

# Cleavage of Peptides from Amphibian Skin Revealed by Combining Analysis of Gland Secretion and in Situ MALDI Imaging Mass Spectrometry

Andrés E. Brunetti,<sup>\*,†,‡,§</sup> Mariela M. Marani,<sup>||</sup> Rafael A. Soldi,<sup>†</sup> Jacqueline Nakau Mendonça,<sup>†</sup> Julián Faivovich,<sup>§,⊥</sup> Gabriela M. Cabrera,<sup>‡,#</sup> and Norberto P. Lopes<sup>\*,†</sup>

<sup>†</sup>Faculty of Pharmaceutical Sciences of Ribeirão Preto, Department of Physics and Chemistry, University of São Paulo, Avenida do Café, s/no, 14040-903 Ribeirão Preto, Brazil

<sup>‡</sup>Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 3° piso, C1428EHA Buenos Aires, Argentina

<sup>§</sup>División Herpetología, Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Avenue Angel Gallardo 490, C1405DJR Ciudad de Buenos Aires, Argentina

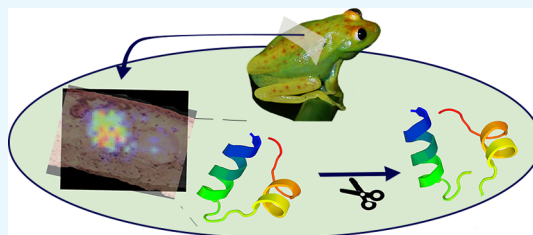
<sup>||</sup>PEEC—CONICET, Consejo Nacional de Investigaciones Científicas y Técnicas, Bvd. Brown 2915, U9120ACD Puerto Madryn, Argentina

<sup>⊥</sup>Facultad de Ciencias Exactas y Naturales, Departamento de Biodiversidad y Biología Experimental, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 4° piso, C1428EHA Ciudad de Buenos Aires, Argentina

<sup>#</sup>Facultad de Ciencias Exactas y Naturales, Unidad de Microanálisis y Métodos Físicos aplicados a la Química Orgánica (UMYFOR), CONICET—Universidad de Buenos Aires, Ciudad Universitaria, Pabellón II, 3° piso, C1428EHA Buenos Aires, Argentina

## Supporting Information

**ABSTRACT:** Peptides from skin secretions of amphibians are considered important components of their immune system and also play a relevant role in their defense mechanism against predators. Herein, by using mass spectrometry (MS), we characterize the sequence of 13 peptides from the gland secretion of the hyloid tree frog, *Boana punctata*. Using in situ matrix-assisted laser desorption ionization imaging MS of a transverse section of the skin tissue, we show that some peptides are stored as longer molecules that are cleaved after being secreted, whereas others do not undergo any modification. Sequence comparison with peptides from other *Boana* species and analysis of the three-dimensional theoretical structure indicate that this cleavage depends on both the presence of a specific sequence motif and the secondary structure. The fact that peptides undergo a rapid cleavage upon secretion suggests that stored and secreted peptides may have distinct roles for anuran survival, including defense against pathogens and predators.



## 1. INTRODUCTION

Mass spectrometry (MS) has become a powerful tool to investigate the chemistry associated with diverse biological systems.<sup>1</sup> This technique allows to detect hundreds to thousands of ions from a single biological sample and also to detect specific classes of compounds.<sup>2</sup> Thus, MS opens exciting perspectives to deeply investigate chemical interactions in nature.<sup>3</sup> Within different biological contexts, MS-based protocols allow a more holistic approach of chemical interactions through proteomics and metabolomics.<sup>2,3</sup> In addition, the improvement of MS-imaging (IMS) reinforces the importance of MS protocols in assisting different fields of biology to assess some of the dogmas of biology.<sup>3</sup>

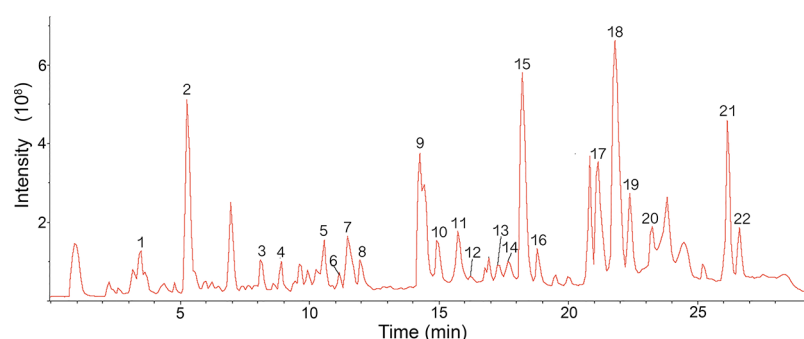
Peptides from skin secretions of anuran amphibians (frogs and toads) have been the subject of numerous studies since the early 90s.<sup>4–6</sup> Much of the interests in anuran peptides are

related to the high concentration in which they are secreted, when compared with analogue substances in mammals,<sup>4,5</sup> as well as to their promising neuroactive and antimicrobial activities.<sup>3</sup> During the last decade, identification of the amino acid sequence of anuran peptides was predominantly conducted through MS techniques, mainly matrix-assisted laser desorption ionization (MALDI), which allowed for characterization of a high diversity of structures in several species from different families.<sup>6</sup> These peptides are stored within serous glands (SGs) and are released on the skin surface under different stress situations.<sup>7</sup> A few studies have suggested that at least some primary peptides are cleaved immediately after secretion.<sup>8–10</sup>

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**Table 1.** Total Ion Chromatogram (TIC) and Compounds Identified in the Gland Secretion of Dorsal Skin of a Male of *B. punctata*<sup>a</sup>

