# Scorpine, an anti-malaria and anti-bacterial agent purified from scorpion venom

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Received 7 March 2000

Edited by Maurice Montal

**Abstract A novel peptide, scorpine, was isolated from the venom of the scorpion *Pandinus imperator*, with anti-bacterial activity and a potent inhibitory effect on the ookinete ($ED_{50}$ 0.7 μM) and gamete ($ED_{50}$ 10 μM) stages of *Plasmodium berghei* development. It has 75 amino acids, three disulfide bridges with a molecular mass of 8350 Da. Scorpine has a unique amino acid sequence, similar only to some cecropins in its N-terminal segment and to some defensins in its C-terminal region. Its gene was cloned from a cDNA library.**



*Key words:* Antibiotic; Malaria; Scorpine; *Pandinus imperator*; *Plasmodium*

## 1. Introduction

Scorpion venoms are rich sources of peptides with a variety of pharmacological functions, specially those that affect membrane permeability for $Na^+$, $K^+$, $Ca^{2+}$ and $Cl^-$ of excitable and non-excitable cells (review [1]). They have been excellent tools to study ion-channel structure and function [2]. However, these venoms contain other interesting peptides, such as phospholipin [3] and imperatoxin-I [4], both with phospholipase activity, and the anti-microbial and anti-malaria peptide described here. Other peptides that could be effective in the control of malaria, a parasitic disease caused by *Plasmodium spp* and transmitted to vertebrates by mosquitoes, were also investigated lately [5,6]. The long range aim of the work is to incorporate into mosquitoes the genes encoding the bioactive peptides to produce transgenic vectors resistant to malaria [7]. Among the peptides isolated from the venom of the African scorpion *Pandinus imperator*, we identified a molecule, named scorpine. Due to the hybrid similarity of this peptide with the amino acid sequence of known cecropins and defensins [8], the idea came of assaying its effect on microbial growth and *Plasmodium berghei* stages that develop in mosquitoes. The description of the structure and biological function of scorpine is the object of this communication.

## 2. Materials and methods

### 2.1. Chemicals and purification procedure

All chemicals were analytical grade reagents, obtained from sources already described [3,4]. The venom of the scorpion was obtained in the laboratory by electrical stimulation, and prepared for chromatographic separations as previously reported [4]. The soluble venom applied to a Sephadex G-50 column (0.9×190 cm) in 20 mM ammonium acetate buffer, pH 4.7, resolved five well-defined fractions, from which fraction number II was subsequently applied to a carboxymethyl-cellulose (CM-cellulose) column, as described earlier for the isolation of imperatoxin-I [3] and phospholipin [4]. Scorpine was finally obtained in homogeneous form by high performance liquid chromatography (HPLC) separation, using a C18 reverse-phase column (Vydac, Hisperia, CA, USA).

### 2.2. Amino acid sequence

Scorpine was sequenced either as a native peptide or after reduction and alkylation, using the method described [3,4]. An aliquot (100 μg) of alkylated peptide was cleaved with endopeptidase AspN and separated by HPLC (data not shown), following the conditions described elsewhere [4]. Each independent sub-peptide was placed into the sequencer for obtaining overlapping amino acid sequences. Microsequence determination was performed on a 6400/6600 Milligen/Biosearch prosequencer, using the peptide adsorbed protocol on CD Immobilon membranes [3,4].

### 2.3. Mass spectrometry determination

The molecular weight of pure scorpine was determined by mass spectrometry, using a Kratos Kompact MALDI 3 V 3.0.2, apparatus.

### 2.4. Cloning and sequencing

Isolation of RNA and preparation of a cDNA library was performed as described [9]. The screening of the library was performed using a degenerated oligonucleotide (ATG GCN AAY ATG GAY ATG CT, where N means any nucleotide and Y means T or C), which encodes for the amino acid sequence of residues 40 to 46 (Met-Ala-Asn-Met-Asp-Met-Leu) of scorpine, synthesized as previously described [6]. The clone detected with this oligonucleotide was amplified by polymerase chain reaction (PCR) using (lambda) gt11 forward and reverse primers. PCR products were subcloned into pBluescript (pKS) phagemid and sequenced using the Sequenase® kit V. 2.0. (US Biochemical corp.). Oligo M13-20 and M13 reverse were used for sequencing [9].

