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Phylloseptins: a novel class of anti-bacterial and anti-protozoan peptides from the *Phyllomedusa* genus

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Abstract

Six novel peptides called phylloseptins (PS-1, -2, -3, -4, -5, and -6) showing anti-bacterial (PS-1) and anti-protozoan (PS-4 and -5) activities were isolated from the skin secretion of the Brazilian tree-frogs, *Phyllomedusa hypochondrialis* and *Phyllomedusa oreades*. Phylloseptins have a primary structure consisting of 19–21 amino acid residues (1.7–2.1 kDa). They have common structural features, such as a highly conserved N-terminal region and C-terminal amidation. Phylloseptin-1 (FLSLIPHAINAVSAIAKHN-NH_2) demonstrated a strong effect against Gram-positive and Gram-negative bacteria (MICs ranging from 3 to 7.9 μM), without showing significant hemolytic activity (<0.6% at the MIC range) towards mammalian cells. Atomic force microscopy experiments indicated that the bacteriolytic properties of these peptides might be related to their disruptive action on the cell membrane, characterized by a number of bubble-like formations, preceding every cell lysis. PS-4 and PS-5 showed anti-protozoan activity with IC_{50} at about 5 μM for *Trypanossoma cruzi*.

Keywords: Antimicrobial peptides; Phylloseptin; *Phyllomedusa oreades*; *Phyllomedusa hypochondrialis*; Atomic force microscopy; Trypanocidal activity

1. Introduction

Bacteria, fungi, and protozoa coexist with all living animals for millions of years. However, considering the number and diversity of these microorganisms and their constant interactions with animals, lethal microbial invasion to the hosts is quite rare [20]. This is mainly due to the highly specific cell-mediated immune responses, and to the broad-spectrum antimicrobial peptides from their innate immune systems. The different families of antimicrobial peptides, which have been classified on the basis of their amino acid sequences, biological activity and secondary structures, are often found in many insects [42], plants [14], amphibians [45], mammals [3,15,32], and microorganisms [6,8]. In amphibians, amphiphilic α-helical antimicrobial peptides such as magainins [17], bombinins [22], buforins [34], and dermaseptins [4,10,30,35] have been a subject of intense research regarding their biosynthesis, activity towards microorganisms, mechanisms of action, and potential clinical applications. In spite of all the effort put on these research lines up to now, it is clear that predicting peptide activity, specificity and 3-D structure from the amino acid sequence alone is not a simple task [23], most peptides without disulfide bonds show no

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defined structure in water, and it is only in a membrane or in a hydrophobic environment that they may fold into α-helices or β-structures [5,19]. It is known that the net positive charge associated with the hydrophobic nature of the polypeptide chain, found in the great majority of the antimicrobials, is the cornerstone feature for the activity and specificity of these molecules. In fact, plasma membrane recognition and binding are the initial steps of the peptide–membrane interaction and a detailed knowledge of these processes and their driving forces are important prerequisites to understand the mechanism of selective lysis of bacterial, fungal and protozoan membranes, which have been reported so far [24,38]. The three-dimensional structures of many antimicrobial peptides demonstrate an amphiphilic motif, where the hydrophilic face is formed by polar and positively charged residues (typically, lysine and arginine) and the hydrophobic face are constituted by non-polar amino acids. This topological arrangement is often induced by the contact of the peptide with hydrophobic environments such as organic solvents, SDS vesicles and phospholipid micelles [36,44]. The lytic activity, on the other hand, appears to be related to the physicochemical properties of the peptide conferred by its amino acid composition and sequence.

For more than two decades the worldwide resurgence of infectious diseases due to evolution of antibiotic-resistant strains is increasing at an alarming pace, as antibiotics progressively loose their effectiveness. In this work, we report a novel family of antimicrobial and anti-protozoan peptide from the skin secretion of two *Phyllomedusa* species (*Phyllomedusa oreades* and *Phyllomedusa hypochondrialis*) named phylloseptins. These new peptides (PS-1, -2, -3, -4, -5, and -6) have molecular mass ranging from 1.7 to 2.1 kDa, and PS-1 showed MICs at about 3 μM for pathogenic bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis* and *Staphylococcus aureus*), and PS-4 and -5 anti-*Trypanosoma cruzi* activity and PS-1 and -2 had negligible effects on red blood cells.

2. Materials and methods

2.1. Amphibians

Frogs skin secretions were obtained from adult specimens of *P. hypochondrialis* ($n = 6$, captured in Brasília, Brazil) and *P. oreades*, a recently described [11] species of the *Phyllomedusinae* sub-family ($n = 4$, captured in Serra da Mesa, Goiás, Central Brazil). Frogs were collected according to the Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis, IBAMA, under the license number 097/96-DIFAS (Process no. 0637/91A.C).

2.2. Peptide purification

Frog secretions were obtained by mild electric stimulation of the skin granular glands of *P. hypochondrialis* and *P. oreades* for a few seconds and freshly collected in distilled water. The water-soluble secretions from each species were filtered by gravity through filter paper, frozen, and lyophilized (Centrivap Concentrator, Labconco). Peptide separation was performed by application (5 mg aliquots each time) of the crude extract to a semi-preparative Vydac reverse-phase (RP) chromatographic column, C_{18}, 5 μm (218TP510; 10 mm × 250 mm) in HPLC system (Shimadzu Co.) Peptides were purified by using linear gradients, initially 0–80% acetonitrile containing 0.1% TFA (trifluoroacetic acid) for 70 min, followed by 80–100% of the same solvent for 20 min. The experiments were monitored at 216 and 280 nm. Fractions were collected manually and lyophilized. The isolated fractions were submitted to another chromatographic step using a Vydac 218TP54, C_{18}, 5 μm (4.6 mm × 250 mm) analytical column, with optimized gradients of acetonitrile in 0.1% TFA over 60 min.

