# Nigriventrine: A low molecular mass neuroactive compound from the venom of the spider *Phoneutria nigriventer*

Paulo C. Gomes [a], Bibiana M. de Souza [a], Nathalia B. Dias [a], Lilian M.M. Cesar-Tognoli [a], Luiz C. Silva-Filho [b], Cláudio F. Tormena [c], Roberto Rittner [c], Michael Richardson [d], Marta N. Cordeiro [d], Mario S. Palma [a,*]

[a] Department of Biology/CEIS, Institute of Biosciences, São Paulo State University (UNESP), Rio Claro, Sao Paulo, Brazil
[b] Department of Chemistry, Faculty of Sciences, São Paulo State University (UNESP), Bauru, Sao Paulo, Brazil
[c] Chemistry Institute, State University of Campinas, Campinas, Sao Paulo, Brazil
[d] Research and Development Center, Ezequiel Dias Foundation (FUNED), Belo Horizonte, Minas Gerais, Brazil

## ARTICLE INFO

## ABSTRACT

Nigriventrine was isolated from the "armed" spider *Phoneutria nigriventer*, in which it constitutes about 0.4% of the total venom content. Its structure was determined to be [1,1′-(1-hydroxyhydrazine-1,2-diyl)bis(oxy)bis(4-hydroxy-2,6-dioxopiperidine-4 carboxylic acid)] by NMR, HR-ES/IMS and MS/MS methods. The intracerebroventricular application of nigriventrine in rat brain, followed by the detection of c-Fos protein expression, indicated that the compound was neuroactive in the motor cortex, sensory cortex, piriform cortex, median preoptic nucleus, dorsal endopiriform nucleus, lateral septal nucleus and hippocampus of rat brain. Nigriventrine causes convulsions in rats, even when peripherally applied.

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## 1. Introduction

Spiders of the genus *Phoneutria* (Aranae, Ctenidae) are commonly known as "armed spiders" or "banana spiders" because of the aggressive attack–defence position they assume when facing their prey or enemies and because of their high incidence in banana plantations. These spiders are widely distributed in the warm regions of South America, and several species have been described (Keyserling, 1891). *Phoneutria nigriventer* is the most common species in the central and southeastern regions of Brazil (Richardson et al., 2006). These spiders are solitary animals that are characterised by wandering habits and are very aggressive. They are also responsible for many severe cases of envenoming, which sometimes results in the death of the victims (Silva et al., 2008). Frequently, the victims of envenomation by *P. nigriventer* show symptoms of neurotoxicity, such as convulsions (Le Sueur et al., 2003).

Spider venoms are considered rich sources of low molecular mass (LMM) compounds, which act mainly on the nervous system and present a wide range of pharmacological effects on synaptic transmission. Spider venoms are complex mixtures of peptides, proteins, and low molecular masses organic molecules. As detailed in Escoubas et al. (2000) the LMM compounds frequently reported in these venoms are free acids (such as citric and lactic), glucose, free amino acids, biogenic amines (such as diaminopropane, putrescine, cadaverine, spermine, and spermidine), and

neurotransmitters (such as aspartate, glutamate, serotonin, histamine, γ-butyric acid, dopamine, and epinephrine). Several of these compounds are neurotransmitters, whereas others block ion channels at the neuronal level. Generally, low molecular mass neurotoxins offer great potential as neurochemical tools to investigate the nervous system. Additionally, they may constitute new models in the drug-screening field for pharmaceutical and agrochemical industries (Palma and Nakajima, 2005). Despite the wide number of LMM compounds already characterised in these venoms, many others remain to be discovered.

Some classes of LMM toxins have been reported in spider venoms, including I) acylpolyamines – isolated from the venoms of orb-web-spiders; some of these are neurotoxic and act as antagonists for different subtypes of ionotropic glutamate receptors, whereas others act on nicotinic acetylcholine receptors (Palma and Nakajima, 2005); II) bis-(agmatine)-oxamide – isolated from the venom of the "fisher-spider", *Plectreurys tristis* (Quistad et al., 1993); III) nucleosides-toxins – mono or disulfated nucleoside compounds that are able to block kainate receptors and act on type-l calcium channels, such as the toxin HF-6 isolated from the venom of *Hololena curta* (Taggi et al., 2004); IV) tetra-hydro-β-carbolines – alkaloid compounds isolated from the venom of the social spider *Parawixia bistriata* (Cesar et al., 2005) and from the web droplets of the orb-web-spider *Nephila clavipes* (Marques et al., 2005); these compounds act as reversible inhibitors of monoamine oxidase (MAO) and are very toxic to insects and are neurotoxic, convulsivant and lethal to rats (Saidemberg et al., 2009).

LMM neurotoxins have been reported in insect venoms, such as the philantho toxins, which are simple types of acylpolyamine toxins isolated from the venom of the solitary wasp *Philanthus triangulum*. These venoms act at the level of both NMDA-dependent glutamate receptors and nicotine acetylcholine receptors (Tikhonov et al., 2004). Polybioside, a histaminyl glucoside compound, was recently isolated from the venom of the social wasp *Polybia paulista* and is neuroactive at the level of AMPA/NMDA-glutamate receptors (Saidemberg et al., 2010).

