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Profiling the short, linear, non-disulfide bond-containing peptidome from the venom of the scorpion *Tityus obscurus*

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ABSTRACT

Many scorpion accidents occur in the Brazilian Amazonian region and are frequently caused by *Tityus obscurus*. Approximately 5% of the crude venom of this species is composed of short linear, non-disulfide-bridged peptides, which have not been intensively investigated. As a consequence, only a few of these peptides have been structurally and functionally characterized to date. In the present paper, the peptide fraction of the venom was subjected to peptide profiling using an LCMS-IT-TOF/MS and MS^n system. The analysis detected 320 non-disulfide bond-containing peptides (NDBPs), of which twenty-seven had their sequences assigned; among them, thirteen peptides were characterized, constituting novel toxins in *T. obscurus* venom. Some of the novel peptides showed similarities to hypotensin-like toxins, while other peptides appear to be natural fragments of neurotoxins. The novel peptides were submitted to a series of bioassays, revealing that many are multifunctional toxins that cause, for example, pain, edema formation and hemolysis to potentiate strong inflammatory processes and alterations in the locomotion and lifting activities in the victims of stinging. Knowledge of the complex matrix of peptides composing the venom of *T. obscurus* will contribute to better understanding of the complex mechanism of envenoming caused by stinging accidents.

Significance: The scorpion *Tityus obscurus* causes many envenoming accidents of medical importance in Brazilian Amazon region; despite to this, very few is known about the toxinology of this animal. The knowledge about the venom composition and mechanisms of action is very important to understand the physiopathology processes related to the envenoming caused by this animal. The proteopeptidomic investigations of scorpion venoms in general have focused mainly the neurotoxins (which are disulfide bonds containing peptides) and large proteins. The short, linear, non-disulfide bonds containing peptides (NDBPs) represent up to 5% of scorpion venom compositions; however, they have been few investigated in comparison with the neurotoxins. The present study used a mass spectrometric approach to detect 320 NDBPs and to sequence 27 of them; pharmacological assays permitted to characterize 13 NDBPs as novel toxins involved with inflammation, pain and edema formation.

1. Introduction

Species belonging to the *Tityus* genus, including the species *T. obscurus* (GERVAIS, 1843) (previously known as *Tityus paraensis* (KRAEPELIN, 1896) and *Tityus cambridgei* (POCOCK, 1897), are responsible for the majority of scorpion accidents in Brazil [1]). *T. obscurus* is responsible for many envenomation accidents, especially in the Amazon region [2].

The number of accidents caused by scorpion stings is increasing due to intensification of human activities in forest regions [3]. Scorpionism is a medical-sanitary problem of great importance, and patients with severe signs of scorpion envenoming primarily present cardiovascular events, including acute heart failure and respiratory distress syndrome. In addition, the victims of *T. obscurus* stings have systemic manifestations, including neurological disorders in 97.2% cases and electric shock-like sensations throughout the body (in 88.9% patients) [4]. In addition, some publications have reported severe health problems following scorpion stinging; Torrez et al. [5] reported 58 accidents presumably caused by *T. obscurus* in the Brazilian Amazon, with symptoms such as cerebellar ataxia, dysdiadochokinesia, dysmetry, dysarthria, nausea and vomiting in most patients; cardiomyopathy is also frequently reported [4,6]. Apparently, the venom from *T. obscurus* is less

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toxic than that of *T. serratus* but is still able to cause lethality in mice, as well other typical effects of *Tityus* venoms with reduced intensities [7]. The biochemical characterization of some toxins of this venom are focused on classical approaches of neurotoxins acting on sodium and/or potassium channels [8–13].

Scorpion venom contains an enormous number of peptide toxins, requiring numerous publications to extensively cover this subject; however, most articles involving scorpion venom investigations primarily describe clinical symptoms and epidemiology of scorpionism and do not provide a detailed characterization of venom composition, structure assignment and the mode of action of the toxins. Peptides represent the most abundant components of scorpion venom [14,15] and can be generically classified into two structural classes: disulfide-bridged peptides (DBPs) and non-disulfide-bridged peptides (NDBPs) [15]. The DBPs are the most abundant components of scorpion venoms and are classically extracted in aqueous solvents and initially fractionated using gel-filtration chromatography; each fraction may be re-chromatographed under RP-HPLC, resulting in pure peptides [16–18]. This experimental approach permits the extraction and purification of many peptides containing 30 to 80 amino acid residues, which constitute the most known scorpion venom toxins to date [19]. The NDBPs are mostly unknown and have been rarely investigated; only a few have been thoroughly characterized [15,20–23]. These peptides are directly extracted with acetonitrile (ACN) from the crude scorpion venoms and purified using RP-HPLC. While most DBPs are neurotoxins, it was reported that most NDBPs are composed of short, linear, polycationic amphipathic peptides, which may present a wide range of biological activities such as antimicrobial action, hemolytic disorders, activation of cell signaling processes, inflammation, immune-modulation and blood pressure changes [15,20–24]. The identification of these toxins is essential for characterizing the pharmacological symptoms observed during the envenoming process [25].

A detailed characterization of animal venom based on the profiling of proteomics and peptidomics has been performed using mass spectrometric analysis. The use of LC-MS and MS/MS has been crucial for detection, sequencing, and identification of the rich composition of peptides/proteins from animal venom, contributing to the assignment of the global proteopeptidomic profiles of various Arthropod venoms such as social wasps [11,26–29], ants [30], honeybees [31], and spiders [32,33]. The assignment of the proteome complement from scorpion venom has also benefited from the use of mass spectrometric analysis; a detailed analysis of *Tityus* spp. venom identified hundreds of components, constituting a complex composition of peptides with molecular masses from 2500 to 8000 Da (mostly DBPs), presenting activity in the modulation of Na^+, K^+, Ca^{++} and Cl^- currents; proline-rich peptides, bradykinin-potentiating peptides (BPP) (NDBPs), proteins such as hyaluronidase, various lysozymes, proteinases (carboxypeptidases, endopeptidases, aminopeptidases), and metalloproteinases were also identified [21,25,34–40]. It was reported that the composition of *Tityus serrulatus* venom is composed of neurotoxins, enzymes, diuretic peptides, bradykinin-potentiating peptides (BPPs) and antimicrobial peptides, among other components [41–44]. However, the peptidome approach has only been applied in a few animal studies [15,20–23].

MALDI-TOF-TOF and de novo sequencing were used to assign the sequence of 28 peptides from 400 Da to 3900 Da in venom from the *T. serrulatus*, mostly as fragments of Pape proteins, corresponding to sequences from the N-terminal region of the TsKβ (scorpine-like) toxin, from potassium channel toxins (other than the k-beta ones), and from hypotensins [45,46].

Nascimento et al. [43] investigated the general profile of polypeptide components from the venom of various scorpion species from 900 Da to 17.000 Da; the authors reported 632 components in *T. stigmurus*, 383 components in *T. serrulatus*, 464 components in *T. bahiensis*, 554 in *Leiurus hebraeus*, and 380 components in *L.q. quinquestriatus*; however, no short, linear peptide sequence was assigned in these studies.

