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Antimicrobial Peptides from Skin Secretions of *Hypsiboas pulchellus* (Anura: Hylidae)

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S *Supporting Information*

ABSTRACT: The skin of many amphibians produces a large repertoire of antimicrobial peptides that are crucial in the first line of defense against microbial invasion. Despite the immense richness of wild amphibians in Argentina, knowledge about peptides with antimicrobial properties is limited to a few species. Here we used LC-MS-MS to analyze samples of *Hypsiboas pulchellus* skin with the aim to identify antimicrobial peptides in the mass range of 1000 to 2000 Da. Twenty-three novel sequences were identified by MS, three of which were selected for chemical synthesis and further studies. The three synthetic peptides, named P1-Hp-1971, P2-Hp-1935, and P3-Hp-1891, inhibited the growth of two ATCC strains: *Escherichia coli* (MIC: 16, 33, and 17 μM, respectively) and *Staphylococcus aureus* (MIC: 8, 66, and 17 μM, respectively). P1-Hp-1971 and P3-Hp-1891 were the most active peptides. P1-Hp-1971, which showed the highest therapeutic indices (40 for *E. coli* and 80 for *S. aureus*), is a proline-glycine-rich peptide with a highly unordered structure, while P3-Hp-1891 adopts an amphipathic α-helical structure in the presence of 2,2,2-trifluoroethanol and anionic liposomes. This is the first peptidomic study of *Hypsiboas pulchellus* skin secretions to allow the identification of antimicrobial peptides.

The emergence of bacterial strains resistant to conventional antibiotics is one of the major causes of inefficient therapy and high mortality rates. In this regard, intensive research efforts are being channeled into developing novel antimicrobials.[1] Historically, bacterial cell walls and protein synthesis have been the major targets of antibacterial drugs.[2]

In spite of the development of new technologies for drug discovery, nature continues to be the most important source of molecules for the development of new therapeutic agents.[3,4] In particular, in the case of compounds with antimicrobial activity, only 20% of marketed products are totally synthetic without inspiration by nature.[5−7] Furthermore, the relevance of peptides in drug discovery programs has recently increased notably.[8,9]

Antimicrobial peptides (AMPs) are produced by all living organisms, including bacteria, fungi, plants, invertebrates, and vertebrates. These natural molecules form a first line of defense against pathogens and are involved in innate immunity.[10,11] In animals, AMPs are produced in the skin, epithelial tissues, and acute inflammatory cells, where they supplement the humoral and cellular immune system of the host.[12,13] AMPs are positively charged (net charge of +2 to +9) and contain a significant proportion of hydrophobic residues.

Initially, cationic AMPs were believed to act only by disrupting the integrity of the bacterial membrane. While mammalian membranes are primarily composed of neutral lipids and contain cholesterol, bacterial membranes are mainly composed of anionic lipids and do not contain cholesterol.[14] Therefore, cationic AMPs are electrostatically attracted to bacterial membranes, and the hydrophobic residues facilitate interactions with the fatty acyl chains.

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Table 1. Sequences and Antimicrobial Activities of AMPs from South American *Hysiboas* Species[a]

peptide	sequences	organism	length	MIC (μM)					HA	ref
				S. aureus	*P. aeruginosa*	*E. coli*	*B. subtilis*	*E. faecalis*		
hylaseptin-P1	GILDAIKAIAKAAG	*Hyla punctata* (spotted tree (frog)	14	8	64	32	ND	ND	−	33
hylin a-1	IFGAILPLALGALKNLIK	*Hypsiboas albopunctatus* (spotted tree frog)	18	8	64	32	8	16	+	34
hylin b-1	FIGAILPAIAGLVHGLINR	*Hyla biobeba* (Brazilian tree frog)	19	ND	ND	ND	ND	ND	+	35
hylin b-2	FIGAILPAIAGLVGGLINR	*Hyla biobeba*	19	ND	ND	ND	ND	ND	+	35
raniseptin 1	AWLDKLKSLGKVVGKVALGVAQNYLNPQQ	*Hypsiboas raniceps* (Chaco tree frog)	29	20	10	5	ND	ND	-	36

[a]The specimens were collected in Brazil (see refs). ND: not determined. HA: hemolytic activity; (+) hemolytic; (−) nonhemolytic.

Additional studies demonstrated that peptides could also translocate across the cytoplasmatic membrane of bacteria to inhibit multiple internal targets including DNA/RNA synthesis, protein folding, cell wall synthesis, cell division, translocation, and protein folding.[15−17]

The skin of many amphibians is associated with a defense system that is effective to varying degrees against predators and pathogenic microorganisms.[14,18] Granular glands (also called poison glands or serous glands) in the dermal layer produce a large repertoire of bioactive substances, which include a variety of defensive principles, such as alkaloids,[19] neurotoxic peptides,[20] gastric disturbance peptides,[12] and AMPs.[14,21] The center of the gland is filled with granules containing active peptides,[22] and when the animal is alarmed or injured, the content is released into skin secretions, often in large amounts.[23,24] Although the original and primary function of AMPs was proposed to be direct antimicrobial activity against bacteria, fungi, parasites, and viruses, more recently these molecules have become increasingly recognized as multifunctional mediators, with both antimicrobial activity and diverse immunomodulatory properties.[1,25]

Hypsiboas pulchellus[26] (Hp) is a common tree frog with a population status categorized as stable.[27] This species is distributed in southern Brazil, eastern and northeastern Argentina, and southern Paraguay, and it is a common anuran species in Argentina.[28] It is a habitat generalist, occurring in natural and anthropogenic environments and reproducing in permanent or semipermanent water bodies.[26] *H. pulchellus* is frequently found in both natural and altered lentic water bodies in agricultural and urban areas, with marginal vegetation composed of small shrubs and riparian trees, interspersed with assemblages of plants belonging to different families such as Poaceae (gramineae or true grasses), Polygonaceae (including buckwheat, dock, knotweed, rhubarb, sea grape, and smartweed), and Cyperaceae (also known as sedges, including water chestnut and papyrus sedge, among others).[29] Moreover, as a result of its wide trophic niche,[30] the diet of this frog includes a diversity of invertebrates, mainly insects, but shows some seasonal variation and a preference for certain types of fly.[31]

The South American hylid frogs of the Phyllomedusinae subfamily (family: Hylidae) produce a rich array of linear antimicrobial peptides. These peptides are endogenously expressed as large precursor molecules, designated the preprodermaseptins, organized at the N-terminal region as a 22-residue signal peptide, followed by a 22−23-residue acidic propeptide domain and a single copy of the biologically active peptide at the C-terminal domain.[32]

