional studies of natural and synthetic peptides have proven to be a robust, cost-effective strategy for peptidomic analysis in species where databases are not available. In addition, these techniques are sensitive enough to generate data with only a few milligrams of material, and this is especially beneficial in the analysis of endangered species where samples are limited. Finally, the discovery of novel natural antimicrobial peptides such as cruzioseptins is a key element in the development of new therapeutic drugs based on the structures of natural compounds.

**ACKNOWLEDGMENTS**
Carolina Proaño-Bolaños is in receipt of a scholarship of the Ecuadorian Secretariat of Science and Technology (SENSCYT). This research was funded by the Natural drug discovery group, School of Pharmacy, Queen’s University Belfast and SENESCYT. The latter also supported field work to CPB. Collection and rearing of frogs in Ecuador were done under permits of the Ecuadorian Ministerio de Ambiente (MAE): 001-13 IC-FAU-DNB/MA, 003-11 IC-FAU-DNB/MA, 005-15 IC-FAU-DNB/MA (Issued to the Centro Jambatu). Exportation of skin secretion samples were done under exportation permits 003-13-EXP-CI-FAU-DNB/MA and 2015-003-FO-DPAP-MA. This research is part of the project “Conservation of Ecuadorian amphibian diversity and sustainable use of its genetic resources”, which involves MAE, Ikiam-Universidad Regional Amazónica, Queen’s University Belfast, and Centro Jambatu, and help of the Global Environmental Facility (GEF) and “Programa de las Naciones Unidas para el Desarrollo” (PNUD).

Thanks to Mayra Rojas and David Narvaez of Universidad de las Américas in Quito, Ecuador, who helped to prepare freeze-dried samples. Elicio E. Tapia aided in collecting specimens in Esmeraldas province. Christian Proy (Austria) and Stassen Raf (Belgium) generously provided access to their Costa Rican pet frogs to obtain samples for this research. Thanks to Dr. Catriona Arlow for the language reviews of the first draft of this manuscript.

We also thank former students (1998-1999) of CS who obtained the unpublished Edman degradation sequences included in this paper.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHORSHIP**
This study was conceived and designed by CS, MZ, TC. Sample collections were performed by CPB and LAC. Data were acquired by CPB. LC-MS/MS analysis was performed by LW. The article was written by CPB and reviewed critically by CS and LAC.
REFERENCES


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

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Table 2. Amino acid sequences of cruzioseptins confirmed by tandem mass spectrometry sequencing. Characteristic motifs of cruzioseptins are highlighted. Amidation was predicted according to the precursor sequence.

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<th>#AAs</th>
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* a= amidation, mc= molecular cloning, ms= tandem mass spectrometry, Ed= Edman degradation sequencing, accession numbers: KX065078-KX065088, COHK07-COHK-T2.
Table 3. Physico-chemical properties of cruzioseptins 1, 2, 3 from *Cruzihyla calcarifer*.

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<tr>
<th>Peptide</th>
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<th>Sequence/Secondary structure*</th>
<th>Theoretical average mass Da</th>
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<th>Hydrophobic moment &lt;br&gt;μD</th>
<th>α-helix (%)</th>
<th>Net charge</th>
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* secondary prediction based on GOR4: h=alpha helix, c=rigid coil, e=extended strand, access: accession numbers K0065078-K0065080.

Table 4. Minimal inhibitory concentrations (MICs) and haemolytic activity of synthetic cruzioseptins from *Cruzihyla calcarifer*.

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<tr>
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<th>MIC µM (mg/L)</th>
<th>MBC µM (mg/L)</th>
<th>Ha mM (mg/L)</th>
<th>Species</th>
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Ha=100% of Haemolytic activity, NA=not available
Figure 1. Nucleotide and translated open-reading frame amino acid sequences of the sense strand of cloned cDNAs encoding cruzioseptins 1 to 9, 11 and 12 from *Cruziohyla calcarifer*. The putative signal peptides are double-underlined, acidic spacers are in italics, the mature peptides are single-underlined and the stop codons are indicated by asterisks. Accession numbers KX065078 and KX065088, respectively.