Considering the medical importance of scorpionism accidents in northern Brazil, especially those caused by *T. obscurus*, as well the inadequate knowledge of venom composition for this scorpion species, we focused the present study on the sequence assignments and functional characterization of NDBPs from the venom of *T. obscurus*. For this purpose, the venom was extracted with acetonitrile (ACN) and subjected to LC-ESI-IT/MS and MS^n analysis to perform peptidome profiling. The sequences of 27 NDBPs were assigned, and thirteen NDBPs were synthesized on in solid phase, purified and assayed for mast cell degranulation, hemolysis, delivery of lactate dehydrogenase activity (LDH) from mast cells, antibiosis, edema formation, and evaluation of locomotion/rearing.

2. Material and methods

2.1. Biological material

Tityus obscurus scorpions were collected in the neighborhood of Santarem, AM, northern Brazil, by the staff of the Butantan Institute (São Paulo, SP, Brazil) with SISBIO/IBAMA authorization (protocols nos. 21483-2 and 20158-1); the animals were maintained in plastic boxes with water ad libitum and regularly fed cockroaches. Access to the genetic patrimony of this scorpion species was formally authorized by CGEN (protocol no. 010803/2013-0). The venom was obtained via electric stimulation of telsons, collected with micropipettes, lyophilized, frozen and stored at − 20 °C. The crude venom was donated to the present study. The venom was solubilized in 50% (v/v) ACN containing a mixture of Protease Inhibitor Cocktail (2 mM AEBSF, 0.3 μM aprotinin, 130 μM bestatin hydrochloride, 1 mM, 2 mM EDTA, 0.1 mM pepstatin A, 14 μM E-64, and 1 μM leupeptin hemisulfate salt, SIGMA-ALDRICH. St. Louis, USA). The extract was centrifuged at 10,000 × *g* for 20 min at 4 °C, and the pellets were discarded and the supernatant was lyophilized and maintained at − 20 °C until use. Three different lots of crude venom were used to prepare three different replicate samples.

2.1.1. Animals

Male Swiss mice weighing between 25 and 30 g were used throughout this study. Mice were housed under controlled humidity at 22 °C ± 1 and subjected to a 12-h light/dark cycle in a sound-attenuated room. Food and water were available ad libitum, and mice were taken to the testing room at least one day before the experiment. All behavioral testing was performed between 9:00 am and 4:00 pm. Each mouse was used only once. All experiments were performed in accordance with the guidelines for the ethical use of conscious animals in pain research published by the National Academy of Sciences (http://www.nap.edu/catalog/5140.html). The procedures were approved by the Institutional Animal Care Committee at São Paulo State University, UNESP campus Rio Claro, SP (CEUA-IB-UNESP-CRC, Protocol no. 1984). Efforts were made to minimize the number of animals used and their suffering.

2.2. LC-MS and $LCMS^n$ analysis

Approximately 50 μg of the peptide-rich fraction from *T. obscurus* venom was solubilized in 100 μL of 50% (v/v) ACN and fractionated in an LC-MS system using an X-Bridge BEH 130 C-18 column (100 mm × 2.1 mm; 3.5 μm) (Waters, Massachusetts, USA) at a flow rate of 200 μL/min. Elution was performed under gradient conditions from 5 to 95% (v/v) ACN (containing 0.1% (v/v) TFA) between 0 and 95 min at 30 °C. The eluent was monitored at 215 nm with a UV-DAD detector, mod. SPD-M10A (SHIMADZU, Kyoto, Japan) coupled to an IT-TOF/MS and MS^n mass spectrometer system equipped with an electrospray ionization source (Shimadzu, Kyoto, Japan).

Spectra were acquired in positive mode, with activation of data-dependent acquisition (DDA), which permits a switch automatically

from MS to MS^2 mode. The electrospray voltage was set to 4.5 kV, the CDL temperature was set to 200 °C, the block heater temperature was adjusted to 200 °C, the nebulizer gas (N2) flow was 1.5 L/min, the trap cooling gas (Ar) flow was 95 mL/min, the ion trap pressure was 1.7×10^{-2} Pa, the TOF region pressure was 1.5×10^4 Pa, and the ion accumulation time was 50 ms. The top five ions from each MS spectrum were selected as precursors (Top N) for fragmentation in MS^2, as typically used in DDA experiments. The collision energy was set at 35% both for MS^2 and MS^3, and the collision gas set to 20%. Autotuning was performed in the presence of Na-TFA solution (TFA 0.1% (v/v) 10 mM NaOH at pH 3.5). The mass spectral resolution was approximately 10,000 FWHM, and error was approximately 3.08 ppm.

2.3. Acetylation of lysine residues

The acetylation of Lys residues was used to distinguish between the quasi-isobaric amino acid residues of Lys and Gln (Lys = 128.09496/Gln = 128.05858) during peptide sequencing by mass spectrometry. The acetylation reagent was prepared by mixing 20 μL of acetic anhydride with 60 μL of methanol, and the peptide of interest (1 nmol solubilized in 20 μL of 50 mM ammonium bicarbonate pH 7.0). Then, 50 μL of the acetylation reagent was mixed with 20 μL of the peptide solution and incubated at 25 °C for 60 min. The mixture was lyophilized to dryness and reconstituted in 50% (v/v) ACN for mass spectrometry analysis.

2.4. Peptide synthesis

The peptides were synthesized by step-wise solid-phase synthesis using N-9-fluorophenylmethoxy-carbonyl (Fmoc) chemistry with Novasyn TGS resin (NOVABIOCHEM, Germany) using an automated peptide synthesizer (PROTEIN TECHNOLOGY INC., mod. Prelude; USA). Side-chain protective groups included *t*-butyl for serine and *t*-butoxycarbonyl for lysine. Cleavage of the peptide resin complexes was performed with trifluoroacetic acid, 1,2-ethanedithiol, anisole, phenol, and water (82.5:2.5:5:5:5 by volume, respectively) for 2 h. The peptides were precipitated with cooled ethyl ether (4 °C). The crude peptides were solubilized in water and purified in a RP-HPLC system mod. LC-8A (SHIMADZU, Kyoto, Japan), using a semi-preparative column (SHISEIDO C-18, 250 mm × 10 mm, 5 mm) under isocratic conditions with 55% (v/v) ACN (containing 0.1% (v/v) TFA) at a flow rate of 2 mL/min. The elution was monitored at 215 nm with a UV-DAD detector, mod. SPD-M10A (SHIMADZU, Kyoto, Japan), and each eluted fraction was manually collected; the purity was checked using mass spectrometry analysis. The synthetic peptides were used to perform all bioassays described below.

2.5. Biological assays

2.5.1. Hemolytic activity

Washed rat red blood cells (WRRBC) were used to evaluate the hemolytic activity of the peptides. WRRBC were prepared by washing Wistar RRBC three times with 50 mL of saline solution (0.85% (w/v) NaCl, containing 10 mM $CaCl_2$), and re-suspended in 50 mL of the same solution. Aliquots of WRRBC (0.5% v/v) were incubated at 37 °C in the presence of each synthetic peptide for 120 min with gentle mixing. Samples were centrifuged, and the absorbance of the supernatants was measured at 540 nm. The absorbance measured from lysed WRRBC in the presence of 1% (v/v) Triton X-100 was considered 100%.