To date, the biochemical characterization of the peptide content of the skin secretions of the large subfamily Hylinae is scarce. A few AMPs from the genus *Hypsiboas* have been reported (Table 1). The peptide hylaseptin P1 (HSP1) isolated from the secretions of *H. punctatus* has no significant hemolytic activity but inhibits the growth of *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*.[33] Hylin a-1, isolated from *H. albopunctatus*, has a broad spectrum of antimicrobial activity against bacteria, yeasts, and fungi, including *E. coli*, *S. aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *P. aeruginosa*, *Candida parapsilosis*, and *Cryptococcus neoformans*.[34] Hylins b-1 and b-2, isolated from *H. biobeba*, are hemolytic peptides that share high sequence similarity with hylin a-1. These were the first examples of bombinin H-like peptides isolated from anuran species not related to *Bombina* species.[35]

A novel family of dermaseptin-related peptides termed raniseptins (1 to 9) has been characterized from the skin secretion of the anuran *H. raniceps*. The organization of the raniseptin preproprecursor resembles that of the precursor molecules of the Phyllomedusinae frogs, and the mature peptides contain 28 or 29 amino acid residues with more than 95% sequence identity. Biological assays demonstrated that raniseptin 1 has antibacterial activity against *E. coli*, *P. aeruginosa*, and *S. aureus* strains, without lytic effects against human erythrocytes.[36] In Argentina, despite the richness of wild amphibians, knowledge about the antimicrobial properties and the presence of alkaloids in amphibian skin is limited to only a few species.[37−41]

■ RESULTS AND DISCUSSION

In previous work, one of our groups established that extracts of Hp obtained by solvent extraction (SE) and transcutaneous amphibian stimulation (TAS) methods inhibited the growth of *E. coli*, *B. cereus*, *S. aureus*, and *Pseudomonas* sp. strains.[42] Herein, samples were analyzed by MS (MS-MS by direct infusion in a Q-TOF mass spectrometer and LC-MS-MS in an Orbitrap Velos system) to identify antimicrobial peptides in the molecular weight range 1000 to 2000 Da.

Mass Spectrometry Analysis of the Complete Extracts of *H. pulchellus*. Peptides were extracted, desalted, and directly analyzed by MS without any further purification. Amino acid sequences of some peptides obtained from LC-MS-MS were inferred through "de novo" analysis with Peaks Studio software. Only those with a "de novo" Peaks Studio score over 85% (ALC $\geq$ 85%) were considered. The application of this strategy required further analysis to determine whether the sequences were novel or corresponded to truncated versions of

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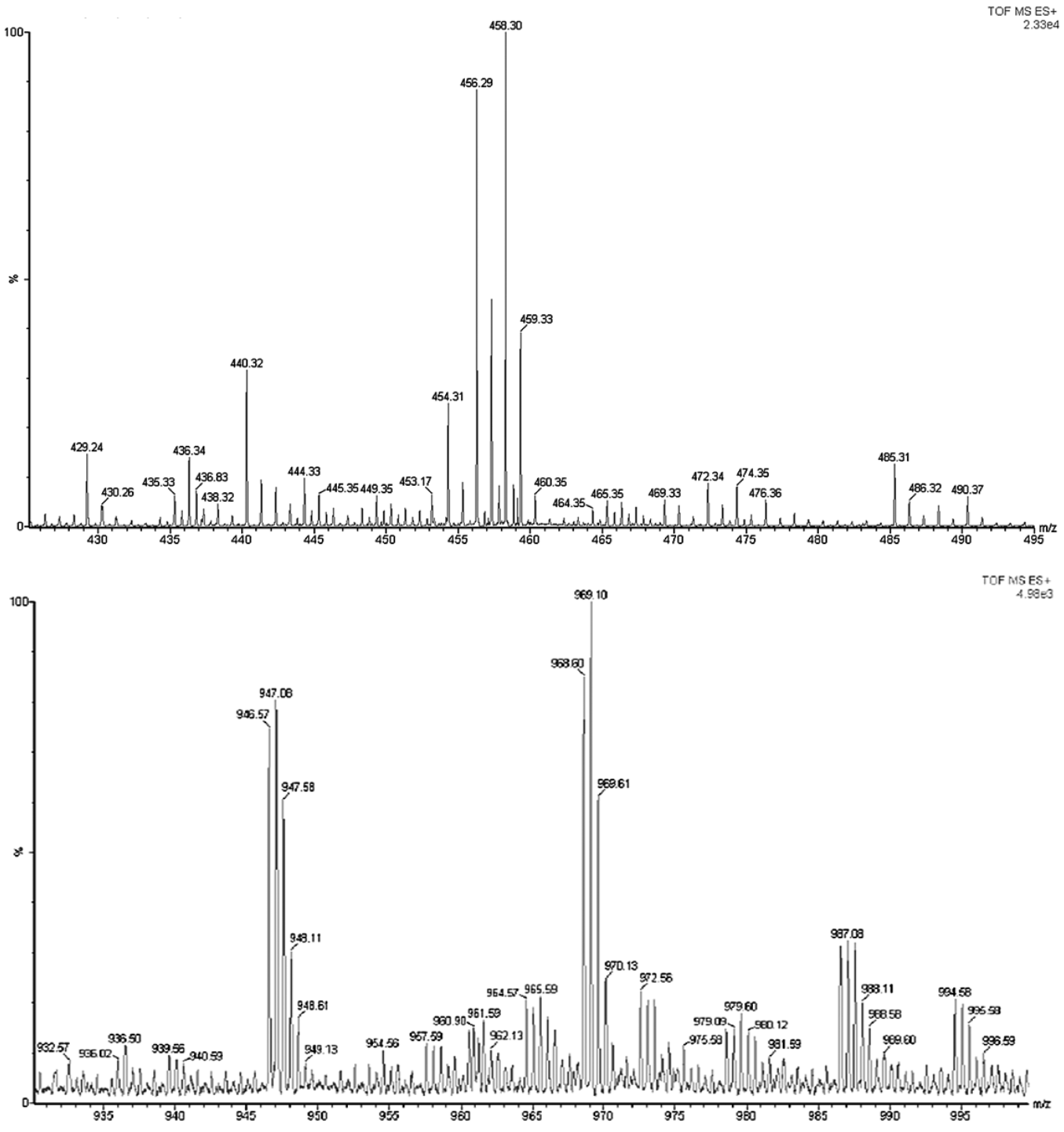

Figure 1. ESIMS spectrum of the complete extract of Hp obtained by SE.

previously reported peptides. The ESI mass spectrum of the complete extract of *H. pulchellus* obtained by solvent extraction is shown in Figure 1, and the identified sequences are listed in Table 2.