peak no.	$T_R$ (min)	compound	ID <sup>c</sup>	sequence <sup>d</sup>	precursor ion		error (ppm)	[M + H] <sup>+</sup> exp
					experimental $m/z$	theoretical $m/z$		
1	3.5	peptide <sup>b</sup>	-	ND	[M + H] <sup>+</sup> = 842.5453	ND	ND	842.5453
2	5.3	peptide	P1_Bpt-1066	<u>A</u> HXAKAAGESX-NH <sub>2</sub>	[M + 2H] <sup>2+</sup> = 533.8045	[M + 2H] <sup>2+</sup> = 533.8038	1.3	1066.6090 <sup>e</sup>
3	8.2	peptide <sup>b</sup>	-	ND	[M + H] <sup>+</sup> = 788.4918	ND	ND	788.4918
4	8.9	peptide	P3_Bpt-561	XGAXTS-OH	[M + H] <sup>+</sup> = 561.3212	[M + H] <sup>+</sup> = 561.3242	2.6	561.3212
5	10.6	peptide	P2_Bpt-1099	<u>K</u> DAXVATAKAX-NH <sub>2</sub>	[M + 2H] <sup>2+</sup> = 550.3457	[M + 2H] <sup>2+</sup> = 550.3453	0.8	1099.6914 <sup>e</sup>
6	11.1	hyloin	Hy-G1	-	-	-	ND	486.3553
7	11.5	hyloin	Hy-G2	-	-	-	ND	488.3702
8	12.0	peptide <sup>b</sup>	-	ND	[M + 2H] <sup>2+</sup> = 572.3177	ND	ND	1143.6355 <sup>e</sup>
9	14.3	peptide	P4_Bpt-1274	KVAXGXAKNFXT-OH	[M + 2H] <sup>2+</sup> = 637.8957	[M + 2H] <sup>2+</sup> = 637.8951	0.9	1274.7915 <sup>e</sup>
10	14.9	peptide	P5_Bpt-1302	KVVXGXAKNFXT-OH	[M + 2H] <sup>2+</sup> = 651.9108	[M + 2H] <sup>2+</sup> = 651.9108	0.0	1302.8216 <sup>e</sup>
11	15.7	peptide	P6_Bpt-1242	<u>K</u> TVVPMXANAXS-NH <sub>2</sub>	[M + 2H] <sup>2+</sup> = 621.8633	[M + 2H] <sup>2+</sup> = 621.8655	3.6	1242.7266 <sup>e</sup>
12	16.2	peptide	P7_Bpt-1432	GFVDTXKKXGKVAG-OH	[M + 2H] <sup>2+</sup> = 716.9307	[M + 2H] <sup>2+</sup> = 716.9297	1.1	1432.8611 <sup>e</sup>
13	17.3	peptide <sup>b</sup>	-	ND	[M + 2H] <sup>2+</sup> = 656.4211	ND	ND	1311.8422 <sup>e</sup>
14	17.7	peptide	P8_Bpt-1300	FXGXAXKXGKAVA-OH	[M + 2H] <sup>2+</sup> = 650.9207	[M + 2H] <sup>2+</sup> = 650.9212	0.7	1300.8414 <sup>e</sup>
15	18.2	peptide	P9_Bpt-1405	GFVDTXKTXGKAG-OH	[M + 2H] <sup>2+</sup> = 703.4065	[M + 2H] <sup>2+</sup> = 703.4061	0.7	1405.8131 <sup>e</sup>
16	18.8	peptide <sup>b</sup>	-	ND	[M + 2H] <sup>2+</sup> = 710.4217	ND	ND	1419.8435 <sup>e</sup>
17	21.1	peptide	P10_Bpt-1340	GXGDXXKNXAKAAG-OH	[M + 2H] <sup>2+</sup> = 670.8985	[M + 2H] <sup>2+</sup> = 670.8984	0.1	1340.7970 <sup>e</sup>
18	21.8	peptide	P11_Bpt-1297	GVXDAXKAXAKAAG-OH	[M + 2H] <sup>2+</sup> = 649.3957	[M + 2H] <sup>2+</sup> = 649.3955	0.3	1297.7915 <sup>e</sup>
19	22.4	peptide <sup>b</sup>	-	ND	[M + 2H] <sup>2+</sup> = 654.4392	ND	ND	1307.8785 <sup>e</sup>
20	23.3	peptide <sup>b</sup>	-	ND	[M + H] <sup>+</sup> = 676.3025	ND	ND	676.3025
21	26.1	peptide	P12_Bpt-1272	FFFDTXKNXAG-OH	[M + 2H] <sup>2+</sup> = 636.8322	[M + 2H] <sup>2+</sup> = 636.8347	4.0	1272.6644 <sup>e</sup>
22	26.6	peptide	P13_Bpt-1661	XXEPXXNXVKGXNX-NH <sub>2</sub>	[M + 2H] <sup>2+</sup> = 831.0394	[M + 2H] <sup>2+</sup> = 831.0398	0.4	1661.0789 <sup>e</sup>

<sup>a</sup>The secretion was obtained by surface electrical stimulation (SES). <sup>b</sup>Compounds were identified as peptides through analysis of MS/MS fragmentation pattern, but the sequences could not be determined due to the low abundance of the ions in the MS/MS spectra. In addition, they may have post-translational modification that hindered the sequence determination. <sup>c</sup>Only sequence-identified peptides were named. <sup>d</sup>The amino acids were identified in one-letter code, except X = L, I. Underlined amino acids correspond to those whose order were not established. Post-translational C-terminal amidation is indicated with -NH<sub>2</sub> at the end of the sequence. <sup>e</sup>Manually calculate from precursor ion [M + 2H]<sup>2+</sup> or [M + 3H]<sup>3+</sup>. ND, not determined.

Whereas the benefits of this cleavage are not understood,<sup>8,10</sup> combined strategies using different MS techniques may be useful to examine the occurrence and potential function of cleavage mechanisms in different species.

Hylidae is one of the largest families of amphibians<sup>11</sup> and is considered one of the most important sources of bioactive peptides.<sup>6</sup> Most research efforts have been focused on the subfamilies, Pelodyadinae and Phyllomedusinae, whereas information on skin peptides in species of the largest subfamily Hylinae (706 species) is scarce. Possible explanations to justify this deficiency include the use of poorly sensitive methodologies in pioneer studies<sup>9</sup> and inadequate bioassays.<sup>6</sup> Nevertheless, of the few reports on cleaved peptides as a result of postsecretory events, most come from studies in the species of the Hylinae tribe Cophomantini.

*Boana punctata* is a common tree frog species with a wide distribution in South America.<sup>12</sup> It is closely associated with emergent aquatic vegetation in large water bodies.<sup>12</sup> This species has become well-known very recently because it is the first species of amphibian known to have natural fluorescence.<sup>13</sup> In addition, it is the first species of the tribe Cophomantini for which the structure of peptides from skin secretion has been described. *B. punctata* is a very interesting model to investigate peptides in anurans from morphological, functional, and evolutionary perspectives because the structure and ultra-structure of the skin has been well-characterized.<sup>14</sup> Moreover, the skin secretion has at least two peptides, one with antimicrobial activity (hylaseptin P1; HSP1), which may actually be a cleavage form of a larger peptide,<sup>9,15</sup> and a second one, which is likely related to an antipredatory function (phenylseptin).<sup>9,10</sup>

Here, we report, on the basis of MS analyses of skin gland secretions and direct skin IMS analysis in transverse tissue sections, compelling evidence for a postsecretory peptide cleavage. Sequence alignment, including all other peptides reported in Cophomantini, suggests that the postsecretory peptide cleavage is site-specific and may occur in other species of the genus *Boana*. Our results provide valuable information on peptide storage and secretion from the amphibian's skin glands and may assist future research on the function and mechanism of action of these substances.

## 2. RESULTS AND DISCUSSION

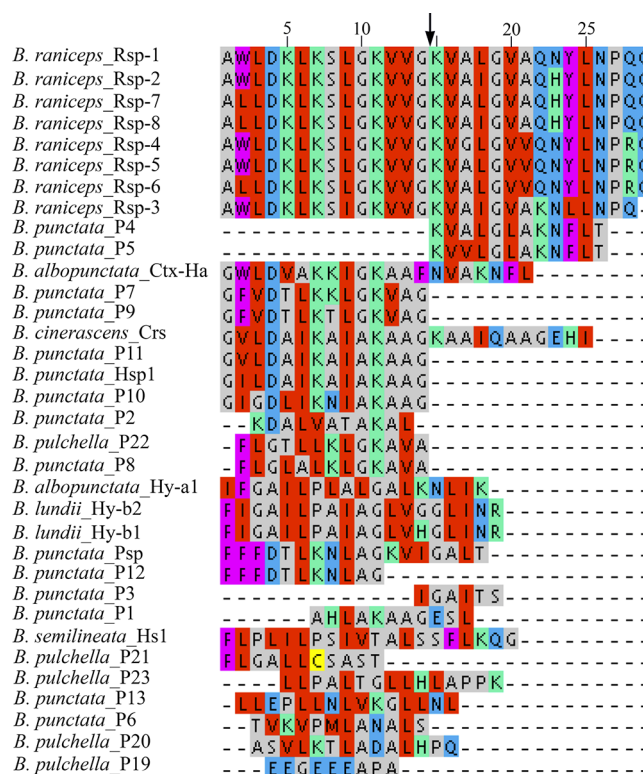
### 2.1. Peptide Identification from Skin Gland Secretion.

