Characterization of Novel Cysteine-rich Antimicrobial Peptides from Scorpion Blood*

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We have isolated, from the hemolymph of unchallenged scorpions of the species Androctonus australis, three distinct antimicrobial peptides, which we have fully characterized by Edman degradation, electrospray ionization mass spectrometry, and matrix-assisted laser desorption/ionization mass spectrometry. Two are novel molecules: (i) androctonin, a 25-residue peptide with two disulfide bridges, active against both bacteria (Gram-positive and Gram-negative) and fungi and showing marked sequence homology to tachypleins and polyphemusins from horseshoe crabs; and (ii) buthinin, a 34-residue antibacterial (Gram-positive and Gram-negative) peptide with three disulfide bridges. The third peptide contains 37 residues and three disulfide bridges and clearly belongs to the family of anti-Gram-positive insect defensins. We have synthesized androctonin and explored its activity spectrum and mode of action.

Within the phylum of the Arthropoda, scorpions (Chelicerata, Scorpionida) are the oldest known terrestrial species. Over 400 million years, they have developed an efficient system to subdue their prey by potent neurotoxic components produced in their venom glands. Scorpion toxins represent a family of small basic polypeptides that act on different types of ion channels and display various degrees of toxicity toward mammals, insects, or crustaceans. For example, the α-toxins from Buthinae venom prolong the Na+ activation phase of the action potential, while the β-toxins from Centrurinae and Tityridae venoms affect the Na+ activation phase (1). Both toxins consist of 60–70 amino acid residues and have four disulfide bridges. Other scorpion toxins with shorter polypeptide chains consist of 60–70 amino acid residues and have four disulfide bridges (2–4). Although the numerous known scorpion toxins differ in size, sequence, and biological activity, they all share a common structural motif consisting of an antiparallel β-sheet linked to an amphipathic α-helix and to an extended N-terminal fragment by three disulfide bridges (5, 6). This motif is also present in insect defensins, a family of inducible antibacterial peptides isolated from a variety of insect species, where they represent a key element of the innate host defense against microorganisms (7). Scorpions, like insects, are particularly resistant to bacterial aggressions, and it appeared of interest to analyze the molecules responsible for this resistance. In particular, we were interested to know whether molecules similar to insect defensins were involved in the scorpion defense against microorganisms. Previous studies from two of our laboratories had indeed shown the presence in the hemolymph of the scorpion Leirus quinquestratus of an antibacterial peptide highly similar to an insect defense isolated from the dragonfly Aeschna cyanea (8). We have now collected hemolymph from a breeding colony of the scorpion Androctonus australis both from untreated and bacteria-challenged individuals. We observed in both types of samples the presence of five distinct antibacterial compounds, three of which could be purified to homogeneity and their sequence established. One of the peptides is a novel member of defensins and shows 95% identity to the defense isolated from L. quinquestratus (8). A second peptide shows a high degree of similarity to the antimicrobial tachypleins and polyphemusins from the horseshoe crabs Tachypleus tridentatus (9) and Limulus polyphemus (10). This peptide has 25 residues and two disulfide bridges and is named androctonin, after the genus Androctonus. We have synthesized androctonin and analyzed its antimicrobial activity. The third peptide has a hitherto unreported sequence with no apparent similarity to other antibacterial peptides. It consists of 34 residues with three disulfide bridges and will be referred to as buthinin, after the family of Buthinae, to which belongs the scorpion Androctonus.

MATERIALS AND METHODS

Animals
Scorpions (Chelicerata, Buthinae, A. australis) were from the Muséum National d’Histoire Naturelle (Paris). From 20 unchallenged animals, 3.8 ml of hemolymph were collected and frozen at −30 °C until use. In parallel, 10 animals were individually injected with a mixture of Micrococcus luteus and Escherichia coli D31 bacteria (2 × 107 bacteria each). The hemolymph (1.9 ml) of these bacteria-challenged scorpions was collected 1 week after injection and centrifuged at 800 × g for 30 min at 10 °C, and the supernatant was frozen at −30 °C until use.