Identifying the neuroactivity of novel natural compounds requires mapping the action of these compounds at the level of the mammalian central nervous system (CNS). Generally, this is done by intracerebroventricular (ICV) application of the compounds in rat brain followed by the use of immunohistochemical methods to detect the expression of c-Fos protein. The expression of c-Fos has been used as a biochemical marker to identify stimulated neurons (Morgan and Curran, 1991). This protein is expressed by the proto-oncogene c-Fos, which is an immediate expression gene and is rapidly activated by neuronal cell stimuli, such as neurotransmitters and trophic factors. The expression of this gene triggers the expression of other specific genes by intracellular secondary messengers, which in turn trigger a series of biochemical events in the cell (Saidemberg et al., 2010). Therefore, c-Fos protein expression is a useful tool for analysing neuronal activation and for determining whether the compound under investigation presents neuroactivity. This method also identifies the brain regions that are the targets of this compound (Lino de Oliveira et al., 2001).

We undertook a chemical study of the LMM compounds present in the venom of the armed spider *P. nigriventer*, which resulted in the isolation and structural elucidation of nigriventrine by ^{1}H and ^{13}C NMR, 2D NMR (gCOSY, gHSQC, and gHMBC), ESI-MS, ESI-MS/MS, and HRESI methods. The ICV administration of nigriventrine in rat brain, the immunohistochemical labelling of CNS neurons for the detection of c-Fos protein and dual-label immunohisto-chemistry for NMDA-GluR1 were indicated that it has neuroactive properties.

## 2. Material and methods

### 2.1. P. nigriventer spiders

The spiders were collected in the region of Santa Barbara (19°34′S, 42°58′W) at Minas Gerais State, Brazil. The spiders were kept in the Scientific Aracnidarium of Fundação Ezequiel Dias (Belo Horizonte, Brazil) in plastic boxes at room temperature with food and water *ad libitum*.

### 2.2. Venom extraction

Venom was extracted by electrical stimulation of the fangs as described by Barrio and Vital Brazil (1949). The venom was immediately transferred to siliconised glass tubes in an ice bath, diluted with the same volume of distilled water and centrifuged at $4.000 \times g$. The supernatant was lyophilised and stored at $-18\,°C$ until use.

### 2.3. Purification

The crude venom of *P. nigriventer* (750 mg) was initially subjected to reverse-phase liquid chromatography (RP-HPLC) in an SHIMADZU instrument, mod. LC10AD, using a semi-preparative column C4 Vydac ($46 \times 250$ mm, 10 μm) under a gradient of acetonitrile (MeCN) from 0 to 70% (v/v) containing 0.1% (v/v) TFA for 150 min. The elution was monitored at 215 nm at a flow rate of 5 mL/min, and the fractions were manually collected into 5 mL glass vials and lyophilised. The fractions eluting between 10 and 15 min were collected, pooled, lyophilised and refractionated under reversed phase in a CapCell Pack-C18 column ($10 \times 250$ mm, 5 μm). The flow rate was 1.7 mL/min for 20 min using a gradient of MeCN from 0 to 30% (v/v) and containing 0.1% (v/v) TFA. The elution was monitored at 215 nm, and the fractions were manually collected into 5 mL glass vials, lyophilised and kept in a freezer at $-20\,°C$ until use.

### 2.4. Mass spectrometry analysis

All of the mass spectrometric analyses were performed in a triple quadrupole mass spectrometer (MICROMASS, mod. Quattro II). The instrument was outfitted with a standard electrospray probe (ESI - Micromass, Altrincham, UK). The samples were injected into the electrospray transport solvent using a micro syringe (500 μL) coupled to a micro infusion pump (KD Scientific) at a flow rate of 200 μL/h. The mass spectrometer was calibrated with a standard mixture of NaI and CsI from *m/z* 22.98 to 772.46. The samples were dissolved in 50% (v/v) acetonitrile [containing

0.1% (v/v) formic acid] and analysed in positive electrospray ionisation (ESI+) mode using the following conditions: a capillary voltage of 3.5 kV, a cone voltage of 40 V, a desolvation gas temperature of 80 °C, a nebuliser gas (nitrogen) flow of 20 L/h and a drying gas (nitrogen) flow of 20 L/h. The spectra were obtained in the continuous acquisition mode scanning from $m/z$ 50 to 3000 at a scan time of 10 s. The acquisition and treatment of data were performed with MassLynx software (Micromass, Altrincham).

### 2.5. MS/MS spectrometry analysis

The HRMS analyses were carried out in an ultrOTOF-Q-ESI-TOF (Bruker Daltonics, Billerica, MA, USA). The instrument was externally and internally calibrated using a 10 mg/mL Na+-TFA solution and by setting the instrument with the following parameters: end plate voltage of 3500 V; capillary voltage of 4000 V; capillary exit voltage of 300 V; skimmer-1 and skimmer-2 voltages of 1:50 V and 1:25 V, respectively.

### 2.6. NMR experiments

The NMR spectra were recorded at 25 °C on a Bruker DRX 500 operating at 500.11 MHz for ^{1}H and 125.08 MHz for [13]C. Measurements were carried out at a probe temperature of 300 K using sample concentrations of 500 μg/cm$^3$ in $D_2O$. Spectra were obtained for about 3 mg of compound in 0.7 mL of solution in $D_2O$, which was used as

a D lock. The samples were filtered to obtain better digital resolution. TMS was used as a reference for ^{1}H and [13]C. The $H_2O$ signal was partially suppressed by applying a presaturation sequence. [1]H, ^{13}C, DEPT, and two-dimensional gCOSY, gHSQC and gHMBC spectra were obtained. The signal for the remaining $H_2O$ was partially suppressed by applying a presaturation sequence (Braun et al., 1998).