The residues Leu/Ile could not be distinguished by mass spectrometry. The near isobaric residues Lys/Gln could not be resolved for Seq 1, 2, and 3, as they were not obtained in a high-resolution mass spectrometer. These residues were inferred considering that Leu, Gly, Ala, and Lys are the most frequently used amino acid residues in amphibian antimicrobial peptides, according to statistical analyses reported by Wang et al.[43]

According to predictive analyses of the secondary structure, five sequences (Seq 3, Seq 9, Seq 10, Seq 12, Seq 17) showed contributions of α-helices and three (Seq 14, Seq 15, Seq 18) of β-structure. However, most of the sequences were considered unstructured by GOR V (http://gor.bb.iastate.edu/) and PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) methods.[44,45] The most cationic and hydrophobic sequences were analyzed through the AMP database APD (http://aps.unmc.edu/AP). The results of the alignments allowed us to determine the most similar peptides in the database.

Seq 1 reached 40% of similarity with nigrocin-2HSa, isolated from *Odorrana hosii*, which belongs to the Ranidae family,[46]

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Table 2. Amino Acid Sequence of Peptides Identified by MS-MS from the Hp Sample Obtained by SE[a]

Seq i.d.	t_R	experimental mass	ion (m/z)	sequence	no.
1		1971	986.5	TKPTLLGLPLGAGPAAGPGKR	21
2		1935.16	968.58	KLSPSLGPVSKGKLLAGQR	19
3		1891	946.5	RLGTALPALLKTLLAGLNG	19
4	22.61	1487.82	496.9399	AHLDLAGSLEGHLR	14
5	22.26	1109.621	555.8107	LSLTGTYDLK	10
6	26.83	1159.6	580.7998	WFYLVYGGR	9
7	24.35	1159.667	580.8333	SYLPLLPTEK	10
8	22.56	1160.648	581.3241	NQVSLTGCLVK	11
9	25.81	1191.589	596.7947	AM(Ox)FYLAAAFAT	11
10	26.9	1229.72	615.86	QGLLPVLESFK	11
11	21.25	1344.652	673.3262	RACSAEYVFATK	12
12	27.38	1385.736	693.8679	VSFLSALEEYKT	12
13	24.85	1477.746	739.873	MYLGYEYVTALR	12
14	23.35	1501.77	751.8851	AMTYSLSSTLTLSK	14
15	21.78	1553.794	777.897	EASVCNVYLQTSLK	14
16	24.88	1611.797	806.8983	LLDNWDSVTSTFSK	14
17	23.08	1631.799	816.8996	FSGSGSGTDFTLTLSR	16
18	24.71	1834.962	918.4812	QEPSQGTTTFAVTTVLR	17

[a]Seq i.d.: sequence identification. t_R: retention time (minutes). no: number of residues.

Table 3. Sequences Identified by MS-MS from the Hp Sample Obtained by TAS[a]

Seq i.d.	t_R	experimental mass	ion (m/z)	sequence	no.
19	15.75	959.395	480.6975	EEGEEEAPA	9
20	24.66	1462.842	488.6139	ASVLKTLADALHPQ	14
21	26.40	1081.571	541.7856	FLGALLCSAST	11
22	23.85	1329.853	665.9266	FLGTLLKLGKAVA	13
23	30.01	1552.979	777.4895	LLPALTGLLHLAPPK	15

[a]Seq i.d.: sequence identification. t_R: retention time (minutes). no: number of residues.

and 40% similarity with leptoglycin isolated from the South American frog *Leptodactylus pentadactylus.*[47] Seq 2 showed 50% similarity with temporin-1TGc, isolated from *Rana tagoi,*[48] and with temporin-1Ska from *R. sakuraii,*[49] while Seq 3 showed 43% similarity with brevenin-1Ja, isolated from *R. japonica,*[50] 35% similarity with hylin b-1 isolated from *Hyla biobeba,*[35] and 26% similarity with hylin a-1, isolated from *Hypsiboas albopunctatus.*[34]

Seq 6 showed 36% similarity with temporin-1KM, temporin-Rb, and temporin-1CSc, all isolated from the genus *Rana,*[51,52] while Seq 8 displayed 45% similarity with JCpep7, a peptide isolated from *Jatropha curcas.*[53]

Seq 11 showed 35% similarity with MP-VB1, an antimicrobial peptide isolated from the venom of *Vespa bicolor fabricius,*[54] and Seq 14 38% similarity with temporin-1Ce, a peptide isolated from the skin of the North American green frog *Rana clamitans.*[55]

The sequences identified from the Hp (TAS) sample are shown in Table 3; only five ions were sequenced with a "de novo" Peaks Studio score higher than 85% (ALC ≥ 85%). According to the predictive analyses of secondary structure, four sequences (Seq 20−23) showed α-helix contributions. Seq 19 had a net charge of −5, and the high content of glutamic acid residues suggests that this sequence may be part of the acidic region of a prepropeptide, as was well studied for hylid and ranid frogs.[32,36,56,57]

Seq 20 comprised 50% hydrophobic amino acid residues and had a tendency to form an α-helix. The analysis done by means of the AMP database (APD) suggested that Seq 20 shares 50% identity with MB-21, a synthetic peptide with helical structure, which was reported to be particularly active against *E. coli* and *S. aureus,* and also has antifungal activity.[58] MB-21 also has demonstrated capacity to lyse liposomes.[59]

Seq 21 comprised 63% hydrophobic residues and showed a tendency to form α-helices. The sequence presented similarity with the temporins 1TSa (46%), PRa (43%), 1M (43%), 1Ec (43%), and 1AUa (43%).[47,60−62]

Seq 22 contained 61% hydrophobic amino acid residues and showed 50% similarity with temporin-1CSd[63] and temporin-1Lb,[64] both peptides that are highly active against Gram (+) and Gram (−) bacteria. On the other hand, Seq 22 displayed 47% similarity with CPF-C1 (C1 fragment of the caerulin precursor) isolated from *Xenopus clivii.*[65]

Seq 23 corresponded to a proline-rich peptide, with 53% hydrophobic amino acid residues. Consulting the APD database, we found that this sequence shares 47% similarity with temporin −1CSC, isolated from *Rana cascadae,*[51] and 47% similarity with temporin C, isolated from *Rana temporaria.*[66] It showed 43% similarity with dahlein 5.5, isolated from *Litoria dahlii,* which belongs to the Hylidae family.[67] According to the data obtained through the APD database, many of the identified sequences showed partial identity with temporins and other amphibian peptides isolated from the genus *Rana*.