Lyophilized dorsal skin secretions of males and females of *B. punctata* were solubilized and analyzed by high-performance liquid chromatography-electrospray ionization MS (ESI-MS) without any previous separation step. Fractionation of the crude extract gave a rich chromatographic profile with more than 22 chromatographic peaks (Table 1). Tandem MS analyses (ESI-MS/MS) allowed identification of 20 peptides, along with the Hyloin fluorophores hyloin G-1 (Hy-G1) and hyloin G-2 (Hy-G2; Table 1).<sup>13</sup> The primary structures of 13 peptides were determined by de novo sequencing through MS/MS analysis of the type *b* and *y* + 2 fragment ions from the precursor ions  $[M + H]^+$ ,  $[M + 2H]^{2+}$ , and  $[M + 3H]^{3+}$  observed in the MS spectrum of each peptide. Table 1 also shows a comparison between *m/z* experimental and *m/z* theoretical data for precursor ions of each sequenced peptide. An example of the sequence process is shown in Figure S1 and Table S1 (under the Supporting Information) for peptide P9, whereas detailed analyses of *b* and *y* + 2 ions observed for each peptide are depicted in Table S2. Because of low abundances of some ions in the MS/MS spectrum, the sequence of the remaining seven peptides could not be identified. For this reason, we do not discard that they contain posttranslational modifications or that they are cyclized.

Four of the 13 identified peptides have an amidated C-terminus, whereas the other nine show the C-terminus in acidic form. The proposed primary structures of the peptides P4, P5, P10, and P11 were confirmed by chemical synthesis. An example of the MS/MS fragmentation comparison between the natural and synthetic form of the same peptide is depicted in Figure S2. The uncertainty owed to the presence of Ile/Leu in the sequence of these peptides was inferred by comparison of retention times of natural and synthetic peptides (Table S4).

**2.2. Skin Peptides in Cophomantini Tree Frogs.** A search in the APD database revealed a 92 and 78% sequence similarity between peptides P11 and P10, identified here in *B. punctata*, and HSP1, an antimicrobial peptide (AMP) identified in this same species from a different locality.<sup>9</sup> A quick comparison with peptides reported in other species of the tribe Cophomantini suggested some sequence similarities (Figure 1). We examined these similarities by aligning all peptide sequences published in species of this tribe, which include eight species from the genus *Boana* that were extracted using surface electrical stimulation (SES).<sup>9,10,15–19</sup> Nevertheless, we have excluded from the analysis 18 peptides identified in *Boana pulchella* having a very low amino acid sequence similarity (data not shown), which were obtained by solvent extraction.<sup>19</sup>

Sequence similarity between the peptides described here and those found in other *Boana* tree frogs suggests putative homologies. For instance, peptide P8 described here shares



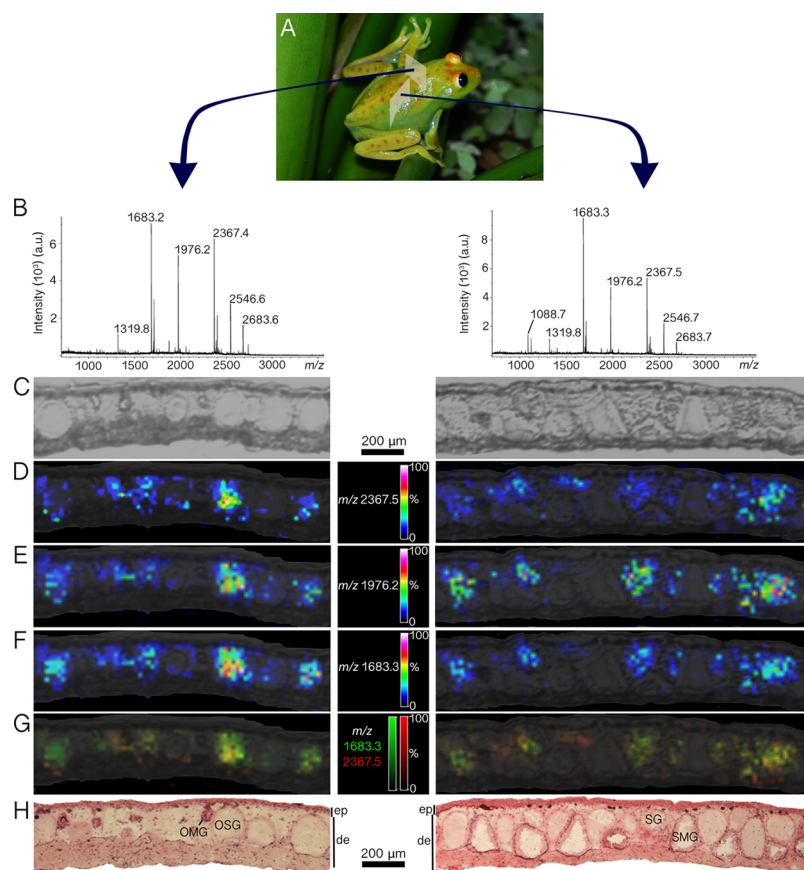
**Figure 1.** Amino acid sequence alignment of the 13 peptides identified in the gland secretion from dorsal skin of *B. punctata* and peptides described in other Cophomantini tree frogs. Arrow indicates the proteolytic cleavage site reported by de Magalhães and co-workers.<sup>18</sup>

85% sequence similarity with peptide P22 described in *B. pulchella*. Additionally, the difference in one amino acid between HSP1 and peptide P11 (current work) described for different populations of *B. punctata* indicates potential differences at population levels. Although some authors<sup>20,21</sup> have proposed that skin peptides profile may be used in combination with other information sources, such as molecular and morphological characters to discriminate hybrids, species, and clades at higher taxonomic levels, no systematic analysis has been performed to assess this suggestion. Furthermore, because peptide profile may vary according to environmental factors,<sup>22</sup> a better understanding of the variability and evolution of these substances represents the necessary previous steps to its use in systematics.

The peptide alignment (Figure 1) shows a partial overlap of some of the sequences reported herein with longer sequences from other *Boana* species. Because the 13 peptides were observed in all samples examined in this study, a likely explanation is the existence of a specific cleavage mechanism. A proteolytic cleavage site was described in *Boana raniceps* for ranisepetins (Figure 1 arrow).<sup>18</sup> As reported by these authors, cleaved peptides were most commonly detected in the gland secretion, whereas full-length ranisepetins were only observed when low temperature and pH conditions were controlled during the extraction procedures. Although we froze the gland secretion at  $-80\text{ }^{\circ}\text{C}$  within 5 min after extraction, a study carried out with *Xenopus laevis*<sup>8</sup> considered a few minutes delay to be enough to alter the relative amounts of intact peptides.

An endopeptidase, specifically a metalloprotease, identified from the skin of *X. laevis* has been described to be responsible for peptide cleavage in this species.<sup>23</sup> According to Giovannini





**Figure 2.** In situ MALDI IMS of dorsal (left panel) and lateral skin regions (right panel) of *B. punctata*. (A) Adult specimen (male). (B) Global MS spectra of detected ions. (C) Scan image of transverse sections. (D–G) MALDI-MS images reconstructed with ions  $m/z$  2367.5 (D), 1976.2 (E), and 1683.3 (F) and colocalization of ions  $m/z$  1683.3 and 2367.5 (G). (H) Light micrographs stained with hematoxylin and eosin. ep, epidermis; de, dermis.

and co-workers,<sup>8</sup> this enzyme would be able to recognize a Xaa-Lys bond (Xaa = Leu, Gly, Ala, or Lys), whereas, according to Resnick and co-workers,<sup>23</sup> the enzyme would hydrolyze peptides on the basis of a specific secondary structure (see below). In *X. laevis*, the enzyme is stored in SGs and is cosecreted with peptides.<sup>23</sup> As it can be observed in Figure 1, raniseptins and cinerascetin show the motif Gly-Lys between residues 14 and 15, suggesting the existence of a putative cleavage site. Partial overlap of peptides P4 and P5, which have Lys in the N-terminal, with the second half of raniseptins and of peptides P7, P9, P10, and P11, and hylaseptin-P1 (HSP-1), which has Gly in the C-terminal, with the first half of raniseptins and cinerascetin, suggests that cleavage of larger intact peptides has occurred in *B. punctata*. Furthermore, phenylseptin, previously described for *B. punctata*, has a Gly-Lys motif at amino acids 10 and 11, which may also explain the presence of the shorter peptide P12, with 100% overlap with the former and a Gly in the C-terminal.