### 2.7. Biological assays

#### 2.7.1. Monitoring the expression of Fos-protein
The method employed was performed as described previously (Gerfen and Sawchenko, 1984; Shu et al., 1988; Sita et al., 2003; Cesar et al., 2005).

Normal adult male Wistar rats weighing 250–300 g were housed two per cage with food and water *ad libitum* in a temperature controlled (21 ± 2 °C) room on a 12 h light–dark cycle from one week prior to experimentation to allow them to acclimate to their new environment. All experiments were carried out in accordance with the guidelines of the Institutional Committee for Research and Animal Care of the University of São Paulo and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996).

The guide cannula was implanted in the lateral ventricle (AP = −0.4; ML = −1.4; DV = −3.4) under anaesthetic action of a cocktail (0.2 mL/100 g) containing ketamine (1 mg), xylazine (5 mg), and acepromazine (0.2 mg) seven days before the application of nigriventrine. The animals
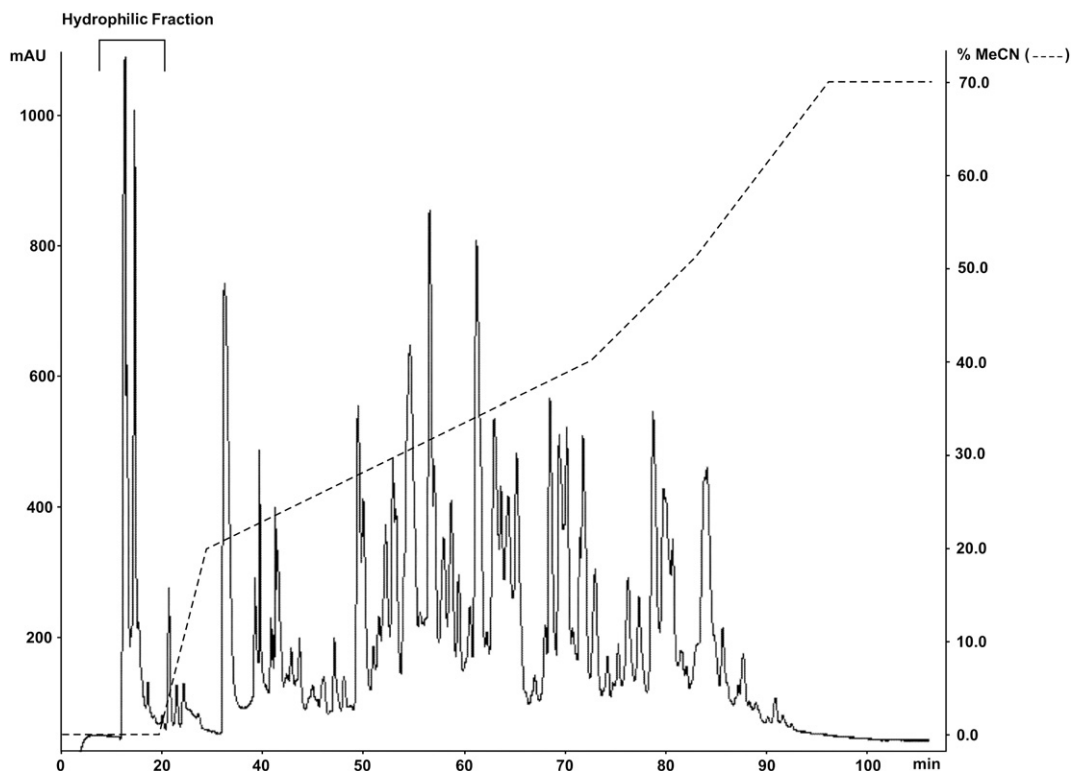


**Fig. 1.** Chromatographic profile of *Phoneutria nigriventer* venom (750 mg) under reverse-phase liquid chromatography (RP-HPLC) in an SHIMADZU instrument, mod. LC10AD, using a semi-preparative column C4 Vydac (46 × 250 mm, 10 μm) under a gradient of acetonitrile (MeCN) from 0 to 70% (v/v) containing 0.1% (v/v) TFA during 150 min. The elution was monitored at 215 nm at a flow rate of 5 mL/min. The fractions were manually collected into 5 mL glass vials and lyophilised.
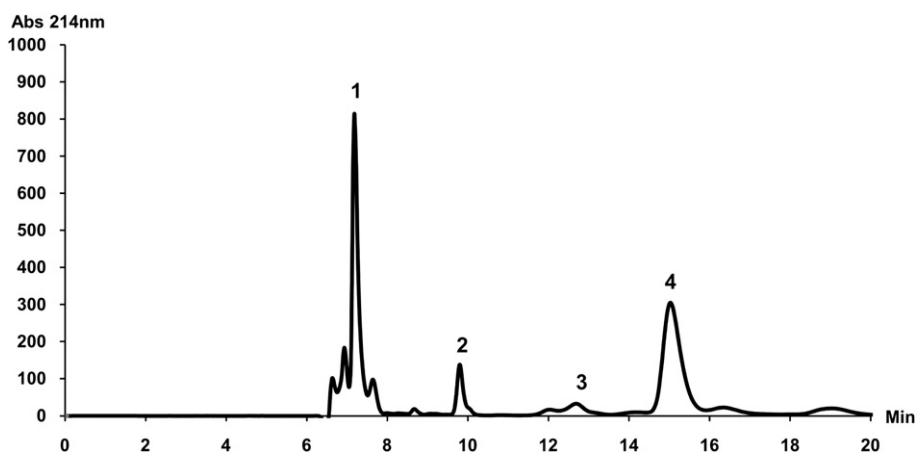
**Fig. 2.** Reversed-phase HPLC chromatogram of the pool of hydrophilic fractions of *Phoneutria nigriventer* venom in a semi-preparative column CapCell Pack-C18 (10 × 250 mm, 5 μm) at a flow rate of 1.7 mL/min using a gradient from 0 to 30% (v/v) of MeCN (containing 0.1% (v/v) TFA).