2.5.2. Measurement of lactate dehydrogenase (LDH) release from mast cells

The release of LDH from mast cell cytoplasm to the surrounding environment is an indicator of mast cell lysis. LDH catalyzes the reversible reduction of pyruvate to lactate with NADH as a coenzyme. The LDH activity was assayed with the supernatant suspension resulting from the peritoneal mast cell degranulation assays as described above. The assay kit UV-LDH (BIOTECNICA, Brazil) was used as follows: 20 μL of peritoneal mast cell supernatant suspension was incubated with 800 μL of 50 mM Tris buffer, pH 7.4, containing 1.2 mM pyruvic acid and 5 mM EDTA (SIGMA-ALDRICH, Saint Louis, USA) for 5 min at 25 °C. The results were initially calculated as catalytic units (mmoles NADH consumed per min at pH 7.4) and converted into relative activity by measuring the total amount of LDH activity of rat mast cells lysed in the presence of 0.1% (v/v) Triton X-100 (this value was considered a reference for 100%). The results were expressed as an average (± SD) of five experiments.

2.5.3. Mast cell degranulation activity

Mast cell degranulation was determined by measuring the release of β-D-glucosaminidase in the presence of synthetic peptides. Mast cells were obtained by peritoneal washing of adult Wistar rats with a solution containing 0.877 g NaCl, 0.028 g KCl, 0.043 g NaH2PO4, 0.048 g KH2PO4, 0.10 g glucose, 0.10 g BSA, 90 mL of a 2 M CaCl2 solution and 50 μL Liquemine (heparin, ROCHE) in 100 mL water. Mast cells were incubated in the presence of peptides for 15 min at 37 °C. After centrifugation, 50 μL of mast cell suspension was added to 50 μL of the substrate [3.4 mg of *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidine (SIGMA-ALDRICH, Saint Louis, USA) dissolved in 10 mL of 200 mM sodium citrate, pH 4.5] and incubated for 6 h at 37 °C. The reaction was stopped by the addition of 150 μL of 0.2 M Tris and the absorbance was measured at 405 nm in a spectrophotometer (mod. Biotrack, AMERSHAM BIOSCIENCES, Sweden). The values are expressed as the percentage of total β-D-glucosaminidase lysed from mast cells in the presence of 0.1% (v/v) Triton X-100.

2.5.4. Antimicrobial activity

The minimal inhibitory concentrations (MIC) of the peptides were determined based on methods described by Meletiadis et al. [47]. The following microorganisms were used: *E. coli* (CCT 1457), *S. aureus* (CCT 6538), *S. pneumoniae* (ATCC11733), and *E. aerogenes* (CCT2572). The experiment was performed in 96-well plates. Bacterial cells were suspended in sterile culture medium; the inoculum size was 1×10^4 cells m/L in Müller–Hinton broth (DIFCO), as confirmed by the use of McFarland scale. From this culture, 50 μL was spread onto the micro-plate previously containing 50 μL of Müller-Hinton broth, resulting in a final cell density of 1.5×10^3 cell/mL. Cells were incubated at 37 °C for 18 h in the presence of 100 μL of each peptide solution in concentrations ranging from 0.8 to 500 μg/mL. After incubation, 10 μL of a triphenyltetrazolium chloride (TTC) (MALLINCKRODT) solution (final concentration 0.05%, w/v) was added to each well. The plate was incubated at 37 °C for 2 h. Live colonies reduce TTC to a dark-red color, while those with reduced respiratory function cannot and remain unchanged. Thus, the results were expressed as the MIC that inhibits all colony forming units. Chloramphenicol (Sigma-Aldrich) was used as a standard antibiotic, and the negative controls were the wells containing only culture medium. The results were expressed as the mean of three experiments.

2.5.5. Hyperalgesic and edematogenic effects

Male Swiss mice weighing between 25 and 30 g were used throughout this study. The housing of mice occurred under controlled humidity (65% ± 5%) and temperature (22 °C ± 1), in a sound-attenuated room subjected to a 12 h light–dark cycle. Food and water were available ad libitum, and mice were taken to the testing room at least 1 h before the experiment. All behavioral testing was performed between 9:00 a.m. and 4:00 p.m. The mice were used only once. All experiments were completed in accordance with the Guidelines for the Ethical Use of Conscious Animals in Pain Research published by the International Association for the Study of Pain [48] and the EC Directive 86/609/EEC for Animal Experiments. The animal manipulation protocols were approved by the Institutional Animals Care Committee (CEUA-IB, protocol 016/2011).

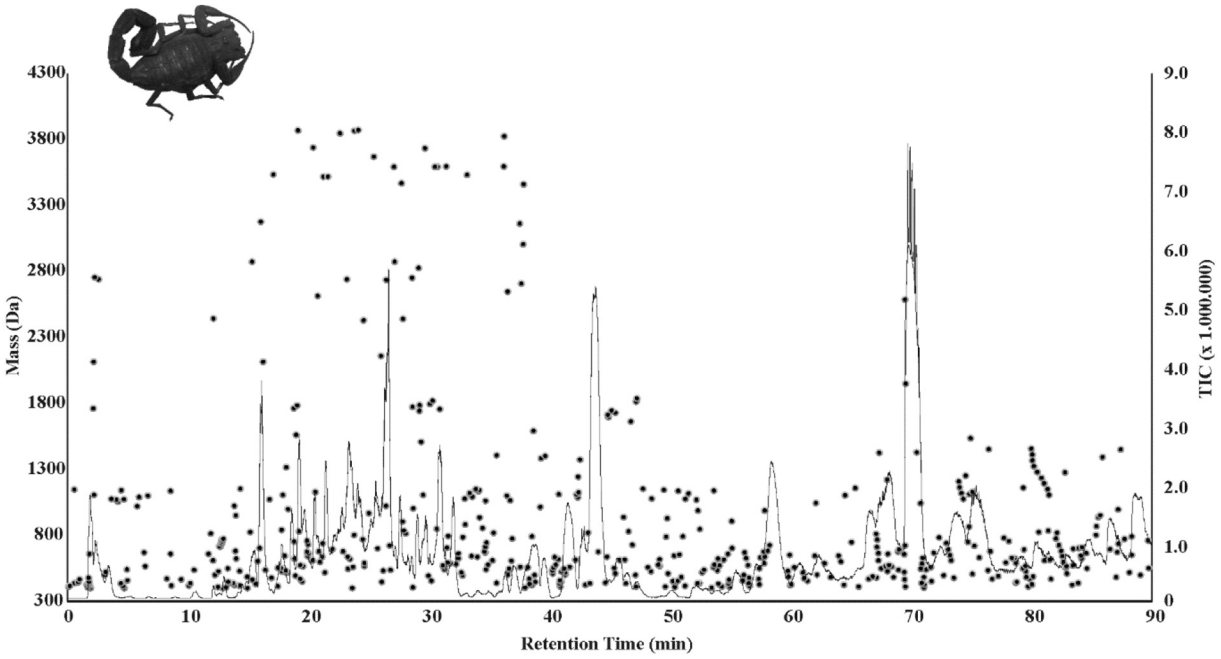

Fig. 1. LC-ESI-MS total ion chromatogram (TIC) and peptide profile (peptide mass vs retention time) of *T. obscurus* venom extracted in 50% (v/v) ACN. The reconstructed masses of each peptide detected are represented as points, according to their retention times in the chromatogram. The TIC was obtained by RP-HPLC with an XBridgeTM BEH 300 C-18 (Waters) column (100 mm × 2.1 mm; 3.5 μm). The elution was performed under a linear gradient condition from 5 to 95% (v/v) ACN (containing 0.1% (v/v) TFA) in the interval from 5 to 90 min. The elution was performed at 30 °C at a flow rate of 200 μL/min.

