

Characterization and Biological Activities of Ocellatin Peptides from the Skin Secretion of the Frog *Leptodactylus pustulatus*

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Supporting Information

ABSTRACT: Eight new peptides were isolated from the skin secretion of the frog *Leptodactylus pustulatus* and their amino acid sequences determined by de novo sequencing and by cDNA cloning. Structural similarities between them and other antimicrobial peptides from the skin secretion of *Leptodactylus* genus frogs were found. Ocellatins-PT1 to -PT5 (25 amino acid residues) are amidated at the C-terminus, while ocellatins-PT6 to -PT8 (32 amino acid residues) have free carboxylates. Antimicrobial activity, hemolytic tests, and cytotoxicity against a murine fibroblast cell line were investigated. All peptides, except for ocellatin-PT2, have antimicrobial activity against at least one Gram-negative strain. Ocellatin-PT8 inhibited the growth of *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella choleraesuis* strains with MICs in the 60–240 μ M range. No significant effect was observed in human erythrocytes and in a murine fibroblast cell line after exposure to the peptides at MICs. A comparison between sequences obtained by both direct HPLC-MS de novo sequencing and cDNA cloning demonstrates the secretion of mature peptides derived from a pre-pro-peptide structure.



Antimicrobial peptides are considered defensive weapons¹ because of their broad spectrum of activity against a range of bacteria, fungi, viruses, and parasites.^{2–5} In addition, other activities such as antioxidant,⁶ antitumor,⁷ enzyme inhibition,⁸ and chemotactic⁸ effects have been reported for these molecules. One of the most important characteristics of their action as antimicrobials is their nonspecific targeting; because the membrane as a whole can be targeted, it has been postulated that the likelihood of resistance being developed by pathogens is reduced compared to antibiotics that have very specific intracellular targets.⁹ Thus, antimicrobial peptides are considered promising alternatives to conventional antibiotics.^{10–12}

The great majority of antimicrobial peptides have been isolated from the granular gland skin secretions of anurans.¹³ The *Leptodactylus* genus represents 40% of the Leptodactylidae family.¹⁴ So far, only eight of the 76 species of frogs from the genus *Leptodactylus* have been examined for bioactive peptides from their skin.

The first report of isolation and characterization of peptides from this genus was made in 2004.¹⁵ The authors described three peptides from *L. ocellatus*, which presented hemolytic activity against human erythrocytes and activity against

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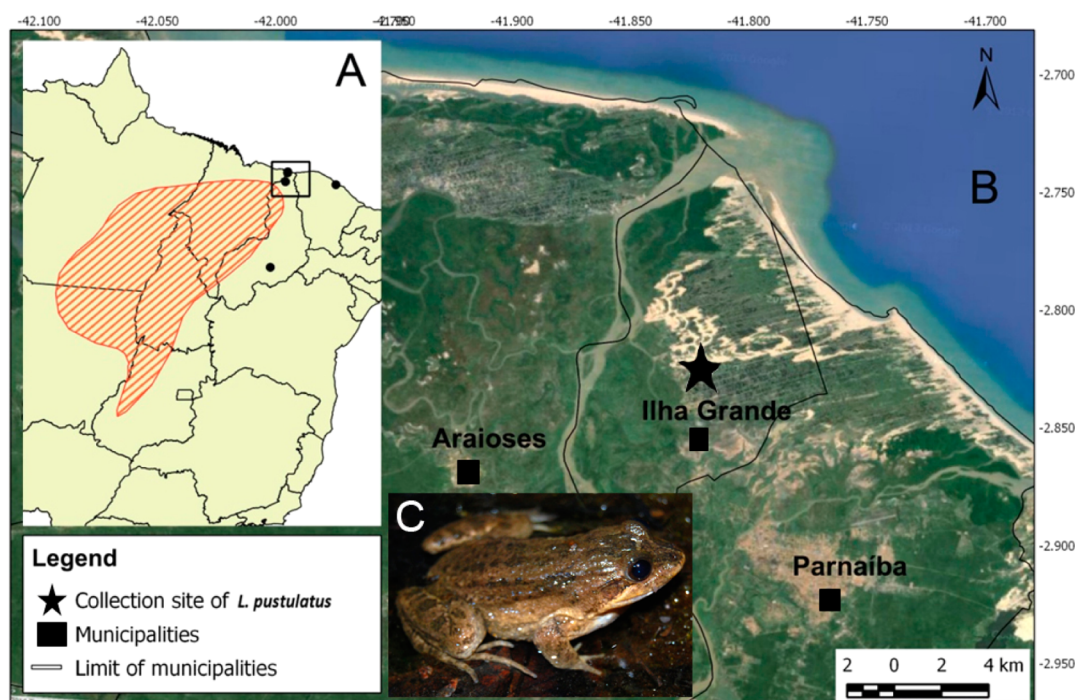


Figure 1. (A) *Leptodactylus pustulatus* distribution from the International Union for Conservation of Nature (IUCN).³² The points outside the shaded region show other published records.^{27–29} (B) Zoomed area shows collection site of *L. pustulatus* specimens for this work (black star). Municipalities near the site are highlighted (black squares). (C) Photograph of a male adult *L. pustulatus* (photo by Etielle Barroso Andrade).

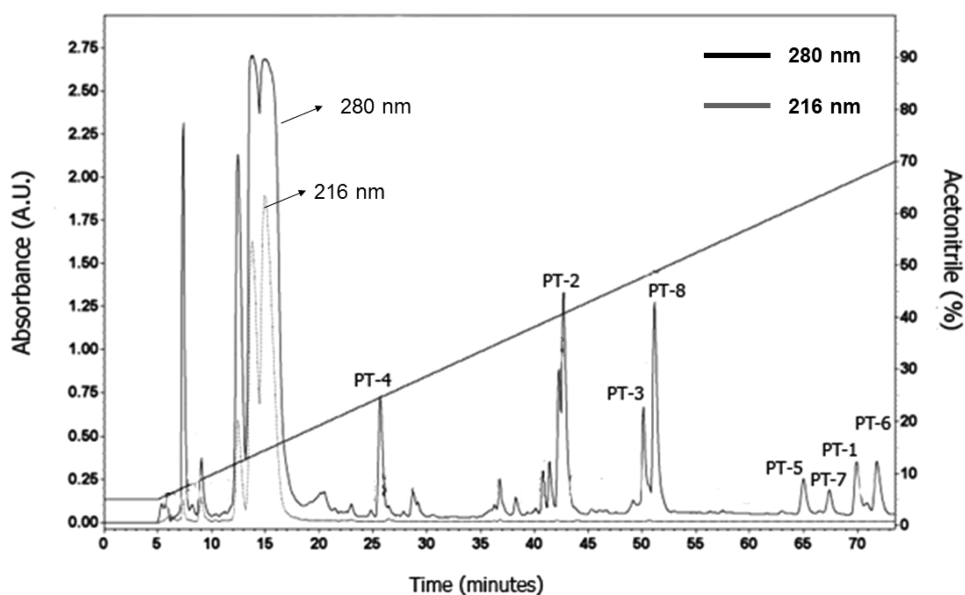


Figure 2. Chromatographic profile of the crude *Leptodactylus pustulatus* skin secretion by semipreparative RP-HPLC. Peaks containing the ocellatin-PT1 to -PT8 peptides are labeled in the chromatogram.