Purification of Antibacterial Peptides

Sep-Pak Preparative—After centrifugation (30,000 × g, 25 min, 4 °C), the cell-free hemolymph was diluted with 0.1% (v/v) trifluoroacetic acid and centrifuged as described above. The supernatant was loaded onto three serially linked Sep-Pak C18 cartridges (Waters Associates) equilibrated with acidified water (0.05% trifluoroacetic acid), and elution was performed with solutions of first 10%, then 40%, and finally 80% acetonitrile in acidified water. All fractions were concentrated in a vacuum centrifuge (Savant Instruments, Inc.) and reconstituted with MilliQ water. The presence of antibacterial activity was detected by liquid growth inhibition assay (test organisms: M. luteus and E. coli D31).

First Step of Purification—The active 40% Sep-Pak fraction was...
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Matrix-assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS)

Further purified by reverse-phase HPLC on an Aquapore OD300 column (220 × 4.6 mm; Brownlee Labs) equilibrated with 2% acetonitrile in acidified water (0.05% trifluoroacetic acid). Elution was performed with a linear gradient of 2–52% acetonitrile in acidified water over 90 min at a flow rate of 1 ml/min. The column was monitored by absorbance at 225 nm. Fractions were hand-collected, and the presence of antibacterial activity was monitored in 10 µl aliquots (equivalent to 260 µl of hemolymph) of each fraction of the chromatogram.

**Final Purification Steps**—Different methods were used for the final purification of peptides A–C: for peptide A, reverse-phase HPLC on a high-pressure inert Delta-Pak C18 column (150 × 3.9 mm; Waters Associates) equilibrated with 2% acetonitrile in 6 mM HCl and developed with a linear basic gradient of acetonitrile in 6 mM HCl from 2 to 11% over 10 min and from 11 to 21% over 50 min at a flow rate of 1.1 ml/min; for peptide B, a reverse-phase HPLC on an Aquapore OD300 column (220 × 4.6 mm) eluted with a linear high-pressure inert Delta-Pak C18 column equilibrated with 5% acetonitrile in acidified water (0.05% trifluoroacetic acid) and elution with a linear basic gradient of acetonitrile in acidified water from 5% to 17% over 10 min and from 17 to 27% over 50 min at 40 °C at a flow rate of 1 ml/min; and for peptide C, a reverse-phase HPLC on an Aquapore OD300 column (220 × 4.6 mm) eluted with a linear basic gradient of acetonitrile in 0.05% trifluoroacetic acid from 2 to 21% over 10 min and from 21 to 36% over 60 min at a flow rate of 1 ml/min. All HPLC purifications were carried out in a Waters HPLC system equipped with a tunable absorbance detector. An all-polyetheretherketone inert system was used for chromatography under HCl conditions.

**Enzymatic Digestions**

Endoproteinase Lys-C Treatment—Peptide A (2 nmol) was treated with endoproteinase Lys-C (Achromobacter protease I, Takara, Otsu, Japan). Digestion was carried out at 37 °C for 16 h under the conditions recommended by the manufacturer. The reaction was stopped by acidification, and the peptide fragments were separated on an Aquapore RP300 C8 column (220 × 4.6 mm; Brownlee Labs) developed with a linear gradient of 2–52% acetonitrile in acidified water (0.05% trifluoroacetic acid) over 120 min at a flow rate of 1 ml/min.

Endoproteinase Asp-N Treatment—Peptide B (80 pmol) was first reduced, alkylated with 4-vinylpyridine, and desalted by reversed-phase HPLC. The pyridylethylated peptide was then treated with endoproteinase Asp-N (Boehringer Mannheim) in 40 µl of 10 mM Tris-HCl (pH 8.0) at an enzyme ratio of 1:100 (w/w). Digestion was carried out at 37 °C for 16 h. The reaction was stopped by acidification, and the peptide fragments were separated as described above.

**Capillary Zone Electrophoresis**

Peptide purity was ascertained by capillary zone electrophoresis. Analysis was performed in a Model 270A-HT capillary electrophoresis system (Applied Biosystems Inc.) equipped with a fused silica capillary (72 cm length, 50-µm internal diameter). Separation was achieved from anode to cathode in 20 mM citrate buffer (pH 2.5) at a voltage of 20 kV. Detection was at 200 nm, the temperature was 30 °C, and the volume injected by vacuum was 2 nL.