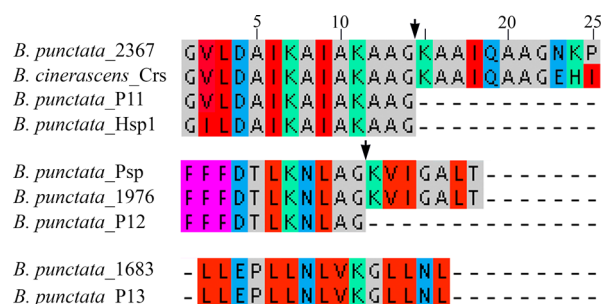
It is also interesting to observe that peptides that end in Gly or Ala have their C-terminal in the form of carboxylic acid, whereas peptides that start with Lys have the C-terminal amidated (P2), suggesting that the latter corresponds to the C-terminal region of an intact mature peptide (exception was observed for P4 and P5). Gly-Lys bonds also occur in position 9–10 in P8 (this work) and P22 (*B. pulchella*) and in position 10–11 in raniseptins, CtL-Ha, P7, and P9. The absence of cleavage peptides at this position may be related to the sequence context next to the potential cleavage site, which is

influenced by the secondary structure of the intact mature peptides.<sup>8,18,24</sup> Gershon<sup>24</sup> has found that residues located proximate to the scissile cleavage site (Lys) can produce an inhibitory effect toward tryptic attack. For instance, residues such as Asp between position –3 and +1, Thr between positions –3 and +2, Ile at positions –2 and –1, and Val at position –1 suppressed the cleavage of Lys-Xaa. These findings may explain the absence of other peptide fragments in the skin secretion analysis of *B. punctata*, despite the occurrence of different putative cleavage sites. For example, although peptide P-1976 has two Lys in its sequence at Gly<sup>11</sup>Lys<sup>12</sup> and Leu<sup>6</sup>Lys<sup>7</sup>, we only observed the peptides derived from the former cleavage. The absence of cleavage in the latter could be explained by the presence of Asp<sup>4</sup> at position –3 of Lys<sup>7</sup>.

**2.3. Spatial Distribution of Peptides in the Skin of Frogs.** Distribution of skin peptides in *B. punctata* was examined by MALDI imaging of transverse sections of dorsal and lateral skin tissues using MS and MS/MS (Figure 2). This sample preparation and a laser scanning resolution of 20 μm allowed discrimination of the precise distribution and sequence of peptides within different gland types occurring in this species. In addition, transverse sections of the same samples were processed by classical histological methods and viewed in a light microscope. Light microscopy (LM) examination along with published detailed structural and ultrastructural analyses of the skin of this species<sup>14</sup> aided in the interpretation of IMS data.

Figure 2A shows an adult male of *B. punctata* and the skin regions dissected for analysis, whereas Figure 2B shows the global mean MS spectra of the dorsal and lateral skins, respectively. Analyses of MALDI-MS and MS/MS IMS data confirmed the presence of at least six peptides corresponding to ions  $m/z$  1319.8,  $m/z$  1683.3,  $m/z$  1976.2,  $m/z$  2367.5,  $m/z$  2546.7, and  $m/z$  2683.7 in both skin regions and the presence of ion  $m/z$  1088.7 exclusively in the lateral region (Figures 2B and S3). The peptides were found within SGs in both lateral and dorsal skins and were not detected either in ordinary mucous glands in the dorsal skin or in specialized mucous glands in the lateral skin (Figure 2D–G). Because of limitations of the hematoxylin and eosin staining, which did not allow distinguishing ordinary SGs from specialized SGs, we were not able to establish the precise location of peptides on SGs (Figure 2H, right side). To the best of our knowledge, this is the first attempt to show the distribution of chemical substances from the skin of amphibians in transverse sections. One of the main advantages of this sample preparation is that it allows for examining the differential distribution of compounds within different gland types.

Direct MS/MS analysis of the skin transverse section enabled determination of the sequence of peptides corresponding to ions  $m/z$  1683.3,  $m/z$  1976.2, and  $m/z$  2367.5 (Figure S3; Table S3), whereas the sequences of remaining ions could only be partially determined. Sequence comparisons with peptides found in the skin gland secretion indicate that ion  $m/z$  1683.3 corresponds to peptide P13, whereas ions  $m/z$  1976.2 and  $m/z$  2367.5 correspond to full-length peptides that were cleaved after the glands secreted their content. Specifically, peptide P12 found in the gland secretion would be the result of cleavage of ion  $m/z$  1976.2 between Gly<sup>11</sup> and Lys<sup>12</sup>, (Figure 3), and



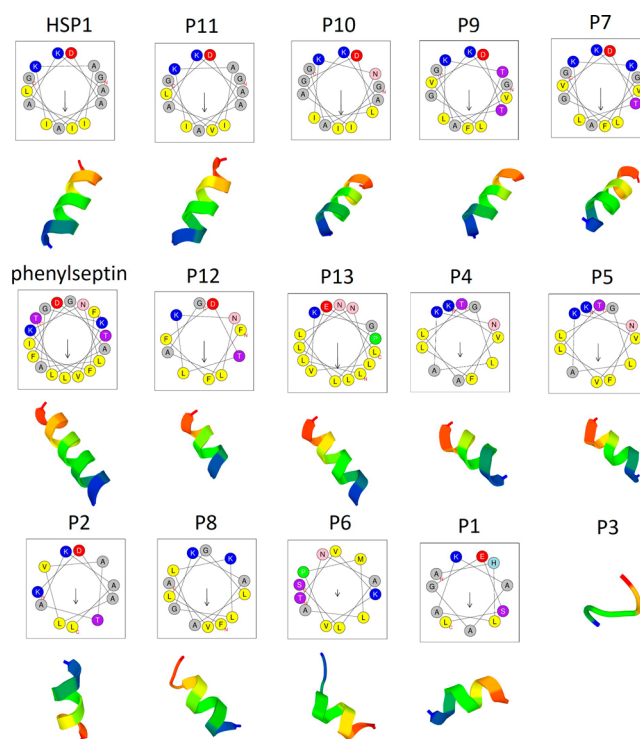
**Figure 3.** Amino acid sequence alignment of three of the peptides identified in situ by MALDI IMS in the transverse section of dorsal and lateral skin samples of *B. punctata* (*B. punctata*\_2367, *B. punctata*\_1976, and *B. punctata*\_1683) and similar or identical peptides identified in the gland secretion of *B. punctata* [*B. punctata*\_P11, *B. punctata*\_P12, and *B. punctata*\_P13 (this article); *B. punctata*\_Hsp9 and *B. punctata*\_Psp10 and *B. cinerascens*\_Crs].<sup>15</sup> Arrows indicate putative proteolytic cleavage site based on sequence identities between peptides identified in situ MALDI IMS and peptides identified in the gland secretion.

