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Isolation and Characterization of Gomesin, an 18-Residue Cysteine-rich Defense Peptide from the Spider *Acanthoscurria gomesiana* Hemocytes with Sequence Similarities to Horseshoe Crab Antimicrobial Peptides of the Tachyplesin Family*

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We have purified a small size antimicrobial peptide, named gomesin, from the hemocytes of the unchallenged tarantula spider *Acanthoscurria gomesiana*. Gomesin has a molecular mass of 2270.4 Da, with 18 amino acids, including a pyroglutamic acid as the N terminus, a C-terminal arginine α -amide, and four cysteine residues forming two disulfide bridges. This peptide shows marked sequence similarities to antimicrobial peptides from other arthropods such as tachyplesin and polyphemusin from horseshoe crabs and androctonin from scorpions. Interestingly, it also shows sequence similarities to protegrins, antimicrobial peptides from porcine leukocytes. Gomesin strongly affects bacterial growth, as well as the development of filamentous fungi and yeast. In addition, we showed that gomesin affects the viability of the parasite *Leishmania amazonensis*.

Antimicrobial peptides are ubiquitously produced throughout the phylogenetic tree. The widespread occurrence of these antimicrobial substances suggests that they play a key role in innate immunity against microorganisms and other pathogens (1–3). Despite the diversity in their structure, most of these antimicrobial substances are small (less than 10 kDa) cationic and amphipathic peptides (for reviews see Refs. 4–6). Over the past 20 years several of these antimicrobial peptides were isolated and characterized from the hemolymph of invertebrates including two chelicerates: horseshoe crabs (merostomata) (for a review see Ref. 7) and scorpions (arachnida) (8, 9). In fact, the hemocytes of horseshoe crabs contain various cysteine-rich cationic peptides (tachyplesin, polyphemusin, tachycitin, tachystatin, and big defensin) with inhibitory effects on the growth of Gram-positive and Gram-negative bacteria and fungi. In addition, two non-cysteine-rich proteins, anti-lipopolysaccharide factor, and factor D, with antimicrobial proper-

ties mainly against Gram-negative bacteria are produced in response to a microbial infection (7). The tachyplesin family of antimicrobial peptides, including polyphemusins, is located in the small granules of the hemocytes, as also are tachycitin and tachystatin, whereas the anti-lipopolysaccharide factor and factor D are found in the large granules and the big defensin in both types of granules. In addition to this group of chelicerates, several antimicrobial peptides were characterized in the hemolymph of the scorpions *Leiurus quinquestriatus* (8) and *Androctonus australis* (9). Among these peptides, two are members of the widespread invertebrate defensin family (for a review see Ref. 10). These scorpion defensins are mainly active against Gram-positive bacteria. Although only a defensin type molecule was found in *L. quinquestriatus* (7), three distinct antimicrobial peptides have been characterized from the hemolymph of unchallenged scorpions of the species *A. australis*: an insect defensin-like molecule (as mentioned above) and two additional cysteine-containing peptides, androctonin and buthinin (9). Androctonin is a 25-residue peptide with four cysteine residues, active against both Gram-positive and Gram-negative bacteria and fungi. Androctonin shows sequence similarities to tachyplesin and polyphemusin. In contrast, buthinin, a 34-amino acid peptide with three internal disulfide bridges, shows no sequence similarities with known antimicrobial peptides. Buthinin was found to be active only against bacteria.

Interestingly, in addition to strictly antimicrobial peptides found in chelicerates, additional molecules with antibacterial properties have been recently isolated from the venom of the wolf spider *Lycosa carolinensis*. In this spider, two toxins (lycotoxins I and II) containing 25–27 amino acids and deprived of cysteine residues exhibit antimicrobial properties. Surprisingly, they show marked sequence similarities to some frog antimicrobial peptides such as magainin, adenoregulin, and dermaseptins. In addition to having antimicrobial activity, they dissipate ion and voltage gradients across the membranes of excitable cells and probably contribute to paralysis of venomous prey (11).

Nevertheless, no report of a strictly antimicrobial peptide from spiders is available in the literature. To understand whether spiders are able to mount an immune response through the production of antimicrobial substances as observed in insects, we started to investigate such possibility in the mygalomorph spider *Acanthoscurria gomesiana*. The characterization of antimicrobial peptides in this class of chelicerates would be of great value to understand the evolutionary aspects of innate immunity. On this basis, the use of mygalomorph spiders as an experimental model is very useful, because they

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The protein sequence reported in this paper has been submitted to the Swiss-Prot Data Bank with accession number P82358.

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are one of the oldest species in the order *Araneae*.

In this paper, we report the isolation and full characterization of a small size antimicrobial peptide from hemocytes of unchallenged *A. gomesiana*. This peptide, named gomesin from the species *gomesiana*, has 18 amino acids and two disulfide bridges. This open-ended cyclic peptide shows marked sequence similarities to tachyplesin and polyphemusin from horseshoe crabs (12, 13) and to androctonin from the scorpion *A. australis* (9). More interestingly, gomesin shares also structural similarities with protegrins, antimicrobial peptides from porcine leukocytes (14). In addition to being effective against the growth of Gram-positive and -negative bacteria, synthetic gomesin was found to be strongly efficient against the growth of filamentous fungi and yeast and moderately active against the viability of the parasite *Leishmania amazonensis*.

EXPERIMENTAL PROCEDURES

Animals and Hemolymph Collection

The common spider (*A. gomesiana*) was obtained from the Arthropods Laboratory of the Butantan Institute (São Paulo, Brazil). *A. gomesiana* is a tarantula spider of the *Theraphosidae* family. The hemolymph (approximately 0.4 ml/spider) from animals of either sex at different stages of development was collected from prechilled animals by cardiac puncture with an apyrogenic syringe. To avoid hemocyte degranulation and coagulation, the hemolymph was collected in the presence of sodium citrate buffer, pH 4.6 (2:1, v/v) (15). The hemocytes were removed from plasma by centrifugation at $800 \times g$ for 10 min at 4 °C. Entire hemocytes were washed once with sodium citrate buffer and lysed by concentration in a vacuum centrifuge (Savant Instruments, Inc). For experimental spider immunization, 50 μ l of the Gram-negative bacteria *Enterobacter cloacae* (2×10^8 cells) were injected into the dorsal abdomen of the animals. The hemolymph was collected 48 h after injection.