**Electrospray Ionization Mass Spectrometry (ES-MS)**

The peptides were dissolved in water/acetonitrile (50:50, v/v) containing 1% formic acid and analyzed in the positive mode in a BioQ triple quadrupole mass spectrometer (Micromass UK Ltd., Altrincham, United Kingdom) at a mass to charge (m/z) range of 4000. The electrospray ionization interface was heated to 70 °C, and electrospray ionization mass spectra were obtained at a sampling cone voltage of 50 V. Sample solutions were introduced into the mass spectrometer source with a Harvard-type 55 1111 syringe pump (Harvard Apparatus Inc., South Natick, MA) at a flow rate of 4 µl/min. Calibration was performed using a linear growth inhibition of horse myoglobin. Scanning was usually performed in the multichannel acquisition mode from m/z 500 to 1500 or m/z 300 to 1300 in 10 s.

1 The abbreviations used are: HPLC, high-performance liquid chromatography; ES-MS, electrospray ionization mass spectrometry; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; ACTH, adrenocorticotropic hormone; FMoc, N-(9-fluorenyl)methoxycarbonyl; MIC, minimal inhibitory concentration.

Purified peptides or enzymatically derived fragments (1 µl) were deposited on a thin layer of a-cyano-4-hydroxycinnamic acid crystals made by fast evaporation of a saturated solution in acetonitrile. The droplet was allowed to dry under gentle vacuum before introduction into the mass spectrometer. MALDI-MS measurement was performed in a Bruker BIFLEXTM time-of-flight mass spectrometer operating in a positive linear mode. Ions were formed by a pulse ultraviolet laser beam (nitrogen laser, λ = 337 nm). Mass spectra were obtained by averaging 50–100 laser shots. External mass calibration was provided by the [M + H]+ ion of angiotensin II (1047.20 Da), ACTH (18–39) (2466.73 Da), and bovine insulin (5734.56 Da) and/or using matrix peaks ([M + H]+: 190.05 Da; [2M + H]+: 379.09 Da).

**Microsequence Analysis**

Automated Edman degradation of native pyridylethylated peptides and peptide fragments and detection of phenylthiohydantoin-derivatives were performed on a pulse liquid automatic sequenator (Applied Biosystems Model 473A).

**Peptide Synthesis**

The peptide was synthesized according to classical Fmoc chemistry on p-sulphonybenzyl alcohol resin on a 25-µmol scale. Automatic peptide assembly and trifluoroacetic acid cleavage protocols have been described in detail (11). Conventional side chain-protecting groups were used 2,5,5,7,8-Pentamethylchroman 6-sulfonyl (Arg), triphenylmethyl (Cys, Asn, and Gln), N,N,N'-triethylenemine (Lys), and t-butyl (Ser and Tyr). Briefly, a standard Fmoc deprotection protocol was used in conjunction with benzotriazol-1-y1-oxys tri(dimethyl氨基)phosphonium hexafluorophosphate/N-hydroxybenzotriazole/diisopropylthylamine. Coupling reactions were allowed to progress for 15 min. After two dimethylformamide washings, a second coupling with the same excesses of reagents was routinely performed. At the end of the synthesis, the resin was washed with dichloromethane and ether and dried under nitrogen. The final trifluoroacetic acid cleavage was performed in the same reaction vessel with 5 ml of the reagent described by King et al. (12) for 150 min. At the end, the peptide was drained in a 40-ml polypropylene centrifuge tube previously filled with 25 ml of cold ether. The peptide was then centrifuged, and the pellet was washed twice with ether. After the last centrifugation, the pellet containing the reduced peptide form was taken up in 0.1 M ammonium acetate buffer (pH 8.5) at a concentration of 35 mg/liter and was allowed to refold by air oxidation for 17 h at room temperature under stirring. The refolded peptide was purified by semipreparative reverse-phase chromatography (Aquapore RP300 column, 250 × 7.0 mm) with a linear gradient of 6–14% acetonitrile in 0.05% trifluoroacetic acid over 40 min at a flow rate 1.5 ml/min.