Escherichia coli. The peptides were termed ocellatins 1, 2, and 3. Other studies involving characterization of leptodactylid antimicrobial peptides with similarities in amino acid sequence to the ocellatins have also been described. Fallaxin (ocellatin-F) from *L. fallax*¹⁶ and from *L. pentadactylus*¹⁷ inhibited, with low potency, the growth of Gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella pneumonia*) and showed very low hemolytic activity. Pentadactylin (ocellatin-P) from *L. pentadactylus* also inhibited the growth of reference strains with low potency.¹⁷ Laticeptin (ocellatin-L1) and ocellatin-L2 from *L. laticeps*^{18,19} did not inhibit the growth

of *E. coli* or *Staphylococcus aureus*. Syphaxins (SPX, also known as ocellatin-S) isolated from *L. synphax*²⁰ inhibited *E. coli* and *S. aureus* strains. Among the peptides tested, a truncated peptide SPX (1–22) was the most effective compared with the conventional antibiotics tested. The ocellatin-V class, isolated from *L. validus*, presents very low antimicrobial potency.²¹ Other ocellatins from *L. ocellatus* have also been described: ocellatin-4²² and ocellatins-5 and -6,²³ which show structural similarity to members of the antimicrobial peptides found in the skin secretion of other leptodactylid frogs. Glycine-leucine-rich peptides were also described from leptodactylid frogs:

Table 1. Sequence, MW, pI, and Hydrophobic Moment of New Ocellatins PT1 to PT8 Isolated from *L. pustulatus*^a

Name	Sequence	MW	pI	µH	Species	Reference
Ocellatin-PT1	G V F D I I K D A G K Q L V A H A M G K I A E K V °	2639.1	8.44	0.267	<i>L. pustulatus</i>	this work
Ocellatin-PT2	G V F D I I K D A G K Q L V A H A T G K I A E K V °	2609.0	8.44	0.272	<i>L. pustulatus</i>	this work
Ocellatin-PT3	G V I D I I K G A G K D L I A H A I G K I A E K V °	2530.0	8.44	0.271	<i>L. pustulatus</i>	this work
Ocellatin-PT4	G V F D I I K G A G K Q L I A H A M G K I A E K V °	2595.1	9.53	0.257	<i>L. pustulatus</i>	this work
Ocellatin-PT5	G V F D I I K D A G R Q L V A H A M G K I A E K V °	2667.1	8.50	0.268	<i>L. pustulatus</i>	this work
Ocellatin-PT6	G V F D I I K G A G K Q L I A H A M E K I A E K V G L N K D G N	3365.9	8.39	0.230	<i>L. pustulatus</i>	this work
Ocellatin-PT7	G V F D I I K G A G K Q L I A H A M G K I A E K V G L N K D G N	3293.8	9.40	0.212	<i>L. pustulatus</i>	this work
Ocellatin-PT8	G V F D I I K G A G K Q L I A R A M G K I A E K V G L N K D G N	3312.9	9.82	0.214	<i>L. pustulatus</i>	this work
Ocellatin-1	G V V D I I K G A G K D L I A H L V G K I S E K V °	2560.0	8.44	0.309	<i>L. ocellatus</i>	15
Ocellatin-2	G V L D I I F K D A A K Q L A H A A E Q I °	2319.1	6.15	0.269	<i>L. ocellatus</i>	15
Ocellatin-3	G V L D I L K N A A K N I L A H A A E Q I °	2202.5	6.75	0.438	<i>L. ocellatus</i>	15
Ocellatin-4	G L L D F V T G V G K D F A Q L I K Q I °	2275.7	5.96	0.473	<i>L. ocellatus</i>	22
Ocellatin-5	A V L D I L K D V G K G L L S H F M E K V °	2312.8	6.80	0.444	<i>L. ocellatus</i>	23
Ocellatin-6	A V L D F I K A A G K G L V T N I M E K V G °	2274.7	8.54	0.344	<i>L. ocellatus</i>	23
Ocellatin-F (falaxin)	G V V D I L K G A A K D A G H L A S K V M N K L °	2549.0	9.53	0.258	<i>L. fallax/L. pentadactylus</i>	16
Ocellatin-L1 (laticeptin)	G V V D I L K G A A K D L A G H L A T K V M N K L °	2563.1	9.53	0.244	<i>L. laticeps</i>	19
Ocellatin-L2	G V V D I L K G A A K D L A G H L A I K V M D K L °	2564.0	8.44	0.247	<i>L. laticeps</i>	19
Ocellatin-P (pentadactylin)	G L L D T L K G A A K N V V G S L A S K V M E K L °	2543.0	9.53	0.324	<i>L. pentadactylus</i>	17
Ocellatin-S (syphaxin)	G V L D I L K G A A K D L A G H V A T K V I N K I °	2545.0	9.53	0.223	<i>L. syphax</i>	20
Ocellatin-V1	G V V D I L K G A G K D L L A H A L S K L S E K V °	2633.1	8.44	0.140	<i>L. validus</i>	21
Ocellatin-V2	G V L D I L K G A G K D L L A H A L S K I S E K V °	2576.0	8.44	0.274	<i>L. validus</i>	21
Ocellatin-V3	G V L D I L T G A G K D L L A H A L S K L S E K V °	2549.0	6.75	0.295	<i>L. validus</i>	21
Ocellatin-K1	G V V D I L K G A A K D L A G H L A S K V M N K I °	2549.0	9.53	0.256	<i>L. knudseni</i>	25
LASP	G L W D D L K A A K K V V S S L A S A A I E - K L °	2513.9	8.43	0.412	<i>L. fallax</i>	36
plasticin-L1	G L V N G L L S S V L G G G Q G G G L L G G I L °	2165.5	5.52	0.367	<i>L. laticeps</i>	18
leptoglycin	G L L G G L L G P L L G G G G G G G L L °	1762.0	5.52	0.295	<i>L. pentadactylus</i>	24

Boxed black letters represent identical amino acid position and boxed gray letters represent conservative substitutions.
^a: C-terminus amidated peptide.