Mice were placed in appropriate acrylic cages with a wire-gridded floor 30 min before the assay. During this adaptation period, the paws of mice were poked 2–3 times. Before paw stimulation, the animals remained quiet, presenting neither exploratory movements nor resting on their paws. In these experiments, an electronic von Frey anesthesiometer, a paw pressure meter fitted with a 0.5 mm^2 plastic tip (IITC Inc., LIFE SCIENCE INSTRUMENTS, Woodland Hills, CA, USA) was used. A mirror fitted below the gridding floor provided a view of hind paw movements. The use of an electronic von Frey instrument (INSIGHT, Ribeirão Preto, SP, Brazil) permitted the automatic register of the force intensity of the stimulus by the time of paw withdraw. The maximal force applied was 18 g. The stimulations were repeated until the mice presented at least two similar measurements. The hyperalgesic effect was induced by injection through the intraplantar (i.pl.) route into one of the hind paws, with either carrageenan (300 μg) or each peptide (25 μg). The results are reported as the D (delta) withdrawal threshold (g).

For evaluation of nociceptive activity, peptides were dissolved in sterile saline (25 μg/50 μL saline) and applied by i.pl. route in one of the hind paws of the mice. The tip of the pressure meter was introduced in the central area of the hind paw (where the compounds were applied with the help of a 26 g needle) to measure the intensity and the time of paw withdraw. Nociceptivity was evaluated at 60, 180 and 300 min after peptide application and compared to the control. Carrageenan (MARINE COLLOIDS, 300 μg/50 μL sterile saline) was used as the positive control, while the negative control was performed with sterile saline.

Edema was induced by the injection of carrageenan (or peptides) into one of the hind paws by the intraplantar (i.pl.) route. The increase in the volume of paws (edema) at the region of tibio-tarsal articulation was measured with a digital paquimeter (MITUTOYO, São Paulo, SP, Brazil). The difference between the values measured in each paw was expressed as the percent increase in paw volume.

2.5.6. Assessment of and evaluation of locomotion/rearing activity (open field test)

An acrylic arena divided into 25 squares was used to evaluate the general movement activity of the animals. Animals were placed in the observation arena for 2 min for acclimatization and then positioned in the center of the field. Two parameters were used: the number of squares crossed and the rearings (number of times the mouse stood on its hind limbs). The data were collected for 3 min after 15 min of i.p. (intra peritoneal) injection of peptide solution (25 μg/50 μL) or saline (50 μL, in the control group).

2.6. Statistical analysis

A two-way analysis of variance (ANOVA) was used to compare the groups and doses over the entire range. Three factors were analyzed: treatments, time and the time vs. treatment interaction. When a significant time vs. treatment interaction was detected, a one-way ANOVA followed by Tukey's test was performed for each time point to distinguish the dose effects [49]. The results with $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Peptidome analysis

To improve our knowledge of the biochemical composition of peptide toxins from *T. obscurus*, venom was submitted to peptide profiling. For this purpose, the soluble fraction from 1 mg of crude venom was extracted with 50% (v/v) ACN, resulting in 50 μg of the short, linear peptide-rich fraction, which in turn was fractionated under RP-HPLC coupled to a LC-ESI-IT-TOF-MS and MS^n system, as described above in the methods section.

LCMS analysis of the sample directly extracted in ACN revealed a rich peptide composition; considering the total number of peptides detected in the total ion chromatogram (TIC) and the accumulated peptides from 400 to 4000 Da, 517 MS/MS spectra were selected and acquired over the 90-min chromatogram (Figs. 1 and 2; Table S1). The DBPs represent the most abundant components of scorpion venoms, which are generally extracted in aqueous buffers (like the proteins) and fractioned under RP-HPLC [16–18]. In the present investigation, the

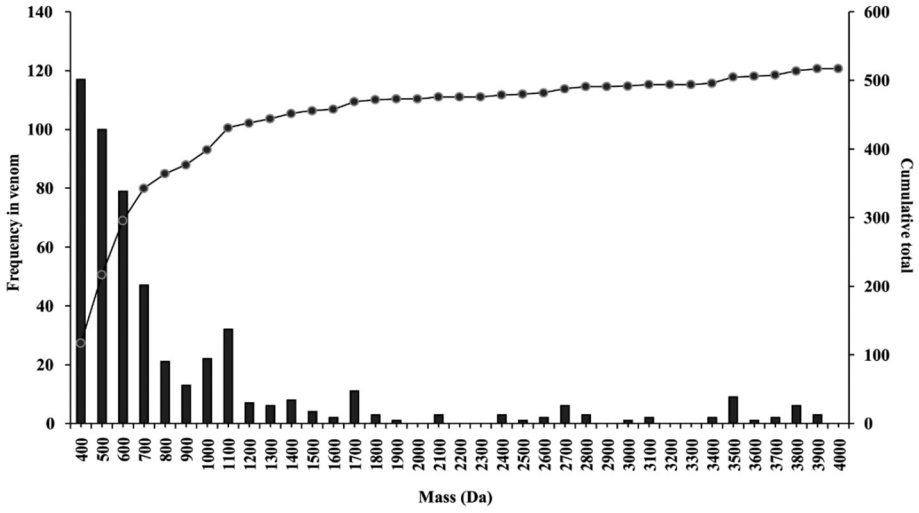

Fig. 2. Histogram of peptide abundance by mass (100 Da bins). Overlaid curves show cumulative total peptide number.

extraction was first performed in 50% (v/v) acetonitrile, so the NDBPs were mostly extracted in relation to the DBPs. A careful observation of Table S1 reveals that from the 517 precursor ions selected for the acquisition of MS/MS spectra, 157 represent analytical redundancies. Thus, a total of 360 peptides were detected in the mass range from 400 to 4000 Da, of which 40 peptides presented molecular masses from 2115 to 3926 Da (26 with molecular masses above 3200 Da and are possibly DBPs/neurotoxins). The other 320 peptides are primarily composed of NDBPs.