In a first stage, we analyzed Hp samples (SE and TAS) by MALDI-TOF-TOF. Only four ions from the SE sample ($[M + H]^+ = m/z$ 856.5, 901.6, 1850.1, and 1892.1) and one from the TAS one ($[M + H]^+ = m/z$ 1517.8) could be fragmented. The MS-MS spectra of the 1850.1, 1892.1, and 1517.8 ions did not give clear enough fragmentation signals to infer a peptide sequence (data not shown).

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Table 4. Sequences and Properties of the Synthetic Peptides

P. i.d.[a]	sequence	secondary structure prediction: PSIPRED	secondary structure prediction: GOR V	net charge at pH = 7	experimental MW[b]	Hy/total aa[c]
P1-Hp-1971	TKPTLLGLPLGAGPAAGPGKR-NH_2	coil	coil	+4	1971.173	7/20 (35%)
P2-Hp-1935	KLSPSLGPVSKGKLLAGQR-NH_2	coil	coil	+5	1935.209	6/19 (31.5%)
P3-Hp-1891	RLGTALPALLKTLLAGLNG-NH_2	α-helix (T_4-L_{17})	α-helix (P_7-A_{15})	+3	1891.241	10/19 (52%)

[a]P. i.d.: peptide identification. [b]Corresponds to the $[M + H]^+$ as determined by MALDI-TOF mass spectrometry. [c]Hy/total aa: % of hydrophobic amino acids in the whole sequence.

The analysis of the samples by LC-MS-MS provided separation of the peptides by reversed-phase liquid chromatography and characterization of more peptide sequences. Moreover, high accuracy in the value of the masses and a better fragmentation of the ions allowed improvement of the signal resolution, thus achieving the characterization of ions that was not possible by MALDI-TOF-TOF.

Solid-Phase Peptide Synthesis (SPPS). Seq 1, 2, and 3 (Table 2) were selected as the first models to study, as these were the largest and most cationic sequences identified in the two samples.

In particular, Seq 1 is a proline- and glycine-rich peptide with a typical extended structure that is not common to frog skin peptides. We therefore considered it of interest to explore the antimicrobial properties of this sequence.

The predictive methods GOR V and PSIPRED indicated that the secondary structure of Seq 3 may correspond to an α-helix. This sequence comprises 52% hydrophobic amino acid residues, which suggests that it may interact with bacterial membranes.

Given that amidation is a common feature of many AMPs isolated from frogs,[68−72] we used SPPS to synthesize these sequences as C-terminal amides. The presence of the amide functional group has been reported to increase the efficiency and selectivity of anticancer and antimicrobial peptides.[73,74]

The corresponding synthetic peptides were named P1-Hp-1971, P2-Hp-1935, and P3-Hp-1891 (Table 4).

Antimicrobial and Hemolytic Activity of the Synthetic Peptides. Table 5 shows the minimal inhibitory concentration (MIC) values of the three synthesized peptides against *E. coli* ATCC 25922 and *S. aureus* ATCC 25929 strains. The most active peptides were P3-Hp-1891 and P1-Hp-1971. However, while the latter was more active against *S. aureus*, the former equally inhibited both strains. Regarding the data reported in the literature about the antimicrobial activity of peptides isolated from amphibians, the results are highly variable.

Table 5. MIC and TI of the Peptides[a]

P. i.d.	MIC (μM): *E. coli*	MIC (μM): *S. aureus*	LHC (μM)	therapeutic index (TI): *E. coli*	therapeutic index (TI): *S. aureus*
P1-Hp-1971	16	8	640	40	80
P2-Hp-1935	33	66	320	9.7	4.9
P3-Hp-1891	17	17	25	1.5	1.5

[a]MIC: minimal inhibitory concentration. P. i.d.: peptide identification. LHC: lowest hemolytic concentration.

Magainin 2 amide (GIGKFLHSAKKFGKAFVGEIMNS) and related peptides isolated from the skin secretions of *Xenopus laevis* are potent antimicrobial compounds with a broad spectrum of activity against many species of bacteria and fungi and also induce osmotic lysis of protozoa.[75] MIC values of around 50 μM against *S. aureus* and *E. coli* were reported for this peptide.[76] By increasing the cationicity by varying the number of lysine residues and maintaining the hydrophobicity, it was possible to optimize the antimicrobial activity of this α-helical peptide, lowering the MIC values more than 10-fold.[77] From these early reports, the antimicrobial activities of other α-helical peptides isolated from amphibians have been documented, such as bombinins, dermaseptins, caerins, maculatins, aureins, and temporins, among others.[78−81]

Table 1 shows the sequences and MIC values reported for several peptides isolated from *Hysiboas* species. Hylin a-1 isolated from *H. albopunctatus* shows considerable antimicrobial activity against *E. coli* (32 μM), *S. aureus* (8 μM), and *E. faecalis* (16 μM), but also high hemolytic activity (50% of hemolysis at 18 μM).[34]

In Figure 2 the hemolysis curves of the peptides are shown. Except for P3-Hp-1891, none of the peptides showed percentages of hemolysis higher than 10%. At a concentration of 320 μM (data not shown), P2-Hp-1935 produced 100% hemolysis. In contrast, P1-Hp-1971 did not exceed 12% hemolysis and was thus considered a nonhemolytic peptide.

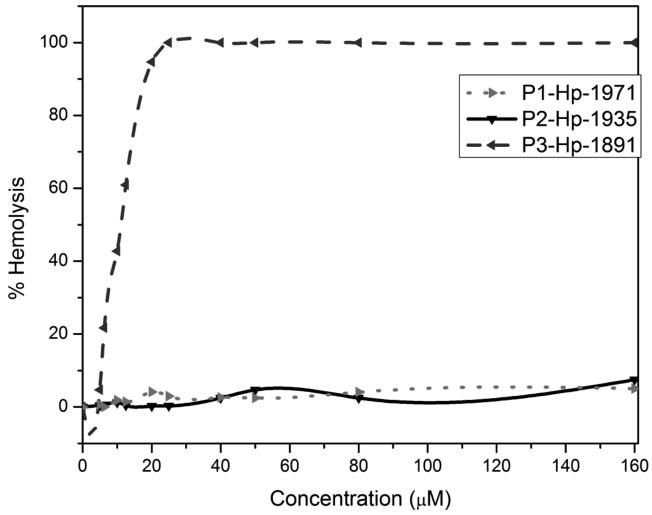

Figure 2. Hemolysis of human erythrocytes as a function of concentration of the three synthetic peptides.