**Microorganisms**—The microbial strains were those used in previous studies (13, 14), with the addition of Clavibacter michiganensis, Pseudomonas syringae pv. syringae, P. syringae, P. syringae phaseoli, Pseudomonas maculicola, Pseudomonas pisi, Pseudomonas valerianella, Xanthomonas campestris pv. campestris, Xanthomonas vesicatoria 687.3, X. vesicatoria B229RI, Alternaria dauci, Fusarium oxysporum M, F. oxysporum L, Verticillium toxicot, Botrytis petunia, and Sclerophyllum (provided by Dr. Guernard, Clause, Paris). Aspergillus fumigatus was a gift from Dr. Koenig (Hôpital Civil, Strasbourg, France).

**Antibacterial Assays**—Antibacterial activity was monitored during the different purification steps by a liquid growth inhibition assay described in (15). Briefly, 100 µl of a suspension of a mid-logarithmic phase culture of bacteria (M. luteus or E. coli D31) at a starting A600 = 0.001 in Poor Broth nutrient medium (1% Bacto-Tryptone and 0.5% (w/v) NaCl) were added to a sodium chloride solution. The bacterial growth was assessed by an increase in A600, after incubation (24 h, 25 °C). The procedure was identical for the determination of the minimal inhibitory concentrations (MICs) of androctonin and of the control antibacterial peptides MSI-94 (a broad-spectrum linear amphipathic magainin) and PGLa (a naturally occurring antibiotic peptide from frog), which were gifts from Dr. M. A. Zasloff (Magainin Scientific Institute, Plymouth Meeting, Philadelphia). MICs are expressed as the interval of concentrations [a] – [b], where [a] is the highest concentration tested at which the bacteria are growing and [b] is the lowest concentration that causes 100% of growth inhibition. The concentrations tested for androctonin, MSI-94, and PGLa were in the range of 0.03–30, 0.028–36, and 0.036–46 µg/ml, respectively.
**RESULTS**

**Isolation of Antibacterial Peptides from the Hemolymph of A. australis**

We have addressed in this study the scorpion species *A. australis* and first collected from unchallenged scorpions (20 individuals) 3.8 ml of hemolymph. After centrifugation, the cell-free hemolymph was purified on Sep-Pak C18 cartridges, and after elution with various concentrations of acetonitrile in acidified water (0.05% trifluoroacetic acid), the bulk of the antibacterial activity was recovered in the 40% acetonitrile fraction. This fraction was further analyzed on a reversed-phase HPLC column using a linear gradient of acetonitrile (Fig. 1). Of all the fractions of the chromatogram, only five corresponding to distinct peaks (A–E) eluting between 17 and 30.5% acetonitrile showed antibacterial activity against *M. luteus* and *E. coli*.

When scorpions were injected with either live or heat-killed bacteria 1 week before collection of the hemolymph, the purification of antibacterial molecules yielded results similar to those obtained with untreated animals. There was no indication that bacterial challenge had induced the appearance of additional antimicrobial substances or increased the amount of the molecules present in untreated animals.

**Purification and Primary Structure Determination of Three Antibacterial Peptides**

The antibacterial compounds present in peaks A–C (Fig. 1) were purified to homogeneity by the following multistep procedure.

**Compound A: Androctonin**—Peak A was subjected to reversed-phase chromatography and eluted with a linear biphasic gradient of acetonitrile under HCl conditions (Fig. 2). A pure antibacterial compound was obtained as judged by capillary zone electrophoresis (data not shown). ES-MS gave a single molecular mass of 3076.7 Da, confirming its purity (Fig. 2, inset). Compound A (25 pmol) was submitted to Edman degradation, which gave the following 25-residue sequence: RSVQRKXRRGGXYYKXYKTNRPY, with four blanks at positions 4, 10, 16, and 20. The attribution of the identified residues was unambiguous, and we postulated that the blanks correspond to cysteine residues. This was in fact corroborated by the molecular mass determination, which gave a value (ES-MS: 3076.7 Da) identical to the calculated molecular mass (3076.7 Da), assuming that the blanks correspond to four cysteine engaged in two disulfide bridges. The sequence of this 25-residue peptide (Fig. 3) appeared to be novel and was named androctonin after the scorpion *A. australis*. Androctonin has a calculated pI of 10.2.

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II) were obtained (Fig. 4), which supports the hypothesis of the linkages Cys-4–Cys-20 and Cys-10–Cys-16. In addition, the molecular masses measured by ES-MS for these fragments (fragment I: 1372.8 Da; and fragment II: 1740.0 Da) were in perfect agreement with the calculated masses of the predicted sequences (fragment I: 1372.6 Da; and fragment II: 1740.0 Da).