peptide P11 would be that of cleavage of ion  $m/z$  2367.5 between Gly<sup>14</sup> and Lys<sup>15</sup>. It is worth noticing that phenylseptin, which was identified in the skin gland secretion from specimens in another population of *B. punctata*,<sup>10</sup> is identical to ion  $m/z$  1976.2, and that the first 16 N-terminal amino acids are identical in both, ion  $m/z$  2367.5 and cinerascetin, found in *Boana cinerascens*.<sup>15</sup>

#### 2.4. Three-dimensional (3D) Theoretical Structure Analysis and Activity Projection. 3D theoretical structure

analysis of the identified peptides in *B. punctata* shows that they all exhibit similar characteristics to those present in active AMPs. The mechanism of action of AMPs is primarily based on the perturbation of the target cell wall membrane.<sup>25</sup> The vast variability of primary structures of antimicrobial sequences found in diverse amphibian species shows that selectivity for different membrane lipid compositions is not due to the linear sequence but due to the 3D structure and the distribution of their residues. Most AMPs are cationic molecules with an amphipathic distribution of the residues that allow them first to approach the membrane through electrostatic interactions and subsequently disrupt the cell wall membrane through hydrophobic interactions.

Twelve of the thirteen peptides identified here in *B. punctata* present a theoretical 3D structure with an  $\alpha$ -helix motif (Figure 4). Schiffer and Edmundson wheel projection diagrams show



**Figure 4.** 3D structure prediction and Schiffer and Edmundson wheel projection diagrams of the peptides identified from the skin secretion of *B. punctata*. Diagram shows  $\alpha$ -helix motifs with amphiphilic peptide structures having a hydrophobic and a hydrophilic region. Amino acid color code: yellow = unpolar/hydrophobic (Leu, Val), gray = Gly, blue = basic (Lys, Arg), purple = polar without charge (Thr), pink = polar without charge (Asn), and green = Pro.

an amphipathic distribution of the amino acid residues (Figure 4),<sup>26</sup> where 9 identified peptides were cationic, with +1 (P2, P6, P9, P10, P11) or +2 (P4, P5, P7, P8) net charges, and 12 peptides have at least 5 hydrophobic residues on the same surface, granting a well-defined hydrophobic side. These characteristics suggest that the peptides could interact with membranes, both in an electrostatically and a hydrophobically way, with the prospect to be AMPs.

Combinatorial techniques and, specifically, the development of soluble synthetic combinatorial libraries with the use of positional scanning deconvolution process, show that there are most important amino acid(s) or building block(s) for every position and many acceptable conservative substitutions that do

not alter the activity potency (e.g., valine for isoleucine and serine for threonine).<sup>27</sup> This observation suggests that P11 will have the same 3D structure and antimicrobial activity described for HSP-1. These peptides differ in only one conservative substitution (Ile<sup>2</sup> for Val<sup>2</sup>). Several techniques such as circular dichroism and antimicrobial assays were employed with *B. punctata* peptides, demonstrating that HSP-1 adopts an  $\alpha$ -helical structure in the presence of a membrane-mimetic ambient and weak antimicrobial activity with no significant lytic effect toward red or white blood cells.<sup>9</sup> This was also observed in homologous sequences described for AMPs in other anuran species.<sup>28</sup> The weak activity projected for P11 is also supported by the minimum inhibitory concentrations (MICs) determined for cinerascetins that this peptide presents MICs in the order of 16 and 10  $\mu$ M for *E. coli* and *S. aureus*, respectively.

Activity of P4 and P5 peptides could be projected taking into account that evaluated for the Rsp-1 and its both halves, Rsp-1 (1–14) and Rsp-1 (15–29). It has been described that mature raniseptins peptides identified in *B. raniceps* are subjected to specific cleavage that gives rise to two half fragments. It was further observed that the C-terminal fragments presented antimicrobial activity at least 30-folds lower than the precursor.

The same analysis could be done with phenylseptin and peptide P12, the latter being identical to the N-terminal region of the former. However, phenylseptin was pondered as possibly responsible for sensorial aversive properties for predators. Therefore, it is plausible that a similar aversive activity could also be performed by P12 as part of a general warning strategy against natural predators of *B. punctata*. In the case of the remaining peptides that presented original sequences, additional work should be carried out to evaluate the biological function.

Peptide secondary structure determines the accessibility of certain residues to proteases.<sup>8,23</sup> Analysis of the 3D theoretical structure shows two  $\alpha$ -helix motifs for raniseptins and Ctx (Ile<sup>21</sup>)-Ha mature peptides, where Gly<sup>14</sup>Lys<sup>15</sup> in raniseptin and Gly<sup>10</sup>Lys<sup>11</sup> in Ctx(Ile<sup>21</sup>)-Ha are placed in between the  $\alpha$ -helix motifs. Considering that in *B. punctata* the same process might occur for mature peptides, we theoretically modeled the peptide sequences corresponding to assembly of P7 or P12 as the N-terminal half and of P4 or P2 as the C-terminal one, as if they were joined in a mature peptide, taking into account the possible existence of a Gly-Lys cleavage site (Figure S4). Two  $\alpha$ -helix bound by a coil with the Gly-Lys motif was found in all tested combinations.

**2.5. Potential Roles for a Postsecretory Cleavage Mechanism of Amphibian Peptides.** Considering the results discussed in sections 2.2 and 2.3, it seems that peptide cleavage depends on various factors, such as the sequence and secondary structure. In the cases in which peptides meet specific conditions, cleavage may be a rapid phenomenon that occurs under a natural state. For instance, the same peptides may be detected in situ and in gland secretions (i.e., peptide P13), whereas in other circumstances, full-length peptides are only found within the gland (i.e., ions  $m/z$  1976.2 and 2367.3) or in the gland secretion when using particular conditions, such as low temperature.<sup>18</sup> Therefore, it seems likely that a rapid enzymatic cleavage mechanism occurs shortly after peptides are secreted. Furthermore, the fact that this mechanism occurs in distantly related groups, such as hylids tree frogs of the genus *Boana* and pipids of the genus *Xenopus*, suggests that peptide cleavage should be relatively common in anurans.

Different hypotheses have been proposed to explain the rapid cleavage of secreted peptides in amphibians. Some suggested that it may function as an “inactivation mechanism,” because prolonged exposure to AMPs present in the secretion would be expected to induce toxicity to the host organism itself.<sup>8,29</sup> If this is the case, SGs in the skin that store full-length peptides should have a protective system against the toxic peptides. Alternatively, peptides with antibiotic activity may be activated by proteolysis.<sup>8</sup> Still, evidences in *Cophomantini* are contradictory: hylaseptin, which is a cleaved peptide, exhibits antimicrobial activity,<sup>9</sup> whereas cleaved raniseptins exhibit much lower or null antimicrobial activity when compared to intact raniseptin. Because the gland content is secreted under stress conditions mostly associated with predation,<sup>7</sup> a rapid endoproteolysis may constitute a line of defense against predators.<sup>18</sup> In fact, it has been shown that protein degradation can generate peptides with diverse biological functions and degrees of bitterness.<sup>30</sup> In this context, we observed that a cleaved form of phenylseptin would be a way to increase the bitterness of this molecule because apparently, the bitter scale of peptides is related with short peptides that share a Phe-Phe motif. Finally, published evidence indicate that amphibian skin peptides have at least two important functions, a host-shielding antimicrobial activity and a toxic effect against predators.<sup>31</sup> Our results, alongside previous research, suggest that a simple and rapid enzymatic cleavage may account for these two important ecological functions for anuran survival.