2.3. Purity and molecular mass determination

The molecular mass and homogeneity of PS-1, PS-2, PS-3, and PS-6 (*P. hypochondrialis*), and PS-4 and PS-5 (*P. oreades*) were determined by MALDI-TOF/MS in a 4700 Proteomics Analyzer with TOF–TOF optics (Applied Biosystems, Framingham, MA). Approximately 20 nmol of lyophilized peptide was dissolved in Milli-Q water and mixed with a saturated matrix solution of α-cyano-4-hydroxycinnamic acid (1:3, v/v) and spotted onto a MALDI-TOF/TOF sample plate, at room temperature.

2.4. Sequence analysis

De novo sequencing of the phylloseptins were performed by MS/MS experiments using argon as collision gas on a Q-TOF Ultima (Waters-Micromass, Manchester, UK) operating in W mode and in a 4700 Proteomics Analyzer with TOF–TOF optics (Applied Biosystems, Framingham, MA). The MS and MS/MS spectra were carried out in reflector mode with external calibration, using the 4700 calibration mixture kit (Applied Biosystems). Peptide de novo sequencing was performed by precursor ion fragmentation, using N_2 as CID gas and the collision cell pressure was kept at 1.8×10^{-6} Torr. Leucine, isoleucine and lysine residues present in each one of the phylloseptin polypeptide chains were assigned based on the high energy fragmentation ions and confirmed by automated Edman degradation on a *PPSQ-23* protein peptide sequencer (Shimadzu Co., Japan). Peptide sequence alignments and similarity searches were performed using the FASTA 3 program on the ExPASy molecular server (http://www.expasy.ch/). Secondary structure prediction was performed using SOPMA, also at this server [21,40].

2.5. Hemolytic assay

Hemolytic assays were performed using human red cells (blood type O^-) in liquid medium, as reported previously

[1]. Serial dilutions of PS-1 and PS-2 (10 μL in PBS) were added to 190 μL of blood and after an incubation at 37 °C for 30 min, the cells were centrifuged and the absorbance of the supernatant was measured at 567 nm. Maximal hemolysis was determined by adding 0.2% Triton X-100 to a cell sample.

2.6. Antibacterial assay

Antibacterial activity of the PS-1 against Gram-positive and Gram-negative bacteria was determined by the broth microdilution assay [31]. The microorganisms used were *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, and *E. faecalis* ATCC 29212. The *P. aeruginosa* wt strain was isolated in the Pathology Laboratory from Hospital Universitário de Brasília, HUB, Brasília, Brazil. Single colonies of bacteria were inoculated into culture medium (3% trypticase soy broth) and cultured overnight at 37 °C. An aliquot of this primary culture was transferred to 50 mL of fresh medium and incubated for additional 3–6 h at 37 °C, in order to obtain the cells in mid-logarithmic phase.

The bioassays were performed by liquid growth inhibition assay as described by Bulet et al. [13]. PS-1 was dissolved in sterile Milli-Q water and diluted eight-fold in TSB (Oxoid, England) broth. The highest concentration used for the assay was 64 μM. The initial inoculum was approximately 1.0×10^5 colony forming units CFU/mL and the limit of detection was 10^2 CFU/mL. The final volume was 250 μL (25 μL of the peptide test in water, 25 μL of the inoculums in TSB, and 200 μL of TSB broth). After incubation for 20 h at 37 °C, the inhibition of bacterial growth was determined by measuring absorbance at 595 nm. The lowest concentration of peptide that completely inhibited growth of the bacteria was defined as the minimal inhibitory concentration (MIC). The MICs were the average values obtained in triplicates, on three independent measurements and they were compared with MICs obtained for conventional antibiotics.

2.7. Atomic force microscopy

P. aeruginosa ATCC 27853 cells in mid-logarithmic phase were prepared as described in the antibacterial assay section of Section 2. *P. aeruginosa* (10^5 CFU/mL) in TBS were incubated with PS-1 (4 μM) at 37 °C for 30 min. After incubation, cells were collected and suspended in 150 mM KCl/20 mM $MgCl_2$/10 mM Tris–HCl, pH 7.8. The bacterial suspension was placed onto freshly cleaved mica and air-dried. The mica was fixed on the specimen holder with a two-sided adhesive tape and was then installed on the top of the scanner for AFM observation. The AFM used in this experiment was a TopoMetrix 2000 Explorer (TopoMetrix, Santa Clara, CA, USA) operating in the contact mode and at ambient air. A piezoelectric hybrid tube scanner with a maximum scanning area of 50 μm^2 and a standard 200 μm V-shaped Si_3N_4 cantilevers with integrated pyramidal tips were used. The nominal spring constant for the contact force of the tip on the specimen surface was set to 0.00 nA. The line scan speed was set to 20 μm/s.

2.8. Antiprotozoa activity

PS-4 and PS-5 were tested in vitro against trypomastigote blood forms of *T. cruzi* Y strain. The bioassays were carried out using fresh blood collected by cardiac puncture of Swiss albino mice during the peak of parasitemia [2]. Non-infected murine blood was added to contaminated erythrocytes up to a final assay concentration of 2.0×10^6 trypomastigote forms/mL. Following dilutions in saline solution, peptide concentrations were measured spectrophotometrically according to the method described by Pace et al. [33]. The bioassays were performed in triplicate on micro titter plates containing 200 μL of mixture/well (150 μL of infected blood and 50 μL of the peptide solution). Aliquots of the PS-4 and PS-5 were added to the *T. cruzi*-infected blood at final concentrations of 0.5, 1.0, 10.0, and 30.0 μg/mL. The plates were incubated at 37 °C during 24 h and the number of parasites determined [12]. Negative controls were the blood of infected mice without any addition, and positive controls were infected blood containing gentian violet at various concentrations [16].

