

## Cinerascetins, New Peptides from *Hypsiboas cinerascens*: MALDI LIFT-TOF-MS/MS *de novo* Sequence and Imaging Analysis

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The continuous search for antimicrobial candidates pushes the pursuit of compounds in the most diverse organisms. Amphibians are known as a prolific source of antibacterial peptides. Based on the rich biodiversity of the Amazon region the unexplored green-tree frog (*Hypsiboas cinerascens*) was studied for its skin secretion peptide content. Chromatographic separations and established tandem mass spectrometry (MS/MS) methods were used for sequencing the primary structures of the purified compounds. *De novo* sequencing lead to the identification of five new peptides related to hylaseptin P1, displaying an aminated C-terminal. Sequencing of the complementary deoxyribonucleic acid (cDNA) analysis allowed the disambiguation of isobaric amino-acids for C-01. Matrix assisted laser desorption ionization (MALDI) was carried out, demonstrating the *in situ* co-occurrence of the identified peptides in the dorsal skin. The major peptide C-01 was synthesized and assayed against a selection of microorganisms displaying minimal inhibitory concentrations (MICs) ranging from 4 to 16  $\mu$ M.

**Keywords:** anuran, antimicrobial peptide, *Hypsiboas cinerascens*, skin secretion

### Introduction

Peptides are short chains of amino acids connected to one another in a sequence by peptide bonds.<sup>1</sup> This type of compounds is widespread through all organisms such as plants, microorganisms and animals.<sup>2</sup> In animals, peptides are involved in the complex coordination of the organism, mainly attributed to hormonal and neural activities.<sup>3</sup> Peptides also are involved in cell growth control that regulate not only cell proliferation but an extraordinary range of cell activities, including matrix protein deposition and resolution, the maintenance of cell viability, cell differentiation, inflammation, and tissue repair.<sup>4</sup>

Beyond these activities, a sub-class of peptides named antimicrobial peptides (AMPs) represents a promising source of active compounds to overcome increasing microbes strains resistant to some antibiotics.<sup>5</sup> Most of these peptides are cationic and hydrophobic possessing the ability to permeabilize microorganisms membranes.<sup>6</sup> The amphipathic nature of AMPs allows the interaction with bacterial membranes through electrostatic bonds, resulting in a disruption process and consequently the cell death.<sup>7</sup> This process can occur by more than one manner (detergent-like, barrel stave and pore toroidal mechanisms)<sup>8</sup> making it difficult for microorganisms to gain resistance to these peptides.<sup>9</sup>

Amphibians represent the main source of AMPs, these compounds are synthesized and stored in the granular glands of the dermal layer of the skin.<sup>10</sup> AMPs and other

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bioactive peptides are synthesized as larger proteins with a signal sequence and an acidic pro-piece that are cleaved to release the mature active peptide before or at the time of secretion from granular.<sup>6</sup> The broad spectrum of pathogenic microorganisms that are inhibited by amphibian AMPs<sup>11</sup> attract attention for the discovery of new structures present in the skin secretions of these vertebrates.

Amphibians belonging to the Hylidae are known as a promising source of AMPs. Members of this family are commonly referred to as “tree-frogs” with adaptations suitable for an arboreal lifestyle, including forward-facing eyes providing binocular vision, and adhesive pads on the fingers and toes.<sup>12</sup> In the non-arboreal species, these features may be greatly reduced, or absent.<sup>12</sup> Several Hylidae genera have been reported as potent AMPs producers, such as *Phyllomedusa*, *Hyla* and *Hypsiboas*, all being found in the Amazon region.<sup>13</sup> *Hypsiboas* is one of the less studied genera found in Brazil, where only *H. punctata* had its peptide content previously evaluated.<sup>13</sup> The green-tree frog (*H. cinerascens*) is a nocturnal and arboreal species largely found in the rain forests of Central and South America<sup>14</sup> not previously studied for its skin secretion’s peptide content.