Compound B: Buthinin—A second antibacterial compound (Fig. 1, peak B) was purified by reversed-phase HPLC using a linear biphasic gradient of acetonitrile in 6 mM HCl. A single peak on the capillary electrophoretogram and a single molecular mass of 3968.5 Da obtained by ES-MS (data not shown) ascertained the purity of the compound. Edman degradation yielded the following N-terminal sequence of 31 residues: SIVPIRCRSNRDCRRFCGFRGRCYTARQCLCGY. No phenylthiohydantoin signal was observed in positions 7, 13, 17, 24, and 30. The mass calculated for the 31-residue peptide, assuming that the five blanks correspond to cysteines, was, however, lower than the measured molecular mass (see above), indicating that the sequence was not complete. To confirm the presence of the cysteines and to determine the C-terminal sequence, we performed reduction of the disulfide bridges of the native peptide and alkylation of thiols with 4-vinylpyridine. The measurement of the molecular mass of the purified pyridylethylated peptide (ES-MS: 4605.0 Da) exceeded that of the native peptide by 636.5 Da, which is attributable to the addition of six pyridylethyl groups (6 × 106 Da) on six oxidized cysteines. We next cleaved the pyridylethylated peptide with endoproteinase Asp-N and obtained two fragments, which were separated by reversed-phase HPLC (data not shown). The molecular masses measured by MALDI-MS for these two fragments were 1404.7 and 3217.2 Da, respectively. The more hydrophobic fragment, submitted to Edman degradation, gave the following sequence: DC*RRFC*GFRGRC*TYARQC*LC*GY, where C* stands for a pyridylethylated cysteine. The calculated molecular mass for this sequence is 3217.8 Da, which is in excellent agreement with the measured molecular mass (see above). The other fragment was identified by MALDI-MS and Edman degradation as the N-terminal peptide SIVPIRC*ESNR. Taken together, these results indicate that compound B is a 34-residue peptide (Fig. 3) with six cysteines engaged in the formation of three intramolecular disulfide bridges. Analysis of data banks showed that...
this sequence is novel, and the peptide was named buthinin. Buthinin has a calculated pI of 10.3.

**Androctonus Defensin**—Three related compounds were isolated from peak C (Fig. 1) after two reversed-phase HPLC steps under trifluoroacetic acid conditions. The major form was submitted to Edman degradation, yielding the following sequence: XRXRSIRRRGGYGYKLKQTXTXY, with six blanks at positions 4, 11, 15, 25, 33, and 35. It is apparent that this sequence represents a new member of the insect defensin family. Assuming that the blanks correspond to cysteines, this *Androctonus* defensin presents only two differences from the defensin of the scorpion *L. quinquestriatus* at positions 6 and 28, where a phenylalanine and a leucine are interconverted (Fig. 3). With the hypothesis that the cysteines are engaged in three intramolecular disulfide bridges, the calculated molecular mass for this sequence is 4205.8 Da, which is close to the molecular mass measured by MALDI-MS (4206.8 Da). The two other peptides exhibited similar chromatographic behaviors and had close molecular masses (MALDI-MS: 4050.7 and 4334.3 Da). We hypothesize that the 4050.7-Da peptide corresponds to a truncated form of *Androctonus* defensin lacking the C-terminal arginine residue and that, in the 4334.3-Da peptide, this arginine residue is followed by a lysine or a glutamine residue. In other words, all three isoforms could be derived from a unique peptide by cleavage of one or two C-terminal residues of the 4334.3-Da *Androctonus* defensin.

**Compounds D and E**—The antibacterial compounds present in peaks D and E were purified to apparent homogeneity by procedures similar to those used above. The purified compounds gave a single peak by capillary electrophoresis. However, in ES-MS, each peak yielded two molecular masses, indicating that they were not pure. Because of lack of sufficient biological material, further purification steps could not be undertaken.