3. Results

3.1. Isolation and characterization of the phylloseptins

The crude skin secretion of *P. hypochondrialis* (Fig. 1A) and *P. oreades* (Fig. 2A) fractionated by semi-preparative

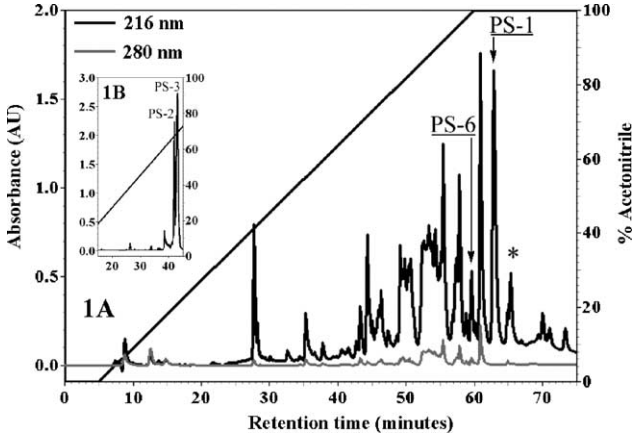

Fig. 1. Fractionation of the total skin secretion lyophilized crude extract from *P. hypochondrialis*. (1A) The skin secretion of *P. hypochondrialis* was loaded onto a C_{18} Vydac 218TP510 (10 mm × 250 mm) column and equilibrated with 0.1% trifluoroacetic acid. The peptide elution was performed using an acetonitrile with 0.1% trifluoroacetic acid solution with 2.5 mL/min flow rate. The absorbance was monitored simultaneously at two wavelengths, 216 and 280 nm. The final purification step of fraction (*) was performed on an analytical Vydac 218TP54 (4.6 mm × 250 mm) column. (1B) PS-2 and PS-3 after re-chromatographic procedure.

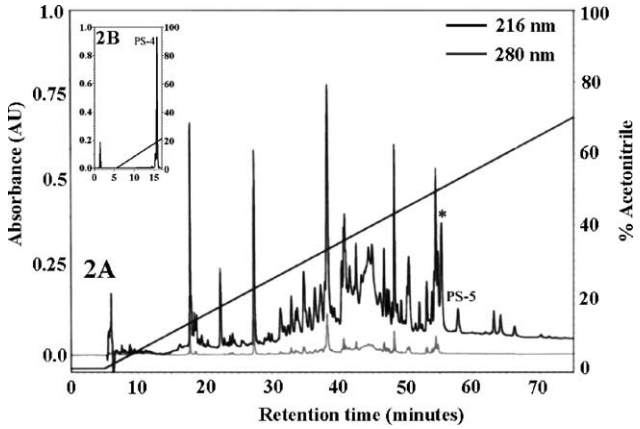

Fig. 2. Fractionation of the total skin secretion lyophilized crude extract from *P. oreades*. (2A) The skin secretion of *P. oreades* was loaded onto a C_{18} Vydac 218TP510 (10 mm × 250 mm) column and equilibrated with 0.1% trifluoroacetic acid. The peptide elution was performed using an acetonitrile with 0.1% trifluoroacetic acid solution with 2.5 mL/min flow rate. The absorbance was monitored simultaneously at two wavelengths, 216 and 280 nm. The final purification step of fraction (*) was performed on an analytical 218TP54 (4.6 mm × 250 mm) column. (2B) PS-4 after a second chromatographic step.

Table 2
Predicted fragment ions from PS-6 matching the observed ones, within 0.1 Da mass tolerances

N-term	Ion	y-Series	b-Series	w-a Ions	w-b Ions	C-term
1	S	1788.02	88.04	–	–	17
2	L	1700.99	201.12	1641.92	–	16
3	I	1587.91	314.20	1542.85	1556.87	15
4	P	1474.82	411.26	–	–	14
5	H	1377.77	548.32	–	–	13
6	A	1240.71	619.35	–	–	12
7	I	1169.67	732.44	1124.62	1138.63	11
8	N	1056.59	846.48	–	–	10
9	A	942.55	917.52	–	–	9
10	V	871.51	1016.58	–	–	8
11	S	772.44	1103.62	–	–	7
12	A	685.41	1174.65	–	–	6
13	I	614.37	1287.74	569.32	583.33	5
14	A	501.29	1358.78	–	–	4
15	K	430.25	1486.87	370.17	–	3
16	H	302.16	1623.93	–	–	2
17	F	165.10	1771.00	–	–	1

N- and C-terminal residue positions in the peptide sequence are shown in columns 1 and 7, while column 2 refers to the corresponding amino acids. Columns 3 and 4 report the parent ion fragmentation pattern, following the y- and b-series. Columns 5 and 6 report the side-chain fragmentations that allow the discrimination of isobaric amino acids.

RP-HPLC yielded a number of unresolved fractions that were better investigated after at least an additional separation experiment. The fractions containing PS-2, PS-3 and PS-4 were submitted to analytical RP-HPLC and are shown in Figs. 1B and 2B, respectively. PS-1 (*P. hypochondrialis*) and PS-5 (*P. oreades*) were isolated independently using a single semi-preparative chromatographic step (Figs. 1A and 2A). The amino acid sequences of the six new polypeptides were aligned in Table 1 using CLUSTAL V multiple sequence alignment software [25] and the C-terminal amidation for each one of these molecules were determined directly by de novo MS/MS sequencing experiments, as it is demonstrated for PS-6 (Fig. 3 and Table 2).