The discovery of new amphibian AMPs requires the use of sensitive analytical techniques, since the amount of material is limited. In this case, mass spectrometry (MS) plays a central role when coupled to chromatographic techniques allowing the identification of several peptides in a single run, and with a restricted amount of material. Ambiguities such as the identification of isobaric residues (leucine and isoleucine) can be overcome by combination with molecular biology tools. Considering the increasing need for new antibiotics and the fact that the amphibians comprise a known source of biologically active molecules,<sup>11</sup> this work aimed for the identification of AMPs from the skin secretion of the tree frog *H. cinerascens*. An approach comprising high performance liquid chromatography (HPLC), MS and complementary deoxyribonucleic acid (cDNA) sequencing was applied enabling the identification of new AMPs named cinerascetins. Additionally, antimicrobial assays were performed for the synthetic peptide C-01.

## Experimental

### Frog skin secretions

Adult specimens of *Hypsiboas cinerascens* (n = 7) whose sex were not determined were captured after acquisition of a license provided by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

(IBAMA, 22533-1) at Adolpho Ducke Reserve (Manaus, Amazonas State, Brazil) in the rainy season of 2010. All frogs were identified at the Department of Biology from the Federal University of Amazonas. Skin secretions were obtained by a gentle transdermal electrical stimulation of the dorsal skin (6 V)<sup>15</sup> and collected in falcon tubes of 15 mL after washing the cutaneous surface of the specimens with distilled water. Skin secretions of all specimens were combined, frozen and lyophilized yielding 1 mg of crude secretion. After collection, the specimens were released, with the exception of two, that were euthanized for cDNA sequencing and imaging analyses. The secretion was kept at 4 °C prior to being snap-frozen with liquid nitrogen, lyophilized and stored at –80 °C prior to analyses.

### Chromatographic analysis and peptide isolation

The total lyophilized skin secretion was dissolved in 0.6 mL of water with 0.1% trifluoroacetic acid and injected into a reverse phase (C18) column (Vydac 218TP 250 × 10 mm<sup>2</sup>, 5 µm particle size). The chromatographic system was a LC10 AD-VP (Shimadzu). Peptides were eluted out by performing a gradient of acetonitrile with 0.1% trifluoroacetic acid ranging from 5 up to 95% over a period of 60 min under flow of 2.5 mL min<sup>-1</sup>. Peptide elution was monitored at 216 and 280 nm.<sup>16</sup> All solvents used for chromatographic and MS techniques were HPLC grade purchased from J. T. Baker and the water was purified by a Milli-Q system.

### Structural characterization

Chromatographic fractions were dissolved in 10 µL of deionized water and mixed with α-cyano-4-hydroxycinnamic acid matrix solution (5 mg of matrix, 250 µL of deionized water, 50 µL acetonitrile with 0.1% trifluoroacetic acid) in a proportion of 1/3 µL followed by spotting matrix assisted laser desorption ionization (MALDI) target plate.<sup>16</sup> For mass spectra acquisition, a MALDI-time-of-flight in tandem (MALDI-TOF-TOF) UltraFlex III mass spectrometer (Bruker Daltonics) was operated in the reflector mode for MALDI-TOF MS peptide mass fingerprint at a range of *m/z* 600-4000 and in the “LIFT™” mode for MALDI-TOF-TOF tandem mass spectrometry (MS/MS) fragmentation experiments, on fully manual mode using FlexControl software v. 2.2. To process the data obtained and perform manual *de novo* sequencing,<sup>17</sup> Flex Analysis v.3.0 software (Bruker Daltonics) was employed. The ambiguities on the fragmentation for peptides containing isobaric amino acids were approached by the molecular cloning of precursor-encoding cDNA.

## Sequence comparison

All obtained sequences were aligned and subjected to similarities search using the FASTA 3 program on the ExPasy Molecular Server ([www.expasy.ch](http://www.expasy.ch)) and online BLAST analysis (blastp) from the National Center for Biotechnology Information (NCBI). Secondary structure prediction was performed using SOPMA, also at this server.<sup>18</sup>