were manipulated twice a day for 10 min to avoid stress on the day of the experiment. The injection cannula was introduced approximately 2 h before the experiment to acclimate the animals and to minimise stress. Nigriventrine was solubilised in 10 μL of saline (0.9% w/v), and the compound was injected by intracerebroventricular (ICV) administration at a concentration of 1 ng/μL. The control group ($n = 6$) received only vehicle injection (saline: 0.9% w/v) to compare the effects of nigriventrine ICV administered in vehicle. Two hours were necessary for effective c-Fos induction. The animals were then anaesthetised with a lethal dose of the same anaesthetic cocktail used in the surgery (3 mL, intraperitoneal application) and perfused via the ascending aorta with cold 0.9% (m/v) saline (100 mL) followed by 4% (m/v) formaldehyde at pH 9.5 and 4 °C (800–1000 mL).

The brains were removed from the skull, post-fixed for 4 h in the same fixative with the addition of 20% sucrose and then transferred to 0.02 M potassium phosphate-buffered saline (KPBS) at pH 7.4 with 20% (m/v) sucrose. The brains were sliced in four series of coronal sections (at bregma 2.70 mm, −0.30 mm, −1.80 mm, and −3.14 mm) at a thickness of 30 μm with the use of a freezing microtome and stored at −20 °C in buffered antifreeze solution (Sita et al., 2003).

One series of each brain slice was stained by immuno-histochemistry as follows: sections were treated in 0.3% (v/v) peroxide in KPBS + 0.3% (v/v) Triton X-100 for 30 min and incubated in primary antiserum anti-c-Fos (PC38T IgG anti-c-Fos (Ab5) (4-17)) rabbit polyclonal antibody (Calbiochem, La Jolla, CA, USA) at 1:5000 and 3% (v/v)

normal goat serum in KPBS + 0.3% (v/v) Triton X-100 for 18 h at room temperature. Sections were rinsed in KPBS and incubated for 1 h in biotinylated secondary antiserum made from goat anti-rabbit antibody (Jackson Labs 1:1000) for one additional hour in avidin–biotin complex (Vector, 1:500). Next, the sections were incubated in dia-minobenzidine tetrahydrochloride (DAB; Sigma Chem Co.) and 0.01% (v/v) hydrogen peroxide dissolved in KPBS. The reaction was terminated after 2–3 min with repeated rinses in KPBS. Sections were mounted on slides and intensified with 0.005% (m/v) osmium tetroxide solution. To aid in the identification of brain regions presenting little or no c-Fos-immunoreactive neurons (mainly in the sections of control brain slices), Nissl method of counterstaining with thionin was used (Windle et al., 1943).

Photomicrographs were acquired through a Spot RT digital camera (Diagnostics Instruments) adapted to a Leica DMR microscope and an Apple Macintosh Power PC computer using the software Adobe Photoshop 5.0. Contrast, sharpness, colour balance and brightness were adjusted and images were combined in plates using Corel Draw 11 software.

### 2.8. Venous catheterisation

For the intravenous administration of nigriventrine, the rats were anaesthetised with chloral hydrate (7%, 350 mg/kg, ip) and submitted for venous catheterisation. A Silastic catheter containing heparinised saline (10 U/mL of pyrogen-free saline, Sigma, St. Louis, MO) was inserted into

**Table 1**
$^1$H and $^{13}$C-NMR data of the nigriventrine in D$_2$O.

| C | $\delta$ C (ppm) | $\delta$ H (ppm) | Multip. | $J$ (Hz) | H/H COSY | gHSQC | gHMBC |
|---|---|---|---|---|---|---|---|
| 1 | 43.729 (CH$_2$) | 2.75 (H$_a$) | D | 15.8 | H$_b$ | H$_a$, H$_b$ | – |
| | | 2.93 (H$_b$) | D | 15.8 | H$_a$ | – | – |
| 2 | 73.698 (C) | – | – | – | – | – | H$_a$, H$_b$ |
| 3 | 173.825 (C) | – | – | – | – | – | H$_a$, H$_b$ |
| 4 | 173.938 (C) | – | – | – | – | – | H$_a$, H$_b$ |
| 5 | 177,246 (C) | – | – | – | – | – | H$_a$ |

HMBC correlations are from the proton(s) stated to the indicated carbon; the other hydrogens could not be correlated with this technique.
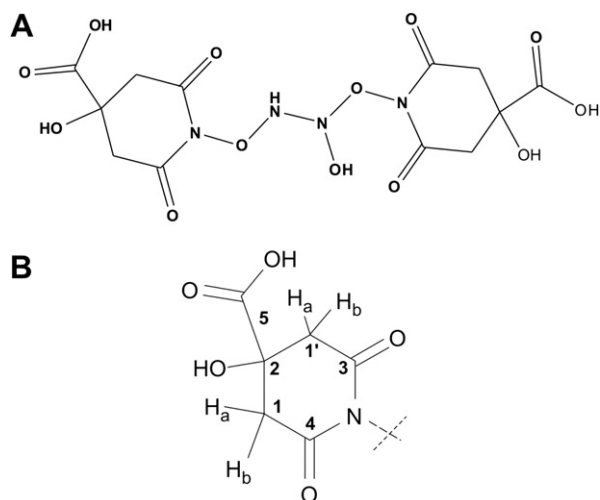
Fig. 3. A) Proposed structure of nigriventrine; B) detail of the piperidinyl moiety, showing the numbering of the carbons of ring ($C_1$, $C_{1'}$, $C_2$, $C_3$, $C_4$ and $C_5$) and assigning the positions of the methylenic hydrogens ($H_a$ and $H_b$).