P3-Hp-1891 produced more than 90% hemolysis at a concentration of 20 μM. This result correlates with the presence of a helical and amphipathic structure (Figure 3 and Table 4), which contained 52% hydrophobic amino acid residues.

A typical example of a high-hemolytic peptide is melittin, a potent toxin isolated from bee venom[82] that produces 50% hemolysis at 0.6 μM.[83] This toxin has a helical structure and high hydrophobicity.

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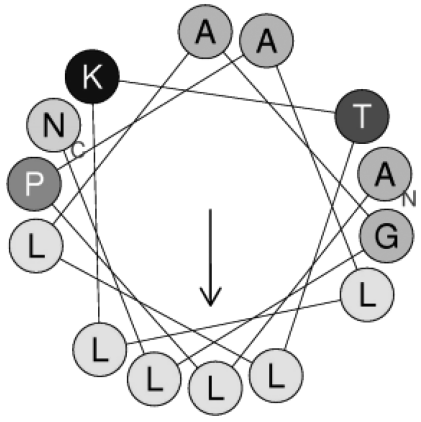

Figure 3. Schiffer–Edmundson wheel projection of P3-Hp-1891. The representation was done using Heliquest software (http://heliquest.ipmc.cnrs.fr/). The window of analyses was limited to the helical region (5–18), determined on the basis of the data obtained by PSIPRED and GOR V. Amphipathic moment (μ): 0.533.

P1-Hp-1971 showed the highest therapeutic index (TI); this finding was expected, as the peptide was not hemolytic (up to 320 μM), and it showed the lowest MIC values (16 μM for *E. coli* and 8 μM for *S. aureus*) (Table 5).

In contrast, the lowest TI values were obtained with P3-Hp-1891. These results are comparable to that found for toxins, such as melittin, for which TI values of 0.6 for Gram (+) and Gram (−) bacteria have been determined.[84]

Secondary Structure Determination by Circular Dichroism (CD). The CD spectra corresponding to the three synthetic peptides are shown in Figure 4. These were obtained in the following experimental conditions: 2,2,2-trifluoroethanol (TFE)/H_2O (50% v/v) and in the presence of dipalmitoylphosphatidylglycerol (DPPG) and dipalmitoylphosphatidylcholine (DPPC) liposomes.

In H_2O, the peptides did not show any preferential conformation, as expected for short linear peptides. These data are consistent with a minimum at 198 nm. In the presence of TFE, P3-Hp-1891 adopted an α-helix conformation, consistent with the presence of two minima at 205–207 nm and 215–220 nm and a maximum near 195 nm. The spectra deconvolution, done with CONTILL and SELCOM 3,[85−87] indicated that P3-Hp-1891 has more than 70% helical structure. P2-Hp-1935 also showed α-helical contributions, while P1-Hp-

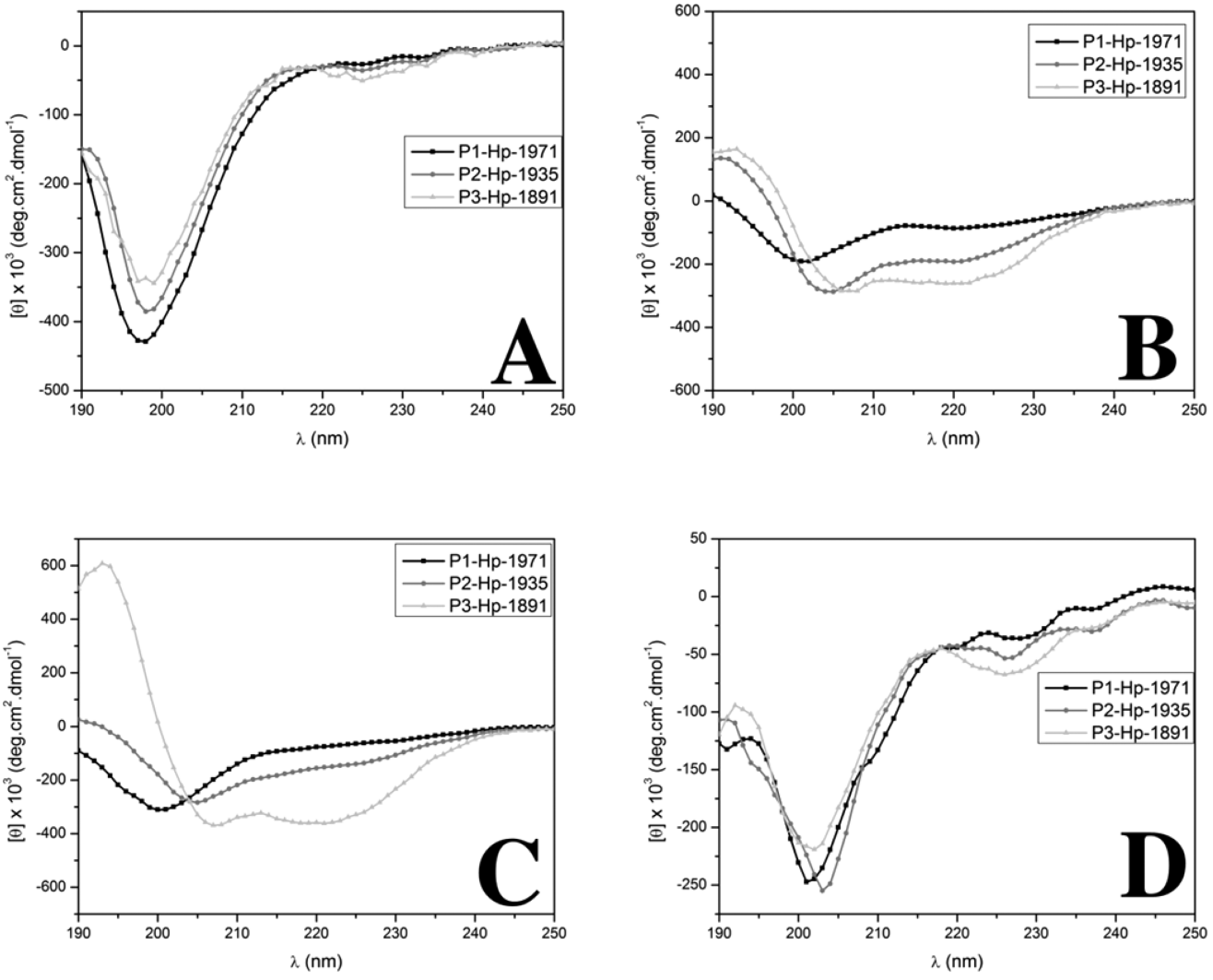

Figure 4. Circular dichroism spectra of the three synthetic peptides: (A) H_2O, (B) TFE/H_2O (50%, v/v), (C) DPPG liposomes, (D) DPPC liposomes. Peptide concentration: 0.2 mg/mL.