Peptide Extraction and Purification

After concentration, the hemocytes collected from the hemolymph (1.8 ml) of unchallenged spiders were homogenized in a Dounce apparatus (maximum, 152 μ m; minimum, 76 μ m) in 1.5 ml of 2 M acetic acid supplemented with aprotinin (20 μ g/ml) as protease inhibitor. A second homogenization was performed by sonication (3×30 s) at medium power (Branson Ultrasonics Annemasse, France) in an ice-cold water bath, and extraction was performed over a period of 30 min at 4 °C under gentle shaking. The supernatant, obtained by centrifugation at $13,800 \times g$ for 30 min at 6 °C, was directly subjected to prepurification by solid phase extraction.

The combined cytosolic and organelle acid extracts were loaded onto serially linked classic Sep-Pak C_{18} cartridges (Waters Associates) equilibrated in acidified water (0.05% trifluoroacetic acid). Three stepwise elutions were successively performed with 5%, 40%, and 80% acetonitrile in acidified water. The 40% Sep-Pak fraction was concentrated in a vacuum centrifuge, reconstituted with MilliQ water, and applied to reversed phase chromatography on a semipreparative Aquapore RP-300 C_8 column (250 \times 7.0 mm, BrownleeTM) equilibrated with 2% acetonitrile in acidified water. Elution was performed with a linear 2–60% gradient of acetonitrile in acidified water over 120 min at a flow rate of 1.3 ml/min.

The active fraction from hemocytes (AGH2) was further purified by size exclusion chromatography using two serially linked HPLC-SEC¹ columns (Ultraspherogel SEC 3000 and SEC 2000 columns, 300 \times 7.5 mm, BeckmanTM) protected by a precolumn (Ultraspherogel SEC, 40 \times 7.5 mm, BeckmanTM). Elution was performed under isocratic conditions with 30% acetonitrile in acidified water at a flow rate of 0.4 ml/min.

HPLC purification was carried out at room temperature on a Beckman Gold HPLC system equipped with a Beckman 168 photodiode array detector. The column effluent was monitored for absorbance at 225 nm. Fractions corresponding to absorbance peaks were hand collected, concentrated under vacuum, and reconstituted in MilliQ water. The presence of antibacterial activity was determined by a liquid growth inhibition assay.

¹ The abbreviations used are: HPLC, high pressure liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MIC, minimal inhibitory concentration; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

Structural Characterization

Capillary Zone Electrophoresis—Peptide purity was ascertained by capillary zone electrophoresis. Analysis was performed in a Model 270A-HT capillary electrophoresis system (Perkin Elmer Applied Biosystems) equipped with a fused silica tube (length, 72 cm; internal diameter, 50 μ m) as described previously (9).

MALDI-TOF-MS and Electrospray Ionization Mass Spectrometry—MALDI-TOF-MS was performed with a Bruker BIFEX[®] (Bremen, Germany) mass spectrometer operating in a positive linear mode as described previously (16).

The electrospray ionization mass spectrometry data were obtained using a Finnigan LCQTMDuo mass spectrometer (ThermoQuest) at a mass to charge (*m/z*) range of 2000. The peptides dissolved in water/acetonitrile (50:50, v/v) were introduced in the spectrometer by flow injection at 5 μ l/min and analyzed in the positive mode. Calibration was performed using the following mixture of standard substances: caffeine (*m/z* 195.2), L-methionyl-arginyl-phenylalanyl-alanine acetate H₂O (*m/z* 524.3), and Ultramark 1621 (*m/z* 1022, 1122, 1222, 1322, 1422, 1522, 1622, 1722, 1822, and 1922).

Reduction and Alkylation—Purified peptide (2 nmol) was subjected to reduction and S-pyridylethylation using a procedure described previously (17). The S-pyridylethylated peptide was desalted/purified on an Aquapore RP300 C_8 column (220 \times 4.6 mm, BrownleeTM) using a linear gradient of 2–60% acetonitrile in acidified water over 120 min at a flow rate of 0.8 ml/min.

Synthetic amidated peptide (90 nmol) was reduced and S-carboxyamidomethylated by iodoacetamide (18). The S-carboxyamidomethylcysteine peptide was desalted/purified on a Vydac C_{18} column (250 \times 4.6 mm, Vydac[®]) using a linear gradient of 2–80% acetonitrile in acidified water over 60 min at a flow rate of 1.0 ml/min.

Pyroglutamate Aminopeptidase Treatment—The pyridylethylated peptide to be cleaved with pyroglutamate aminopeptidase (Roche Molecular Biochemicals) was dissolved in 100 mM sodium phosphate buffer containing 10 mM EDTA, 5 mM dithioerythritol and 5% glycerol (v/v), pH 8, using the procedure recommended by the manufacturer. The deblocked peptide was purified by HPLC onto a microbore reversed phase column (100 \times 1 mm, Aquapore RP-300 C_8 , BrownleeTM) using a linear 2 to 60% acetonitrile gradient in acidified water over 60 min at a flow rate of 80 μ l/min at 35 °C. The eluted peaks were analyzed by MALDI-TOF-MS, and the fraction corresponding to the deblocked peptide was subjected to sequencing by Edman degradation.

Determination of the Cysteine Arrangement by Trypsin Digestion—Native peptide was treated with trypsin (Roche Molecular Biochemicals) at an enzyme/substrate ratio of 1:20 (w/w) in a 40- μ l reaction medium containing 0.1 M Tris-HCl, pH 7.5. Incubation was carried out overnight at 37 °C. The reaction was stopped by acidification with formic acid (final concentration, 3%). Peptide fragments were separated by HPLC on a narrowbore reversed phase column (Delta Pak HPI C_{18} , 2 \times 150 mm; WatersTM) using a linear 2–80% acidified acetonitrile gradient over a period of 90 min at a flow rate of 200 μ l/min at 30 °C. The various peptide fragments obtained were analyzed by MALDI-TOF-MS, and the fragments of interest were subjected to microsequencing by Edman degradation.

Microsequence Analysis—Automated Edman degradation of the deblocked pyridylethylated peptide and of the peptide fragments and detection of phenylthiohydantoin-derivatives were performed using a pulse liquid automatic sequencer (Perkin Elmer 32 Applied Biosystems model 473A).

Peptide Synthesis

Nonamidated and amidated peptides were synthesized according to a procedure previously described (9). Briefly, the peptides were synthesized according to classical Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. At the end of the process, the peptides (25 μ mol) were taken up to 2.5 liters in 0.1 M ammonium acetate buffer, pH 8.5, and allowed to renature by air oxidation overnight at room temperature under stirring. After renaturation, the pH was adjusted to 4 with acetic acid and the peptide solution loaded onto Sep-Pak Vac C_{18} cartridges (Waters Associates) equilibrated with acidified water. The peptides were eluted with 60% acetonitrile in acidified water, concentrated in a vacuum centrifuge and purified to homogeneity by reversed phase chromatography using a preparative column (Aquapore RP 300 C_8 , 150 \times 10 mm, BrownleeTM).