the femoral vein and sutured in place. The free end of the catheter was passed under the skin of the back, exteriorised between the scapulae, and plugged with a sterile wire stylet. A week later, nigriventrine (100 ng kg$^{-1}$) was intravenously applied.

### 2.9. Statistical analysis

For the quantitative analysis of c-Fos-ir and/or NMR1-ir cells, three representative slices of each brain region were chosen for each rat. All of the areas expressing c-Fos/NMR1 protein were included in the analysis. Three different animals were used in this protocol. The number of cells was counted in a defined area as follows: 0.25 mm$^2$ for the piriform cortex, 0.5 mm$^2$ for the lateral septal nucleus dorsal, paraventricular nucleus of the hypothalamus, dorsomedial hypothalamic nucleus, reuniens nucleus, central medial nucleus, dorsal intermediate nucleus, and 1 mm$^2$ for the paraventricular thalamic nucleus and the pre-limbic cortex. The statistical analyses were performed using SigmaStat software and Student's $t$-test was used for comparisons between groups ($p < 0.05$).

### 3. Results and discussion

#### 3.1. Structural characterisation

The crude venom of *P. nigriventer* was initially fractionated under RP-HPLC in a C18 column and resulted in the elution of 55 fractions (Fig. 1), as previously reported by Richardson et al. (2006). Since we were interested in LMM hydrophilic compounds, the first two fractions that eluted between 10 and 15 min (assigned as hydrophilic fractions in Fig. 1) were collected, pooled, lyophilised and then refractionated in a CapCell Pak C18 column under a binary gradient of water-acetonitrile, which resulted in the elution of four fractions (Fig. 2). The ESI-MS analysis of these fractions revealed that only fraction 4 was pure enough (not shown results) to be chemically characterised. Thus, ESI-MS spectrum of the compound present in fraction 4 revealed a molecular ion of $m/z$ 423.0631 as [M + H]$^+$ (Fig. S1), which indicated that the molecular mass of the compound was 422.0631 Da. In order to carry out the structural elucidation of the purified compound, $^1$H and $^{13}$C NMR spectroscopy and HRESI-MS/MS were performed.

The NMR spectra of fraction 4 are presented in the supplemental information (Figs. S2–S5), while the spectroscopic data are represented in Table 1. In the $^1$H NMR
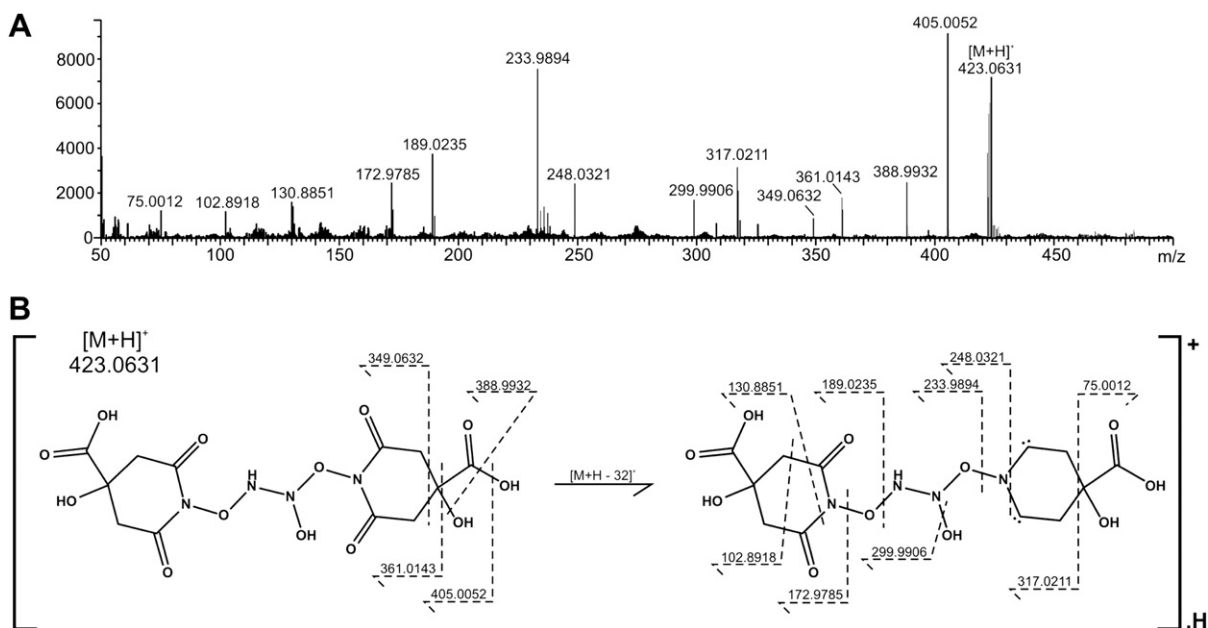