## Molecular cloning of precursor-encoding cDNA

One specimen was euthanized by injection of a 2% lidocaine solution directly in the brain. Immediately, the skin was removed, frozen with liquid nitrogen and mechanically pulverized. The ribonucleic acid (RNA) isolation of approximately 10 mg of the skin was performed using trizol reagent (Invitrogen). Spectrophotometric analysis ensured the purity and quantity of RNA. The protocol for gene cloning and cDNA sequencing was carried according to Brand *et al.*<sup>19</sup> Briefly, the total RNA (1 µg) was used for the first strand cDNA synthesis using a superscript reverse transcriptase kit (Invitrogen) and an oligo(dT)-anchor primer (5'-GACCACGCGTATCGATGTGCGACTTTT TTTTTTTTTTTT-3').<sup>20</sup> The cDNA amplification reactions employed the four degenerated 5' primers PPS-1 (5'-ATGGCTTTCCTGAARAARTCBCTTTT YCTT GTACTATTCCTTGS-3'), PPS-1A (5'-ATGGCTTTCCTGAAGAAATCTCTTTT CCTTG TACTATT CCTTGG-3'), PPS-2 (5'-ATGGCTTTCCTGAARAARTCBCTTT TYCTTGTATTATTTCTCGG-3') and PPS-2A (5'-ATGGCTTTCCTGAAGAAATCTCTTTT CCTTG TATTATTTCTCGC-3') that were designed based on the highly conserved 5'-signal regions of previously described AMPs cDNAs of anurans of the Hylidae family.<sup>21</sup>

## Imaging analysis

The same euthanasia process was carried for other specimen, and immediately the whole dorsal skin was surgically removed. The dorsal skin was stretched over a glass plate and dried at room temperature. After dryness, the dorsal skin was fixed in a MALDI plate and covered with a thin layer of matrix solution (5 mg of  $\alpha$ -cyano-4-hydroxycinnamic acid, 250 µL of deionized water, 50 µL acetonitrile with 0.1% trifluoroacetic acid). The imaging analysis was performed on an Ultraflex III instrument on a reflective positive mode, with laser intensity at 30% and 200 µm of distance from each acquisition point. The data acquisition and post analysis was performed using

Flex-imaging 3.0 software (Bruker Daltonics) which was programmed to map molecular components ranging from 600 to 4000 *m/z*. The software Biomap was used to perform the ion co-localization analysis. The complete preparation of the dorsal frog skin fragment for MALDI imaging analysis has been described elsewhere.<sup>19</sup>

## Solid phase peptide synthesis

The peptide C-01 was manually synthesized by the solid phase approach using the 9-fluorenylmethoxycarbonyl (Fmoc)/*t*-butyl chemistry according to the previously employed methodology.<sup>22</sup> After chemical de-protection and lyophilization, the peptide was purified by reverse phase HPLC.

## Antimicrobial assay

*Candida albicans* American Type Culture Collection (ATCC) 90028, *Cryptococcus neoformans* ATCC 28957, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used to evaluate the antimicrobial activity of C-01. Dermaseptin-1 (DS01) peptide, a known antimicrobial peptide isolated from frog skin, was used as positive control and pure water was used as negative control. The minimal inhibitory concentrations (MICs) were evaluated by broth microdilution test according to Clinical and Laboratory Standards Institute (CLSI) protocols.<sup>23</sup> For yeasts, CLSI M27-A3 protocol was used. Briefly, yeast cell suspensions from 48 h old Sabouraud dextrose agar (SDA) were prepared in 0.85% (v/v) sodium chloride and diluted at appropriate densities in Roswell Park Memorial Institute (RPMI) 1640 broth (with *L*-glutamine, without bicarbonate, pH 7.0). A total of  $1 \times 10^3$  cells were mixed at different peptides concentration ranging from 256 to 0.5 µM at a final volume of 100 µL. Experimental tests were incubated at  $35 \pm 2$  °C for 48 and 72 h for *C. albicans* and *C. neoformans*, respectively. MIC was determined as the concentration that no visible cell growth was observed after incubation period. All tests were performed in triplicate.