### Chemical Synthesis of Androctonin

To further investigate the mode of action and the activity spectrum of androctonin, we synthesized, renatured, and purified to homogeneity 16 mg of this 25-residue peptide (yield: 21%). The identity of the synthetic refolded peptide was confirmed by Edman degradation and ES-MS, and by the use of endoproteinase Lys-C, the disulfide array was shown to be identical to that of the native peptide (i.e. Cys-4–Cys-20 and Cys-10–Cys-16).

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**Activity Spectrum of Androctonin against Various Microbial Strains (Table I)**

In the liquid growth inhibition assay, androctonin had a marked activity (MIC < 3 µM) against the Gram-positive bacteria *Aerococcus viridans*, *M. luteus*, and *Bacillus subtilis*. It was moderately active against *Staphylococcus aureus* and *C. michiganensis* (MIC < 30 µM) and inactive against *Bacillus cereus* and *Bacillus thuringiensis up to a concentration of 30 µM*. Among the Gram-negative bacteria that were tested, the most sensitive strains were the phytopathogenic *P. syringae*, *P. syringae phaseoli*, and *V. xesicatoria B229RI*. In general, the two control antibacterial peptides used

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### Table I

**Antimicrobial activity spectrum of androctonin**

| Gram-positive bacteria | Androctonin | MSI-94 | PGLa |
|-----------------------|------------|-------|-----|
| *M. luteus* | 0.6–1.5 | 0.45–0.9 | 0.3–0.6 |
| *A. viridans* | 0.3–0.6 | 0.45–0.9 | 0.6–1.15 |
| *B. cereus* | ND | ND | ND |
| *B. subtilis* | 1.5–3.0 | 0.9–1.8 | 0.6–1.15 |
| *B. thuringiensis* | ND | 1.8–3.6 | 4.6–46 |
| *S. aureus* | 15–30 | 0.9–1.8 | 1.15–2.3 |
| *C. michiganensis* | 6–15 | 0.2–0.45 | 1.15–2.3 |

**Gram-negative bacteria**

| *E. coli D31* | 3–6 | 0.45–0.9 | 1.15–2.3 |
| *E. coli D22* | >30 | 0.9–1.8 | 1.15–2.3 |
| *E. coli 1106* | 6–15 | 0.9–1.8 | 0.6–1.15 |
| *E. cloacae β12* | ND | 0.9–1.8 | 4.6–46 |
| *E. carotovora carotovora* | ND | 0.9–1.8 | 2.3–4.6 |
| *S. typhimurium* | 3–6 | 0.9–1.8 | 4.6–46 |
| *K. pneumoniae* | ND | 1.8–3.6 | 4.6–46 |
| *P. cepacia* | ND | 0.2–0.45 | 1.15–2.3 |
| *P. syringae* | 1.5–3.0 | 0.2–0.45 | 1.15–2.3 |
| *P. syringae pv. syringae* | 15–22 | 0.45–0.9 | 1.15–2.3 |
| *P. syringae phaseoli* | 1.5–3.0 | 0.2–0.45 | 1.15–2.3 |
| *P. pisi* | 6–15 | 0.2–0.45 | 0.6–1.15 |
| *P. maculicola* | 3–6 | ND | ND |
| *P. valerianella* | 15–22 | 0.9–1.8 | ND |
| *X. campestris pv. oryzae* | ND | 0.9–1.8 | 4.6–46 |
| *X. campestris pv. campestris* | 3–6 | 0.2–0.45 | 0.3–0.6 |
| *X. vesicatoria 687.3* | 1.5–3.0 | 0.45–0.9 | 0.6–1.15 |
| *X. vesicatoria B229RI* | 1.5–3.0 | 0.2–0.45 | 0.6–1.15 |

**Fungi**

| *A. dauci* | 8–16 |
| *A. brassicola* | 3–6 |
| *Stemphylium* | 4–8 |
| *F. culmorum* | 3–6 |
| *F. oxysporum* | 6–12 |
| *F. oxysporum M.* | 2–4 |
| *F. oxysporum L.* | 2–4 |
| *N. crassa* | 6–12 |
| *B. cinerea* | 6–12 |
| *B. petunia* | 4–8 |
| *N. haematococca* | 6–12 |
| *T. viride* | 6–12 |
| *V. tircis* | 2–4 |
| *A. fumigatus* | 25–50 |

*ND, not detected in the range of dilutions tested.*
in this study, MSI-94 and PGLa, were more active against the various strains tested, with the exception of A. viridans.