Sequence similarity searches using FASTA 3 program revealed low identity scores (around 21%) with temporins, 13-residue C-terminal amidated peptides isolated from the Asian frog *Rana ornativentris* [26], and with a cDNA sequence named PBN1 from *Phyllomedusa bicolor* (about 55%), that has been regarded as an antimicrobial peptide based only on its similarities with the preprodermaseptins cDNAs [43]. All six new members of the PS family can be fitted into an α-helix, using theoretical information predicted by SOPMA program (data not shown) and Edmundson wheel projections [39]. When plotted as an α-helical wheel (Fig. 4), all peptides showed distinguishable hydrophobic and hydrophilic domains in which the charged and the hydrophobic residues are placed on opposed sides of the cylindrical surface. Moreover, predictions of the secondary structures of these peptides indicate a high content of α-helix that may range from 80% to 100%.

3.2. Hemolytic and antibacterial activity

Both PS-1 and PS-2 did not exhibit significant hemolytic activity on human erythrocytes, even at 64 μM (Table 3). PS-1 was assessed for its antimicrobial activity on two

Table 1
The amino acid sequences, molecular masses, and some physical–chemical features of the phylloseptin peptides

Peptides	Primary structure[a]	pI[b]	H[c]	Mt[d]	Me[d]
PS-1	FLSLIPHAINAVSAIAKHN-NH_2	8.76	0.78	2016.14	2015.25
PS-2	FLSLIPHAINAVSTLVHHF-NH_2	7.02	1.10	2116.17	2115.26
PS-3	FLSLIPHAINAVSALANHG-NH_2	6.92	0.92	1945.07	1944.05
PS-4	FLSLIPHAINAVSTLVHHSG-NH_2	7.02	0.85	2113.16	2112.18
PS-5	FLSLIPHAINAVSAIAKHS-NH_2	8.76	0.92	1989.13	1988.15
PS-6	SLIPHAINAVSAIAKHF-NH_2	8.51	0.85	1789.11	1788.11

[a] Maximized pair-wise and multiple sequence alignments of phylloseptins performed by CLUSTAL V.
[b] Theoretical pI (isoelectric point) calculated according to Bjellqvist et al [7].
[c] GRAVY: grand average of hydropathicity (H); the GRAVY value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence.
[d] Mt and Me corresponded as theoretical mass and experimental monoisotopic mass, respectively.