Bioassays

The microbial strains were those used in previous studies (19–22). The following additional fungal and bacterial strains were generous

gifts from different colleagues: *Enterococcus faecalis*, *Staphylococcus epidermidis*, *S. hemeolyticus* (H. Monteil, Institute of Bacteriology, University of Strasbourg, France), *Nocardia asteroides* (Y. Piémont, Institute of Bacteriology, University of Strasbourg, France), *Serratia marcescens* Db11 (H.G. Boman, Department of Microbiology, University of Stockholm, Stockholm, Sweden), *Beauveria bassiana* (INRA, St-Christol-les-Ales, France), and *Tricophyton mentagrophytes* and *Candida tropicalis* (H. Koenig, Laboratory of Mycology, University of Medicine, Strasbourg, France).

During the purification procedure, antibacterial activity was monitored by a liquid growth inhibition assay against *Micrococcus luteus* and *Escherichia coli* SBS363 as described in Ref. 23. The MIC of the synthetic peptides was determined against fungi and bacterial strains using a procedure described previously (9). When the antifungal activity assay was performed in the presence of salt, the culture medium was prepared in phosphate-buffered saline, 137 mM NaCl. MICs are expressed as the [a] – [b] interval of concentrations, where [a] is the highest concentration tested at which the microorganisms are growing and [b] is the lowest concentration that causes 100% growth inhibition.

The bactericidal assay was monitored against *M. luteus* and *E. coli* D22 as described in Ref. 9. The final concentration of amidated gomesin tested was 10 μ M. For the fungicidal assay, spores of *Neurospora crassa* were incubated in the presence of 10 μ M amidated gomesin. After 0.5, 1, 2, 4, and 8 h, the culture medium containing the peptide was removed and replaced with fresh medium. After 48 h the cultures were examined microscopically and spectrometrically.

The hemolytic activity assay was monitored against human erythrocytes. Two times serial dilutions of synthetic gomesin from concentrations of both forms of gomesin of 0.1 to 100 μ M were incubated in Eppendorf tubes with a suspension of erythrocytes (0.4%) in phosphate-buffered saline. As a positive control (100% lysis), the erythrocytes were incubated with 0.1% SDS. After 1 h at 37 °C, the samples were centrifuged (320 \times g, 5 min), and the absorbance of the supernatant (50 μ l) was measured at 405 nm using a microtiter plate reader.

For the antiparasitic assay, cell viability of *L. (Leishmania) amazonensis* (MPRO/BR/72/M 1841-LV-79) was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as described (24) and adapted for *Leishmania* spp. (25). Briefly, 5 \times 10⁶ stationary phase promastigotes suspended in buffered saline (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM D-glucose, 10 mM MOPS, pH 7.2) were incubated with two times serial dilutions of synthetic gomesin and hemoglobin fragment (33–61 of bovine α -hemoglobin) (26) from 0.3 to 80 μ M. After a 1-h incubation at 22 °C, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was added to a final concentration of 1 mg/ml, and the reaction was measured after 40 min in a microtiter plate reader at 595 nm.

RESULTS

Purification and Primary Structure Determination of Gomesin—We first performed a pilot experiment to compare the antibacterial activity against *M. luteus* of crude extracts of hemocytes from unchallenged and bacteria-challenged *A. gomesiana* spiders. Because no increase in antibacterial activity was found in the immunized spiders, we decided to purify the antimicrobial compounds from native spider hemocytes.

The antimicrobial compounds from the hemocytes were extracted under acidic conditions after sonication. The acidic extract was prepurified by solid phase extraction. The three eluted fractions obtained (5, 40, and 80%) were submitted to reversed phase HPLC and to the antibacterial assay. Among the different fractions obtained from the 40% Sep-Pak fraction, three were found to have activity against *M. luteus* and *E. coli*, the two bacterial strains used for the bioassay (Fig. 1A). In the present study, we focused our attention on fraction AGH2, which has activity against both strains of bacteria. MALDI-MS analysis of fraction AGH2 revealed the presence of three different molecules with molecular masses of 413.4, 2270.4, and 7179.8 Da. To separate these molecules, the second step of purification was performed using size exclusion chromatography (Fig. 1B). Among the different fractions obtained, only one peak was found to be active against *M. luteus* and *E. coli*. Because a single molecular mass of 2270.4 Da was obtained by

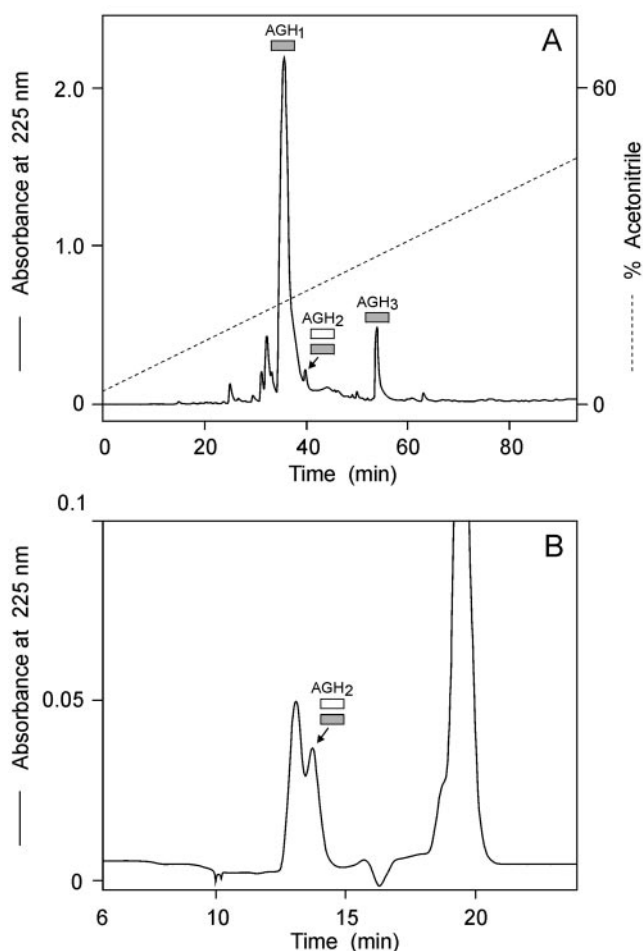


FIG. 1. Purification of gomesin from spider hemocytes by reversed-phase and size exclusion HPLC. An acidic extract obtained from *A. gomesiana* hemocytes was submitted to solid phase extraction on Sep-Pak C₁₈ cartridges. The fraction eluted with 40% ACN was analyzed on an Aquapore RP-300 C₈ column with a linear 2 to 60% acetonitrile gradient in acidified water over 120 min (dotted line), at a flow rate of 1.3 ml/min (A). Three fractions were active against *E. coli* SBS363, AGH 1–3 (shaded rectangle). Gomesin present in fraction AGH2, also active against *M. luteus* (open rectangle), was purified by size exclusion chromatography using two serially connected Ultraspherogel SEC 3000 and SEC 2000 columns. Elution was performed under isocratic conditions with 30% acetonitrile in acidified water at a flow rate of 0.4 ml/min (B). Absorbance was monitored at 225 nm, and antibacterial activity was detected by liquid growth inhibition assay.

mass spectrometry, the molecule was considered to be pure enough for sequencing by Edman degradation.