Table 2. Amino Acid Sequences of the Pre-pro-ocellatins from *Leptodactylus pustulatus*

peptide	signal peptide	acidic piece	mature peptide	body region
ocellatin-PT1	MAFLKKSFLVFLGFLVLSIC	DEEKRQDEDDDDDDDEEKR	GVFDIIKDAGKQLVAHAMGKIAEKV ^a	dorsal and ventral
ocellatin-PT2	MAFLKKSFLVFLGFLVLSIC	DEEKRQDEDDDDDDDEEKR	GVFDIIKDAGKQLVAHATGKIAEKV ^a	dorsal
ocellatin-PT3	MAFLKKSFLVFLGFLVLSIC	DEEKRQDEDDDDDDDEEKR	GVIDIIKGAGKDLIAHAIGKLAEKV ^a	dorsal
ocellatin-PT4	MAFLKKSFLVFLGFLVLSIC	DEEKRQDEDDDDDDDEEKR	GVFDIIKGAGKQLIAHAMGKIAEKV ^a	dorsal
ocellatin-PT5	MAFLKKSFLVFLGFLVLSIC	DEEKRQDEDDDDDDDEEKR	GVFDIIKDAGRQLVAHAMGKIAEKV ^a	dorsal
ocellatin-PT6	MAFLKKSFLVFLGFLVLSIC	DEEKRQDEDDDDDDDEEKR	GVFDIIKGAGKQLIAHAMEKIAEKVGLNKDGN	dorsal
ocellatin-PT7	MAFLKKSFLVFLGFLVLSIC	DEEKRQDEDDDDDDDEEKR	GVFDIIKGAGKQLIAHAMGKIAEKVGLNKDGN	dorsal
ocellatin-PT8	MAFLKKSFLVFLGFLVLSIC	DEEKRQDEDDDDDDDEEKR	GVFDIIKGAGKQLIARAMGKIAEKVGLNKDGN	ventral

^aC-terminus-amidated peptide.

plasticin-L1 from *L. laticeps*,¹⁸ similar to plasticins previously identified only in phyllomedusid frogs (Hylidae), with no activity against *E. coli* or *S. aureus*, and leptoglycin, an antimicrobial peptide from *L. pentadactylus* that was able to inhibit the growth of Gram-negative bacteria but did not show antimicrobial activity against Gram-positive bacteria, yeast, or the dermatophyte fungi tested.²⁴ The most recently described peptide of the genus *Leptodactylus* was named ocellatin-K1 from *L. knudseni*.²⁵

Leptodactylus pustulatus (Peters, 1870) is one of the most distinctive species in the genus because of its ventral pattern of large bright red to yellow spots on a dark background and its complex calls.²⁶ It has been found in the central Brazilian cerrado biome, in the Brazilian states of Goiás, Mato Grosso, Tocantins, Pará, and Maranhão (Figure 1); however it was also recorded in Piauí and Ceará states.^{27–30} It is classified as having a stable population trend by the International Union for Conservation of Nature (IUCN).³¹

Bioprospection of new natural molecules is a demanding science, taking into account the utility of natural products as sources of novel structures, although not necessarily the final drug entity,^{33,34} to produce therapeutic agents. We describe the purification, identification, and characterization of eight new peptides isolated from *L. pustulatus* skin secretions. These peptides were termed ocellatins-PT1 to -PT8, following the proposed nomenclature for antimicrobial peptides from frogs of the genus *Leptodactylus*.³⁵ Antimicrobial activity against *E. coli*, *S. aureus*, *K. pneumoniae*, and *Salmonella choleraesuis* and hemolytic activity and cytotoxicity against a murine fibroblast cell line were tested to evaluate their potential use as antimicrobial agents. We describe the pre-pro-peptide sequences obtained by cDNA cloning and compare them with the sequences obtained by direct HPLC-MS de novo sequencing. We also observed the effect of one of the peptides on *E. coli* cell structure by atomic force microscopy (AFM).

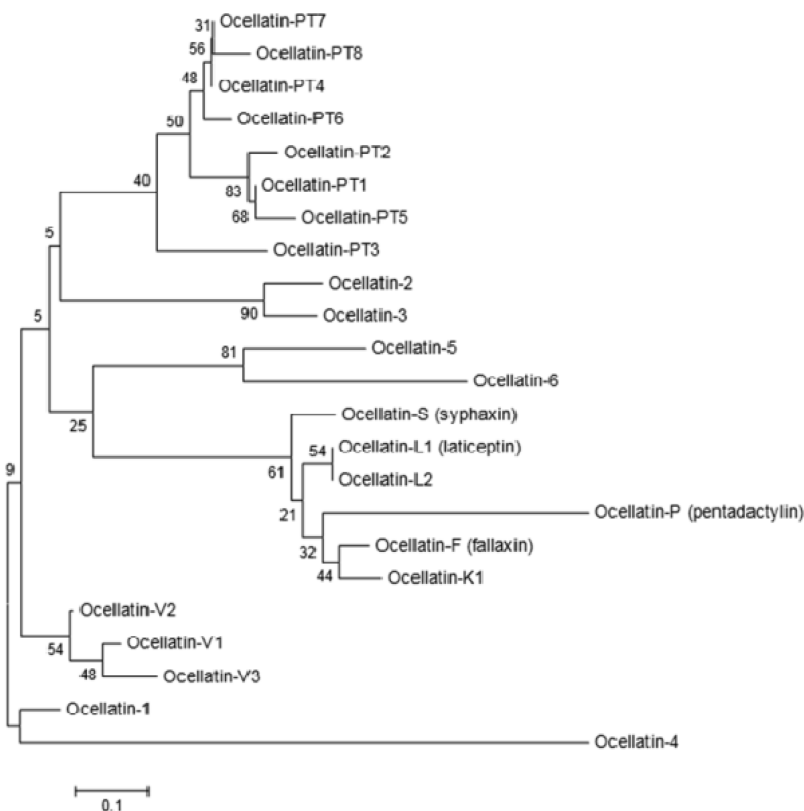


Figure 3. Phylogram built using the ClustalW tool to display similarity in peptides. The phylogram estimates phylogeny among peptides, and the branch lengths are proportional to the amount of inferred evolutionary change. The peptides found in *Leptodactylus pustulatus* (ocellatins-PT1 to -PT8) form a cluster.