### 3. CONCLUSIONS

Resistance to conventional antibiotics by certain pathogenic microorganisms is an increasingly serious problem and many infections are no longer easily cured, leading to expensive and prolonged treatment as well as a higher risk of death (press release, World Health Organization, World Health Day 2011).<sup>32</sup> AMPs from amphibians gland secretions were demonstrated to have notable activity against a wide variety of pathogens, including antibiotic-resistant bacteria. Moreover, Raaymakers and co-workers have recently shown that AMPs also act as a toxin delivery system against predators.<sup>31</sup> Although many species of amphibians have already been studied worldwide, our knowledge of the diversity, evolution, and biological functions of peptides in this group of vertebrates is limited. Still, much of the null effects reported several years ago for a defensive role of related compounds could be associated with the use of inadequate bioassays and less sensible methodologies. Our findings call for a reinterpretation and reanalysis of the roles of skin peptides in these species of amphibians. The cleavage mechanism proposed herein, taken together with evidences in distantly related species, suggest that this mechanism may be widespread in amphibians.

### 4. MATERIALS AND METHODS

**4.1. Amphibian Skin Secretions.** Female and male specimens of the tree frog *B. punctata* were collected at night in the outskirts of the city of Santa Fe, Santa Fe, Argentina, and next to Forte Coimbra, Mato Grosso do Sul, Brazil. Frog secretion was obtained by mild electric stimulation of the dorsal skin region (3–6 V, 1–2 min), collected in distilled water, frozen within the first 5 min after collection, and lyophilized.

Collection permits were issued by Secretaria de Medio Ambiente, Ministerio de Aguas, Servicios Públicos y Medio Ambiente, Province of Santa Fe, Argentina (021-2011 and 063-



2013) and Instituto Chico Mendes de Conservação da Biodiversidade SISBIO (Permit 50071-1). All procedures involving animals were carried out according to the regulations specified by the Institutional Animal Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (Res C/D 140/00) and those specified by Conselho Nacional de Controle de Experimentação Animal, Ministério da Ciência, Tecnologia e Inovação, Brazil. Authorization access in SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado). Voucher specimens of *B. punctata* are housed at the herpetological collection of the Museo Argentino de Ciencias Naturales “Bernardino Rivadavia”, Buenos Aires, Argentina (MACN 40110-17, 43337-48) and Coleção Celio F.B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil (CFBH 40991-94).

**4.2. Peptide Separation and Sequencing.** Liquid chromatography (LC)-diode array detector (DAD)-time-of-flight (TOF) high-resolution MS analyses were performed using an Agilent 1200 series LC with a DAD coupled with a QqTOF Mass Spectrometer (Bruker Daltonics, Billerica—USA). Skin secretion was dissolved in ultrapure cold water, filtered, and injected into a C18-Luna column (Phenomenex—100 mm  $\times$  2.0 mm  $\times$  3  $\mu$ m) with temperature adjusted to 30 °C. The mobile phase (flow 0.3 mL min<sup>-1</sup>) consisted of water/formic acid 0.1% (v/v) (A) and methanol (B) in the following gradient: 0.0–25.0 min (10–75% B) and 25.0–35.0 min (75–100% B). Q-TOF acquisition parameters were as follows: capillary 3.5 kV, end plate offset 500 V, and capillary temperature at 200 °C; N<sub>2</sub> was used as the dry gas at 6.0 L min<sup>-1</sup> and nebulizer pressure was 3 bar; drying gas temperature was 200 °C; positive ESI mode. All peaks were automatically selected for fragmentation during the LC-ESI-MS/MS experiments. Fragmentation was performed by collision-induced dissociation using ultrahigh pure argon as the collision gas, with a collision energy of 35 eV for each precursor ion. The collision cell quadrupole was set to an energy of 7.0 eV, the cell radiofrequency was set at 800 V<sub>pp</sub>, and the gas flow rate at 30%. Accurate masses were obtained by multipoint mass calibration using sodium formate as the internal standard. Further details of MS/MS conditions used in LC-ESI-MS/MS experiments are depicted in Table S5.

The molecular mass determination of compounds presented in the gland secretion was performed by selecting the ions [M + H]<sup>+</sup>, [M + H]<sup>2+</sup>, and/or [M + 3H]<sup>3+</sup>. De novo peptide sequencing was performed through manual interpretation of tandem mass spectra (MS/MS) of fragments *b* and *y* + 2 of ions [M + H]<sup>+</sup> and [M + 2H]<sup>2+</sup>. Data acquisition and interpretation were performed using the software Bruker Compass Data Analysis v. 4.0. Fragment analysis was carried out following the Biemann nomenclature.<sup>33</sup> The MS/MS spectra were uploaded to Global Natural Products Social Molecular Networking (GNPS).<sup>34</sup>

The amino acid sequence similarity search was carried out using NCBI nonredundant database through the PSI-BLAST algorithm.<sup>35</sup> The program Compute pI/Mw Tool was used for theoretical pI and molecular mass calculation. ClustalW tool was used to calculate the identity and similarity scores among the peptides.<sup>36</sup>

**4.3. Solid-Phase Peptide Synthesis.** Peptides were manually synthesized by the solid-phase approach using Fmoc/*t*-butyl chemistry.<sup>37</sup> Peptide elongation was carried out in polypropylene syringes fitted with a polyethylene porous

disk. Solvents and soluble reagents were removed by suction. A Wang Resin and Rink amide MBHA Resin (Peptides International) were used for C-terminal carboxylated and amidated peptide synthesis respectively. Samples were treated with trifluoroacetic acid (TFA)/triisopropylsilane/water (95:2.5:2.5) for removal of the protecting group and cleavage. Identity of the peptide was confirmed by electrospray MS on a MicroTOF Q II (Bruker Daltonics).

**4.4. Sequence Comparison within Cophomantini Tree Frogs.** The search for similar sequences was performed employing an online database of AMPs.<sup>38</sup> Because of the Leu/Ile uncertainty in some of the determined sequences, the search for sequences similar to those of *B. punctata* was done using two extreme approaches, assigning all uncertainties (X) to Leu or to Ile. The similarity in the sequences of peptides identified in this work were compared, employing the alignment of multiple sequences, on the online server MAFFT.<sup>39</sup> This alignment included sequences described in *B. punctata*<sup>9,10</sup> and in all species of the tribe Cophomantini, subfamily Hyliinae. The specific sequences included were the following: eight peptides of *B. raniceps* (raniseptins),<sup>18</sup> peptides of *Boana albopunctata* (hylin-1a and Ctx-Ha),<sup>16</sup> peptides of *Boana lundii* (hylin-b1 and hylin-b2),<sup>17</sup> one peptide of *Boana semilineata* (Hs-1),<sup>40</sup> and one peptide of *B. cinerascens* (Cinerascetin-01).<sup>15</sup> For comparison, uncertainties (X) in the peptide sequence identified in our study in *B. punctata* were either left as Leu or were tentatively assigned to Leu or to Ile based on similarities with the amino acid sequence identified in the same or related species and also on comparisons with synthetic analogues (Table S4).

**4.5. Sample Preparation for MALDI IMS.** The frog skin sections preparation for the MALDI imaging studies was the challenging part of this work. The skins were very thin and fragile, making it almost impossible to obtain transverse sections without suitable embedding. We tried several embedding materials, among which we found that 2% carboxymethyl cellulose worked the best while eliminating several problems in ionization.