Because no PTH-derivative signal was observed in the 25 cycles of Edman degradation, this molecule was supposed to be a N-terminally blocked peptide. To verify this hypothesis, the reduced and S-pyridylethylated molecule was subjected to pyroglutamate aminopeptidase digestion to remove a potential pyroglutamic acid. After purification by reversed phase HPLC of the digestion products, two fractions were obtained (data not shown). Mass spectrometry analysis revealed a difference of 111 Da between the two molecules that corresponded to the exact molecular mass of a pyroglutamic acid. For this reason, only the compound with the smallest molecular mass (2583.8 Da) was subjected to Edman degradation. The following 17-residue sequence was obtained: C*RRLC*YKQRC*VTYC*RGR, where C* stands for a pyridylethylated cysteine residue. The identification of the four pyridylethylated cysteine residues after Edman degradation is in agreement with the increased mass (424.3 Da) of the native peptide following reduction/alkylation with vinyl pyridine (2694.7 Da). From the primary se-

quence obtained and assuming pyroglutamic acid (Z) as the first residue and four cysteine residues involved in two disulfide bridges, the mass calculated as 2269.7 Da differs only by approximately +1 Da from the mass measured by MALDI-TOF-MS (2270.4 Da). This observation indicates a possible amidation of the C-terminal arginine of the peptide.

To define the cysteine array, the native molecule (ZC*RRLC*YKQRC*VTYC*RGR) was subjected to tryptic digestion. Following digestion, several fragments were separated by reversed phase HPLC. A peak with a molecular mass at 1006.9 Da was sequenced by Edman degradation, and the following sequence was obtained: Leu-Val-(Tyr/Thr)-(Tyr/Lys). The presence of two clear PTH-derivatives at positions 3 and 4 at the same intensity clearly demonstrated the presence of a double sequence in this fraction. This observation, associated with the primary structure established, is in agreement with the linkage of the tetrapeptide Leu⁵-Cys⁶-Tyr⁷-Lys⁸ to the sequence Cys¹¹-Val¹²-Thr¹³-Tyr¹⁴. Therefore, the two intramolecular disulfide bridges are between Cys²¹ and Cys¹⁵⁴ and between Cys⁶² and Cys¹¹³.

To confirm our hypothesis concerning the terminal amidation of this peptide, amidated and non-amidated molecules were prepared chemically. After renaturation and purification to homogeneity, the identity and integrity of both compounds were confirmed by mass spectrometry and capillary zone electrophoresis (data not shown). When the native molecule and the two synthetic forms were analyzed by MALDI-TOF-MS, the monoisotopic molecular mass of the native molecule (2270.9 Da) was in perfect agreement with the monoisotopic molecular mass of the synthetic amidated molecule (2270.5 Da) compared with the molecular mass of the non-amidated form (2271.6 Da). This confirmed that the native peptide presents a C-terminal arginine α -amide. This 18-residue amidated peptide was named gomesin in reference to the species name of the spider *A. gomesiana*.

Antimicrobial Activity Spectrum of the Amidated Gomesin—The MIC of gomesin purified from spider hemocytes against *M. luteus* was 0.25–0.5 μ M, similar to the MIC found for the synthetic peptides (0.4–0.8 μ M) (Table I). The amidated synthetic gomesin had a strong activity against almost all Gram-positive bacteria tested (MICs < 3.15 μ M), except *Pediococcus acidolactici* (MIC = 3.15–6.25 μ M) and *Bacillus cereus* and *E. faecalis* (MIC = 6.25–12.5 μ M). Therefore, the synthetic gomesin can be considered equivalent to the native one. Synthetic gomesin showed also high activity against almost all Gram-negative bacteria tested with the exception of the insensitive strain *Agrobacterium tumefaciens* and the entomopathogenic bacteria *S. marcescens*. The most sensitive strains were *E. coli*, *Pseudomonas aeruginosa*, and *Salmonella thyphimurium* (MICs < 3.15 μ M). The other strains were affected by gomesin at MICs between 3.15 and 6.25 μ M. Only a partial growth inhibition of *Alcaligenes faecalis* was observed at concentrations of 50–100 μ M.

All filamentous fungi tested were highly sensitive to gomesin (MIC < 3.15 μ M), with the exception of *B. bassiana* (MIC = 12.5–25 μ M). Gomesin was also found to have a marked activity against most of the yeast strains tested such as *Candida albicans*, *C. tropicalis*, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* (MIC < 6.25 μ M) and moderate activity against *C. glabrata* (MIC = 12.5–25 μ M). When the antifungal activity assay was performed under salt conditions (137 mM ion strength), the activity was found to be identical to that recorded under normal conditions (data not shown).

To ascertain the role of the disulfide bonds on gomesin antibacterial activity, we have determined the antibacterial activity of the reduced/S-carboxyamidomethylated peptide. The mo-

TABLE I
Antimicrobial activity spectrum of gomesin

The activity of amidated gomesin was compared to a non-amidated form (gomesin-fa) and to androctonin a four cysteine-rich antimicrobial peptide from the scorpion *A. australis* (9).