RESULTS AND DISCUSSION

Peptide Purification, Mass Spectrometry Analysis, and de Novo Sequencing. Fractionation by RP-HPLC of the crude skin secretion of *L. pustulatus* (Figure 2) yielded 12 fractions. Each fraction was collected and analyzed by MALDI-TOF-TOF MS (Figure S1 Supporting Information). Mono-isotopic molecular masses of the new ocellatins were determined as follows: ocellatin-PT1 $[M + H]^+ = 2638.24$ Da, ocellatin-PT2 $[M + H]^+ = 2607.68$ Da, ocellatin-PT3 $[M + H]^+ = 2528.53$ Da, ocellatin-PT4 $[M + H]^+ = 2593.46$ Da, ocellatin-PT5 $[M + H]^+ = 2666.45$ Da, ocellatin-PT6 $[M + H]^+ = 3365.74$ Da, ocellatin-PT7 $[M + H]^+ = 3293.69$ Da, ocellatin-PT8 $[M + H]^+ = 3312.71$ Da. MS/MS spectral analysis allowed peptide primary structure determination by de novo sequencing (Figure S2). The sequences obtained are presented in Table 1.

Identification of cDNA Encoding Ocellatins. cDNA sequences, corresponding to mRNA of ocellatins expressed in *L. pustulatus* skin, were cloned and sequenced (Table 2). The obtained sequences confirmed the primary structures determined by isolation, purification, and de novo sequencing of peptides present in the skin secretion of *L. pustulatus*.

The deduced amino acid sequences of the precursors show a tripartite structure as previously described for antimicrobial peptides:^{37–40} a signal peptide, an acidic part, and a progenitor sequence coding for the mature molecule. All identified ocellatins contained a signal peptide of 22 amino acid residues, defined as a potential signal for endoplasmic reticulum membrane translocation, ending with a Cys residue.⁴¹ This signal peptide is immediately followed by an acidic sequence of 19 amino acids, which ends with two consecutive basic amino

acids (Lys40-Arg41) described previously for dermaseptins as a pro-hormone processing signal⁴⁰ (Table 2). The mature peptides are at the C-terminus and consist of 25 or 32 amino acids that differ little compared to other peptides found in skin secretions of other members of the *Leptodactylus* genus.

Sequence Analysis. The primary structures of the new ocellatin peptides from *L. pustulatus* obtained by de novo sequencing are described in Table 1 and are identical to the sequences obtained by cDNA sequencing (Table 2). Sequence similarity searches between ocellatins-PT1 and -PT5 show similarity ranging from 97% to 72%. They differ in one to seven amino acids, but in most cases substitutions are conservative (Table 1). Ocellatin-PT7 incorporates the full 25 amino acids of ocellatin-PT4, but has an additional seven amino acids at the C-terminus.

The similarities among all 23 ocellatin-leptodactylid antimicrobial peptide sequences described to date were supported by a ClustalW phylogram (Figure 3). The amino acid sequences of the peptides from *L. pustulatus* show similarity with peptides found in the skin secretion of other leptodactylid frogs (Table 1), probably due to a consequence of multiple duplication of an ancestral gene that existed before the radiation of the species and within individual species, as hypothesized by Conlon et al.¹⁹

The ocellatin-PT1 to -PT8 sequences all include a Glu23, as also found in ocellatin-1, ocellatin-P, and ocellatins-V1, -V2, and -V3 from *L. ocellatus*, *L. pentadactylus*, and *L. validus*, respectively. The presence of this amino acid contrasts with the Asn23 present in ocellatin-F, ocellatin-L1, ocellatin-S, and ocellatin-K from *L. falax*, *L. laticeps*, *L. syphax*, and *L. knudseni*, respectively. This reinforces the proposal that ocellatin-L2,

Table 3. Antimicrobial (MIC Determination) and Hemolytic Assays of Ocellatins from *Leptodactylus pustulatus*

peptide name	<i>E. coli</i>		<i>S. aureus</i>		<i>K. pneumoniae</i>		<i>S. choleraesuis</i>		hemolytic activity at the MIC ^a	hemolytic activity at 800 µg/mL
	ATCC 25922		ATCC 29313		ATCC 700603		ATCC 14028			
	MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC		
	(µg/mL)	(µM)	(µg/mL)	(µM)	(µg/mL)	(µM)	(µg/mL)	(µM)	(% lysis)	(% lysis)
ocellatin-PT1	800	300	>800	>300	>800	>300	>800	>300	1.98 ± 0.08	1.98 ± 0.08
ocellatin-PT2	>800	>310	>800	>310	>800	>310	>800	>310	0	0
ocellatin-PT3	800	320	>800	>320	>800	>320	>800	>320	0.69 ± 0.04	0.69 ± 0.04
ocellatin-PT4	200	80	>800	>310	800	310	800	310	0.19 ± 0.09	4.29 ± 0.23
ocellatin-PT5	800	300	>800	>300	>800	>300	>800	>300	4.05 ± 0.19	4.05 ± 0.19
ocellatin-PT6	400	120	>800	>240	>800	>240	>800	>240	0	0.69 ± 0.04
ocellatin-PT7	200	60	800	240	>800	>240	800	240	0.06 ± 0.06	13.72 ± 1.29
ocellatin-PT8	200	60	800	240	800	240	800	240	0	8.09 ± 0.52

^aMIC observed for *E. coli*.