### 2.5. Anti-bacterial assays

The classical method of Fleming was used for testing the anti-bacterial activity of scorpine. *Escherichia coli* ATCC 25922, *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Klebsiella pneumoniae*, were initially assayed. Inhibition zones were recorded around wells in thin agar plates with bacteria, as described by Hultmark et al. [15].

### 2.6. Parasites

The gametocyte-producing clone of *P. berghei* Anka strain 2.34 (kindly provided by R.S. Sinden, Imperial College of Science Technology and Medicine, UK) was used. Parasites were maintained in BALB/c mice by mechanical passage. Mice, with parasitemias ranging between 50 and 60% and gametocytemia around 10% were bled by heart puncture using heparin (100 IU/ml blood). This usually occurred after 8–10 days post-infection.

### 2.7. Susceptibility of P. berghei sexual stages to scorpine

Ookinete cultures were carried out as described [5]. Leucocyte-de-

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PII: S0014-5793(00)01384-3

pleted infected-mouse blood was suspended 1:5 in culture medium and tested in 100 μl aliquots in flat-bottom 96-well plates. Scorpine at concentrations of 0.049 to 100 μM was added to triplicate wells and the number of rosettes, indicative of active fecundation, was assessed by bright field microscopy between 10 and 30 min. later. The number of ookinetes were assessed 24 h later in Giemsa-stained blood smears.

## 3. Results and discussion

Fig. 1 shows the profile of CM-Cellulose separation of fraction 5, from the soluble venom of *P. imperator* after gel filtration on Sephadex G-50 [4]. This chromatogram shows a better resolution of that earlier reported [4], because it was applied in a much greater amount of protein and separated with a slightly different gradient. The component eluting at about 0.35 M NaCl (labeled with an asterisk) was further applied to a C18 reverse phase column providing homogeneous scorpine (marked with an asterisk in the inset-figure). Small contaminants with weak phospholipase activity were eliminated by this HPLC step. Scorpine purified by this procedure corresponds to approximately 1.4% of the total venom.

Direct automatic Edman degradation of native scorpine permitted the identification of the first 38 amino acid residues of the N-terminal segment, whereas a sample of scorpine reduced and carboxymethylated, permitted the unequivocal assignment of the first 47 amino acid residues of the primary structure, as shown in Fig. 2A. Two additional sub-peptides obtained by HPLC, after digestion with AspN endopeptidase (data not shown), allowed the identification of the C-terminal part of the molecule (residues in position 44–75), as well as an extended overlapping region of scorpine (residues from positions 14 to 43). Additional segments with excellent overlapping positions were also obtained, but not included in Fig. 2A, for simplification of the picture. The completion of the sequence was confirmed by the results of molecular weight determination using mass spectrometry analysis. The molecular mass of scorpine, 8350 is compatible both with the direct amino acid sequence obtained from the sequencer, and that deduced from the cloned gene. Fig. 2B shows the nucleotide sequence of a gene cloned using the internal oligonucleotide for hybridization and the (lambda) gt11 forward and reverse primers for amplification. A clone containing 487 nucleotides was sequenced. The internal segment contains the information (indicated by capital letters) that encodes the amino acids in position 1–75 of mature scorpine, ending in the stop codon at position 76. Two additional stretches of nucleotides were also found, one situated at the 5′ region, where a putative signal peptide containing the information for 19 amino acids is shown, and another at the 3′ untranslated region, where a putative adenylation site was identified (see lower case letters in bold, in Fig. 2B).