Microorganisms	Minimal inhibitory concentration		
	Gomesin	Gomesin-fa	Androctonin
	μ M		
Gram-positive bacteria			
<i>Aerococcus viridans</i>	0.8–1.6	0.8–1.6	0.3–0.6
<i>Bacillus cereus</i>	6.25–12.5	12.5–25	ND ^a
<i>Bacillus megaterium</i>	0.2–0.4	0.4–0.8	NM ^b
<i>Bacillus thuringiensis</i>	1.6–3.15	3.15–6.25	ND
<i>Enterococcus faecalis</i>	6.2–12.5	NM	NM
<i>Listeria monocytogenes</i>	0.8–1.6	3.15–6.25	NM
<i>Micrococcus luteus</i>	0.4–0.8	0.4–0.8	0.6–1.5
<i>Pediococcus acidolactici</i>	3.15–6.25	3.15–6.25	NM
<i>Staphylococcus aureus</i>	1.6–3.15	3.15–6.25	15–30
<i>Staphylococcus epidermidis</i>	0.8–1.6	3.15–6.25	NM
<i>Staphylococcus haemolyticus</i>	0.8–1.6	1.6–3.15	NM
<i>Staphylococcus saprophyticus</i>	0.8–1.6	1.6–3.15	NM
<i>Streptococcus pyogenes</i>	1.6–3.15	3.15–6.25	NM
<i>Nocardia asteroides</i>	1.6–3.15	3.15–6.25	NM
Gram-negative bacteria			
<i>Agrobacterium tumefaciens</i>	ND	ND	ND
<i>Alcaligenes faecalis</i>	>100	NM	NM
<i>Escherichia coli</i> 1106	0.8–1.6	1.6–3.15	6–15
<i>Escherichia coli</i> D22	0.4–0.8	0.4–0.8	6–15
<i>Escherichia coli</i> D31	0.8–1.6	0.8–1.6	3–6
<i>Escherichia coli</i> SBS363	0.4–0.8	0.4–0.8	NM
<i>Erwinia carolovorana carolovorana</i>	3.15–6.25	3.15–6.25	ND
<i>Enterobacter cloacae</i> β 12	3.15–6.25	6.25–12.5	ND
<i>Klebsiella pneumoniae</i>	3.15–6.25	6.25–12.5	ND
<i>Pseudomonas aeruginosa</i>	1.6–3.15	1.6–3.15	NM
<i>Salmonella thyphimurium</i>	0.8–1.6	0.8–1.6	3–6
<i>Serratia marcescens</i> Db11	ND	ND	ND
<i>Xanthomonas campestris</i> pv. orizae	3.15–6.25	6.25–12.5	ND
Fungi			
<i>Alternaria brassicola</i>	0.4–0.8	0.4–0.8	3–6
<i>Aspergillus fumigatus</i>	1.6–3.15	1.6–3.15	25–50
<i>Beauveria bassiana</i>	12.5–25	25–50	NM
<i>Fusarium culmorum</i>	0.4–0.8	0.4–0.8	3–6
<i>Fusarium oxysporum</i>	0.4–0.8	0.8–1.6	6–12
<i>Neurospora crassa</i>	0.4–0.8	0.4–0.8	6–12
<i>Nectria haematococca</i>	0.2–0.4	0.2–0.4	6–12
<i>Tricoderma viridae</i>	0.4–0.8	0.4–0.8	6–12
<i>Trichophyton mentagrophytes</i>	0.8–1.6	0.8–1.6	NM
Yeasts			
<i>Candida albicans</i>	0.15–0.3	0.15–0.3	1.6–3.15
<i>Candida glabrata</i>	12.5–25	12.5–25	NM
<i>Candida tropicalis</i>	3.15–6.25	NM	NM
<i>Cryptococcus neoformans</i>	0.8–1.6	NM	NM
<i>Saccharomyces cerevisiae</i>	1.6–3.15	1.6–3.15	NM

^a ND means not detected in the range of the concentrations tested up to 100 μ M (gomesin) and 50 μ M (androctonin).

^b NM means not measured.

lecular mass measured by electrospray ionization mass spectrometry showed that 100% of amidated gomesin was reduced and alkylated after treatment with dithiothreitol and iodoacetamide (data not shown). Using a calibrated solution of synthetic peptide, the amount of reduced/alkylated gomesin has been estimated by HPLC and the MICs determined against *M. luteus* and *E. coli* SBS363. The MICs observed for both strains at 12.5–25 μ M are significantly lower than the one observed for the non-modified gomesin (0.4–0.8 μ M; Table I).

Bactericidal and Fungicidal Effect of Gomesin—When synthetic gomesin was incubated with *M. luteus* and *E. coli* D31 at concentrations 12.5 times higher than the respective MICs, no living bacteria were detected after a 1-min incubation with both tested strains (Fig. 2). In the presence of synthetic gomesin at a concentration 12.5 times higher than the respective MIC, no growth of *N. crassa* spores was observed after a 30-min incubation (data not shown).

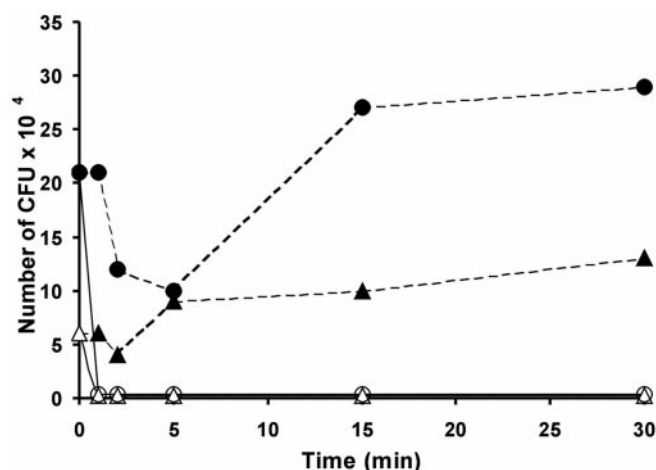


FIG. 2. Kinetics of *M. luteus* and *E. coli* D22 killing by gomesin. Synthetic gomesin (10 μM , solid lines) or water (control, dotted lines) was added to an exponential phase culture of *M. luteus* (circle) or *E. coli* D22 (triangle). Aliquots were removed at various times, and the number of colony forming units (CFU) was determined on Luria Bertani agar plates after overnight incubation at 37 $^{\circ}\text{C}$.

Activity of Gomesin against Eukaryotic Cells—To determine the effect of gomesin against eukaryotic cells, various concentrations of synthetic peptide (0.1–100 μM) were tested against the parasite *L. (L.) amazonensis* and human erythrocytes using viability and hemolytic assays, respectively. After 1 h of incubation, the viability of the parasite was found to be dependent on the gomesin concentration used (Fig. 3). Using as control a hemoglobin fragment (33–61 of bovine α -hemoglobin) (26), which exhibits some effect on the viability of the parasite at high concentrations (>40 μM), gomesin was found to strongly reduce viability even at concentration as low as 2.5 μM . In addition, in an hemolytic assay against human erythrocytes, the percentage of cell lysis was found to be dependent of the gomesin (amidated and non-amidated) concentrations tested. From 0.1 μM (the lowest concentration tested) up to 0.2 μM , the percentage of hemolysis remains rather low (<5%), whereas increasing the peptide concentration up to 1 μM results in a rapid and significant elevation of the percentage of cell lysis (up to approximately 16%). However, an increase in the concentration of gomesin of 2 orders of magnitude (final concentration tested of 100 μM) has only little effect on human erythrocyte lysis (Fig. 4). This contrast to the gomesin activity against the parasite *L. (L.) amazonensis* where cell viability was found to be almost linearly dependent of the peptide concentration (see Fig. 3).