which contains Asp23, is encoded by a gene that has arisen from a duplication of the ocellatin-L1 gene in *L. laticeps*.¹⁸ Ocellatin-PT3 shows 76% sequence identity with ocellatin-V1, differing in six amino acids, of which four are conservative substitutions. Ocellatin-PT3 also shows 72% sequence homology with ocellatin-1, ocellatin-V2, and ocellatin-V3, differing in seven amino acids, of which six, five, and four are conservative substitutions, respectively. Ocellatin-PT4 shows 72% sequence homology with ocellatin-1 and ocellatin-V2 with five and two conservative substitutions, respectively. One distinctive difference of ocellatins-PT1 to -PT8, except ocellatin-PT3, compared to all the *Leptodactylus* genus peptides described to date is the presence of the aromatic amino acid Phe3 instead of Leu or Val, although this is a conservative change because all those amino acids are nonpolar (hydrophobic). Three-dimensional structural model predictions of the new peptides (Figure S4) show that the presence of a Phe3 favors α -helix formation at the N-terminal region, together with Gly8 (ocellatins-PT4, -PT6, -PT7, and -PT8), while the presence of Ile3 (ocellatin-PT3) or Asp8 (ocellatins-PT1 and -PT2) decreases the α -helix content (Figures S4 and S5A). This could be related to antimicrobial activity against *E. coli*, as peptides with Phe3 and Gly8 present slightly lower MICs against this strain (Table 3). The presence of the amino acids Asp8 (in ocellatins-PT1, -PT2, and -PT5), Asp12 (ocellatin-PT3), and Glu19 (ocellatin-PT6) influences the pI value as well, which could be related to activity against other strains. Ocellatins-PT4, -PT7, and PT8, which have pI values of 9.53, 9.40, and 9.82, respectively, not only displayed lower MICs (200 µg/mL) against *E. coli* but also had activity against *S. choleraesuis* and/or *K. pneumoniae* and *S. aureus*. According to the phylogram (Figure 3), all new ocellatins identified in this work form a cluster, raising the possibility that gene duplications occurred after the divergence from the other species. However, additional molecular information would be necessary to fully understand the phylogenetic relationships between members of this genus.

Another modification that could be altering peptide structures and consequently their activity is the presence of Thr18 instead of Met18. Its presence produced an alteration in the helix prediction structure (Figure S4). All new ocellatins have a predicted helix structure at the C-terminal region that involves amino acids between the 11th and 15th position, with the exception of ocellatin-PT2, which presents a Thr18 and is the only peptide that has no activity against *E. coli*.

By analyzing the wheel projections, considering the helical segments predicted in 3D structures (Figure S5A and B), we noticed conserved structures at the hydrophobic region of the amphipathic helix projection not only between the *L. pustulatus* ocellatins but also compared with other ocellatins. This is consistent with similar values found in the hydrophobic moment predictions and with the suggestion by King et al.²¹ that differences in hydrophobicity are not of major importance in accounting for the antimicrobial potencies. However, the hydrophilic regions are more variable, which could be related to the differences found in the antimicrobial activity, as mentioned above, despite the high percentage of similarity in the sequences (Table 3), as also found by other authors.^{19,21,22} Nevertheless, even though the hydrophobic region is variable, we observe that they have conserved polar charged residues that could be part of a recognition motif for some other protein/complex. There are two independent groups of charged polar residues (DxxKxxxK and KxxEK) near each end of the peptide on opposing sides (Figure S5). These hydrophobic domains may represent aggregation motifs, as helical segments in these peptides are relatively short to traverse a membrane. This suggests multiple binding partners for these peptides, perhaps as part of a larger complex.

Antimicrobial Assays. All new ocellatins were manually synthesized, purified, and quantified in order to enable antimicrobial testing (Figure S3). Between 12 and 24 mg of each peptide was obtained. All peptides synthesized, with the exception of ocellatin-PT2, showed activity against *E. coli* (MICs between 60 and 320 µM) (Table 3). Ocellatins-PT4 and -PT8 also have activity against two more Gram-negative strains, *K. pneumoniae* (producing extended spectrum beta-lactamase, ESBL) and *S. choleraesuis*, with MICs between 240 and 310 µM. Ocellatin-PT7 also showed activity against *S. choleraesuis* (240 µM). Regarding the Gram-positive strain tested, only ocellatins-PT7 and -PT8 showed antibacterial activity (240 µM).

The results show that small differences in the sequence can lead to important differences in the activity spectra of the peptides. According to the Shai–Matsuzaki–Huang (SMH) model of antimicrobial peptide action mechanisms, a positive net charge is needed to interact with the bacterial membranes.^{1,42–44} The presence of negatively charged lipopolysaccharide (LPS) molecules in the outer membrane of Gram-negative bacteria oriented toward the exterior can favor the interaction of peptides with these organisms.^{22,45,46} In this

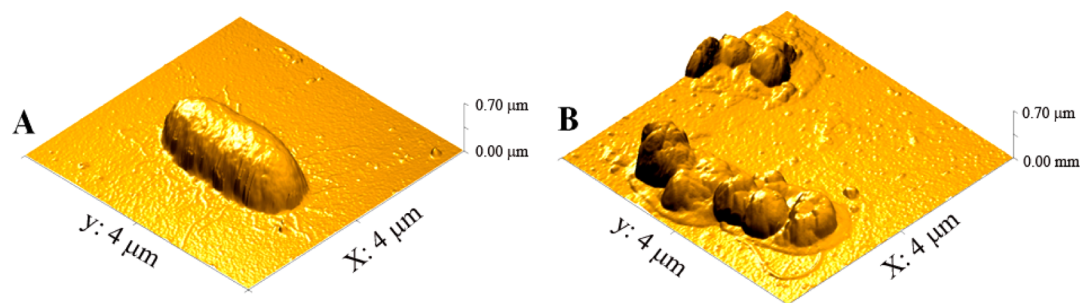


Figure 4. Representative AFM height images of the antimicrobial effect of ocellatin-PT7 on *E. coli* ATCC 25922: (A) untreated; (B) after 24 h of ocellatin-PT7 treatment at the MIC.