For bacteria, M07-A9 protocol was used.<sup>24</sup> Prior to testing, bacteria were transferred to inclined casoy agar and incubated at  $35 \pm 2$  °C for 24 h. Bacteria cells suspension were prepared in 0.85% (v/v) NaCl, diluted and adjusted to 0.5 McFarland ( $1 \times 10^8$  colony-forming unit (CFU) mL<sup>-1</sup>).<sup>25</sup> Muller Hinton broth was used for antibacterial test. The final volume of 100 µL, containing different peptides concentration and 5 µL of bacteria inoculum were incubated at  $35 \pm 2$  °C for 24 h. The MIC was determined as the concentration that no visible cell growth was observed after incubation period. All tests were performed in triplicate.<sup>26</sup>

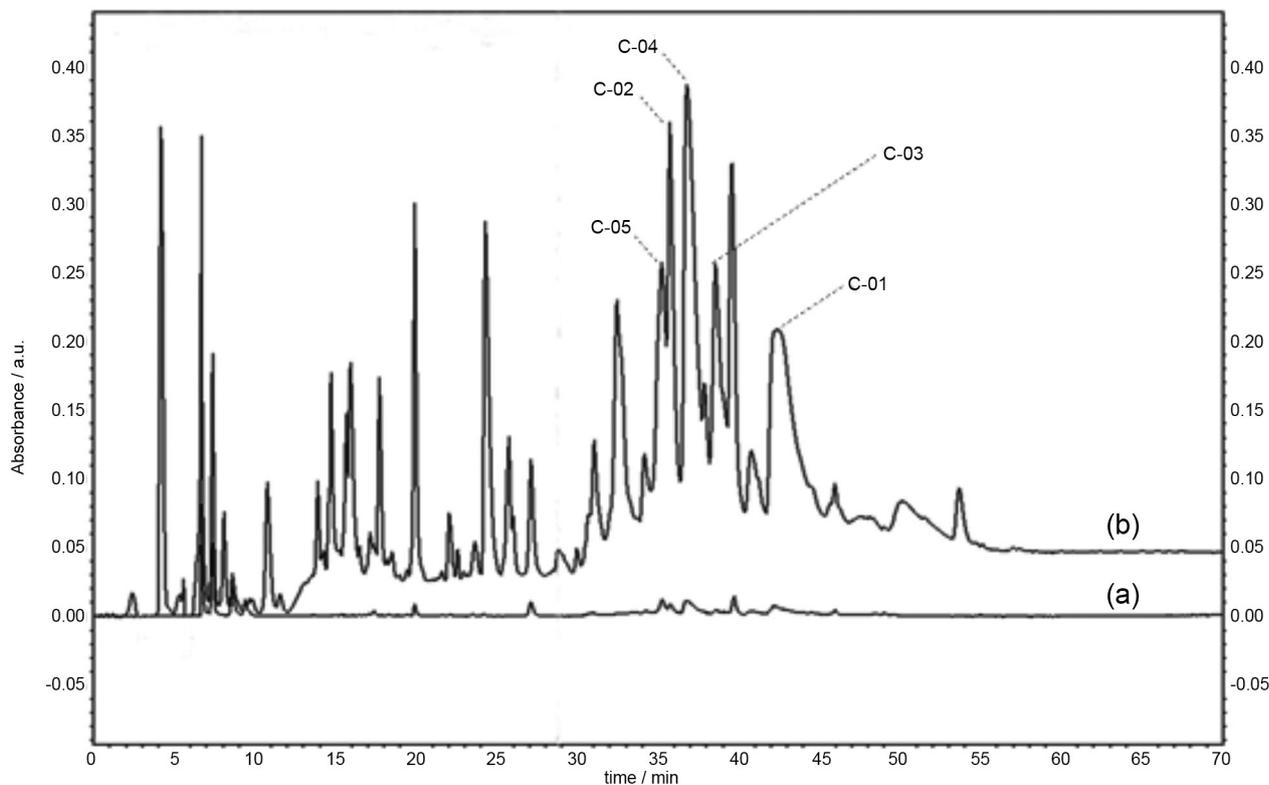
## Results and Discussion

### Peptide identification

The pooled skin secretion obtained from *H. cinerascens* was purified by reverse phase HPLC (Figure 1). Twenty fractions were manually collected and aliquots of each one containing the major peaks in the chromatogram were subjected to MALDI LIFT-TOF-MS/MS analysis. Chromatographic fractions eluting at 42.41, 34.90, 37.94, 36.64 and 34.90 min (Figure 1) displayed ions whose monoisotopic masses were  $m/z$  2386.43, 2395.25, 2165.30, 2393.43 and 2490.50, respectively. MS/MS experiments allowed *de novo* sequencing of the major peaks. The manual interpretation of the product spectra displayed similar fragmentation behaviors, consistent with peptides sharing similar sequences named cinerascetins (C-01 to C-05). The chemical similarities among the isolated peptides demanded a carefully inspection of the fragmentation pattern.