The frog skins were removed, immediately embedded, and frozen. Samples were sectioned in slices of 10  $\mu$ m width in a cryotome (CM1860 Cryostat, Leica Biosystem, Nussloch, Germany) at -20 °C. Sections were mounted on an indium tin oxide-coated glass slide for MALDI imaging (Bruker Daltonic, Bremen, Germany), followed by matrix coating in Imageprep (Bruker Daltonic, Bremen, Germany). Sixty cycles from the solution of 7 mg mL<sup>-1</sup> of  $\alpha$ -cyano-4-hydroxy-cinnamic acid matrix in 50% acetonitrile/H<sub>2</sub>O with 0.2% TFA was applied. Each cycle was based on spraying, filling chamber with nitrogen, and drying. After that, the tissue sample was subjected to analysis by MALDI imaging.

**4.6. MALDI IMS.** Analyses were performed using a MALDI-TOF/TOF (Bruker Daltonics, UltrafleXtreme, Bremen, Germany) system equipped with a smartbeam-II laser system controlled by a FlexControl v.3.3 (Bruker Daltonics, Bremen, Germany), employing AutoXecute mode. IMS data were analyzed and normalized using FlexImaging v.2.1 (Bruker Daltonics). The settings of the instrument were as follows: positive ion reflector mode; ion source 1, 25.00 kV; ion source 2, 22.40 kV; lens, 8.30 kV; pulsed ion extraction, 120 ns; laser frequency, 100 Hz; and matrix suppression mass cutoff, *m/z* 600. The spectra were recorded across a mass range of *m/z* 700–4000 and accumulate 500 shots per spectrum. The images were collected from a transverse section of frog skin at 20  $\mu$ m

spatial resolution in both  $x$  and  $y$  directions using MS positive-ion mode. A peptide calibration standard II (Bruker Daltonics, Bremen, Germany) was used as the external calibration of the mass spectrometer. The average mass deviation was below 10 ppm. MS/MS analysis directly from frog skin was performed to confirm assignments and identify the major peptides in the positive-ion reflector mode. The ions selected were accelerated to 19 kV in the LIFT cell for MS/MS analysis, where the settings of the instrument were as follows: pulsed ion extraction, 110 ns, laser frequency, 100 Hz, and 500 shots. De novo peptide sequencing was performed through manual interpretation of tandem mass spectra (MS/MS) of fragments  $b$  and  $y + 2$  of  $[M + H]^+$  ions. Data acquisition and interpretation were performed using the software, Bruker Flex Analysis v. Fragment analysis was carried out following Biemann nomenclature.<sup>33</sup> The MS/MS spectra were uploaded to PeptideAtlas.<sup>41</sup> Accession number: PASS0118.

**4.7. Light Microscopy Images.** For LM images, 10  $\mu\text{m}$  sections of dorsal and lateral skin samples were cut before and after the sections were used for MALDI IMS. These sections were mounted onto microscope slides, stained with hematoxylin and eosin, mounted with glycerol, covered with quartz coverslips, and observed using LM. These images were compared with those detailed by Brunetti and co-workers.<sup>14</sup>

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsomega.7b02029](https://doi.org/10.1021/acsomega.7b02029).

Three MS/MS spectra obtained from LC-ESI-MS/MS experiments and MALDI MS/MS experiments, example analysis of the peptide sequencing process, detailed analyses of ions  $b$  and  $y + 2$  from 13 peptides present in the gland secretion and obtained in LC-ESI-MS/MS experiments, detailed analyses of ions  $a$  and  $y + 2$  from 3 peptides present within the gland and obtained in MALDI-MS/MS experiments, retention time comparison of natural peptides and synthetic analogues, MS/MS conditions used in LC-ESI-MS/MS experiments, and 3D prediction structure of peptides indicating putative cleavage sites (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Authors

\*E-mail: [andresbrunetti@gmail.com](mailto:andresbrunetti@gmail.com) (A.E.B.).

\*E-mail: [npelopes@fcfrp.usp.br](mailto:npelopes@fcfrp.usp.br) (N.P.L.).

### ORCID

Norberto P. Lopes: 0000-0002-8159-3658

### Notes

The authors declare no competing financial interest.

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## ■ REFERENCES

- (1) Aksenov, A. A.; da Silva, R.; Knight, R.; Lopes, N. P.; Dorrestein, P. C. Global chemical analysis of biology by mass spectrometry. *Nat. Rev. Chem.* **2017**, *1*, 0054.
- (2) Ernst, M.; Silva, D. B.; Silva, R. R.; Vêncio, R. Z. N.; Lopes, N. P. Mass spectrometry in plant metabolomics strategies: From analytical platforms to data acquisition and processing. *Nat. Prod. Rep.* **2014**, *31*, 784–806.
- (3) Brunetti, A. E.; Neto, F. C.; Vera, M. C.; Taboada, C.; Pavarini, D. P.; Bauermeister, A.; Lopes, N. P. An integrative omics perspective for the analysis of chemical signals in ecological interactions. *Chem. Soc. Rev.* **2018**, *47*, 1574–1591.
- (4) Bevins, C. L.; Zasloff, M. Peptides from frog skin. *Annu. Rev. Biochem.* **1990**, *59*, 395–414.
- (5) Ersamer, V. Bioactive secretions of the amphibian integument. In *Amphibian Biology*; Heatwole, H., Bartholomew, G. T., Eds.; Surrey Beatty & Sons: Chipping Norton, 1994; pp 178–350; Vol. 1: The Integument.
- (6) König, E.; Bininda-Emonds, O. R. P.; Shaw, C. The diversity and evolution of anuran skin peptides. *Peptides* **2015**, *63*, 96–117.
- (7) Brunetti, A. E.; Hermida, G. N.; Iurman, M. G.; Faivovich, J. Odorous secretions in anurans: Morphological and functional assessment of sereous glands as a source of volatile compounds in the skin of the treefrog *Hypsiboas pulchellus* (Amphibia: Anura: Hylidae). *J. Anat.* **2016**, *228*, 430–442.
- (8) Giovannini, M. G.; Poulter, L.; Gibson, B. W.; Williams, D. H. Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones. *Biochem. J.* **1987**, *243*, 113–120.
- (9) Prates, M. V.; Sforça, M. L.; Regis, W. C. B.; Leite, J. R. S. A.; Silva, L. P.; Pertinhez, T. A.; Araújo, A. L. T.; Azevedo, R. B.; Spisni, A.; Bloch, C. The NMR-derived solution structure of a new cationic antimicrobial peptide from the skin secretion of the anuran *Hyla punctata*. *J. Biol. Chem.* **2004**, *279*, 13018–13026.
- (10) de Magalhães, M. T. Q.; Barbosa, E. A.; Prates, M. V.; Verly, R. M.; Munhoz, V. H. O.; de Araujo, I. E.; Bloch, C., Jr Conformational and functional effects induced by D- and L-amino acid epimerization on a single gene encoded peptide from the skin secretion of *Hypsiboas punctatus*. *PLoS One* **2013**, *8*, e59255.
- (11) Frost, D. R. *Amphibian Species of the World: An Online Reference*, version 6.0. Electronic Database accessible at <http://research.amnh.org/herpetology/amphibia/index.html>; American Museum of Natural History, New York, USA, April, 2018.
- (12) Prado, C.; Haddad, C.; Uetanabaro, M. Breeding activity patterns, reproductive modes, and habitat use by anurans (Amphibia) in a seasonal environment in the Pantanal, Brazil. *Amphibia-Reptilia* **2005**, *26*, 211–221.
- (13) Taboada, C.; Brunetti, A. E.; Pedron, F. N.; Neto, F. C.; Estrin, D. A.; Bari, S. E.; Chemes, L. B.; Lopes, N. P.; Lagorio, M. G.; Faivovich, J. Naturally occurring fluorescence in frogs. *Proc. Natl. Acad. Sci. U.S.A.* **2017**, *114*, 3672–3677.
- (14) Brunetti, A. E.; Hermida, G. N.; Faivovich, J. New insights into sexually dimorphic skin glands of anurans: the structure and ultrastructure of the mental and lateral glands in *Hypsiboas punctatus* (Amphibia: Anura: Hylidae). *J. Morphol.* **2012**, *273*, 1257–1271.
- (15) Almeida, R. A.; Gordo, M.; Silva, F. M. A.; Araújo, R. C.; Ramada, M. H. S.; Abrão, F. Y.; Costa, T. O. G.; Koolen, H. H. F.; Souza, A. D. L.; Bloch, C., Jr. Cinerascetins, New peptides from *Hypsiboas cinerascens*: MALDI LIFT-TOF-MS/MS de novo sequence and imaging analysis. *J. Braz. Chem. Soc.* **2015**, *26*, 2290–2297.
- (16) Castro, M. S.; Ferreira, T. C. G.; Cilli, E. M.; Crusca, E.; Mendes-Giannini, M. J. S.; Sebben, A.; Ricart, C. A. O.; Sousa, M. V.; Fontes, W. Hylin al, the first cytolytic peptide isolated from the