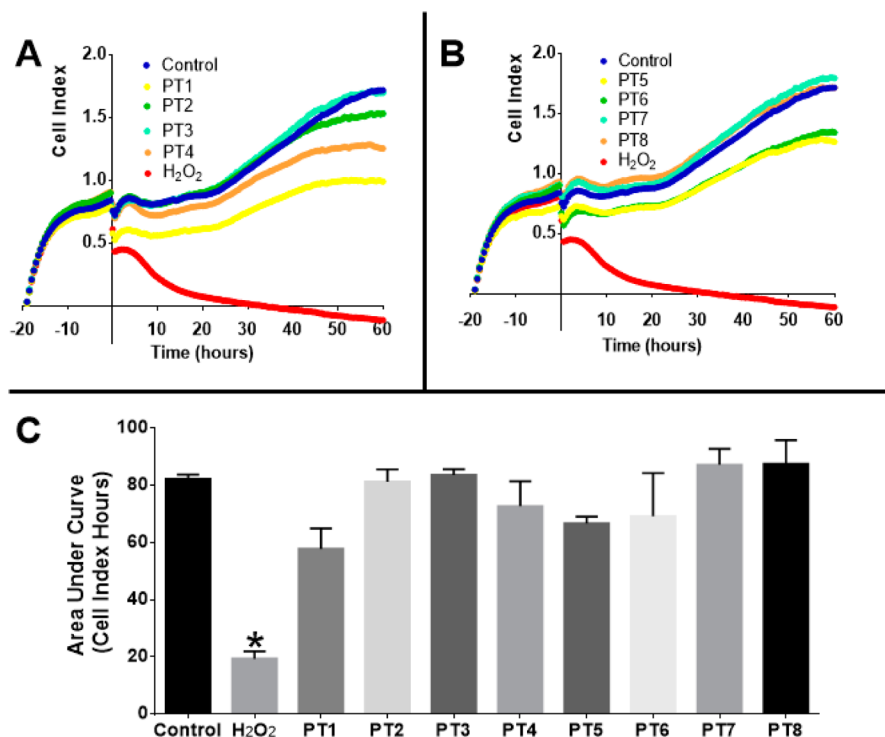


Figure 5. Cell growth curves of NIH-3T3 fibroblast cells exposed to peptides (A) ocellatins-PT1–PT4 and (B) ocellatins-PT5–PT8 and both positive (H₂O₂) and negative (sterile growth media) controls. Treatment solutions (including negative control) were administered at 0 h. (C) The area under the curve values of the NIH-3T3 cell proliferation curves presented in A and B.

sense, the presence of a negative charge in the “polar” sector of the amphipathic helix (Asp8 in ocellatin-PT1, -PT2, and -PT5, Asp12 in ocellatin-PT3, and Glu19 in ocellatin-PT6) could be the cause of the low activity in comparison with ocellatins-PT4, -PT7, and -PT8 (Table 3 and Figure S4). This difference is reflected in the predicted pI values, where ocellatins-PT1, -PT2, -PT3, -PT5, and -PT6 have pI values around 8.4, while ocellatins-PT4, -PT7, and -PT8 have pI values between 9.4 and 9.8 (Table 1). A similar case was noticed comparing ocellatin-L1 and ocellatin-L2, whose sequences differ only in one position, where the substitution of a neutral amino acid (Asn23) by an acidic residue (Asp23) prevents ocellatin-L2 from inhibiting *E. coli* growth.¹⁸

Gram-positive bacteria such as *S. aureus*, however, do not have an outer membrane or LPS. Teichoic acid-rich peptidoglycan, a major component of their surface structure, lowers their sensitivity to cationic antibacterial peptides. The potency of a particular peptide against a microorganism is determined by a complex interaction between factors such as conformation (degree of helicity), net charge, hydrophobicity,

and amphipathicity.⁴⁷ In this case, the small differences in the hydrophilic regions of the molecules would seem to be the major cause of their differences in activity. The low potency of these antimicrobial peptides could be related to the hypothesis proposed by Conlon⁴⁸ that antimicrobial peptides, together with cutaneous symbiotic bacteria, had a supplementary role in the frog-skin’s defense against pathogenic microorganisms. Another hypothesis for the low antimicrobial activity observed is that, in vivo, ocellatins could act synergistically with the various peptides typically present in the skin or with other secreted molecules, as previously described for other peptides.^{23,42} However, in our tests, these molecules being absent from our purified samples would not be able to modulate the peptide’s activity. Further work is needed to evaluate this hypothesis.

Atomic Force Microscopy. Figure 4 shows representative images of the ocellatin-PT7 effect on *E. coli* ATCC 25922 cells at the MIC concentration of 625 μg/mL (190 μM). Differences in cell envelope morphology between treated (Figure 4B) and untreated bacteria (Figure 4A) are clearly visible. Figure 4A

shows a representative nontreated *E. coli* cell, with a typical rod-shaped morphology and large number of pili. Figure 4B shows a representative image of an affected *E. coli* cell as a consequence of ocellatin-PT7 action at the MIC concentration. A reduction in the number of pili and a dramatic structural perturbation of the cell are observed, along with what appears to be cytoplasmic content release. This effect was also observed by other authors after *E. coli* treatment with the peptide PGLa⁴⁹ and with the peptides BP100 and pepR.⁵⁰ Disturbance of the outer membrane caused by ocellatin-PL7 interaction may permit the access of the peptide to the interstitial space and therefore the interaction with the inner membrane causing leakage of the cytosol and eventual lysis and cell death. In fact, the overall shape was greatly changed, making recognition of the cells difficult. However, the overall rod shape is maintained in the collapsed membrane seen around the cell, and one filamentous structure (pili) remains, allowing their identification in the AFM image. It is important to note that the observation of bacteria using AFM was performed in air, as it shows better resolution than in water,⁵¹ and they were obtained without the use of fixing agents, to ensure that there is no effects on bacterial physiology, as was reported by Colville et al.⁵² for the poly(L-lysine).

Cytotoxicity. Preliminary tests of cytotoxicity were performed with all new ocellatins (ocellatins-PT1 to -PT8) on erythrocytes and a normal animal cell line (murine fibroblast cell line, NIH-3T3) at peptide concentrations corresponding to the MIC observed for *E. coli*.

All of the peptides present little or no hemolytic activity at the MIC observed for *E. coli* or even at the highest concentrations tested for each peptide (Table 3).

Figure 5 presents the proliferation pattern of the NIH-3T3 fibroblast cells under different treatment conditions. The graphs (Figure 5A and B) show cell growth before treatment (−20 to 0 h) and after peptide exposure (0 to 60 h). All of the groups treated with peptide samples (ocellatins-PT1 to -PT8) at *E. coli* MIC concentrations presented a significant positive cell growth during the experimental time. The positive control group (H₂O₂) was the only one that presented a negative cell growth pattern. Figure 5C presents the area under the curve (AUC) of the cell proliferation tests. Only the positive control group presented a statistically significant AUC decrease compared to the control group. None of the peptide-treated groups presented a significant difference ($p > 0.05$) compared to the control group. In addition, no significant difference was observed between the peptide-treated groups.

These results using human red blood cells and a fibroblast cell line as mammalian models indicate that the antimicrobial peptides tested may have an effect that is selective toward bacterial cells. On the basis of the biochemical characterization (Table 1) of the peptide samples (ocellatins-PT1 to -PT8), all of them have isoelectric points higher than 8, which suggests a residual cationic superficial charge. In other words, at physiological pH, these peptides have a tendency to be protonated, explaining this positive superficial charge. These biochemical aspects may contribute to low cytotoxic activity of these peptides due to a more negative superficial charge of bacterial cells compared to eukaryotic cells, contributing to the therapeutic potential for these peptides.