The main peak observed at the chromatographic step (C-01) displayed the  $m/z$  2386.4328 (-1.5 ppm) whose primary structure presented a sequence with 25 amino acid residues (GVI/LDAI/LKAI/LAKAAGKAAI/LQAAGEHI/L-NH<sub>2</sub>). The monoisotopic mass of C-01 it showed to

be 1 Da below the theoretical value (2387.3696). The observed  $m/z$  suggested a post-translational modification. Based on the sequencing and the recorded protonated peptide we proposed the existence of a carboxyamided C-terminal residue. C-02 eluted at 34.90 min ( $m/z$  2395.2525, +1.3 ppm) displaying a similar sequence to C-01, differing only at the residue number 18, where a histidine residue is found instead of a glutamine/lysine. The peptide C-03 ( $m/z$  2165.2939, 3.6 ppm) eluted at 37.94 min and its sequence is composed of 24 amino acid residues (GVSVI/LAI/LAGTI/LAKAAGKAAI/LEAAI/L-NH<sub>2</sub>). The remaining peptides C-04 ( $m/z$  2393.4300, -2.6 ppm) and C-05 ( $m/z$  2490.5047, 3.3 ppm) eluted in 36.64 and 34.50 min, respectively, containing 25 and 26 amino acid residues characterized as GVI/LDKI/LKAI/LAKAAGKAAI/LKAAGESI/L-NH<sub>2</sub> and GVVI/LDKI/LKAI/LAKAAGKAAI/LQAAGEVV-NH<sub>2</sub>. For C-02 to C-05 the same 1 Da shifts on the recorded masses were observed, indicating that all identified peptides include a carboxyamided C-terminal residue. AMPs with such modifications are largely found in Anuran. For the studied genus, *H. pulchellus* displayed peptides with the same post-translational modification.<sup>27</sup> Disambiguation of isobaric residues and confirmation of the post-translational modifications were carried by the cDNA analysis.



**Figure 1.** Chromatographic profile of the total skin secretion obtained from *H. cinerascens*. The fractions containing cinerascetins-01 to 05 (C-01 to C-05) were collected at 42.41, 34.90, 37.94, 36.64 and 34.50 min, respectively. The absorbance was monitored at 216 and 280 nm ((b) and (a) lines, respectively).

### Cloning of cinerascetins biosynthetic precursor-encoding cDNAs

In order to establish the isobaric residues such as leucine and isoleucine and residues with near masses (glutamine/lysine) the biosynthetic precursors were cloned. Four different cinerascetin-encoding sequences were consistently cloned from the skin secretion cDNA library. Unfortunately, from the MS-based identification only C-01 was encoded (Figure 2), whereas the remaining ones represent peptides not identified by MS. Those peptide sequences were named C-06, C-07 and C-08, since they displayed similar sequences when compared with the MS-identified ones. The ambiguities were resolved for C-01, where the obtained sequence was GVLDAIKAIKAAGKAALQAAGEHI-NH<sub>2</sub>. All encoded peptides displayed a glycine at the end of the mature peptide followed by a stop codon, which is a confirmation of C-terminal amination during the peptide cleavage. NCBI-BLAST comparisons were performed with these four encoded peptides revealing that they possess novel sequences with high identities to known AMPs from *Hypsiboas punctatus*: 93% of identity with hylaseptin-P1;<sup>28</sup> *Phyllomedusa distincta*: 65% with dermadistinctin-K;<sup>29</sup> and *Phyllomedusa sauvagii*: 71% with dermasseptin-S5.<sup>30</sup> On the other hand, cinerascetins displayed different sequences when compared with peptides reported from *H. semilineatus*,<sup>31</sup> *H. albopunctatus*,<sup>32</sup> *H. biobeba*,<sup>33</sup> *H. raniceps*,<sup>21</sup> and *H. pulchellus*<sup>27</sup> (Table 1).