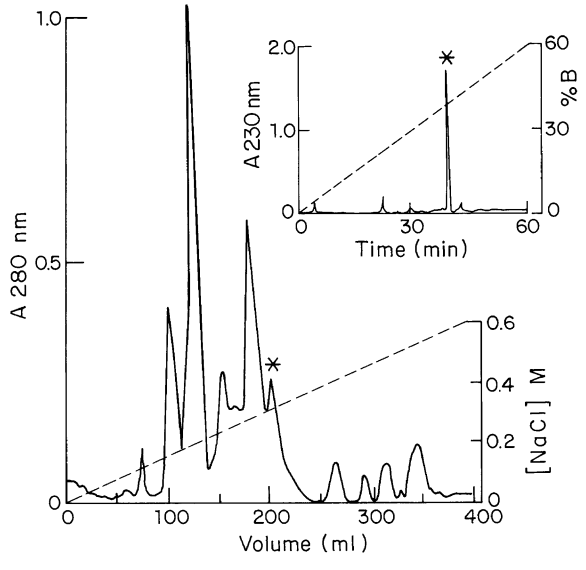


Fig. 1. Purification of scorpine. Fraction II (63 mg) of the venom from *P. imperator* separated by Sephadex G-50 gel filtration (see figure 1 in [4]) was loaded into a CM-cellulose chromatographic column (0.9×30 cm) dissolved in 20 mM ammonium acetate buffer, pH 4.7. The column was eluted with a linear gradient of sodium chloride, from 0.0 to 0.6 M salt. The fraction labeled with an asterisk contained anti-bacterial activity, and some residual phospholipase activity and was further purified by HPLC in a C18 reverse-phase column, as shown in the inset. Scorpine corresponds to the major component, labeled with an asterisk. A linear gradient was used from solvent A (0.12% trifluoroacetic acid in water) to 60% B (0.10% trifluoroacetic acid in acetonitrile).

When the amino acid sequence of scorpine was compared with other known peptides, using data banks (SwissProt and FASTA program), none of them showed substantial similarities to that of scorpine, except for a small stretch of the C-terminal segment, that contains three-disulfide bridges like those of defensins [10,11]. Also, the N-terminal region was similar to cecropins [12–14]. Fig. 3C shows the comparison of the primary structure of scorpine with these peptides (only representative examples were chosen for this figure). It is clear that scorpine is a unique peptide, much longer than other anti-bacterial peptides (practically double size). It seems to be a hybrid of cecropin and defensin, showing some identical amino acids (labeled with an asterisk in Fig. 2C) in certain stretches of the sequence, when appropriate gaps are manually introduced in order to increase similarities.

Due to the fact that the N-terminal segment of scorpine showed similarity to cecropins, the first idea was to test its effect against bacteria.

The method described by Hultmark et al. [15] was used to access the effect of scorpine on the inhibition of bacterial growth. Clear inhibition was obtained for *B. subtilis* (Minimum inhibitory concentration (MIC) around 1.0 μM) and for *K. pneumoniae* (MIC of approximately 10 μM), respectively.

The biological function of scorpine in *P. imperatur* awaits investigation. It is possible that it may function as microbicide within the venom gland which is open to the exterior and in contact with the hemocele of the scorpion prey during the sting.

Transmission of malaria parasites by mosquito vectors is dependent on the successful development of *Plasmodium* infective forms following ingestion of a blood-meal infected with gametocytes. This process is complex and includes a series of morphological and physiological transformations within the mosquito midgut (gametogenesis, fecundation, and ookinete formation) [16]. We previously observed in in vitro and in vivo tests that 100 μM Shiva-3, a synthetic cecropin, inhibited *P. berghei* ookinetes development [5–7]. The similarity of scorpine to cecropin prompted us to investigate its effect on the same *P. berghei* sexual stages. Our results showed that scorpine completely inhibited both fecundation and ookinete formation at 50 and 3 μM, respectively (Fig. 3), and that it had a toxic effect on gametes and on ookinetes at