Fig. 4. A) Interpretation of the fragmentation pattern of HRESI-MS/MS for nigriventrine; B) HRESI-MS/MS spectrum for nigriventrine.
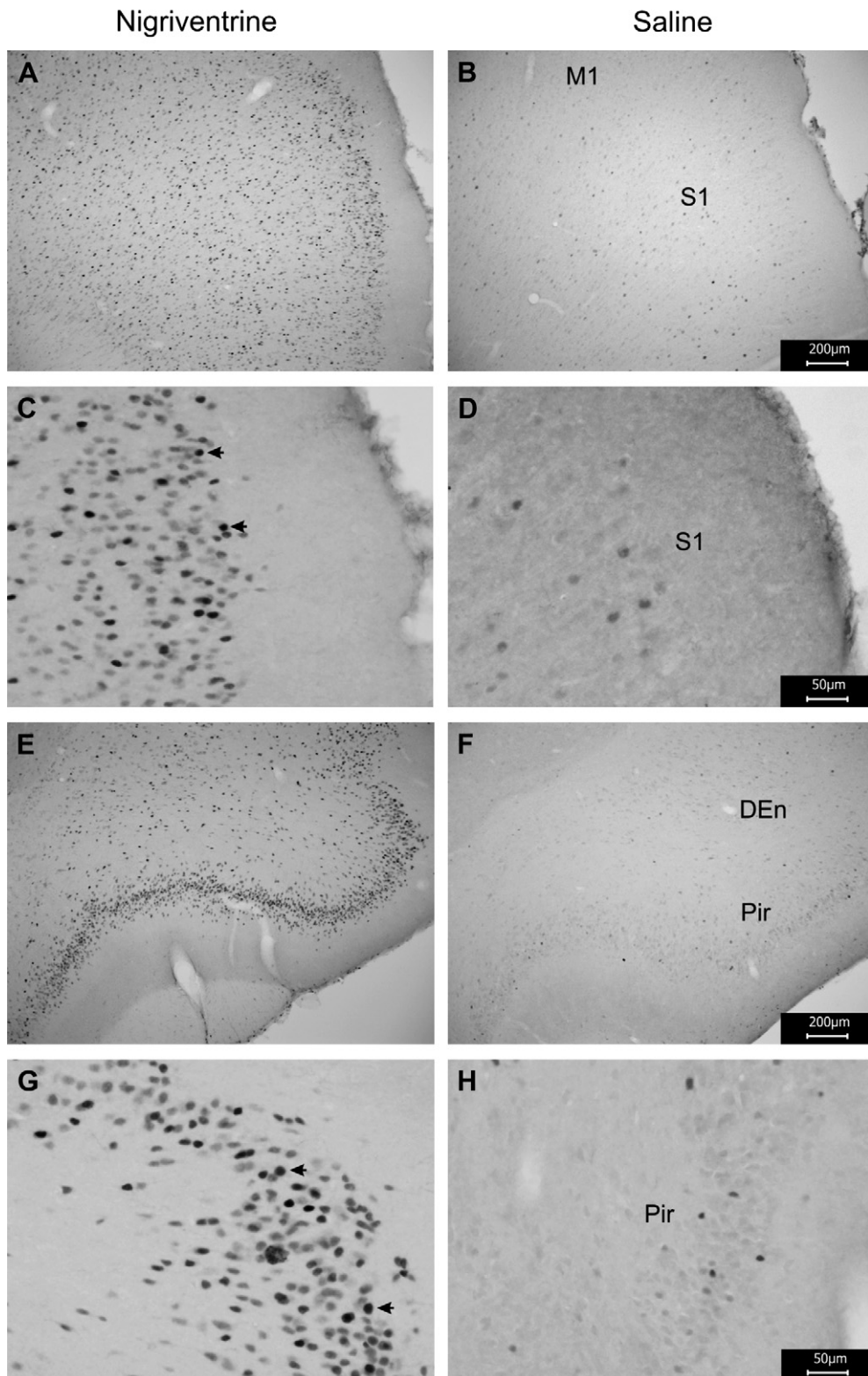
**Fig. 5.** Bright-field photomicrograph showing *Fos*-ir neurons following ICV administration of nigriventrine; the nuclei of Fos-ir neurons are stained in black. A and B: sensory and motor cortex; C and D; magnification of the sensory cortex to show some details of the Fos expression in these regions; E and F: piriform cortex and dorsal endopiriform nucleus; G and H: magnification of the piriform cortex to show details of Fos expression in these regions. A, C, E and G represent photomicrographs of rat brain regions under the effect of nigriventrine, whereas B, D, F and H correspond to the control regions (saline-treated). Abbreviations: M1: Motor cortex; S1: Sensory cortex; DEn: Dorsal Endopiriform nucleus; Pir: Piriform cortex. Scale bar = 50 μm in C and D/G and H and 200 μm in A and B/E and F.
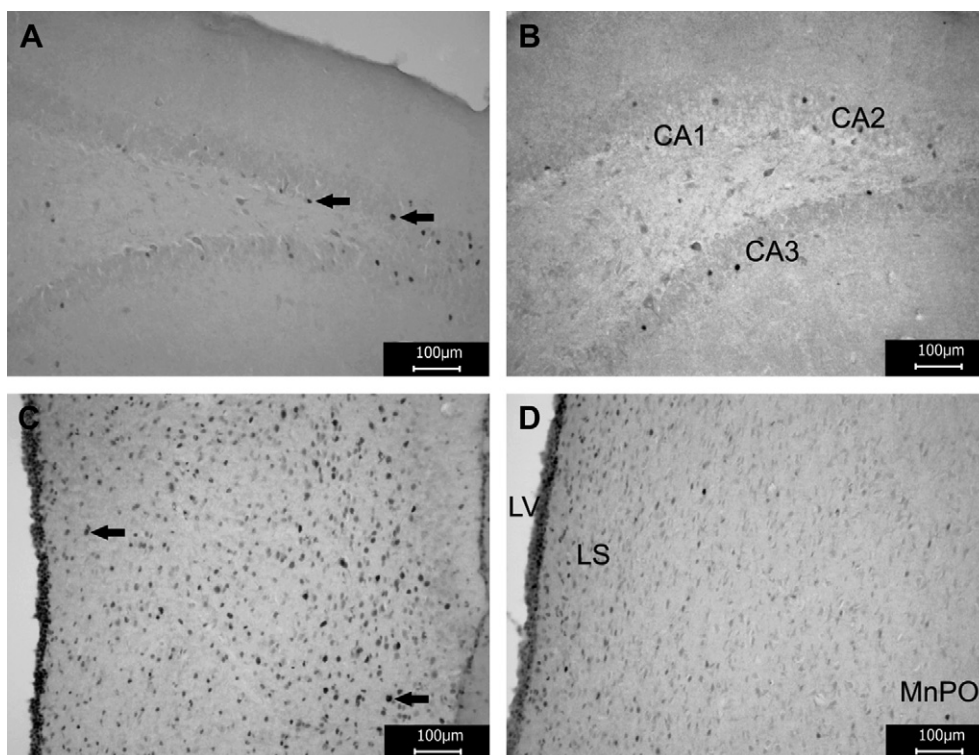
**Fig. 6.** Bright-field photomicrograph showing *Fos*-ir neurons following ICV administration of nigriventrine; the nuclei of Fos-ir neurons are stained in black. A and B: hippocampus; C and D; lateral septal nucleus and median preoptic nucleus. A and C represent photomicrographs of rat brain regions under the effect of nigriventrine, whereas B corresponds to the control regions (saline-treated). Abbreviations: CA1, CA2 and CA3: hippocampal region; LV: Lateral Ventricle; LS: Lateral septal nucleus; MnPO: Median preoptic nucleus. Scale bar = 100 μm.