### Imaging analysis

Imaging of biological tissues enables the molecular mapping of ions under almost native conditions, preserving morphological and molecular informations. Aiming to

	<u>M</u>	<u>A</u>	<u>F</u>	<u>L</u>	<u>K</u>	<u>K</u>	<u>S</u>	<u>L</u>	<u>F</u>	<u>L</u>	<u>V</u>	<u>L</u>	<u>F</u>	<u>L</u>	<u>G</u>
1	ATGGCTTTC	CTGAAAAG	TCGCTTTT	CTGTACTA	TTCCTTGA										
	TACCGAAG	GACTTTTC	AGCGAAAA	GAACATGAT	AAGGAACCT										
	<u>I</u>	<u>V</u>	<u>S</u>	<u>L</u>	<u>S</u>	<u>I</u>	<u>C</u>	<u>Q</u>	<u>D</u>	<u>E</u>	<u>K</u>	<u>R</u>	<u>E</u>	<u>G</u>	<u>D</u>
51	ATAGTTTCT	CTGTCCATC	TGTCAAGAT	GAGAAAAGA	GAGGGAGAC										
	TATGAAAGA	GACAGGTAG	ACAGTTCTA	CTCTTTTCT	CTCCCTCTG										
	<u>E</u>	<u>Q</u>	<u>Y</u>	<u>E</u>	<u>E</u>	<u>E</u>	<u>E</u>	<u>N</u>	<u>E</u>	<u>E</u>	<u>A</u>	<u>S</u>	<u>E</u>	<u>E</u>	<u>K</u>
101	GAGCAGTAT	GAAGAGGAA	GAAAATGAA	GAGGCAAGT	GAGGAAAAG										
	CTCGTCATA	CTTCTCCTT	CTTTTACTT	CTCCGTCA	CTCCTTTTC										
	<u>R</u>	<u>G</u>	<u>V</u>	<u>L</u>	<u>D</u>	<u>A</u>	<u>I</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>A</u>	<u>K</u>	<u>A</u>	<u>A</u>	<u>G</u>
151	AGAGGAGTA	CTCGATGCA	ATTAAGGCC	ATAGCGAAA	GCAGCTGGT										
	TCTCCTCAT	GAGTACGT	TAATTCGG	TATCGCTTT	CGTCGACCA										
	<u>K</u>	<u>A</u>	<u>A</u>	<u>L</u>	<u>O</u>	<u>A</u>	<u>A</u>	<u>G</u>	<u>E</u>	<u>H</u>	<u>I</u>	<u>G</u>			*
201	AAAGCCGCT	CTTCAAGCT	GCTGGTGAA	CACATAGGT	ACTCGTTAA										
	GGGGCGGA	GAAGTTCGA	CGACCATT	GTGTATCCA	TGAGCAATT										
251	TGATTCATC	TCTATGGAA	CATAACTGT	TAGTTGTGT	CAGACATAT										
	ACTAAGTAG	AGATACCTT	GTATTGACA	ATCAACACA	GTGTGTATA										
301	AATAAAGCA	TATTATCTG	AAAAAANA	AAAAAANA	TCGACATCG										
	TTGTTTCGT	ATAATAGAC	TTTTTTTT	TTTTTTTT	AGCTGTAGC										
351	ATAGCGGTG														
	TATGCGCAC														