DISCUSSION

To gain information on the involvement of antimicrobial peptides in arachnid immunity, we started to investigate the antimicrobial peptides of the tarantula spider *A. gomesiana*, which can live over 23 years (Butantan Institute, São Paulo, registration book). We have purified and characterized from *A. gomesiana* hemocytes an antimicrobial peptide that we named gomesin. The level of antibacterial activity found in the hemocytes has not changed in bacteria-challenged spiders, such as scorpions (9), ticks (26), shrimps (27), and bivalve mollusks (28), suggesting that the antimicrobial peptides are in fact stored in the spider hemocytes and may be released into the plasma upon infection, as established in mollusks (28). This remains to be confirmed by gomesin mRNA quantification.

Gomesin is an 18-residue peptide with four cysteine residues engaged in disulfide bridges, a pyroglutamic acid as the N terminus, and an Arg α -amide as C-terminal residue. The

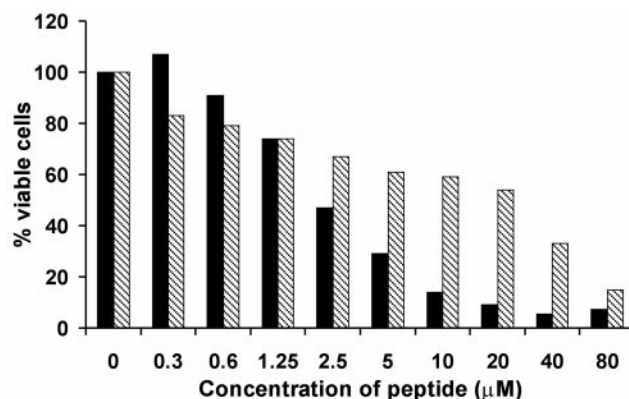


FIG. 3. Activity of gomesin against *L. (L.) amazonensis* promastigotes. The synthetic gomesin (solid bar) and a hemoglobin fragment (33–61 of bovine α -hemoglobin) (hatched bar) used as positive control were incubated with the parasites for 1 h at 22 $^{\circ}\text{C}$ at concentrations ranging from 0.3 to 80 μM . Parasite viability (%) was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as described previously (24) and adapted for *Leishmania* spp. (25).

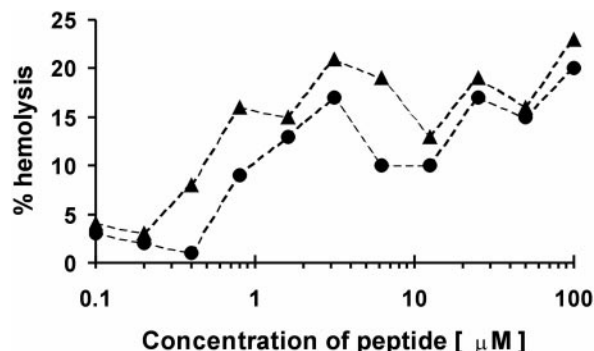


FIG. 4. Activity of gomesin against human erythrocytes. Both forms of synthetic gomesin: amidated (circle) and nonamidated (triangle) were incubated with human erythrocytes at concentrations ranging from 0.1 to 100 μM for 1 h at 37 $^{\circ}\text{C}$. The hemolysis percentage was expressed in relation to a 100% lysis control (erythrocytes incubated with 0.1% SDS).

presence of two internal disulfide bridges plus a C-terminal amidation certainly contributes to the stability of the peptide to proteases within the hemocytes or if released into the hemolymph. Gomesin has six positively charged residues (five Arg and one Lys for a calculated pI of 12.7) and showed similarities to (i) antimicrobial peptides from horseshoe crabs: tachyplesins and polyphemusins (50%, determined by using Clustal V method with PAM250 residue weight table) (12, 13), (ii) androctonin (23%), from scorpions (9), and finally (iii) protegrins (17%), porcine leukocyte antimicrobial peptides (14) (Fig. 5). In gomesin, the four cysteine residues form two disulfide bridges linking Cys² to Cys¹⁵ and Cys⁶ to Cys¹¹. Such a disulfide array (Cys1-Cys4 and Cys2-Cys3) is identical for all antimicrobial peptides (Fig. 5). Interestingly, in gomesin an equal number of three residues is observed on each segment upstream and downstream the disulfide bridge Cys2-Cys3 (Arg³-Arg⁴-Leu⁵ and Val¹²-Thr¹³-Tyr¹⁴, gomesin numbering) as in tachyplesin I and polyphemusins II. A similar balance in the number of amino acids between the two disulfide bridges is also found in protegrin PG-1 (one residue in each segment). Unlike gomesin, the horseshoe crab peptides and protegrin PG-1, androctonin presents an unequal number of residues: five residues (Arg-Gln-Ile-Lys-Ile) upstream Cys2 and three amino acids (Tyr-Tyr-Lys) downstream Cys3. This suggests that gomesin will adopt a β -hairpin structure close to the one found in tachyplesin (29,

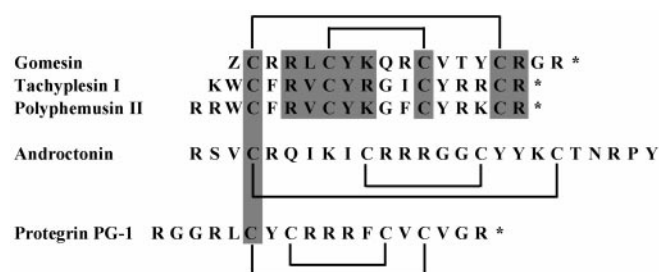


FIG. 5. Amino acid sequence comparison of gomesin to vertebrate and invertebrate antimicrobial peptides. Gomesin was compared with (i) tachyplesin I and (ii) polyphemus II from the horseshoe crabs *Tachyplesus tridentatus* and *Limulus polyphemus*, respectively (12, 13); (iii) androctonin from the scorpion *A. australis* (9); and (iv) protegrin PG-1 from porcine leukocytes (14). Z stands for pyroglutamic acid, and asterisks indicate a C-terminal α -amide. Identical and conserved residues are shaded.