spectrum (Fig. S3), two signals were observed and were confirmed by *g*-HMQC and COSY experiments (Figs. S4 and S5). These peaks corresponded to the methylene hydrogens (2.75 and 2.93 ppm), and their coupling constants (15.8 Hz) were characteristic of vicinal hydrogens. The $^{13}C$ NMR spectrum showed five signals: 43.7 ppm and 73.7 ppm signals, corresponding to methylene carbon and quaternary carbon, respectively. The signals 173.8 ppm, 173.9 ppm



**Fig. 7.** Number of *Fos*-ir neurons in each region of nigriventrine- and saline-treated (control) rat brain. Abbreviations: motor cortex (M1), sensory cortex (S1), piriform cortex (Pir), median preoptic nucleus (MnPO), dorsal endo-piriform nucleus (DEn), lateral septal nucleus (LS) and hippocampus (HIP). Results are reported as means (±S.E.M.); $p < 0.05$ as compared to the respective saline-treated group.

and 177.2 ppm (Table 1; Fig. S2) corresponded to carbonyl carbons of amide or acid functions.

The correlation between methylene hydrogens ($H_a$ and $H_b$) and all carbons ($C_1$, $C_1$, $C_2$, $C_3$, $C_4$ and $C_5$) was investigated in the gHMBC spectrum (Fig. S4), which indicated that a correlation did not exist between $H_b$ and $C_5$. This was due to the conformational arrangement of dihedral angles formed between $H_b$ and $C_5$, which were close to 90° according to the Karplus diagram (Jackman and Sternhell, 1978).

The interpretation of the spectroscopic data indicated that the compound of fraction 4 corresponds to the hydroxyl-hydrazyl-dioxopiperidine [1,1′-(1-hydroxyhydrazine-1,2-diyl)bis(oxy)bis(4-hydroxy-2,6-dioxopiperidine-4 carboxylic acid)], which was generically named nigriventrine (Fig. 3A); Fig. 3B details the structure numbering of the piperidinyl moiety.

The formula $C_{12}H_{14}N_4O_{13}$ was determined by HRESIMS ($m/z$ 423.0631 as $[M + H]^+$; calcd. 422.0508). The ESI-MS/MS spectrum in the positive mode for nigriventrine revealed main fragment ions with $m/z$ 405.0052, 388.9932, 361.0143, 349.0632, 317.0211, 299.9906, 248.0321, 233.9894, 189.0235, 172.9785, 130.8851, 102.8918, and 75.0012 as $[M + H]^+$ (Fig. 4A). The pattern of fragmentation revealed that the ions of $m/z$ 349.0632, 361.0143, 388.9932 and 405.0052 resulted from the fragmentation of the intact compound, whereas the ions of $m/z$ 75.0012, 102.8918, 130.8851, 172.9785, 189.0235, 233.9894, 248.0321, 299.9906 and 317.0211 resulted from the fragmentation of the molecule that lost two oxygens from
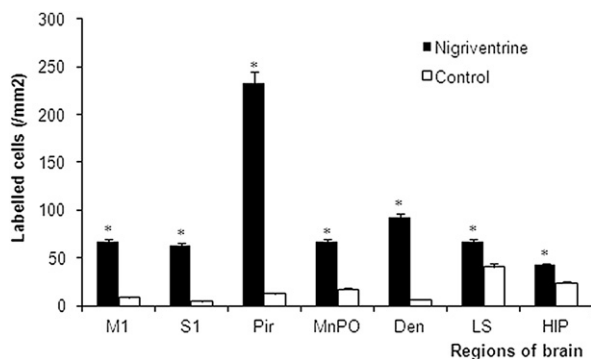
one of the piperidinyl moieties [M + H − 32] (*m/z* 370.0631), as represented in Fig. 4B. The pattern of fragmentation proposed in Fig. 4B fitted well with the chemical structure proposed for nigriventrine in Fig. 3A and corroborated the structure proposed by NMR analysis.