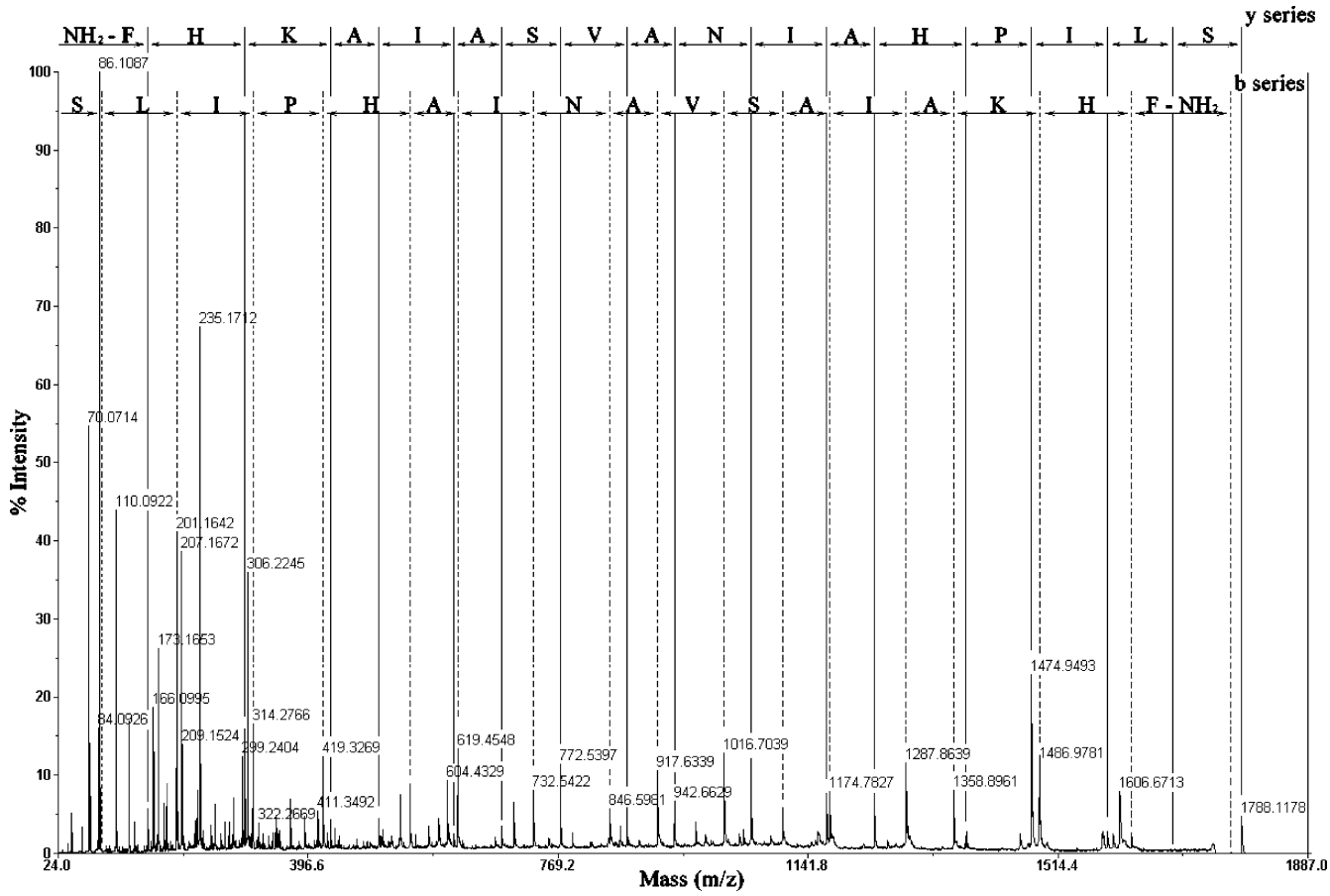

Fig. 3. De novo sequencing of the peptide PS-6 (*P. hypochondrialis*) [M + H] = 1788.13 in an ABI 4700 Proteomics Analyzer with TOF–TOF optics using N_2 as CID gas. The observed fragments allowed complete assignment of the major y and b ion series. The peptide sequence using one-letter code following the y and b series orientation is shown on the top part of the graph.

Gram-positive (*S. aureus* and *E. faecalis*) and three Gram-negative (*E. coli*, *P. aeruginosa*, and *P. aeruginosa* wt) bacteria. As it is shown in Table 4, MICs for PS-1 were in the range of 3.0–7.9 μM, depending on the tested microorganism. MICs of PS-1 showed a stronger anti-bacterial effect against all microorganisms when compared to conventional antibiotics such as chloramphenicol, gentamicyn, ampicilyn, and polimixyn B (Table 4).

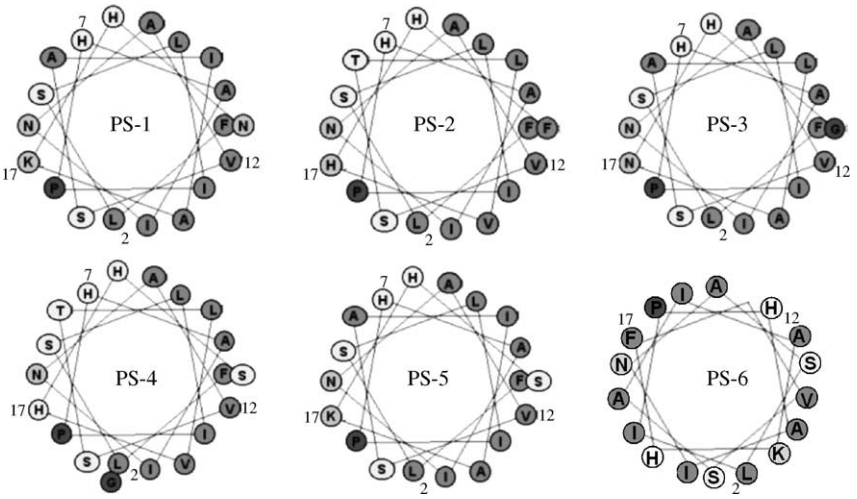

Fig. 4. The α-helical wheel plots of the phylloseptins showing their amphiphilic structure prediction. In this conformation, periodic variation in the hydrophobic values of the residues along the peptide backbone with a 3.6 residues per cycle period characterizes an α-helical conformation (Schaffer and Edmondson [39]).

Table 3
Hemolytic activity assay of PS-1 and PS-2. The total hemolytic control experiment was obtained by the addition of 50 μL Triton X-100 in 150 μL of red blood cell suspension

Concentration (μM)	Hemolysis of human red blood cells (%)	
	PS-1	PS-2
0.5	0.00	0.00
1.0	0.00	0.00
2.0	0.00	0.10
4.0	0.30	0.28
8.0	0.57	0.70
16.0	0.60	0.80
32.0	0.98	1.05
64.0	1.98	2.05

Positive control: Triton X-100 (0.2%) treated red blood cells.

3.3. Atomic force microscopy

The effects of PS-1 on cells of *P. aeruginosa* ATCC 27853 were examined under AFM. Bacterial colonies treated with PS-1 using the same concentration determined by the MIC experiments revealed several bubble-like structures on the surface of *P. aeruginosa* (Fig. 5b, d, and f) and changes in the morphological dimensions, particularly in the cell height and width, producing an overall conformational flattening on the PS1-treated bacteria. These results are in very close agreement with the AFM recent observations reported by Silva and Teschke, when similar phenomenon is described for *E. coli* cells incubated with the antimicrobial peptide PGLa [41].

3.4. Trypanocidal assay

Anti-protozoan bioassays revealed that PS-4 and PS-5 are active against *T. cruzi* at low micromolar levels. The IC_{50} values determined for PS-4, PS-5 and for gentian violet, used as drug of reference, with a starting inoculum of 2.0×10^6 trypomastigote forms/mL, were 5.1, 4.9 and 76 μM, respectively (Table 5). These results indicate a better quantitative response against *T. cruzi*, especially if one ought to compare them to the IC_{50} values determined for trypanocidal drugs derived from benzotropolone esters and diethyl azodicarboxylate, which were in the range of 15.8–41.0 μM, obtained in bioassays that used parasite's inoculum of 10^4 forms/mL [16]. However, IC_{50} values for PS-4 and PS-5 could be comparable to dermaseptin 01 (DS 01, $IC_{50} = 8$ μM), a 28-residue cationic C-terminal amidated peptide isolated skin secretion from *P. oreades* [10].