30) and protegrin (31, 32) rather than the one observed for androctonin (33). In fact, the main differences that can strongly modify the properties (physico-chemical and bioactivity) are located at (i) the level of the turn, between Cys2 and Cys3 and (ii) at the N-terminal segment upstream the first cysteine residue, whereas the C-terminal has almost the same physico-chemical properties with the exception of androctonin (Fig. 5). In the turn, the net charge is rather different from one molecule to the other and this can modify the activities since this is exposed to the external environment as pointed out by the different three-dimensional structures available (29–33). The size of the N-terminal segment upstream Cys1 varies from one amino acid (pyroglutamic acid) for gomesin to two and three amino acids for tachyplesin and polyphemus/androctonin, respectively and up to five residues for the longest one in PG-1 (Fig. 5). Gomesin has no charge in this segment, whereas one positive charge is observed in androctonin and tachyplesin and two in polyphemus and protegrin. Comparison of gomesin to the horseshoe crab antimicrobial peptides shows that the four cysteine residues, three basic residues (Arg⁴, Lys⁸, and Arg¹⁶, gomesin numbering), one hydrophobic residue (Leu⁵ in gomesin versus Val in the horseshoe crab peptides), and a tyrosine residue (Tyr⁷) are conservative and that most conserved residues are arranged in a same cluster (Arg⁴ to Lys⁸) in gomesin and tachyplesin/polyphemus. Finally, all peptides except androctonin have a C-terminal arginine α -amide. This amidation of the basic residue increases the electropositivity of the peptide and probably facilitate the interaction of the peptide with the membrane of the microorganisms.

Gomesin is effective against the majority of the bacterial strains tested. 24 of the 27 bacterial strains tested were susceptible to gomesin at MICs (i) below 1.6 μ M for half of them, (ii) between 1.6 and 6.25 μ M (42%), and (iii) between 6.25–12.5 μ M for two strains (*B. cereus* and *E. faecalis*). Gomesin is bactericidal on *M. luteus* and *E. coli* within 1 min (Fig. 2), and this differs from androctonin (10 min against *M. luteus* and over 2 h against *E. coli*) (9). Such a bactericidal effect was also observed for tachyplesins and polyphemusins, on *S. aureus* and *E. coli* (34) and for protegrin PG-1 on methicillin-resistant *S. aureus* and *P. aeruginosa* (35). This “fast killing” of gomesin may offer significant advantages over conventional nonpeptide antimicrobial agents that require longer periods of exposure.

In addition to being antibacterial, gomesin has marked activity against a variety of fungi at a rather low concentration (<3.15 μ M), the most resistant strain being the entomopathogenic fungus *B. bassiana* and the filamentous yeast *C. glabrata* (Table I). Tachyplesins and polyphemusins have also a pronounced activity against yeast (13) and a lower activity

against filamentous fungi (36). When compared with androctonin, the antimicrobial activity of gomesin is always higher, with the exception of *A. viridans* (Table I). Interestingly, the activity of gomesin on *N. crassa* was not affected at physiological salt concentrations. In addition, the synthetic amidated gomesin is slightly more active than the non-amidated form, suggesting that amidation has no effect on the activity of the peptide (Table I).

Finally, the pronounced decrease in activity observed against *M. luteus* and *E. coli* following reduction/alkylation of the gomesin disulfide bridges suggests the importance of the disulfide bridges for the biological activity of the molecule. Similarly, studies with tachyplesin analogs, where the SH groups were chemically protected to prevent cyclization or when cysteine residues were replaced by alanine residues, suggested that the disulfide bridge were also essential for antibacterial activity (37). In fact, replacements of the cysteine residues by various amino acids were in favor of the necessity to maintain the hydrophobic-hydrophilic balance in the peptide to retain its biological activity (36). Protegrin and its reduced form were active in the absence of high salt at comparable concentrations, but the disulfide bridges were essential for activity in media containing salt concentrations comparable with those found in sera and extracellular fluids (38).

Moreover, even if gomesin has evident hemolytic activity against human erythrocytes at a rather low concentration (16% at 1 μ M), the effect on these human cells does not increase, whereas the peptide concentration reaches 100 μ M. This is in contrast to the hemolytic effect of tachyplesin, a response which was found to be linearly dependent on the peptide concentration tested, 5% up to 20 μ M up to 100% hemolysis at 100 μ M (34). Surprisingly, Rao (36) observed only 30% hemolysis with 100 μ M of tachyplesin. On the other hand, the scorpion androctonin is not hemolytic even at high 150 μ M (9). The differences in hemolytic activity observed between androctonin and gomesin and tachyplesin, could be attributed to (i) a longest C terminus and (ii) charge differences in this section of the molecule. Gomesin was found to be more toxic to *Leishmania (L.) amazonensis* promastigote than to human erythrocytes. In fact in the presence of 2.5 μ M gomesin only 50% of promastigotes were viable. Such effect was also observed with the cecropin A-melittin hybrid peptide when tested against *Leishmania donovani* promastigotes (39). This activity on *Leishmania* promastigotes could be attributed to a higher percentage of anionic phospholipids in their plasma membrane of compared with the plasma membrane of mammalian cells and of ergosterol instead of cholesterol (40). The presence of a large number of positively charged amino acids (five Arg and one Lys) in gomesin contributes to a higher specificity of the peptide for prokaryotic cells because of a higher electronegativity of prokaryotic cells when compared with the eukaryotic cell membrane. The participation of hydrophobic residues in the interaction may occur during later events, resulting in bacterial lysis. To obtain a better understanding of the mode of action of gomesin, its three-dimensional structure should be elucidated using NMR spectroscopy.

In conclusion, gomesin appears to have an interesting potential for therapeutic application because of its strong and broad activity spectrum (affecting bacteria, fungi, yeasts, and eukaryotic parasites) and structural properties conferring high stability to the molecule. In addition, the conservation of the activity in a physiological condition supports the possibility to develop this peptide as a new drug for human therapy. Despite hemolytic activity of the gomesin against human erythrocytes, this activity could be decreased or abolished through the modifica-

tion by molecular biology and/or chemical approach as obtained in tachyplesin analogs (41).