Figure 2. Reverse phase HPLC chromatogram of *Cruziohyla calcarifer* skin secretion fractionated over 240 min with dual UV detection at 214 nm (red line) and 280 nm (green line). Arrows denote retention times of fractions with antimicrobial activity. Cruzioseptin-1 was identified in fraction 171 and Cruzioseptin-2 in fraction 162.

Figure 3. Mass analysis of antimicrobial HPLC fraction with retention time 171 min containing Cruzioseptin 1. A) The arrow denotes a singly-charged ion of m/z 2117.54 obtained by MALDI-TOF MS analysis. B) LCQ MS ESI denotes precursor ions of m/z 2+ 1059.75 and 3+ 706.67 corresponding to CZS-1.

Figure 4. Mass analysis of antimicrobial HPLC fraction with retention time 162 min containing Cruzioseptin 2. A) Arrow denotes a singly charged ion of m/z 2427.42 obtained by MALDI-TOF MS analysis B) LCQ MS ESI denotes precursor ions of m/z 2+ 1215.08, 3+ 810.50, and 4+ 316.25 corresponding to CZS-2.
**Figure 5.** Synthetic cruzioseptins 1, 2, and 3 produced by SPPS and purified by RP-HPLC.

A) Cruzioseptin-1 single charge ion of m/z 2117.63. B) Cruzioseptin-2 single charged ion of m/z 2427.38. C) Cruzioseptin-3 single charged ion of m/z 2400.61.

**Figure 6.** LCQ MS/MS Sequencing of Cruzioseptin-1 (A), Cruzioseptin-2 (B), and Cruzioseptin-3 (C). Each table contains the predicted b and y ions from each sequence. Observed ions are underlined in blue and red typefaces.

**Figure 7.** Predicted alpha helical wheel plots of cruzioseptins 1, 2, and 3. Basic residues are in blue and acid residues are in red. The basic amino acid histidine is in light blue as its charge depends on pH. Non polar residues are in yellow and polar residues are in purple. Uncharged residues of glycine and alanine are in grey and asparagine and glutamine are in pink. The arrow points to the hydrophobic face.

**Figure 8.** Haemolytic activity of Cruzioseptins 1, 2 and 3.
**Figure 1**

A) **Cruzioseptin-1**

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C) **Cruzioseptin-3**

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EMVNQAEQ*

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G) Cruzioseptin-7

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MAYLKKSLFLVLFLGLLV

```
  51 51 CTCTCTTTTCG ATCTGTGAAG AAGAGAAAAG AGAAGAGGAG AATGAGGAGG
  101 TACAAGAGA TGATGAATCAA AGTGAAGAGA AGAGAGCTT CCTGATGTA
```

IATHVGAKAVLNAV

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```

EMVNQAEQ*

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CAAAAAAAGAAAAAAA AAAAA

H) Cruzioseptin-8

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MAYLKCLFLVLFLGLLV

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I) Cruzioseptin-9

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KRFLDVITHVGKAVGK

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ACTGAAATAAAAAA AAAAAAAA AAAAA

J) Cruzioseptin-11

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  1 101 ATGGGTTTAAAC TGAAGAAATC TCTTTTCTTT GTGATTTCG TTGGATTGGT
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MVKLKLFSFLFLFLGLLV

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KRFLDVITHVGKAVGK
29 of 21

1

\text{Q E D D D Q S E E K R G F L D I}

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3

\text{V K H V G K A A G K A A L N A V T.}

4

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\text{E M V N Q G E Q *}

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8

\text{301 GCAATTTGGAAA CAAAAA AAAAAAA AAAAAA}

9

\text{K) Cruzioseptin-12}

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11

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37
Figure 2
Figure 3

A)

B)

171 V3-7 RT: 0.02-0.05 AV: 5 NL: 6.66E2
T (TMS + p ESI Full ms) [150.00-2000.00]
Figure 4

A)

B)
Figure 5

A)

B)

C)
# Figure 6

## A) Cruizinsephtin-1

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Figure 7

A) CZS-1
GFLDIVKVGGKVALGAVSKLF

B) CZS-2
GFLDVKHVGKAALGVVTHLINC

C) CZS-3
GFLDVVKHIGKAALGVVTHLINC

[Diagram of the structures with amino acid sequences]
Figure 8

A)

B)

C)