4. Discussion

The present work reports the purification and characterization of six new antimicrobial peptides named phylloseptins (PSs), present in the skin secretion of two different species of Brazilian tree-frogs, *P. hypochondrialis* and *P. oreades*. Phylloseptins are highly conserved peptides with not more than 20 amino acid residues showing amidation at the C-terminal. Like several amphiphilic amphibian skin peptides, PS-1 has a broad-spectrum activity against Gram-positive, Gram-negative bacteria and PS-4 and -5 protozoans, PS-1 and -2 with negligible hemolytic effect. Conversely, these molecules show either a one or a zero net positive charge as the great majority of the amphibians' antimicrobial peptides do, it seems that the conserved proline and histidine residues and the amidation at the C-terminal could play that role under certain conditions whilst the amphiphilic feature is preserved. Nevertheless, the antibacterial assays (Table 4) showed a close quantitative range of activities against the tested bacteria comparable to a number of results present in the literature [3,10,15,32,34]. PS-1 was marginally less effective against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (MICs of 7.9 μM) than the inhibitory potency determined for *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, and *P. aeruginosa* wt, where the MIC values were around 3.0 μM. The majority of the tested microorganisms susceptible to PS-1 are not only resistant to a number of conventional antibiotics but they are also important opportunistic or pathogenic agents involved in serious problems of human and animal health (e.g., *P. aeruginosa* in cystic fibrosis and tuberculosis, and *S. aureus* in tuberculosis).

Atomic force microscopy (AFM) has been used extensively to study a variety of materials [29], biological samples

Table 4
The antibacterial activity assay for the peptide PS-1. Minimal inhibitory concentration (MIC) was the average values obtained in triplicates on three independent measurements

Microorganism	MIC[a] (μM)				
	PS-1	Chloramphenicol	Gentamycin	Ampicilyn	Polimixyn B
Gram-positive bacteria					
S. aureus ATCC	7.9	99.9	[b]	[b]	[b]
E. faecalis ATCC	4.0	99.9	[b]	52.3	[b]
Gram-negative bacteria					
E. coli ATCC	7.9	99.9	133.0	260.2	58.1
P. aeruginosa ATCC	4.0	[b]	133.0	[b]	14.5
P. aeruginosa wt	3.0	[b]	133.0	[b]	14.5

[a] MICs are expressed as the lowest concentration of peptide that completely inhibited growth of the bacteria.
[b] No activity was detected at the highest antibiotic concentration tested.

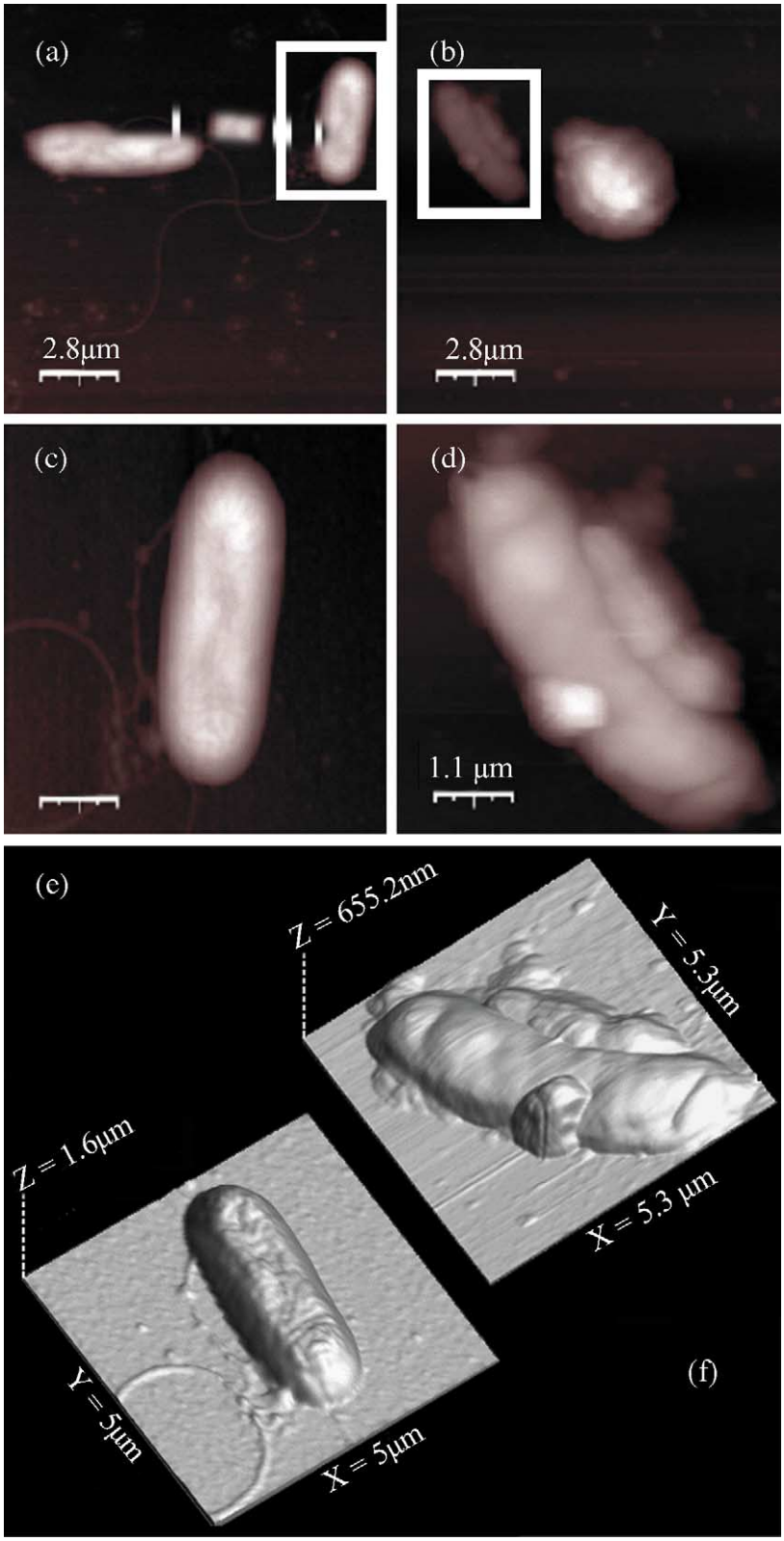

Fig. 5. Atomic force microscopy topographical images of *P. aeruginosa* ATCC 27853 cells. Common morphology of the control *P. aeruginosa* cells (a, c, e) and treated with the peptide PS-1 (b, d, f). *P. aeruginosa* in mid-logarithmic growth at 10^5 cells/mL was incubated with the phylloseptin 1 for 30 min (PS-1 = 4 μM).