Androctonin was also found to be active against fungi. The level of activity was high (MIC < 4 μM) against V. torellis and F. oxysporum M. and L. It was somewhat lower against Alternaria dauci, A. brassicola, Stemphylium, Fusarium culmorum, F. oxysporum, N. crassa, Botrytis cinerea, B. petunia, Nectria haematococa, and Trichoderma viride (MIC < 16 μM). A. furmigatus had a low sensitivity (MIC = 25–50 μM). When compared with thanatin (14) and drosomycin (16), two antifungal peptides isolated from Podisus maculiventris and Drosophila melanogaster, respectively, androctonin showed a similar level of activity on A. brassicola and F. culmorum, but a lower activity on the other strains.

**Bactericidal Effect of Androctonin**

Androctonin was incubated for various times with the test organisms M. luteus and E. coli D31 at concentrations 10 times higher than the respective MICs (0.6–1.5 μM for M. luteus and 3–6 μM for E. coli D31). As shown in Table II, androctonin was found to kill 80% of growing cells of M. luteus after a 4-min incubation, while the bactericidal effect was slower (>2 h) on E. coli D31 (Table III). No colony-forming unit was detected after incubations of 10 min with M. luteus and 24 h with E. coli D31.

**Fungicidal Effect of Androctonin**

Spores of N. crassa were incubated in the presence of various concentrations of androctonin (0.03–30 μM). After 48 h, the medium containing the peptide was removed and replaced with fresh medium. At androctonin concentrations of 12 μM and higher, no growth recovery occurred after 48 h, indicating that androctonin is fungicidal at this concentration.

**Hemolytic Activity of Androctonin**

When assayed under conventional conditions, androctonin at a concentration of up to 150 μM did not exhibit hemolytic activity on porcine or bovine erythrocytes.

**DISCUSSION**

We report here the isolation of five antimicrobial molecules from the cell-free hemolymph of unchallenged A. australis scorpions (Chelicerata, Scorpioniidae). Three peptides (A–C) were purified to homogeneity and fully characterized at the level of their amino acid sequence using Edman degradation, ES-MS, and MALDI-MS. Comparison of the cell-free hemolymph from bacteria-challenged and control scorpions indicates that all antibacterial molecules are constitutively present and neither induced nor up-regulated by this immune challenge.

Peptide B, which was named buthinin, is active against M. luteus and E. coli. It is a 34-residue peptide with six cysteines engaged in three intramolecular disulfide bridges and has no clear-cut sequence similarity to any antimicrobial peptide reported so far (17). However, buthinin contains the consensus motif Cys-(Xaa)-Cys-Xaa-Xaa-Cys-(Xaa)-Cys-Xaa-Cys-(Xaa)-Cys-Xaa-Cys, which is present in insect defensins and scorpion toxins (5, 6). In addition, comparison of the sequence of buthinin with that of various scorpion toxins shows interesting similarities to short chain toxins (Fig. 5). In particular, buthinin has 44–50% sequence similarity (including conservative replacements) to agitoxins and kaliotoxins, which are blockers of the voltage-dependent K⁺ channels (5, 18, 19). It has 35–40% sequence similarity to iberiotoxin and charybotoxin, which are blockers of the voltage-dependent Ca²⁺ channels (3, 20, 21). Finally, it exhibits 33% similarity to leuriotoxin and PO₄, which block the amdin-sensitive Ca²⁺-activated K⁺ channels (22–24). Interestingly, the positively charged residue N-terminal to the fourth cysteine (Arg in buthinin and Lys in scorpion toxins), which is thought to interact directly with K⁺ in the ion conduction pore (25), is preserved in all the molecules.

Peptide C was identified as a new member of the insect defensin family. Androctonus defensin is almost identical to Leirius defensin, with a single conservative replacement of two residues (Phe and Leu) at positions 6 and 28 (Fig. 3). As already discussed, these scorpion defensins have similarities to insect defensins, but are closer to the defensin of Aeschna, an insect belonging to the ancient order of Odonata (8, 26). Taken together, these results call for several remarks. 1) As already observed for Leirius, scorpion defensins are constitutively present in the hemolymph, and their concentration does not increase after bacterial challenge, in contrast to insect defensins. 2) As opposed to the situation observed in Leirius, Androctonus defensin is not the only antimicrobial peptide present in the blood of this scorpion, which contains at least four additional antimicrobial molecules. 3) Scorpion defensins, like insect defensins, seem to be a common element in the antibacterial defense of arthropods.