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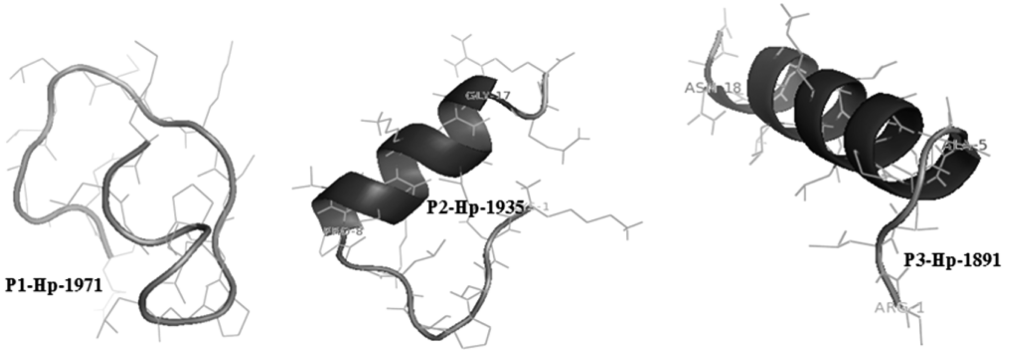

Figure 5. Structural models of the synthetic peptides P1-Hp-1971, P2-Hp-1935, and P3-Hp-1891 The pdb secondary structure models were obtained using the Web resources PEP-FOLD (http://bioserv.rpbs.univ-paris-diderot.fr/cgi-bin/PEP-FOLD) and Spark X (http://sparks.informatics.iupui.edu/).

1971 was the least ordered in the presence of TFE (unordered 40%).

In the presence of DPPG liposomes (Figure 4C), P3-Hp-1891 adopted an α-helical conformation. Spectra deconvolution suggested the contribution of more than 70% α-helix. In these conditions P2-Hp-1935 showed 36% α-helix, 24% turn, and 40% unordered structure, while P1-Hp-1971 displayed 35−40% turn and 40% unordered structure, in concordance with the prediction data (Table 4).

In the presence of DPPC liposomes, P1-Hp-1971 and P2-Hp-1935 were unordered. Nevertheless, for P3-Hp-1891 the spectrum deconvolution indicated that the peptide is partially in an α-helical conformation (approximately 30%).

In accordance with the CD results, the 3D models of P2-Hp-1935 and P3-Hp-1891 suggest the presence of extended α-helical structures. The structure of P1-Hp-1971 is highly flexible, with regions of turns and random coils (Figure 5).

Structure−Activity Relationships. Several studies have revealed that, in spite of the wide diversity in the amino acid sequences of amphibian AMPs, in most cases they adopt amphipathic α-helical structures in the presence of membrane-mimetic micelles, liposomes, and organic solvent mixtures. Nevertheless, we found that only five of the 23 identified sequences of *H. pulchellus* form an α-helix, according to the predictive methods.

P3-Hp-1891 showed high antimicrobial activity and a similar capacity to inhibit the growth of *S. aureus* and *E. coli* strains. The sequence contained 52% hydrophobic amino acid residues, had a net charge of +3, and adopted an extended amphipathic helical structure in the presence of TFE and anionic liposomes. The membrane selectivity was very low, as it was markedly hemolytic at concentrations near the MIC (about 48% hemolysis).

Studies with model α-helical peptides have demonstrated a direct correlation between hydrophobicity and cytolytic activity, particularly against mammalian cells such as erythrocytes. Other parameters, such as helicity, amphipathicity, hydrophobic moment, and the size of the hydrophobic/hydrophilic domain, influence membrane interaction and selectivity.[14,88,89]

The reduced antimicrobial activity of P2-Hp-1935 was associated with a low content of hydrophobic amino acid residues (31.5%) and reduced helicity, despite its good cationicity (net charge +4). This peptide was more active against *E. coli* than against *S. aureus*.

It has been proposed that a stabilized amphipathic α-helical structure is a requirement for cidal activity against Gram (+) bacteria and fungi. The structural requirements for activity against several Gram (−) bacteria were instead considerably less stringent, so that activity persisted in peptides in which the formation of α-helical structure and/or amphipathicity were impeded.[90]

Although there is no universal agreement regarding the precise mechanism of action of AMPs, it is accepted that cytolytic activity is not mediated by interaction with specific receptors. Many studies have shown that peptide−lipid interactions lead to membrane permeation and play a major role in the activity of AMPs. Membrane permeation by amphipathic α-helical peptides has been proposed to occur via one of two general mechanisms: transmembrane pore formation via a "barrel-stave" mechanism, and membrane destruction/solubilization via a "carpet" mechanism.[14,81,91−95]

The "barrel-stave" model describes the formation of transmembrane channels/pores by bundles of amphipathic α-helices, such that the hydrophobic surfaces interact with the lipid core of the membrane and their hydrophilic surfaces point inward, producing an aqueous pore.[91] Peptides with cytolytic properties toward both bacteria and mammalian cell membranes are considered to act following the "barrel-stave" model.[92]

Temporins have the propensity to form a stable amphipathic α-helix in a membrane-mimetic environment. The lack of selectivity of these peptides may be related to the low number of positive charges, and the binding to the membrane is due mostly to hydrophobic interactions; thereby, Mangoni et al.[81] suggested the occurrence of a "barrel-stave" mechanism for these molecules.