Table 5
The trypanocidal activity assay for the peptides PS-4 and PS-5

Concentration (μg/mL)	% Lysis (±S.D.)	
	PS-4	PS-5
0.5	26.4 ± 15	33.3 ± 18
1.0	42.0 ± 9	37.7 ± 9
10.0	52.4 ± 15	51.5 ± 15
30.0	100	100

IC_{50}, μg/mL (μM)	
PS-4	PS-5
2.5 (5.1)	2.4 (4.9)

Positive control: gentian violet at 250 μg/mL (IC_{50} = 76 μM); negative control: blood infected.

[28], and it has been proving its invaluable importance in bacterial topology identification and plasma membrane analysis [9]. Although the lytic modes of actions of amphiphilic antimicrobial peptides against microbial cells are not yet fully understood, the results obtained here by AFM suggest that the cytoplasmic membrane may possibly be the primary target of the PS-1, since the treated bacterial cells exhibited several bubble-like projections and an overall conformational flattening (Fig. 5b, d, and f), resulting in severe membrane alteration, that could progressively lead to an increased permeability and cell lysis. These observations are in accordance with the current understanding of the lipid bilayer membrane destabilizing effect produced by detergent-like peptide structures [18,27,37].

PS-4 and PS-5 (*P. oreades*) showed low micromolar cytolytic activity towards *T. cruzi* trypomastigote form comparable to small molecule drugs [12,16]. Because these PSs act by directly killing trypomastigote forms, they prevent *T. cruzi* from invading host cells and, thus, may be useful in preventing *T. cruzi* transmission from blood transfusions. It is of particular interest that the PSs probably exert a common mechanism of action on a protozoan parasite, *T. cruzi*, and on prokaryotes without damaging host cells. For further research, the differences in membrane lipid composition between *T. cruzi* and mammalian host cells may be exploited in the development of new trypanocidal targeting agents.

In summary, this work demonstrates that among all the six new sequences reported here, the phylloseptins PS-1, PS-4 and PS-5 represent the first peptides directly isolated from the skin secretion of two different *Phyllomedusa* species and properly tested against various microorganisms. Phylloseptin-like peptides have also been identified by our group in the skin secretions and cDNA's of *P. burmaister*, *P. distincta*, *P. rohdei*, *P. tarsius*, and *P. tomopterna* (data not shown) reinforcing the hypothesis of a new emerging class of antimicrobial peptides present in the Phyllomedusinae subfamily. Each one of the these peptides, together with PS-2, PS-3 and PS-6, is to be synthesized chemically, tested against microorganisms and structurally investigated.

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References

[1] Abaudy Y, Mendelson E, Shalit I, Bessalle R, Fridikin M. Activity of two synthetic amphiphilic peptides and magainin-2 against herpes simplex virus types 1 and 2. Int J Peptide Protein Res 1994;43:573–82.
[2] Bastos JK, Albuquerque S, Silva ML. Evaluation of the trypanocidal activity of lignans isolated from the leaves of *Zanthoxylum naranjillo*. Planta Med 1999;65(6):541–4.
[3] Batista CV, Scaloni A, Rigden DJ, Silva LR, Rodrigues-Romero A, Dukor R, et al. A novel heterodimeric antimicrobial peptide from the tree-frog *Phyllomedusa distincta*. FEBS Lett 2001;494(1–2):85–9.
[4] Batista CV, Silva LR, Sebben A, Scaloni A, Ferrara L, Paiva GR, et al. Antimicrobial peptides from the Brazilian frog *Phyllomedusa distincta*. Peptides 1999;20:679–86.
[5] Bello J, Bello HR, Granados E. Conformation and aggregation of melittin: dependence on pH and concentration. Biochemistry 1982;21:461–5.
[6] Bevins CL. Antimicrobial peptides as agents of mucosal immunity. Ciba Found Symp 1994;186:250–60.
[7] Bjellqvist B, Hughes GJ, Pasquali Ch, Paquet N, Ravier F, Sanchez J-Ch, et al. The focusing positions of polypetides in the immobilized pH gradients can be predicted from their amino acid sequence. Electrophoresis 1993;14:1023–31.
[8] Boman HG. Peptide antibiotics and their role in innate immunity. Annu Rev Immunol 1995;13:61–92.
[9] Braga PC, Ricci D. Atomic force microscopy: application to investigation of *Escherichia coli* morphology before and after exposure to cefodizime. Antimicrob Agents Chemother 1998;42(1):18–22.
[10] Brand GD, Leite JRSA, Silva LP, Albuquerque S, Prates MV, Azevedo RB, et al. Dermaseptins from *Phyllomedusa oreades* and *Phyllomedusa distincta*. Anti-*Trypanosoma cruzi* activity without cytotoxicity to mammalian cells. J Biol Chem 2002;277:49332–40.
[11] Brandão RA. A new species of *Phyllomedusa* Wagler, 1830 (Anura: Hylidae) from central Brazil. J Herpetol 2002;36(4):571–8.
[12] Brener Z. Therapeutic activity and criterion of cure on mice experimentally infected with *Trypanosoma cruzi*. Rev Inst Med Trop 1962;4:389–96.