CONCLUSIONS

In the present study we report the isolation, identification, and antimicrobial and cytotoxic activities of eight new ocellatins

isolated from the skin secretions of *L. pustulatus*. All of the new ocellatins, except ocellatin-PT2, inhibit one or more Gram-negative bacterial strains, and ocellatins-PT7 and -PT8 present antimicrobial activity against a Gram-positive strain at low antimicrobial potency. Membrane perturbation of *E. coli* was also demonstrated by AFM images. Skin secretions of *L. pustulatus* showed a set of antimicrobial peptides that differ by only a few amino acid substitutions, with different bactericidal activities. This could be part of a multidrug defense system that minimizes the chance of microorganisms developing resistance to any one individual peptide.⁴⁰

As demonstrated in this work, although they exhibit low antimicrobial potency, all of these peptides are selective for microorganisms versus human (erythrocytes) or animal (murine fibroblast) cells at MIC concentrations, which is an important requirement from the perspective of potential applications. Combination therapy with antibiotics is a topic that could be explored, as they could act as an enhancing agent of membrane permeability to allow antibiotics easier access to the cytoplasmic target. This interaction was demonstrated by Iwasaki et al., who used a synthetic peptide derived from insect defensin that exhibited a synergistic effect when combined with antibiotics.⁵³ Although peptides are currently the subject of much speculation regarding potential therapeutic uses, significant work is still required to explore possible harmful in vivo effects and to design combinations of antimicrobial peptides and antibiotics or to incorporate moieties that increase the functionality of the antimicrobial molecules.

EXPERIMENTAL SECTION

General Experimental Procedures. A MALDI-TOF/TOF mass spectrometer (matrix-assisted laser desorption ionization–time of flight/time of flight–Ultraflex II, Bruker Daltonics) and an automatic sequencer (PPSQ-23 Shimadzu Co.) were used for purity, mass determination, and de novo sequencing. An RP-HPLC system (Shimadzu Co., model LC-10VP) using a semipreparative column (Vydac C18 218TP510, 5 μ m, 10 \times 250 mm, 300 Å , Hesperia) was used for peptide purification.

An ABI Prism 3700 DNA analyzer system with BigDye terminator, POP-5 polymer (Applied Biosystems), and the Lasergene sequence analysis software (DNASTAR, Inc.) were used for ocellatins' cDNA sequencing.

A TT-AFM microscope from AFM Workshop and the Gwyddion v.2.33 software were used to obtain and analyze the images of the effect of ocellatin-PT7 on *E. coli* cells.

A real-time impedance-based analyzer (xCELLigence RTCA DP instrument, Roche) was used to quantify the cell proliferation assay.

Skin Secretions. Seven specimens of *Leptodactylus pustulatus* were collected near the Ilha Grande municipality, in the Piauí state of Brazil, during January and February 2012 (Figure 1). Collecting permission was provided by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, IBAMA (SISBIO/17687-1). Frog skin secretions were obtained by mild electric stimulation of the dorsal region, collected in ultrapure water, frozen, and lyophilized.⁵⁴ Specimens are deposited in the herpetological collection, voucher number CZDP 302 a 308, Coleção Zoológica do Delta do Parnaíba (CZDP), Campus de Parnaíba, Universidade Federal do Piauí (UFPI), Brazil.

Peptide Purification. The skin secretion (5 mg) was dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) aqueous solution (500 μ L) and filtered (Millex 0.22 μ m). The filtered extract was submitted to an RP-HPLC system using a linear gradient of CH₃CN/TFA 0.1% (v/v) rising from 0% to 60% over 60 min using a semipreparative column (Vydac C18 218TP510, 5 μ m, 10 \times 250 mm, 300 Å , Hesperia). An optimized linear gradient was used in analytical mode (Vydac C18 218TP54, 5 μ m 4.6 \times 250 mm, 300 Å , Hesperia). Eluents were

monitored by UV absorbance at 280 and 216 nm. Fractions were manually collected and vacuum-dried.

Peptide Sequencing. Purity and molecular mass determination of ocellatins-PT1 to -PT8 were performed by using a MALDI-TOF/TOF mass spectrometer. One microliter aliquots of the chromatographic fractions dissolved in an α -cyano-4-hydroxycinnamic acid matrix solution (1:3, v/v) were applied on a stainless steel plate and dried at room temperature for 15 min. The peptide monoisotopic mass was obtained in reflector mode with external calibration, using the Peptide Calibration Standard for Mass Spectrometry mixture (up to 4000 Da mass range, Bruker Daltonics). Isolated peptides were submitted to an automatic sequencer for de novo sequencing.

Gene Cloning and cDNA Sequencing. *L. pustulatus* cDNA was performed as previously described.⁴¹ Briefly, the dorsal and/or ventral region skin sections were frozen in liquid nitrogen and pulverized in a mortar. Aliquots of the resulting powder were submitted to total RNA extraction using Trizol reagent (Invitrogen). The quality and quantity of the resulting RNA were checked by agarose gel electrophoresis and by spectrophotometric analysis, respectively. One microgram of total RNA was applied as the template for first-strand cDNA synthesis using a SuperScript reverse transcriptase kit (Invitrogen). Reactions of 3'RACE were performed for the second strand cDNA synthesis using as primers PPS-2 (5'-ATGGCTTTCCTGAARAARTCBCT-TTTYCTTGATATTCTCGG-3') and a reverse primer NOT-1-REV (5'-TCGCGAGCGGCCGCCCTTTT-3'). The cycling parameters were as follow: four cycles of 94 °C/30 s and 72 °C/120 s; 25 cycles of 94 °C/30 s and 68 °C/180 s. PCR products, ranging from 300 to 400 bp, were purified using the Wizard SV Gel Clean-up System (Promega) after agarose gel electrophoresis. Purified fragments were used in a ligation reaction with the pGEM-T Easy vector system (Promega), following the manufacturer's instructions. After dialysis of the ligation mixture, aliquots were used for *E. coli* (DHS α) transfections. Following the selection and growth of bacterial colonies, the resulting plasmids were subjected to sequencing carried out by the dideoxy chain-termination method,⁵⁵ using an ABI Prism 3700 DNA analyzer system and universal M13 forward or reverse primers. Sequences were analyzed using the Lasergene sequence analysis software (DNASTAR, Inc.).