The mass spectrometric analysis was performed using ESI as an ionization source in the positive and negative acquisition mode; however, because the peptide fraction of this venom is composed of polycationic components, only data obtained in the positive mode are presented in this publication, resulting in much more intense peaks than the negative mode data. The venom sample components were distributed over the molar mass range of 400–4000 Da, which together comprise up to 5% of the dry weight of the venom. This analysis allowed the preparation of the profile of mass distribution shown in Fig. 2. It may be observed that the abundance of mass values is in the interval between 400 Da and 700 Da. Generally, the large peptides elute on RP-HPLC columns; the hydrophilic ones elute first under mobile phases rich in water, while the hydrophobic ones elute later, under mobile phases rich in ACN. However, in the present study, the NDSBs appear to elute throughout the chromatogram. To emphasize this feature, Table 1 also shows the GRAVY Index for the NDBPs from *T. obscurus* venom, calculated with the tool ProtoParam (http://web.expasy.org/protoparam/). Algorithms used to calculate the average hydropathy depend on amino acid sequences and are based on the work of Kyte and Dolittle [50,51]; a positive index indicates a hydrophobic protein/peptide, while a negative index indicates hydrophilic character. It is expected that peptides with negative indexes should elute first, while those with positive indexes should elute later. However, a detailed observation of Table 1 reveals that the NDSB peptides elute throughout the chromatogram. To explain this chemical behavior of the NDSB peptides, it must be acknowledged that the GRAVY index tends to underestimate hydrophobicity when the elements of secondary structure bury charged residues; in addition, the index does not work well for too-short sequences (less than five residues) [52,53]. In addition, other factors may influence the retention times of short, linear peptides: i) the stability of elements of secondary structure generally results in higher retention times than predicted by the index, as the polarity of peptide backbone is minimized [50,51]; ii) it has been shown that the detection of peptides in RP-HPLC coupled to an ESI/MS system tends to demonstrate a counter-intuitive preference for some peptide conformations in relation to other proline-containing peptides [52], which generally suffer slow conformational changes under equilibrium due to the cis-trans isomerism of Pro-residues, with reasonable changes in retention times for the same peptides, which may elute with different retention times in the same chromatogram in RP-HPLC [53]. A careful observation of Table 1 reveals that 18 of the 27 peptides presented Pro residues in their sequences, which may interfere with their conformations, their interactions with the stationary phase of the RP-column and therefore their retention times.

In the present study we focused on the short, linear NDSB peptides; we considered the analysis of 320 tandem mass spectra obtained for these peptides. Most did not present analytical features to render their interpretation in reliable amino acid sequences. The reasons for the selection of the 27 peptides in the present study were i) high abundances (as indicated by spectral counting of each peptide component detected) (Table 1), which resulted in relatively high peak intensities (in tandem mass spectra); ii) reliable mass spectra under CID conditions, with a large number of sequence-related fragment ions; iii) the presence of two residues of Pro in sequences, which frequently causes difficulties in spectral interpretation due the occurrence of gaps in the spectra. Despite the high abundance of some peptides, their tandem mass spectra did not result in reliable mass spectra due to high hydrophobicity and therefore poor ionization and overall fragmentation pattern with a poor series of sequence-related fragment ions. Meanwhile, other peptides occurred in reduced abundance but ionized quite well and fragmented under CID conditions, resulting in large series of sequence-related fragment-ions. Table 1 shows some examples of peptides occurring in reduced abundance (reduced spectral counts) with intense signals and a large series of sequence-related fragment ions. We selected the 27 tandem mass spectra that permitted unambiguous sequence assignment of NDBPs.

The interpretation of MS^2 spectra obtained under CID conditions across the mass chromatogram was used to assign the peptide sequence through the subtraction of the *m/z* values between consecutive *b*-ions (or *y*-ions), permitting the assignment of peptide sequences. This procedure allowed the almost complete sequence assignment of many peptides, a few apparent ambiguities in relation to the isobaric residues I/L and K/Q remain. The ambiguities I/L for residues positioned inside the peptide chain were solved using ion fragments of the *d*- and/or *w*-type, specific for the fragmentation of the side chains of I and L residues. This was initially done by simulating the “candidate sequences” (containing I or L residues) fragmentation under CID conditions using the algorithm Protein Prospector—MS Product (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct); the *m/z* values of virtual *d*- and *w*-fragment ions were manually searched for in the experimental MS^2 spectra to assign I or L residues where necessary; *m/z* values with signal/noise above 5 were accepted. The N-terminal residue ambiguity was solved by synthesizing two sequences per peptide (with I

Table 1
Profile of peptides sequenced from venom of the scorpion *Tityus obscurus*.

Peptide number	Amino acid sequences	Molecular mass (Da)	Retention time (min)	Spectral count	GRAVY index
Pep-1	AEIDFSGIPEDIIKQI-OH	1786.94	34.2	22	0.181
Pep-2	GCDALLSGDHGGLLSANGC-OH	1758.77	51.3	15	0.342
Pep-3	LIPNDQLRSI-OH	1167.63	74.5	66	− 0.080
Pep-4	NIALGQDQSGR-NH2	1156.74	18.1	54	− 0.909
Pep-5	KIKEKLIEA-OH	1070.67	52.0	23	− 0.456
Pep-6	WPNKIEPGK-OH	1067.57	37.4	216	− 1.644
Pep-7	KETNAKPPA-OH	954.51	8.1	34	− 1.678
Pep-8	KIITPPIR-OH	936.53	34.8	116	0.150
Pep-9	KPVEPVG-OH	724.47	20.5	176	− 0.371
Pep-10	SESNTCG-OH	696.26	26.8	496	− 1.029
Pep-11	NAKPPA-OH	596.31	78.2	202	− 1.167
Pep-12	ILTGKLKCK-OH	1002.60	28.4	60	0.200
Pep-13	ILPNDK-OH	698.39	20.1	372	− 0.700
Pep-14	VYWLPAVLGSLLGFTP-OH	1731.99	45.3	25	1.281
Pep-15	RPPHNPGFLTVYN-OH	1510.71	29.6	32	− 0.854
Pep-16	PHWLFFGVSVLC-OH	1403.75	39.1	76	1.433
Pep-17	VTLTLPPAES-NH2	1025.67	14.5	22	0.470
Pep-18	KETNAKPP-OH	883.48	8.7	116	− 2.112
Pep-19	LFGAFALV-OH	837.45	81.0	960	2.575
Pep-20	HERLLGP-OH	820.45	35.4	199	− 0.800
Pep-21	TVPDLEM-NH2	802.52	87.3	94	0.086
Pep-22	HGCIGCGR-NH2	800.36	6.0	32	0.075
Pep-23	PCFTLPG-OH	733.38	26.5	900	0.686
Pep-24	NAKPP-OH	525.29	61.4	324	− 1.760
Pep-25	K/QPVVG-OH	498.31	33.2	298	0.500/0.590
Pep-26	SILK-OH	459.32	45.8	638	n.d.*
Pep-27	KPPA-OH	411.24	3.6	210	n.d.*

(*) The sequence must have at least 5 residue to have the GAVY Index calculated by ProtoParam algorithm.

or L positioned at the N-terminus), which in turn were submitted to LCMS-IT-TOF/MS analysis. The molecular masses and retention times of synthetic and natural peptides were compared to each other. To solve the K/Q ambiguity, the samples were derivatized with acetic anhydride and submitted to mass spectrometric analysis under CID conditions. The ε-amino group from the side chain of K residues and the α-amino group of the N-terminal residue of each peptide become acetylated, contributing increments of 42 mass units per acetyl group incorporated into the peptide chain. The side chain of the Q residue does not react with acetic anhydride. To save space in the manuscript and to present the data more objectively and less repetitively, we decided to present and discuss the complete set of sequencing data in the manuscript body for a typical peptide, while data for the remaining peptides are presented in the Supplementary section.