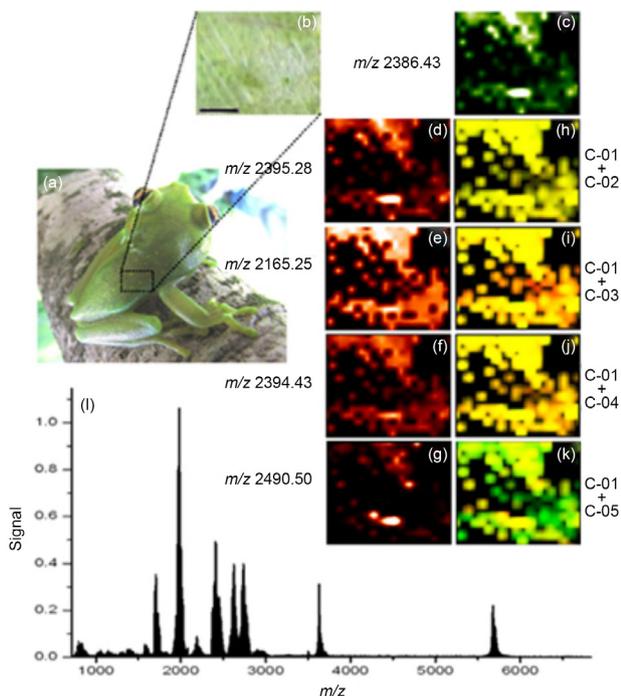
**Figure 2.** Nucleotide and translated amino acid sequences of cloned skin secretion-derived cDNA encoding the biosynthetic precursor of C-01. The signal peptide is shown double-underlined, mature peptide in single-line underline and stop codon is indicated with asterisk. Glycine amino acid residue indicates a possible amination.

map the cinerascetins on the skin tissue and to compare their distribution on the skin, the total dorsal tissue of *H. cinerascens* was submitted to imaging acquisition. To facilitate observation of results, a representative fragment of analyzed skin is demonstrated in Figure 3. Pictorial representation of C-01 ( $m/z$  2386.43), C-02 ( $m/z$  2395.25), C-03 ( $m/z$  2165.29), C-04 ( $m/z$  2393.43) and C-05 ( $m/z$  2490.50) are shown in Figures 3c to 3g, respectively. Co-localization between C-01 (green) and the other related peptides (red) are shown in Figures 3h to 3k, respectively. The areas shared between two ions are shown in yellow. The technique was able to detect all reported cinerascetins in this work. The results indicate that C-01 is present in a great area of the dorsal tissue and that the other related peptides shared the same area. Thus, based on the primary structure and spatial location, the peptides C-02, C-03, C-04 and C-05 probably have similar biological role as C-01.

**Table 1.** General characteristic of antimicrobial peptides derived from *Hypsiboas* species

Organism	Peptide	Sequence	No. <sup>b</sup>	Mass <sup>c</sup>
<i>H. cinerascens</i>	cinerascetin-01	GVLDAIKAIKAAGKAALQAAGEHI-NH <sub>2</sub>	25	2386.43
<i>H. semilineatus</i>	Hs-1	FLPLILPSIVTALSSFLKQG	20	2144.60
<i>H. albopunctatus</i>	hylin-1a	IFGAILPLALGALKNLIK	18	1864.37
	Ctx-Ha	GWLDVAKKIGKAAFVAKNFL/I	21	2289.73
<i>H. biobeba</i>	hylin-b1	FIGAILPAIAGLVHGLINR	19	1945.36
<i>H. punctata</i>	hylaseptin-P1	GILDAIKAIKAAG	14	1311.80
	phenylseptin	FFFDLTKNLGKLVIGALT-NH <sub>2</sub>	18	1954.20
<i>H. raniceps</i>	Rsp-1 <sup>a</sup>	AWLDKLSLGVVGVKVALGVQNYLNPQQ	29	–
<i>H. pulchellus</i>	P1-Hp-1971	TKPTLLGLPLGAGPAAGPGKR-NH <sub>2</sub>	21	1971.35

<sup>a</sup>Determined only by cDNA analysis; <sup>b</sup>No.: number of residues; <sup>c</sup>monoisotopic protonated peptide [M + H]<sup>+</sup>; <sup>d</sup>structure confirmed by cDNA sequencing.



**Figure 3.** Imaging analysis of *H. cinerascens* skin. (a) Adult specimen of *H. cinerascens*; (b) analysis of the skin fragment; (c)-(g) pictorial representation of C-01 to C-05; (h)-(k) colocalization between C-01 and the other peptides; (l) global spectrum of detected ions.