Sequence Analysis. New ocellatin sequences were compared with all peptides found in the skin secretions of other leptodactylid frogs in the literature and databases. The ClustalW tool was used to calculate identity and similarity scores among the peptides. Evolutionary relationship prediction among similar peptides was done using the ClustalW software, using the neighbor joining method.⁵⁶ The program ProtParam⁵⁷ was used for computation of physical and chemical parameters (MW, theoretical pI, instability index, aliphatic index, and grand average of hydrophobicity, GRAVY). The HeliQuest program was used to calculate hydrophobic moments (μ H) and helix wheel projections.^{58,59} Three-dimensional structural model predictions of the new peptides were obtained using the Web resource PEP-FOLD.⁶⁰

Solid-Phase Peptide Synthesis and Purification. All of the peptides were manually synthesized using a solid-phase approach using Fmoc/*tert*-butyl chemistry.⁶¹ 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 (Peptides International) was used for the synthesis of the peptides, except when the amidated N-terminal peptides were produced, for which a Rink amide MBHA resin (Peptides International) was used. Samples were treated with trifluoroacetic acid/triisopropylsilane/water (TFA/TIS/H₂O) (95:2.5:2.5) for removal of the protecting group. Peptide purification was carried out by preparative RP-HPLC (Phenomenex columns Kinetex 5 μ m C₁₈ 50 \times 21.20 mm) using a Shimadzu Prominence instrument. Each peptide was dissolved in H₂O/CH₃CN (6:4) and submitted to an RP-HPLC system using a gradient of CH₃CN, starting with H₂O/0.1% TFA and rising to 100% CH₃CN over 15 min. The formula $(A_{215}-A_{225}) \times 144$ (μ g/mL) was applied for peptide quantification.⁶²

Antibacterial Activity. The bacterial strains *E. coli* ATCC 25922, *S. aureus* ATCC 29313, *K. pneumoniae* ATCC 700603, and *S. choleraesuis* (Typhimurium) ATCC 14028 were used for antimicrobial

activity determination. All bacterial strains were grown at 37 °C in Mueller-Hinton broth until the logarithmic phase was reached (1×10^8 colony forming units (CFU)/mL).

Minimum inhibitory concentration (MIC) determination was performed according to the CLSI protocol (2012). Briefly, 2-fold serial dilutions of each peptide were made in Mueller-Hinton broth with concentrations ranging from 6.25 to 800 μ g/mL. In each well the final inoculum concentration was 5×10^5 CFU/mL. After incubation for 24 h at 37 °C in aerobic conditions, the MIC was defined as the lowest concentration of peptide that inhibited the visible bacterial growth (absorbance lower than 0.05 at 600 nm, in a Spectra Max-190 spectrophotometer, Molecular Devices). Standard antibiotics were also used as controls (meropenem for *E. coli*, *K. pneumoniae*, and *S. choleraesuis*; oxacillin for *S. aureus*). This experiment was performed in triplicate in two independent experiments.

Atomic Force Microscopy. The MIC value of ocellatin-PT7 against *E. coli* was determined as described above with concentrations of peptide ranging from 156 to 5000 μ g/mL, but here using a larger inoculum of 1×10^8 CFU/mL in each well, to facilitate observation by AFM. After incubation for 24 h, 30 μ L of the culture media containing the MIC-treated or untreated bacteria was deposited onto a clean glass surface followed by drying in a bacteriological incubator at 35 °C for 10 min. The samples were then gently rinsed twice with 1 mL of deionized water to remove salt crystals and dried again under the same conditions, before AFM analysis. All samples were prepared at the same time, exposed to the same conditions, and examined within 8 h of deposition. The analysis of the effect of ocellatin-PT7 on *E. coli* cells was carried out in vibrating mode, using NSG10 cantilevers (NT-MDT) with a resonant frequency of approximately 280 kHz. Images were analyzed using Gwyddion software 2.33. Multiple areas of each sample were examined, and selected representative images are shown.

Hemolytic Assays. Human red blood cells (RBCs), collected in EDTA (1.8 mg/mL), washed three times, and resuspended with sterile saline solution (0.85%), were used for the hemolytic activity test of peptides. The RBC suspension was added to an equal volume of each peptide solution at different concentrations (100–800 μ g/mL). The mixtures were incubated for 1 h at 37 °C and centrifuged at 10000g for 1 min. The supernatant absorbance (A) value at 550 nm was then measured. Saline solution and 0.1% (v/v) Triton-X were used as negative and positive hemolysis controls, respectively. The hemolysis percentage was calculated as follows:

$$\left[\frac{(A_{\text{peptide}} - A_{\text{saline}})}{(A_{\text{Triton}} - A_{\text{saline}})} \right] \times 100$$

Cell Culture and Proliferation Assay. The murine fibroblast cell line NIH-3T3 (ATCC number 1658) was used for the proliferation assays. Cells were routinely maintained in 25 cm² culture flasks (TPP) at 37 °C, in 5% (v/v) CO₂, in DMEM (Dulbecco's modified Eagle medium/Sigma-Aldrich) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 100 IU/mL penicillin, and 100 μ g/mL streptomycin.

For proliferation assays 5000 cells/well were seeded on E-plates, and a real-time impedance-based analyzer (xCELLigence RTCA DP instrument, Roche) was used to quantify cell proliferation for 80 h, with 20 h for cell attachment, followed by 60 h of cell exposure to the different peptide samples with the following final peptide concentrations: ocellatin-PT1 (800 μ g/mL); ocellatin-PT2 (400 μ g/mL); ocellatin-PT3 (800 μ g/mL); ocellatin-PT4 (200 μ g/mL); ocellatin-PT5 (800 μ g/mL); ocellatin-PT6 (400 μ g/mL); ocellatin-PT7 (200 μ g/mL); ocellatin-PT8 (200 μ g/mL). As negative and positive controls, cells were treated with sterile media and media supplemented with hydrogen peroxide (H₂O₂, 1% v/v), respectively. The results are represented with a cell index, which is directly related to the number of cells attached to the plate bottom, and used to indicate cell proliferation. A larger cell index indicates a greater number of cells detected. Treatments were conducted in quadruplicate for each experimental group. For the statistical analyses, the AUC of the cell index versus time graph from each experimental group was calculated, and analysis of variance (ANOVA) followed by Dunnett's test ($p < 0.05$) was calculated among the experimental groups.

■ ASSOCIATED CONTENT

■ Supporting Information

This material includes MALDI-TOF-TOF MS spectra and MS/MS spectra analysis, to determine de novo sequencing of the new peptides, RP-HPLC chromatograms of synthetic peptide purification, 3D structural model predictions and wheel projections of new and some previously described ocellatins. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/np500907t.

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Notes

The authors declare no competing financial interest.

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