Thus, the peptide eluting at a retention time of 32.4 min was selected as an example of the application of mass spectrometry as an analytical strategy for sequencing purposes (Fig. 3A and B), while sequencing data for the remaining peptides were presented in Supplementary figures (Figs. S1 to S26). Fig. 3A shows the MS^1 spectrum of this peptide, with a very intense peak corresponding to the ion of *m/z* 894.472 as $[M + 2H]^{+2}$. The deconvolution of these data indicates that the peptide presents a molecular mass of 1786.94 Da, and the presence of the complete series of *b*-type fragment ions (from b_2 to b_{15}), can be used to assign the sequence of this peptide as AEIDFSGIPEDIIKQI (Fig. 3B). Considering this sequence and the value of the molecular mass mentioned above, it may be suggested that the C-terminal residue of this peptide is in the acidic form; therefore, the complete sequence of the peptide eluted at 34.2 min is AEIDFSGIPEDIIKQI-OH. This component was designated Pep 1.

The complete amino acid sequences of twenty-seven peptides were assigned using ESI-IT-TOF-MS and MS^n analysis and are shown in a summarized form in Table 1 considering their presentation in decreasing order of molecular mass. A careful observation of Table 1 reveals that Pep-4 (NIALGQDQSGR-NH2), Pep-17 (VTLTLPPAES-NH2), Pep-21 (TVPDLEM-NH2), and Pep-22 (HGCIGCGR-NH2) present their C-terminal residues in the amidated form. This is a characteristic feature of many peptide toxins from animal venoms; it is generally essential for their activities and important for their biostabilities [42,51]. It requires a Gly residue neighbor to the residue that will be the C-terminus; the Gly is used as a substrate for the amidation of the C-terminus, catalyzed by the enzyme alpha-peptidyl glycine amidating monooxidase (PAM). The terminal amidation of the C-terminal residue of some scorpion venom peptides has been reported as part of the process of toxin maturation [42,54] C-terminal amidation in scorpion venom peptides have been reported by many authors [55–59].

Venom was extracted in the presence of a cocktail of proteinase inhibitors, indicating that the peptides sequenced did not result from artifacts of sample manipulation, such as uncontrolled proteolysis of high weight molecular proteins during venom extraction. Thus, the twenty-seven peptides sequenced were apparently physiologically produced, representing natural compounds present in the venom of *T. obscurus*. Once the peptide sequences were assigned, it was necessary to verify whether each peptide could be functionally identified by individual sequences; this was achieved using the sequence alignment tool BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). The searches were performed using the non-redundant protein sequences DB, considering the taxa Scorpiones (taxid: 6855), and using the algorithm blastp (protein-protein BLAST); searches with 100% coverage, 100% identity, and E-values below 1e-04 were considered to correspond to the protein/peptide indicated in the hit, presenting the parameters mentioned above. The searches identified Pep 1 (AEIDFSGIPEDIIKQI-OH) and Pep 7 (KETNAKPPA-OH) as corresponding to fragments 1–16 of hypotensin-1 (100% coverage, 100% identity, and an E-value of 1e-13), and 17–25 of the hypotensin-2 (100% coverage, 100% identity, and an E-value of 8e-05), respectively, that were isolated from *T. serrulatus* venom. The peptide AEIDFSGIPEDIIKQIKETNAKPPA-OH was initially identified as fragment 1–25 of hypotensin-1 (100% coverage, 100% identity, and an E-value of 8e-24), previously identified as TsHpt-Ib in the venom of *Tityus serrulatus* [21]; this peptide was characterized as a bradykinin-potentiating peptide (BPP), i.e., an agonist of the B2 receptor of kinin, which does not inhibit the angiotensin-converting-