#### Solid phase synthesis and antimicrobial assay

Based on the structure of peptides characterized here and knowledge about the amphibian peptides antimicrobial potential, C-01 was selected to be manually synthesized for its antimicrobial evaluation. The peptide was prepared on a solid phase system and the purified peptide assayed against the yeast *Cryptococcus neoformans*, the fungus *Candida albicans*, the gram-positive bacteria *Staphylococcus aureus* and the gram-negative bacteria *Escherichia coli*, all human pathogens.<sup>34</sup> The synthetic peptide was also evaluated against *Xanthomonas axonopodis* pv. *glycines*, a plant pathogenic bacteria. The peptide dermaseptin-01 (DS01) obtained from *Phyllomedusa hypochondrialis* was used as positive control due to its antimicrobial properties.<sup>15</sup>

The peptide C-01 was able to inhibit the growth of all tested microorganisms with MIC values ranging from 4 to 16  $\mu\text{M}$  (Table 2). The uncommon broad spectrum activity of C-01 was shown to be more active than peptides previously described in *Hypsiboas* such as Rsp-1 (MIC of 20  $\mu\text{M}$  against *S. aureus*)<sup>21</sup> and hylaseptin-P1 (MIC of 24.2  $\mu\text{M}$  against *E. coli*).<sup>28</sup> Over the last years a wide range of antimicrobial cationic peptides have been shown to play a significant role in host defenses.<sup>35</sup> The mode of action of these peptides against gram-positive bacteria is well known,<sup>36</sup> where the ability to form channels in lipid bilayer

membranes seems to be most accepted theory.<sup>35</sup> On the other hand the mechanism against gram-negative bacteria and fungi still remains unclear.<sup>36</sup> The cationic nature of C-01 must be taken into account to determine the possible mechanism (76% of  $\alpha$ -helix according to self-optimized prediction method with alignment (SOPMA) prediction). Similar anuran peptides such as dermaseptin-01 are also cationic, and it has been suggested that the membrane surface in the presence of negatively charged lipids is associated with a “carpet-like” manner, breaking the lipid bonds due the reduction of repulsive electrostatic forces between positively charged peptides.<sup>37</sup> The similarities among dermaseptin-01 and C-01 may be the key to establish the mechanism of action, however, more studies to confirm this hypothesis are needed. Additionally, the presence of a carboxyamidated C-terminal moiety has been shown as a factor of increasing the antimicrobial activity, possibly by a decrease of degradation rate by carboxypeptidases.<sup>38</sup>

**Table 2.** Antimicrobial activities of C-01

Pathogen	MIC / $\mu\text{M}$ <sup>a</sup>		
	C-01 <sup>b</sup>	DS01 <sup>c</sup>	Water <sup>d</sup>
<i>C. albicans</i>	10.00	7.64	n.d. <sup>e</sup>
<i>C. neoformans</i>	16.00	8.00	n.d. <sup>e</sup>
<i>E. coli</i>	16.00	1.00	n.d. <sup>e</sup>
<i>S. aureus</i>	10.00	8.00	n.d. <sup>e</sup>
<i>X. axonopodis</i> pv. <i>glycines</i>	< 4.00	n.d. <sup>e</sup>	n.d. <sup>e</sup>

<sup>a</sup>MIC ( $\mu\text{M}$ ): minimal peptide concentrations required for total inhibition of cell growth in liquid medium; <sup>b</sup>C-01: synthetic peptide; <sup>c</sup>DS01: positive control; <sup>d</sup>water: negative control; <sup>e</sup>n.d.: not detected.

#### Conclusions

Using chromatography and MS it was possible to identify and characterize five new peptides. These compounds displayed sequences with 24 to 26 amino acid residues. Molecular cloning of precursor-encoding cDNA was performed and four sequences were identified, being one previously detected by MS. Imaging analysis with the dorsal skin allowed the localization of the MS identified peptides throughout the whole tissue. These peptides were assigned as new peptides related to hylaseptin-P1, displayed the C-terminal region with amination as post translational modifications. The synthetic peptide C-01 displayed *in vitro* antimicrobial activity against the tested organisms. The presented results are part of the continuous search for novel substances that can serve as models for new antibiotics. This work constitutes the first report on the peptide constitution of *H. cinerascens*.

## Supplementary Information

Supplementary data (imaging and MALDI LIFT-TOF-MS/MS spectra of C-01, C-02, C-03, C-04 and C-05 and sequences of cDNA of C-06, C-07 and C-08) are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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