arboreal South American frog *Hypsiboas albopunctatus* ("spotted treefrog"). *Peptides* **2009**, *30*, 291–296.

(17) Castro, M.; Matsushita, R.; Sebben, A.; Sousa, M.; Fontes, W. Hyalins: bombinins H structurally related peptides from the skin secretion of the Brazilian tree-frog *Hyla biobeba*. *Protein Pept. Lett.* **2005**, *12*, 89–93.

(18) Magalhães, B. S.; Melo, J. A. T.; Leite, J. R. S. A.; Silva, L. P.; Prates, M. V.; Vinecky, F.; Barbosa, E. A.; Verly, R. M.; Mehta, A.; Nicoli, J. R.; Bemquerer, M. P.; Andrade, A. C.; Bloch, C., Jr Post-secretory events alter the peptide content of the skin secretion of *Hypsiboas raniceps*. *Biochem. Biophys. Res. Commun.* **2008**, *377*, 1057–1061.

(19) Siano, A.; Húmpola, M. V. N.; de Oliveira, E.; Albericio, F.; Simonetta, A. C.; Lajmanovich, R.; Tonarelli, G. G. Antimicrobial peptides from skin secretions of *Hypsiboas pulchellus* (Anura: Hylidae). *J. Nat. Prod.* **2014**, *77*, 831–841.

(20) Pukala, T. L.; Bowie, J. H.; Maselli, V. M.; Musgrave, I. F.; Tyler, M. J. Host-defence peptides from the glandular secretions of amphibians: structure and activity. *Nat. Prod. Rep.* **2006**, *23*, 368–393.

(21) Jackway, R. J.; Pukala, T. L.; Donnellan, S. C.; Sherman, P. J.; Tyler, M. J.; Bowie, J. H. Skin peptide and cDNA profiling of Australian anurans: Genus and species identification and evolutionary trends. *Peptides* **2011**, *32*, 161–172.

(22) Wabnitz, P. A.; Bowie, J. H.; Tyler, M. J.; Wallace, J. C.; Smith, B. P. Differences in the skin peptides of the male and female Australian tree frog *Litoria splendida*. *Eur. J. Biochem.* **2000**, *267*, 269–275.

(23) Resnick, N. M.; Maloy, W. L.; Guy, H. R.; Zasloff, M. A novel endopeptidase from *Xenopus* that recognizes  $\alpha$ -helical secondary structure. *Cell* **1991**, *66*, 541–554.

(24) Gershon, P. D. Cleaved and missed sites for trypsin, Lys-C, and Lys-N can be predicted with high confidence on the basis of sequence context. *J. Proteome Res.* **2014**, *13*, 702–709.

(25) Bechinger, B. Insights into the mechanisms of action of host defence peptides from biophysical and structural investigations. *J. Pept. Sci.* **2011**, *17*, 306–314.

(26) Gautier, R.; Douguet, D.; Antonny, B.; Drin, G. HELIQUEST: A web server to screen sequences with specific  $\alpha$ -helical properties. *Bioinformatics* **2008**, *24*, 2101–2102.

(27) Blondelle, S. E.; Pérez-Payá, E.; Houghten, R. A. Synthetic Combinatorial Libraries: novel discovery strategy for identification of antimicrobial agents. *Antimicrob. Agents. Chemo.* **1996**, *40*, 1067–1071.

(28) Marani, M. M.; Dourado, F. S.; Quelemes, P. V.; de Araujo, A. R.; Perfeito, M. L. G.; Barbosa, E. A.; Vêras, L. M. C.; Coelho, A. L. R.; Andrade, E. B.; Eaton, P.; Longo, J. P. F.; Azevedo, R. B.; Delerue-Matos, C.; Leite, J. R. S. A. Characterization and Biological Activities of Ocellatin Peptides from the Skin Secretion of the Frog *Leptodactylus pustulatus*. *J. Nat. Prod.* **2015**, *78*, 1495–1504.

(29) Carvalho, K. M.; Joudiou, C.; Boussetta, H.; Leseney, A.-M.; Cohen, P. A peptide-hormone-inactivating endopeptidase in *Xenopus laevis* skin secretion. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 84–88.

(30) Maehashi, K.; Matsuzaki, M.; Yamamoto, Y.; Udaka, S. Isolation of peptides in enzymatic hydrolysate of food proteins and characterization of their taste properties. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 555–559.

(31) Raaymakers, C.; Verbrugghe, E.; Hernot, S.; Hellebuyck, T.; Betti, C.; Peleman, C.; Claeys, M.; Bert, W.; Caveliers, V.; Ballet, S.; Martel, A.; Pasmans, F.; Roelants, K. Antimicrobial peptides in frog poisons constitute a molecular toxin delivery system against predators. *Nat. Commun.* **2017**, *8*, 1495.

(32) World Health Organization (WHO); World Health Day, 2011. Accessible at: <http://www.who.int/world-health-day/2011/en/>.

(33) Biemann, K. Mass spectrometry of peptides and proteins. *Annu. Rev. Biochem.* **1992**, *61*, 977–1010.

(34) Wang, M.; et al. Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nat. Biotechnol.* **2016**, *34*, 828–837. Accessible at: <https://gnps.ucsd.edu/ProteSAFe/static/gnps-splash.jsp>.

(35) Altschul, S. F.; Madden, T. L.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402.

(36) Gasteiger, E.; Gattiker, A.; Hoogland, C.; Ivanyi, I.; Appel, R. D.; Bairoch, A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* **2003**, *31*, 3784–3788.

(37) Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis: A Practical Approach (The Practical Approach Series)*; Oxford University Press: USA, 1989; p 203.

(38) Wang, G.; Li, X.; Wang, Z. APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res.* **2016**, *44*, D1087–D1093.

(39) Katoh, K.; Standley, D. M. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* **2013**, *30*, 772–780.

(40) Nacif-Marçal, L.; Pereira, G. R.; Abranches, M. V.; Costa, N. C. S.; Cardoso, S. A.; Honda, E. R.; de Paula, S. O.; Feio, R. N.; Oliveira, L. L. Identification and characterization of an antimicrobial peptide of *Hypsiboas semilineatus* (Spix, 1824) (Amphibia, Hylidae). *Toxicon* **2015**, *99*, 16–22.

(41) Desiere, F.; Deutsch, E. W.; King, N. L.; Nesvizhskii, A. I.; Mallick, P.; Eng, J.; Chen, S.; Eddes, J.; Loevenich, S. N.; Aebersold, R. The peptideatlas project. *Nucleic Acids Res.* **2006**, *34*, D655–D658.