In addition to buthinin and Androctonus defensin, we have also isolated and fully characterized a novel 25-residue antibacterial peptide, named androctonin (peptide A). This peptide has four cysteines engaged in two intramolecular disulfide bridges, with Cys-4 linked to Cys-20 and Cys-10 linked to Cys-16. Androctonin is highly cationic (with a calculated pI of 10.2) and contains eight positive charges, including a cluster of three Arg residues linked to two Gly residues in the central part of the molecule. Interestingly, this motif is also observed in both Leirius and Androctonus defensins. Androctonin has no sequence similarity to other insect defense molecules. However, it shows a high degree of similarity to a family of cationic antimicrobial peptides from other arthropods (Meropeptin)
such as tachyplesins (27) from T. tridentatus (Japanese horseshoe crabs) and polyphemusins (28) from L. polyphemus (American horseshoe crab). These peptides were isolated from hemocyte lysates and consist of 17–18 residues with four cysteines engaged in two intramolecular disulfide bridges. The overall sequence similarity, including conservative replacements, to androctonin is close to 65% with tachyplesin I and to 60% with polyphemusin I (Fig. 4). In contrast to tachyplesins and polyphemusins, which possess a C-terminal arginine α-amide following the most C-terminal cysteine residue, androctonin presents a free C-terminal extension of five residues. In addition, the disulfide array (Cys–1–Cys–4 and Cys–2–Cys–3), which is responsible for the fairly rigid conformation of tachyplesin I (29), is identical in androctonin and the tachyplesin family, suggesting a similar three-dimensional structure.

Tachyplesins and their analogues, polyphemusins, are strongly active against both Gram-positive and Gram-negative bacteria (MIC = 0.35–2.8 μM) and also against the two fungi Candida albicans M9 and Cryptococcus neoformans IMF40040 (MIC = 0.7–2.6 μM) (28, 30). Moreover, at a higher concentration (50–100 μM), tachyplesin I is hemolytic on human erythrocytes (31). For these peptides, androctonin was found to have an activity against both Gram-positive and Gram-negative bacteria (20 out of the 27 bacterial strains tested were susceptible). In particular, a large variety of phytopathogenic bacterial strains were affected by androctonin, namely A. tumefaciens, E. coli, and S. aureus (20 out of the 27 bacterial strains tested were susceptible). The availability of large amounts of androctonin obtained by chemical synthesis will warrant a detailed analysis of its mode of action on M. luteus and E. coli.

In addition to the sequence similarity to tachyplesins and polyphemusins, androctonin was also found to have some structural similarities to α-conotoxin SII (32), a small peptide isolated from the venom of the marine mollusc Conus striatus, which is a blocker of the nicotinic acetylcholine receptor (32). In the family of α-conotoxins, this 19-residue peptide has the peculiarity of possessing six cysteines (instead of four for other α-conotoxins) and an additional disulfide bridge between the first and the sixth cysteine (33). Thus, as compared with α-conotoxin SII, androctonin has the same pattern of cysteines (i.e., the same number of residues between the four common cysteines), and one of the disulfide bridges is in a similar position. These similarities prompted us to investigate whether androctonin and α-conotoxin SII have similar binding properties. While α-conotoxin SII was reported to have an IC50 of 8 μM for the Torpedo nicotinic acetylcholine receptor (32), androctonin was found to bind to this receptor with a comparable affinity. 2

In conclusion, this report demonstrates the presence, in the hemolymph of naïve A. australis scorpions, of several molecules that may serve as elements in the defense of this arthropod against invading microbes. Of special interest is the identification of the tachyplesin-related androctonin, which shows a remarkably large spectrum of antimicrobial activities directed against both bacteria and fungi. The successful chemical synthesis of this peptide paves the way to future studies on the potential use of this molecule in therapy and agronomy. Finally, our results underline several structural similarities between antimicrobial peptides and toxins in scorpions and raise the tantalizing question of whether these peptides have evolved from a common ancestral molecule.

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