It has also been reported that ceratotoxins, which are cationic and α-helical peptides isolated from *Ceratitis capitata* (fruit fly), and the peptide Ctx-Ha, isolated from *Hypsiboas albopunctatus* and sharing sequence similarity with ceratotoxins, also act by this mechanism.[93,94]

In the "carpet" model proposed by Shai and co-workers,[92,95] the membrane is permeabilized in a "detergent-like" manner. The peptides are in contact with the lipid head groups during membrane permeation and do not insert into the hydrophobic core of the membrane. This mechanism explains the mode of action of a range of α-helical AMPs from frog skin that show selective activity against bacteria compared to eukaryotic cells.

In previous studies, we demonstrated that a bacteriocin named Plan149a acts by the "carpet" mechanism.[96−98]

In this context, and according to the CD spectroscopy results, the antimicrobial activity of P3-Hp-1891 could be

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explained by the formation of transmembrane pores, in light of the α-helix folding observed in the presence of negatively charged vesicles. However, the same folding was not observed for the purely zwitterionic vesicles (mimicking a eukaryotic membrane). The low cationicity and high hydrophobicity were not sufficient to provide the peptide with a good selectivity for negatively charged membranes, and this explains the high hemolytic activity of P3-Hp-1891.

More recently, it has been shown that AMPs may act by other mechanisms, such as producing membrane perturbation by formation of specific lipid−peptide domains and even the formation of nonlamellar lipid phases.[99]

Buforin II is an α-helical AMP that kills a microorganism by entering the cell without membrane permeabilization, so it seems that the target is the cytoplasm of the cell.[100]

Peptides enriched in specific amino acids may translocate to the cytoplasm without disrupting the membrane. Linear peptides enriched in arginine and/or proline have been evaluated as cell-penetrating peptides (CPPs). These molecules have generated considerable attention on the part of the research community because of their potential for drug delivery systems. The most frequently used CPPs are polyarginines, Tat, penetratin, and calcitonin-derived peptides, among others.[101,102]

There are a few reports about proline-rich peptides isolated from amphibians.[103−106] Highly cationic fragments of the AMP Bac 7 have been reported to cross the cell membrane and reside within the nucleus. A common characteristic shared by the cell-permeant Bac(1−24) fragments is their high proline content.[103] PR-bombesin is a bombesin-like peptide derived from the skin of the Chinese red belly toad, *Bombina maxima*, that exhibited a broad-spectrum of antibacterial activity and contains a proline-rich segment (−KKPPRPP−) on the N-terminal region of this peptide.[104]

Another example of a proline-rich peptide is PhypoXa, isolated from the skin secretion of *Phyllomedusa hypochondrialis* (pEFRPSYQIPP). This peptide is able to potentiate bradykinin activities in vivo and in vitro, in addition to efficiently and competitively inhibiting the angiotensin converting enzyme (ACE).[105]

The secretion of the frog *Litoria rothii* contains a series of new peptides, including a new proline-rich peptide, named rothein 4.1 (AEILFGDVRPPWMPPPIFPEMP-OH), which shows neither antimicrobial nor neuronal nitric oxide synthase activity.[106]

Peptide P1-Hp-1971 is a proline-rich peptide and was the most active synthetic AMP, being more active against *S. aureus* than against the *E. coli* strain. Its sequence suggests entry based on folding around the polar portion, likely complexed with a counterion for the arginine. A detailed analysis of CD spectra shows that the minimum at 198 nm, characterizing the spectrum collected in H_2O (unstructured), shifted to higher wavelengths in the presence of both kinds of liposomes, and negative ellipticity decreased. These spectral modifications could suggest either folding of the peptide in a PP-II-like structure in the presence of the vesicles or the equilibrium between the unfolded and a partially helical folded state. The presence of PP-II conformation in proline-rich peptides and proteins has been reported.[107,108]

Consequently, it is probable that P1-Hp-1971 acts differently from the other two peptides, and its mechanism of action cannot be explained by permeabilization of the bacterial membrane. Therefore, other possible actions such as membrane depolarization, anionic lipid clustering, and translocation should be considered in future studies to determine the mechanism of action of this peptide. Our findings highlight anuran amphibians from Argentina as a potential source of new AMPs.

EXPERIMENTAL SECTION

General Experimental Procedures. Seq 1, 2, and 3 were determined by direct infusion into a Q-TOF Global mass spectrometer (Micromass/Waters). For the rest of the sequences we used a nanoAcquity liquid chromatograph (Waters) integrated with an LTQ-OrbitrapVelos (Thermo Scientific) mass spectrometer.

Far-UV CD measurements were taken on a Jasco J-810 CD spectrometer (Tokyo, Japan) in a 0.1 cm path quartz cuvette (Hellma) and recorded after accumulation of five runs.

Synthetic peptides were purified by HPLC (Gilson, France) using a semipreparative reversed-phase C_{18} column (Jupiter-Proteo Phenomenex, 10 μm, 90 Å, 250 × 10 mm). The purified peptides were analyzed by analytical RP-HPLC using a Jupiter (Phenomenex) C_{18} column (5 μm, 300 Å, 250 × 4.60 mm). The peptides were eluted with a linear gradient of 5−80% of CH_3CN with 0.1% TFA for 33 min at a flow rate of 0.8 mL/min. Absorbance was measured at 220 nm. The experimental molecular weights were determined by MALDI-TOF/TOF in an Ultraflex II Bruker Daltonics mass spectrometer.

Collection of Amphibian Specimens. Adult specimens of the common tree frogs, *Hypsiboas pulchellus* (N = 7) (Anura: Hylidae), were collected in the northern access to the city of Paraná (province of Entre Ríos, Argentina) during the summer months, in the period 2006−2010. Voucher specimens are deposited in the herpetological collection, Laboratory of Ecotoxicology, Faculty of Biochemistry and Biological Sciences, Santa Fe, Argentina.

Methods for Biological Sampling. Biological samples from each species were collected by electrical stimulation of the granular glands using the transcutaneous membrane stimulator previously described by Tyler et al.[109] and by extraction with acidified EtOH from the skin.

Transcutaneous Amphibian Stimulation. The instrument for electric stimulation was built following the literature.[109,110] Secretions were obtained by moistening the skin with Milli-Q H_2O and then gently massaging the frogs laterally and dorsally from the neck to the thigh with activated TAS electrodes for 20−30 s with a maximum voltage of 20 V. During the electrical stimulation the skin was rinsed with Milli-Q H_2O, and the aqueous solution was collected in a suitable vessel. No adverse events were observed in the specimens after stimulation.