### 3.2. Mapping the neuronal activity of nigriventrine

Nigriventrine was ICV administered to male Wistar rats, and the c-Fos-immunoreactive (ir) neurons were counted in all active brain regions. Examination of the four coronal sections sliced from the rat brains revealed that seven brain regions expressed the c-Fos protein; therefore, the Fos-ir neurons of all these regions were mapped (Figs. 5 and 6) and counted (Fig. 7). Comparing the counting of nigriventrine-treated and saline-treated neurons revealed that the brain areas stimulated by nigriventrine were the motor cortex, sensory cortex, piriform cortex, median preoptic nucleus, dorsal endopiriform nucleus, lateral septal nucleus and hippocampus. The counting of Fos-ir neurons in these regions indicated that the stimulation of the piriform cortex was particularly high compared to the other regions (Fig. 5E and F; Fig. 7).

The widespread activation of c-Fos by nigriventrine in different populations of neurons of rat brain could be due to secondary actions resulting from the activation of specific brain regions because of the connectivity and network structure between spatially distributed brain areas. This finding has been previously reported for the spatiotemporal spreading of Fos induction by different types of stimuli (McIntosh et al., 2003; Tchelingerian et al., 1997). Different brain regions present different propensities for generating epileptiform activity in the presence of convulsant stimuli. The piriform cortex and the hippocampus have strong tendencies to generate epileptiform events. Specifically, the piriform cortex has a propensity to generate spontaneous interictal spikes, which in turn may result in epileptic events (Namvar et al., 2008; Rigas and Castro-Alamancos, 2004). It is interesting to note that the piriform cortex was the most intensely labelled region of c-Fos expression in the rat brain after treatment with nigriventrine (Fig. 7). Thus, this region may be the primary target of nigriventrine action, which may generate epileptic events that propagate through the brain to several other regions upon activation.

The toxicity of nigriventrine to rats was not evaluated due to the limited amount of material. However, animal behaviour under the effects of the nigriventrine was observed after ICV and peripheral application of the compound. The rats showed light convulsions 10 min after ICV application (10 ng kg$^{-1}$) of nigriventrine and were characterised by tonic–clonic crises that lasted up to 5 min. The animals' fur looked bristled, with partially diffuse piloerection localised around the neck and on the head. During the subsequent 15 min, the eyelids appeared partially closed with porphyrin accumulation around the eyes. The observed effects were transient, and the rats recovered fully after 30 min. In order to evaluate whether nigriventrine crosses the blood–brain barrier, it was peripherally administered to the animals (100 ng kg$^{-1}$). The same clinical signs as reported above were observed by peripherical administration of nigriventrine, although they appeared in a milder form. These results suggested that nigriventrine might cross the blood–brain barrier. This type of incident was relatively common with *P. nigriventer* bites and could explain some of the convulsive effects reported after this type of accident.

### 4. Conclusion

The compound hydroxyl-hydrazyl-dioxopiperidine [1,1′-(1-hydroxyhydrazine-1,2-diyl)bis(oxy)bis(4-hydroxy-2,6-dioxopiperidine-4 carboxylic acid)], generically named nigriventrine, was isolated and structurally characterised from the hydrophilic fraction of the venom from the "armed" spider *P. nigriventer*. It is a novel natural compound not previously reported amongst the venoms of venomous Arthropods.

The dioxopiperidine moiety is uncommon amongst the LMM compounds from animal venoms. It has already been reported as a basic building block of analgesic, anti-anxiety and anti-psychotic synthetic drugs (Gittos, 1989). This was the first report of a natural compound of animal origin presenting this type of chemical structure.

The neuroactivity of nigriventrine in rat brain was investigated through the monitoring of the pattern of expression of c-Fos protein, which is an inducible transcription factor whose activation is an important tool and a well-established marker to identify activated neurons in the autonomous or central nervous system after physical, chemical and/or biological stimuli (Kobelt et al., 2004). This assay revealed that nigriventrine acted in seven different brain regions: the motor cortex, sensory cortex, piriform cortex, median preoptic nucleus, dorsal endopiriform nucleus, lateral septal nucleus and hippocampus. In summary, nigriventrine may be considered a novel class of LMM spider venom toxin, belonging the group of hydroxyl-hydrazyl-dioxopiperidine compounds, which seems to be neuroactive at different rat brain regions. This the first LMM toxin reported in the venom of the "armed" spider *P. nigriventer* and must be more deeply investigated in the near future.

### Acknowledgments

### Appendix. Supplementary material

Supplementary material related to this article can be found online at doi:10.1016/j.toxicon.2010.11.021.

### Conflict of interest

The authors have no conflict of interest.

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