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REFERENCES

- Boman, H. G. (1995) *Annu. Rev. Immunol.* **13**, 60–65
- Ganz, T., and Lehrer, R. I. (1999) *Mol. Med. Today* **5**, 292–297
- Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999) *Science* **284**, 1313–1318
- Hwang, P. M., and Vogel, H. J. (1998) *Biochem. Cell Biol.* **76**, 235–246
- Andrew, D. (ed) (1998) *Biopolymers (Peptide Sci.)* **47**, 415–491
- Bulet, P., Hetru, C., Dimarcq, J. L., and Hoffmann, D. (1999) *Dev. Comp. Immunol.* **23**, 329–344
- Iwanaga, S., Kawabata, S., and Muta, T. (1998) *J. Biochem. (Tokyo)* **123**, 1–15
- Cociancich, S., Goyffon, M., Bontems, F., Bulet, P., Bouet, F., Menez, A., and Hoffmann, J. A. (1993) *Biochem. Biophys. Res. Commun.* **194**, 17–22
- Ehret-Sabatier, L., Loew, D., Goyffon, M., Fehlbaum, P., Hoffmann, J. A., Van Dorsselaer, A., and Bulet, P. (1996) *J. Biol. Chem.* **271**, 29537–29544
- Dimarcq, J. L., Bulet, P., Hetru, C., and Hoffmann, J. A. (1998) *Biopolymers (Peptide Sci.)* **47**, 465–477
- Yan, L., and Adams, M. (1998) *J. Biol. Chem.* **273**, 2059–2066
- Nakamura, T., Furunaka, H., Miyata, T., Tokunaga, F., Muta, T., Iwanaga, S., Niwa, M., Takao, T., and Shimonishi, Y. (1988) *J. Biol. Chem.* **263**, 16709–16713
- Miyata, T., Tokunaga, F., Yoneya, T., Yoshikawa, K., Iwanaga, S., Niwa, M., Takao, T., and Shimonishi, Y. (1989) *J. Biochem. (Tokyo)* **106**, 663–668
- Kokryakov, V. N., Harwig, S. S., Panyutich, E. A., Shevchenko, A. A., Aleshina, G. M., Shamova, O. V., Korneva, H. A., and Lehrer, R. I. (1993) *FEBS Lett.* **327**, 231–236
- Söderhall, K., and Smith, V. J. (1983) *Dev. Comp. Immunol.* **7**, 229–239
- Uttenweiler-Joseph, S., Moniatte, M., Lambert, J., Van Dorsselaer, A., and Bulet, P. (1997) *Anal. Biochem.* **247**, 366–375
- Bulet, P., Cociancich, S., Reuland, M., Sauber, F., Bischoff, R., Hegy, G., Van Dorsselaer, A., Hetru, C., and Hoffmann, J. A. (1992) *Eur. J. Biochem.* **209**, 977–984
- Aitken, A., and Learmonth, M. (1996) in *The Protein Protocols Handbook* (Walker, J. M., ed) pp. 339–340, Humana Press, Totowa, NJ
- Bulet, P., Cociancich, S., Dimarcq, J.-L., Lambert, J., Reichhart, J.-M., Hoffmann, D., Hetru, C., and Hoffmann, J. A. (1991) *J. Biol. Chem.* **266**, 24520–24525
- Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W. F., Hetru, C., and Hoffmann, J. A. (1994) *J. Biol. Chem.* **269**, 33159–33163
- Cociancich, S., Dupont, A., Hegy, G., Lanot, R., Holder, F., Hetru, C., Hoffmann, J. A., and Bulet, P. (1994) *Biochem. J.* **300**, 567–575
- Lamberty, M., Ades, S., Uttenweiler-Joseph, S., Brookhart, G., Bushey, D., Hoffmann, J. A., and Bulet, P. (1999) *J. Biol. Chem.* **274**, 9320–9326
- Bulet, P., Dimarcq, J. L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A., and Hoffmann, J. A. (1993) *J. Biol. Chem.* **268**, 14893–14897
- Mosmann, T. (1983) *J. Immunol. Methods* **65**, 55–63
- Kiderlen, A. F., and Kaye, P. M. (1990) *J. Immunol. Methods* **127**, 11–18
- Fogaça, A. C., da Silva, P. I., Jr., Miranda, M. T., Bianchi, A. G., Miranda, A., Ribolla, P. E., and Daffre, S. (1999) *J. Biol. Chem.* **274**, 25330–25334
- Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J., and Bachere, E. (1997) *J. Biol. Chem.* **272**, 28398–28406
- Mitta, G., Vandenbulcke, F., Hubert, F., and Roch, P. (1999) *J. Cell Sci.* **112**, 4233–4244
- Kawano, K., Yoneya, T., Miyata, T., Yoshikawa, K., Tokunaga, F., Terada, Y., and Iwanaga, S. (1990) *J. Biol. Chem.* **265**, 15365–15367
- Tamamura, H., Kuroda, M., Masuda, M., Otaka, A., Funakoshi, H., Nakashima, N., Yamamoto, N., Waki, M., Matsumoto, J. M., Lancelin, D., Kohda, D., Tate, S., Inagaki, F., and Fujii, N. (1993) *Biochim. Biophys. Acta* **1163**, 209–216
- Aumelas, A., Mangoni, M., Roumestand, C., Chiche, L., Despau, E., Grassy, G., Calas, B., and Chavanieu, A. (1996) *Eur. J. Biochem.* **237**, 575–583
- Fahrner, R. L., Dieckmann, T., Harwig, S. S., Lehrer, R. I., Eisenberg, D., and Feigon, J. (1996) *Chem. Biol.* **3**, 543–550
- Mandard, N., Sy, D., Maufrais, C., Bonmatin, J., Bulet, P., Hetru, C., and Vovelle, F. (1999) *J. Biomol. Struct. Dyn.* **17**, 367–380
- Katsu, T., Nakao, S., and Iwanaga, S. (1993) *Biol. Pharm. Bull.* **16**, 178–181
- Steinberg, D. A., Hurst, M. A., Fujii, C. A., Kung, A. H., Ho, J. F., Cheng, F. C., Loury, D. J., and Fiddes, J. C. (1997) *Antimicrob. Agents Chemot.* **41**, 1738–1742
- Rao, A. G. (1999) *Arch. Biochem. Biophys.* **361**, 127–134
- Tamamura, H., Ikoma, R., Niwa, M., Funakoshi, S., Murakami, T., and Fujii, N. (1993) *Chem. Pharm. Bull.* **41**, 978–980
- Harwig, S. S. L., Waring, A., Yang, H. J., Cho, Y., Tan, L., and Lehrer, R. I. (1996) *Eur. J. Biochem.* **240**, 352–357
- Díaz-Achirica, P., Ubach, J., Guinea, A., Andreu, D., and Rivas, L. (1998) *Biochem. J.* **330**, 453–460
- Wassef, M. K., Fioretti, T. B., and Dwyer, D. M. (1985) *Lipids* **20**, 108–115
- Tam, J. P., Lu, Y.-A., and Yang, J.-L. (2000) *Biochem. Biophys. Res. Commun.* **267**, 783–790

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