Solvent Extraction. The specimens were euthanized following the recommendations of the American Society of Ichthyologists & Herpetologists[111] and with the approval of the Animal Ethics Committee of FBCB−UNL (Santa Fe, Argentina).

The skin of each frog was removed and triturated, and a solution of EtOH/H_2O (60:40) was added. Aliquots of acetic acid were added for the extraction and solubilization of compounds. The solution was kept under constant agitation at 0 °C for 2 h, followed by centrifugation at 10 000 rpm at 4 °C, and the process was repeated twice. The supernatant was separated and concentrated under reduced pressure on a rotary evaporator.

Samples obtained by both methods were lyophilized and stored at −20 °C.

Mass Spectrometry Analysis. Seq 1, 2, and 3 were ionized in an emitter needle (picoTip, New Objective) with a voltage of 2 eV. The collision energy was 35−40 eV, and the collision gas was argon. The cone voltage was 70 eV. Resolution was 10 000 at m/z 400. For "de novo" analysis of those sequences, Peptide Sequencing (PepSeq), a tool in Masslynx software (Waters), was used (error tolerance of the peptide: 50 ppm; error tolerance of the fragment: 0.1 Da).

For the rest of the sequences, aliquots of the resuspended samples were injected for chromatographic separation in a C_{18} column (75 μm × 10 cm, 1.7 μm BEH column, Waters). Solvents: A, 0.1% formic acid in H_2O; B, 0.1% formic acid in CH_3CN. The following gradient elution was used: 1% to 40% of B in 20 min, followed by 40% to 60% of B in 5 min, flow rate 250 nL/min. The eluted peptides were ionized

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by applying an electrical potential with a nano-ES needle. The applied voltage was 2 kV. The masses of the peptides were measured in full scan MS (Orbitrap at a resolution of 60 000 fwhm at 400 m/z). Up to five of the most abundant peptides (minimum intensity of 3000 counts) were selected in each MS analysis to be fragmented in the high energy collision-induced dissociation (HCD) trap with helium as the collision gas, with normalized collision energy of 40%. Data were acquired with Thermo Xcalibur software (v.2.1.0.1140) in raw format.

For the "de novo" analyses, Peaks Studio v5.2 software was used (error tolerance of the peptide: 10 ppm; error tolerance of the fragment: 0.1 Da). The average local confidence (ALC) reflects the average correct ratio for each amino acid in the sequence and was used as a "de novo" Peaks Studio score.

Structure Analysis. Each sequence identified by MS-MS was analyzed by GOR V and PSIPRED predictive methods.[44,45] Some sequences were also analyzed through the Web resources for 3D structure prediction named PEP-FOLD (http://bioserv.rpbs.univ-paris-diderot.fr/cgi-bin/PEP-FOLD) and Spark X (http://sparks.informatics.iupui.edu/). This approach allowed us to obtain a predictive structural model for the most studied sequences.[112−115]

Circular Dichroism Analyses. CD analyses were recorded in the presence of DPPG and DPPC vesicles. For the preparation of small unilamellar vesicles, the lipid dispersion in Milli-Q H_2O was sonicated, using a tip-sonicator, until the solution became transparent. The final lipid concentration was 3 mM, and the peptide concentration was 0.2 mg/mL in all samples. Spectra were corrected for background scattering caused by the vesicles by subtracting the spectrum of a single vesicle solution from that of the peptides in the presence of vesicles.[116] Additional spectra were obtained in H_2O and in the presence of TFE [50% TFE (v/v)]. The final peptide concentration was 0.2 mg/mL in all cases. Deconvolution of CD spectra was performed by means of the CDPro software package (Colorado State University) using the SELCOM 3 and CONTILL methods.[85−87]

Peptide Synthesis. Peptides were synthesized as C-terminal amides by Fmoc solid-phase peptide synthesis. Couplings were performed by *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide and diisopropylethylamine. Fmoc removal was done with 20% piperidine in DMF (v/v). Final cleavage from the resin was achieved by a mixture of TFA/H_2O/1,2-ethanedithiol/triisopropylsilane (94.5:2.5:2.5:0.5) (v/v). After 3 h, the resin was filtered off and the crude peptide was precipitated in dry, cold diethyl ether, centrifuged, and washed several times with cold diethyl ether until the scavengers were removed. The product was then dissolved in H_2O and lyophilized twice.

Minimal Inhibitory Concentration Determination. MIC determinations were performed by the modified microtiter dilution assay, following the procedures proposed by the R.E.W. Hancock Laboratory for testing AMPs (http://cmdr.ubc.ca/bobh/methods/MODIFIEDMIC.html). The target strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25929 were activated by culture for 24 h at 37 °C in Mueller-Hinton broth (MHB) (Biokar Diagnostics). An inoculum was taken and adjusted to cellular concentrations of 5×10^7 cfu/mL. These inocula were used to perform the assay using diluted MHB and were incubated from 18 to 24 h at 37 °C.[117−119] The MIC was the lowest peptide concentration that inhibited the growth of each bacterial strain.

All of the peptides were dissolved in 36 μL of Milli-Q H_2O with the addition of 10% acetic acid in order to favor their solubilization and were then further diluted to the highest concentration of the assay (1280 μg/mL).

Hemolysis Assays. The assay was performed using human red blood cells and following previously described protocols.[98,120] Briefly, human erythrocytes were isolated from heparinized blood by centrifugation (3000 rpm for 10 min), after washing three times with saline solution. Erythrocyte solutions were prepared at a concentration of 0.4% (v/v) in isotonic-saline solution. Test tubes containing 1 mL of erythrocyte solution were incubated with 1 mL of increasing concentrations of each extract (from 0.4 to 4.4 mg/mL) for 60 min at 37 °C. After centrifugation at 3000 rpm for 5 min, the supernatant absorbance was measured at 405 nm. Lysis induced by 1% Triton X-100 was taken as the 100% reference value.

Therapeutic Index Calculation. The therapeutic index or specificity is defined as the relationship between the lowest hemolytic concentration (LHC is the lowest peptide concentration that produces 100% hemolysis) and the MIC. When 100% of hemolysis was not detected at 320 μM, a value of 640 μM was used to calculate the TI. The index was calculated for each peptide and bacterial strain tested.

■ ASSOCIATED CONTENT

Supporting Information

Analysis of the MS-MS spectra of sequences 1, 2, and 3 (natural peptides, Table 2) are included as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All the authors contributed to the writing of this manuscript and approved the final version.

Notes

The authors declare no competing financial interest.

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