[13] Bulet P, Cociancich S, Dimarcq JL, Lambert J, Hoffmann D, Hoffmann JA. Insect immunity. Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. J Biol Chem 1991;236:24520–5.
[14] Cammue BP, De Bolle MF, Terras FR, Proost P, Van Damme J, Rees SB, et al. Isolation and characterization of a novel class of plant antimicrobial peptides form *Mirabilis jalapa* L. seeds. J Biol Chem 1992;267:2228–33.
[15] Cole AM, Hong T, Boo LM, Nguyen T, Zhao C, Bristol C, et al. Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. Proc Natl Acad Sci USA 2002;99(4):1813–8.
[16] Da Silva LH, Nussenzweig V. Sobre uma cepa de *Trypanosoma cruzi* virulenta para o camundongo branco. Folia Clínica et Biologica 1953;20(3):191–208.
[17] Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C. Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. Appl Environ Microbiol 1991;57:3450–5.
[18] Dathe M, Wieprecht T. Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. Biochim Biophys Acta 1999;1462(1–2):71–87.
[19] Falla TJ, Karunaratne DN, Hancock REW. Mode of action of the antimicrobial peptide indolicidin. J Biol Chem 1996;271:19298–303.
[20] Gabay JE. Ubiquitous natural antibiotics. Science 1994;264:373–4.
[21] Garnier J, Osguthorpe D, Robson B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 1978;120:97–106.
[22] Gibson BW, Tang D, Mandrell R, Kelly M, Spindel ER. Bombinin-like peptides with antimicrobial activity from skin secretions of the Asian toad, *Bombina orientalis*. J Biol Chem 1991;266:23103–11.
[23] Hancock REW, Chapple DS. Minireview: peptide antibiotics. Antimicrob Agents Chemother 1999;43:1317–23.
[24] Hancock REW, Falla T, Brown MH. Cationic bactericidal peptides. Adv Microb Physiol 1995;37:135–75.
[25] Higgins DG, Sharp PM. Fast and sensitive multiple sequence alignments on a microcomputer. Comput Appl Biosci 1989;5(2):151–3.
[26] Kim JB, Iwamuro S, Koop FC, Conlon JM. Antimicrobial peptides from the skin of the Japanese mountain brown frog, *Rana ornativentris*. J Pept Res 2001;58:349–56.
[27] Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J Mol Biol 1982;5:105–32.
[28] Lacava BM, Azevedo RB, Silva LP, Lacava ZGM, Skeff Neto K, Buske N, et al. Particle sizing of magnetite-based magnetic fluid using atomic force microscopy and birefringence. Appl Phys Lett 2000;77(12):1876–8.
[29] Leite JRSA, Silva LP, Taveira CC, Teles RCL, Freitas SM, Azevedo RB. Topographical analysis of *Schizolobium parahyba* chymotrypsin inhibitor (SPCI) by atomic force microscopy. Protein Peptide Lett 2002;9:179–84.
[30] Mor A, Nicolas P. The NH_2-terminal alpha-helical domain 1–18 of dermaseptin is responsible for antimicrobial activity. Eur J Biochem 1994;219:145–54.
[31] National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Susceptibility Testing. 4th ed. Wayne, PA: NCCLS; 1999. Approved standard M100-S9.
[32] Nicolas P, Mor A. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu Rev Microbiol 1995;49:277–304.
[33] Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. Protein Sci 1995;4:2411–23.
[34] Park CB, Kim MS, Kim SC. A novel antimicrobial peptide from *Bufo bufo gargarizans*. Biochem Biophys Res Commun 1996;218(1):408–13.
[35] Prates MV, Bloch Jr C. Peptídeos Antimicrobianos: uma alternativa no combate a microorganismos resistentes. Biotecnologia 2000;8:30–6.
[36] Prates MV, Sforca ML, Regis WC, Leite JRSA, Silva LP, Pertinhez TA, et al. The NMR-derived solution structure of a new cationic antimicrobial peptide from the skin secretion of the anuran *Hyla punctata*. J Biol Chem 2004;279:13018–26.
[37] Richard M, Epand, Vogel HJ. Diversity of antimicrobial peptides and their mechanisms of action. BBA: Biomembranes 1999;1462(1–2):11–28.
[38] Saberwal G, Nagaraj R. Cell-lytic and antibacterial peptides that act by perturbing the barrier function of membranes: facets of their conformational features, structure-function correlations and membrane-perturbing abilities. Biochem Biophys Acta 1994;1197:109–31.
[39] Schiffer M, Edmundson AB. Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. Biophys J 1967;7(2):121–35.
[40] Segrest JP, De Loof H, Dohlman JG, Brouillette CG, Anantharamaiah GM. Amphipathic helix motif: classes and properties. Proteins 1990;8:103–17.
[41] Silva Jr A, Teschke O. Effects of the antimicrobial peptide PGLa on live *Escherichia coli*. Biochim Biophys Acta 2003;1643(1–3):95–103.
[42] Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 1981;292:246–8.
[43] Ivanhoe D, Ruston F, Nicolas P, Amice M. Antimicrobial peptides from hyoid and rain frogs originated from a 150-million-year-old ancestral precursor with a conserved signal peptide but a hyper mutable antimicrobial domain. Eur J Biochem 2003;270(9):2068–81.
[44] Westerhoff HV, Diuretic D, Handler RW, Castoff M. Magainins and the disruption of membrane-linked free-energy transudation. Proc Natl Acad Sci USA 1989;86:6597–601.
[45] Castoff M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci USA 1987;84:5449–53.