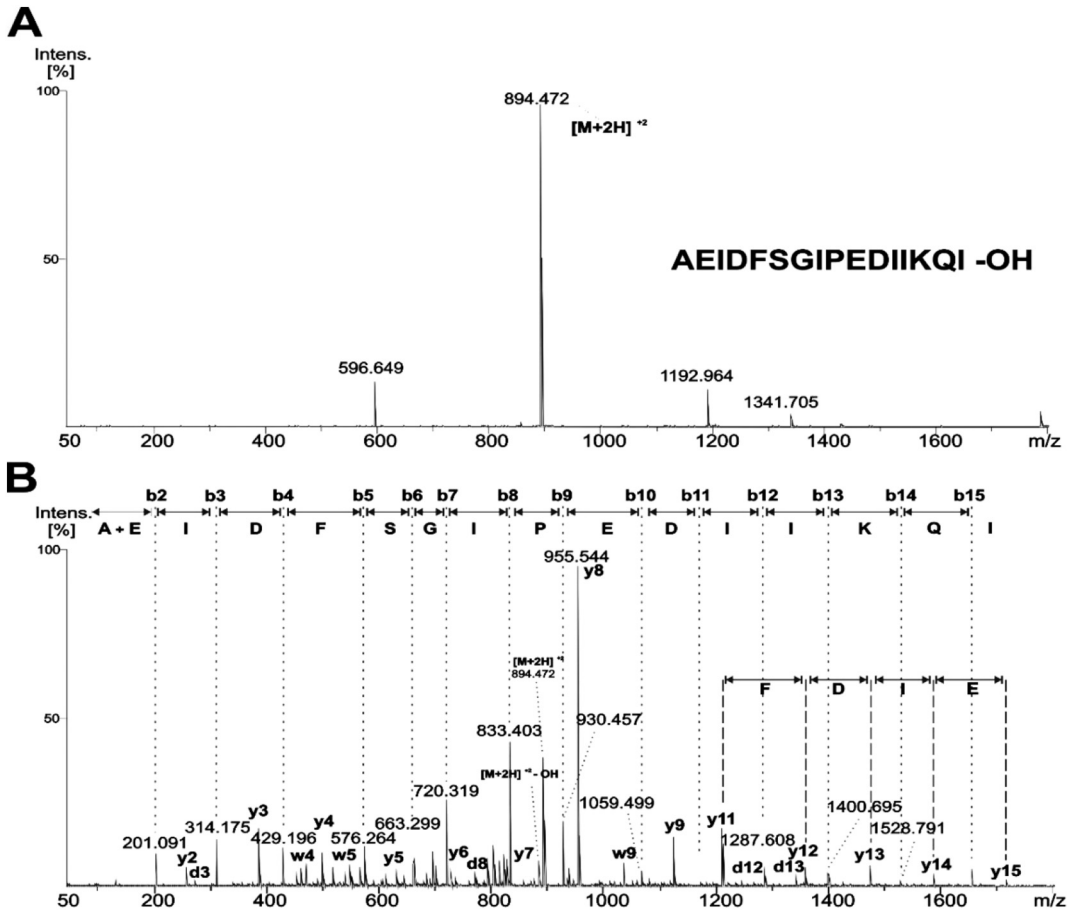

Fig. 3. (A): ESI-MS spectrum obtained in positive mode for peptide eluted at retention time of 34.2 min, showing the ion of *m/z* 894.472 as $[M + 2H]^{+2}$ and the complete sequence of the peptide; (B): MS^2 spectra obtained under CID conditions obtained by selecting the precursor-ion of *m/z* 894.472 as $[M + 2H]^{+2}$ for fragmentation, showing the *b* and *y* series of fragment ions used to assign the peptide sequence, as well the *w*-ions used to diagnostically eliminate the ambiguity between the isobaric residues I/L. This component was designated Pep 1.

enzyme (ACE). Later, Verano-Braga et al. [60] described the analogs of TsHpt-Ib presenting the motif Lys-Pro-Pro in their sequences, which still maintained partial native biological activity of TsHpt-Ib, even in the short sequences. Therefore, Pep 1 (AEIDFSGIPEDIIKQI-OH), Pep 7 (KETNAKPPA-OH), Pep 11 (NAKPPA-OH), Pep 18 (KETNAKPP-OH), Pep 24 (NAKPP-OH) and Pep 27 (KPPA-OH) are partially conserved in the relation to TsHpt-Ib. Despite the use a cocktail of protease inhibitors during the extraction of these peptides from the crude venom, they can be considered fragments of TsHpt-Ib that already existed before peptide extraction from crude venom. Pep 1, Pep 7, Pep 11, Pep 18, Pep 24, and Pep 27 may have actions related to the induction of hypotension in victims of *Tityus* scorpion envenoming.

Pep 4 (NIALGQDQSGR-NH2) corresponds to the fragment 48–58 of the antigen 5-like protein, a cysteine-rich allergen reported in the venom of *T. serrulatus* venom (100% coverage, 100% identity, and E-value 1e-06); thus, Pep 4 may have action related to the antigen 5 function, i.e., a component with a potent effect on the activation of the immunological system of the victims, as also previously observed in patients allergic to the venom of social wasps [61]. Pep 3 (LIPNDQLRSI-OH) corresponds to the fragment 32–41 of tityustoxin Kα (TsTX-Kα) (100% coverage, 100% identity, and E-value 1e-06; accession code Q0GY46). TsTX-Kα selectively blocks voltage-gated non inactivating K^+ channels in synaptosomes (IC_{50} values of 8 nM and 30 nM, respectively) [46,61–64]. Pep 5 (KIKEQLIEA-OH) corresponds to the fragment 36–42 of the potassium channel toxin TtrKIK (100% coverage, 100% identity, and an E-value of 1e-06; accession code Q0GY45); this toxin also blocks voltage-gated potassium channels. These peptides may have action related to potassium channel impairment. TsTX-Kα and TtrKIK appear to be involved in processes of regulation of cell excitability and physiology, such as heart-beating regulation, neurotransmitter release, signal transduction and cell proliferation [65].

Rates et al. [42] reported a series of linear peptides in *T. serrulatus* venom, from which twenty-eight components corresponded to fragments of Pape proteins; ten corresponded to fragments of the N-terminal region of the TsKb toxin (scorpine-like), potassium channel toxins (other than the k-beta type) and hypotensins-1 and -2. In the present investigation, component Pep 5 (KIKEKLIEA-OH) is likely a fragment from the pro-peptide region of the β-KTx neurotoxin, while Pep 26 (SILK-OH) seems to be a fragment of the C-terminal region of the peptide KLVALIPNDQLRSILKAVVH, which was previously reported in *T. serrulatus* venom [42] without any known functional role.

Recently, Pucca et al. [46] reported three novel NDBPs from *Tityus serrulatus* venom: RIRSKGKK, RIRSKG, and KIWRS. None of these peptides presented analogous components in *T. obscurus* venom. These peptides were able to modulate macrophage responses, increasing the production of IL-6, and inhibiting the activity of the angiotensin-converting enzyme. The short, linear peptides (generally shorter than eight amino acid residues) are too reduced to generate reliable identification as natural peptides, and their searches usually result in very redundant protein identification. Only eight of twenty-seven peptides were identified by their sequences; nineteen were not functionally identified based on their amino acid sequences. Thus, we selected Pep 1 to Pep 13 to be synthesized and functionally assayed.

3.2. Bioassays

As most NDBPs assigned in the present study could not be identified by their sequences, we decided to synthesize Pep 1 to Pep 13 in the

Table 2
Profile of biological activities of short and linear peptides from venom of the scorpion *T. obscurus*.*

Peptides	Sequences	Hemolysis	Mast cell degranulation	LDH release	MIC** (μg/μL)
Pep-1	AEIDFSGIPEDIIKQI-OH	+ +	−	−	> 500
Pep-2	GCDALLSGDHGGLLSANGC-OH	+	−	+	> 500
Pep-3	LIPNDQLRSI-OH	−	−	−	> 500
Pep-4	NIALGQDQSGR-NH2	−	−	+	> 500
Pep-5	KIKEKLIEA-OH	+ +	−	+	> 500
Pep-6	WPNKIEPGK-OH	+	−	−	> 500
Pep-7	KETNAKPPA-OH	+	−	+	> 500
Pep-8	KIITPPIR-OH	−	−	−	> 500
Pep-9	KPVEPVG-OH	+ +	−	+	> 500
Pep-10	SESNTCG-OH	+ +	−	−	> 500
Pep-11	NAKPPA-OH	+ +	−	+	> 500
Pep-12	ILTGKLKCK-OH	−	−	−	> 500
Pep-13	ILPNDK-OH	−	−	−	> 500

(*) All biological activities were compared against a standard compound as reference for each assay and expressed as percentages in relation to these standards; (−) means no activity was detected for the peptide, while (+), (+ +), and (+ + +) represent up to 40%, from 41 to 70%, and from 71% to 100% of standard compounds activities, respectively. (**) Values of MIC were observed for both Gram-positive and Gram-negative bacteria.

solid phase, purify them, and submit them to a series of functional bioassays such as hemolysis, delivery of LDH from mast cells, mast cell degranulation, antibiosis, nociception, edema formation, and evaluation of locomotion/rearing.

The results for the tests of biological activities were expressed compared to the standard compounds used in the assay of hemolysis, release of LDH from mast cells, mast cell degranulation, (Figs. S27 to S29), and antibiosis (Table 2). To summarize these results, it was established that an activity was considered weak (+), moderate (+ +), or strong (+ + +) when it represented 20% to 40%, 41% to 70%, or ≥ 71%, respectively, of the activity of the standard compound of each assay (Table 2).