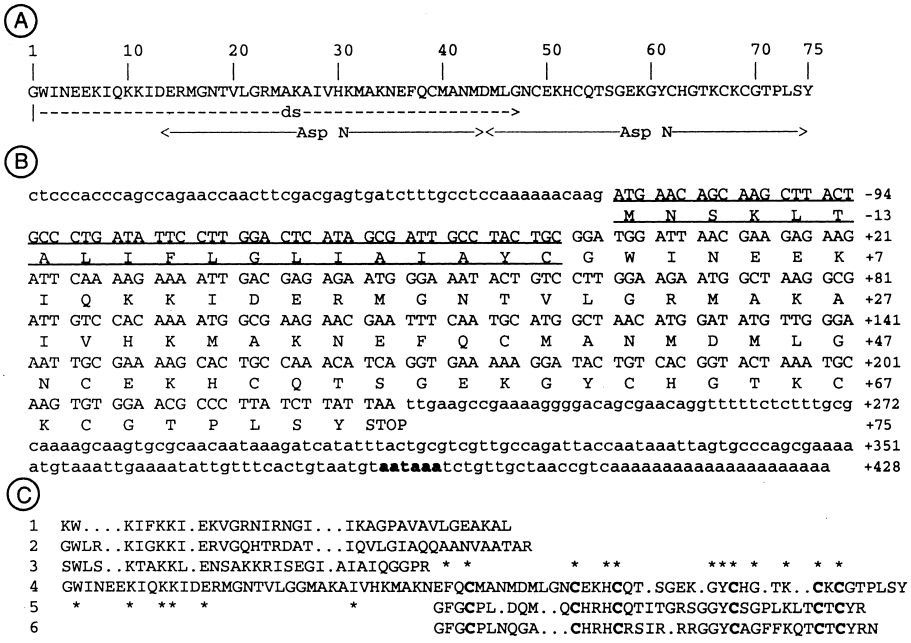


Fig. 2. Amino acid and nucleotide sequence of scorpine. A: Primary structure of scorpine as determined by direct sequencing the native peptides (underlined DS) and fragments produced by enzymatic hydrolysis with endopeptidase AspN (labeled ASP-N). B: Nucleotide sequence written from the 5′ to the 3′ end of the clone that encodes scorpine. The sequence corresponding to the putative signal is underlined. The mature peptide is indicated from residues 1–75, ending with the stop codon. The lower case letter in bold at the 3′ region indicates the possible poly-adenylation site, whereas lower case letters at the extreme 5′ and 3′ ends the non-coding region of the gene. Numbers on the right side of the page correspond to the positions of nucleotides from the signal peptide to the stop codon (upper lines) and to their corresponding amino acids (lower lines). C: Amino acid sequence comparison of scorpine with that of cecropin and defensin antibiotic peptides. The sequences were aligned with extended blank spaces, because of the difference in size of the peptides and lack of extended similarities. The segments with some consensus sequences are shown pairwise with artificial blank spaces (small dots, to enhance similarity) and whenever an identical residue is situated in equivalent position, an asterisk is placed either down or up to the sequence of scorpine. The sequences compared are: cecropin B from *Antheraea pernyi* [12]; sarcotoxin Ic from *Sarcophaga peregrina* [13]; cecropin P1 from *Sus scrofa* [14]; scorpine from this work; defensin from *Aeschna cyanea* [10] and a defensin from the scorpion *Leiurus quinquestriatus hebraeus* [11].

lower concentration than that of Shiva-3. The calculated $ED_{50}$ were 10 μM for the gamete and 0.7 μM for the ookinete stages of development. These results indicate that scorpine could be a better candidate than Shiva-3, to be incorporated into the genome of genetically engineered malaria-resistant anophelines.

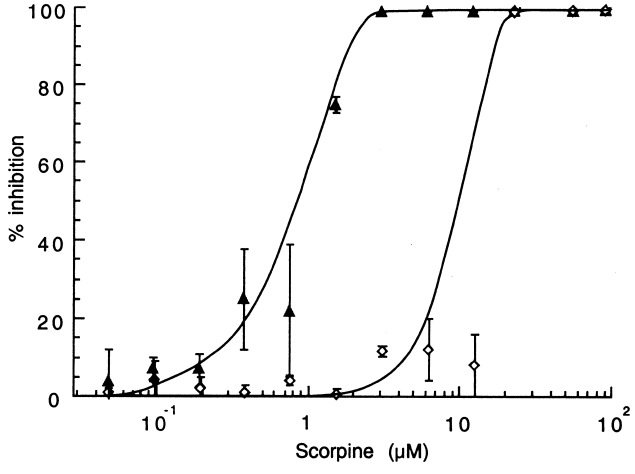


Fig. 3. Dose response curve of the effect of scorpine on the ookinete and fecundation (rosette) phases of the development of *P. berghei*. Inhibition of the ookinete development (closed triangles) and inhibition of rosette formation (open rhomboids) of *P. berghei* in vitro by the scorpine peptide. For the ookinete phase, at least 10000 red blood cells were counted, whereas for the rosette formation a minimum of 10 microscopic fields were directly examined, for each scorpine concentration.

*Acknowledgements:* This work was supported in part by a Grant from Howard Hughes Medical Institute (No. 75197-527107) to LDP, WHO/TDR (ID 930812), and CONACyT-Mexico (G021-M9607) to MHR and LDP. R. Conde was a fellow of the N.G.O. (Service Civil Internacional, branche Belge) of the Belgium Government. The technical assistance of Mr. Fredy Coronas and Jorge A. Torres is acknowledged.

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