In general, no peptide presented mast cell degranulation (Fig. S29) and antimicrobial effects (Table 2). The peptides Pep 1, Pep 5, Pep 9, Pep 10 and Pep 11 presented moderate hemolytic activity at physiological concentrations (micromolar range) (Fig. S27), suggesting that these peptides interact with the plasma membrane of erythrocytes, disturbing their structure and causing hemoglobin delivery. The same peptides presented no antibiosis, indicating that they have no affinity for the anionic membrane of bacteria. Meanwhile, the peptides Pep 2, Pep 4, Pep 5, Pep 7, Pep 9 and Pep 11 presented a weak releasing effect of LDH from mast cells at physiological concentrations (Fig. S28). If it is considered that these peptides have very reduced or no activity of LDH delivered from mast cells, these results together suggest that the interaction of Pep 1, Pep 5, Pep 9, Pep 10, and Pep 11 with the zwitterionic lipids of the plasma membrane from the erythrocyte membrane are highly selective (or at least have high affinity). The inactivity of these peptides in relation to mast cell degranulation indicates that none presented any affinity for G-protein coupled receptors responsible for mast cell exocytosis.

The synthesized peptides were evaluated for mechanical algesia (Fig. S30) and edema formation at three different times (60, 180, and 300 min) after peptide administration (Fig. S31). Table 3 summarizes the results. The signal (*) indicates the statistical significance: *$p < 0.5$, **$p < 0.01$, and ***$p < 0.001$; (−) indicates no significance, as determined by two-way ANOVA. The detailed graphic data for these assays is shown in the supplementary data (Figs. S30 to S31). The peptides Pep 1, Pep 7, Pep 9, Pep 10 and Pep 11 caused a significant increase in nociceptive sensibility during all test times; peptides Pep 4, Pep 5, Pep 6, and Pep 8 showed a moderate increase in nociceptive sensibility over a short time, and this activity appears to disappear for up to 300 min; the other peptides presented no significant response in this test (Fig. S30). Fig. S31 shows that most peptides produced weak edema; Pep 5 and Pep 11 caused moderated edema between 60 and 300 min.

Table 3 summarizes edema formation; it was observed in the presence of Pep 1, Pep 3, Pep 4, Pep 5, Pep 11 and Pep 13 (Fig. S31). Pep 11 shows more intense activity ($p < 0.01$) during the 300 min of the assay. The other peptides caused very reduced or no edema formation. Meanwhile, Pep 6, Pep 7, Pep 9, Pep 10, Pep 11, Pep 12, and Pep 13 caused hypernociception in the animals.

Comparing the activities of peptides with some conservation of their sequences, such as Pep 7 (KETNAKPPA-OH) and Pep 11 (NAKPPA-OH), the presence of some extra amino acids in the sequence of Pep 7 makes it less hemolytic and causes no edema but has a greater effect on nociception than Pep 11 (Tables 2 and 3). Both peptides must present a net charge of +1 at pH 7.0. The effects on nociception may be related to a better fit of the longer chain of Pep 7 to the pain receptors than Pep 11.

The open field test is used to evaluate the effects of the compounds in the pattern of the movements of the model animals. The open field tests access the exploratory activity of mice, trying to correlate the sequences of some repeated motions with inputs acquired during the exploration of new environments [66]. In this test, the locomotion of an anxious animal is strikingly diminished; in contrast, animals that are comfortable generally spend more time in the peripheral areas, and some alterations can occur, such as a reduction in rearing and grooming [67,68]. In this test, an increase in the number of squares (central and peripheral) of the observation arena represents an anxiolytic activity. The general activity shows that peptides Pep 4, Pep 5 and Pep 9 cause a significant alteration in rearing, while Pep 1, Pep 2, Pep 3, Pep 7, Pep 8 and Pep 11 reduce locomotion (Figs. S32 to S33). Table 3 summarizes the results of mechanical hyperalgesia, edema formation and the effects observed in open field tests (rearing and locomotion).

4. Conclusions

T. obscurus is an endemic species from North Brazil, where it is responsible for many scorpion incidents every year that result in severe envenoming. The major portion of this venom (approximately 70%) is composed of peptides, but NDBPs correspond to 5% of the crude venom. Despite the high number of NDBPs detected, only a few peptides have been structurally and functionally characterized. In the present study, *T. obscurus* venom was submitted to mass spectrometric analysis to profile its peptidome. Twenty-seven major peptides among the NDBPs were sequenced, and thirteen were synthetized and functionally characterized. Some of the novel peptides showed similarity to hypotensins, potassium channel toxins and the allergen 5 protein, but most do not match any known toxin.

Despite the prey-killing function, scorpion venom is generally not lethal to predators (humans, monkeys, birds, bears, and other animals). The venom is principally used to promote actions in victims due to

Table 3
Profile of oedematogenic, nociceptive, rearing and locomotion activities of short and linear peptides from venom of scorpion *T. obscurus*.

Peptides	Sequences	Oedema formation			Nociception			Rearing	Locomotion
		60 min	180 min	300 min	60 min	180 min	300 min	–	–
Pep-1	AEIDFSGIPEDIIKQI-OH	*	–	**	***	***	***	–	***
Pep-2	GCDALLSGDHGGLLSANGC-OH	–	–	–	–	–	–	–	*
Pep-3	LIPNDQLRSI-OH	*	***	**	–	–	–	–	***
Pep-4	NIALGQDQSGR-NH2	***	**	–	*	–	–	***	–
Pep-5	KIKEKLIEA-OH	***	*	**	*	–	–	***	–
Pep-6	WPNKIEPGK-OH	–	–	–	***	**	–	–	–
Pep-7	KETNAKPPA-OH	–	–	–	***	***	***	–	***
Pep-8	KIITPPIR-OH	*	–	–	*	–	–	–	***
Pep-9	KPVEPVG-OH	–	–	–	**	***	*	***	–
Pep-10	SESNTCG-OH	–	–	–	***	***	***	–	–
Pep-11	NAKPPA-OH	***	***	***	*	**	***	–	***
Pep-12	ILTGKLKCK-OH	**	–	–	***	***	***	–	***
Pep-13	ILPNDK-OH	**	–	***	*	**	***	–	***

(−) no significance.
* $p < 0.5$.
** $p < 0.01$.
*** $p < 0.001$.

uncomfortable pain and inflammatory actions caused by venom toxins, which are mainly peptides.

According to the results above, the NDBPs of this venom act synergistically and are multifunctional toxins, causing a series of actions in the victims/prey of *T. obscurus* stings. This potentiates inflammatory processes; some peptides may also alter rearing or locomotion of victims/prey. Undoubtedly, information about the complexity of the peptides that constitute the venom of *T. obscurus* will contribute to a better understanding of the complex mechanism of envenoming pathogenesis caused by this venom.

Transparency document

The http://dx.doi.org/10.1016/j.jprot.2017.09.006 associated with this article can be found, in online version.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2017.09.006.

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