

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/41450175>

Polybioside, a Neuroactive Compound from the Venom of the Social Wasp *Polybia paulista*

ARTICLE in JOURNAL OF NATURAL PRODUCTS · FEBRUARY 2010

Impact Factor: 3.8 · DOI: 10.1021/np900424t · Source: PubMed

CITATIONS

5

READS

38

5 AUTHORS, INCLUDING:



Daniel Menezes Saidemberg

Shimadzu do Brasil

21 PUBLICATIONS 192 CITATIONS

SEE PROFILE



Luiz Carlos Da Silva-Filho

São Paulo State University

25 PUBLICATIONS 181 CITATIONS

SEE PROFILE



Claudio Tormena

University of Campinas

144 PUBLICATIONS 1,259 CITATIONS

SEE PROFILE



Mário S Palma

São Paulo State University

211 PUBLICATIONS 2,890 CITATIONS

SEE PROFILE

Polybioside, a Neuroactive Compound from the Venom of the Social Wasp *Polybia paulista*

Daniel M. Saidemberg,[†] Luiz C. da Silva-Filho,[‡] Lilian M. M. Cesar Tognoli,[†] Claudio F. Tormena,[§] and Mario S. Palma^{*,†}

Laboratory of Structural Biology and Zoochemistry, Department of Biology/CEIS, Institute of Biosciences, São Paulo State University (UNESP), 13506-900, Rio Claro, SP, Brazil, Department of Chemistry, São Paulo State University (UNESP), P.O. Box 473, 17033-360, Bauru, São Paulo, Brazil, and Chemistry Institute, State University of Campinas, P.O. Box 6154, 13084-971, Campinas, SP, Brazil

Received July 13, 2009

Polybioside (**1**) was isolated from the venom of the social wasp *Polybia paulista*, and its structure was assigned as 3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl 3-(1H-imidazol-4-yl)propanimidate by NMR and MS protocols. The application of polybioside in rat brain, followed by the detection of c-Fos protein expression in some brain regions, indicated the compound is neuroactive in a number of brain areas. Polybioside causes convulsions in rats, even when peripherally applied.

The Hymenoptera (bees, wasps, and ants) evolved their venoms and stinging apparatuses according to their biology and behavior mainly for targeting the nervous systems of victims as a defense and/or prey capture strategy. *Polybia paulista* is a very aggressive social wasp, endemic to Southeast Brazil, that causes thousands of stinging incidents every year.¹ Frequently, the victims of envenomation by social wasps show symptoms of neurotoxicity, such as convulsions and amnesia.^{2–6}

The low molecular weight (LMW) fraction from wasp venom contains a series of biological amines (histamine, serotonin, spermine, spermidine, adrenaline, and noradrenaline), some free amino acids (aspartic and glutamic acids), nucleotide triphosphates, nucleosides, and free sugars.^{7,8} Several of these compounds are neurotransmitters, while others block ion channels at the neuronal level.⁹ Wasp venom is considered a rich source of LMW compounds that act on several biological systems. They act mostly on the nervous system and show a wide range of pharmacological effects on synaptic transmission,⁶ however, there are still many other LMW compounds to be discovered in these defensive secretions.¹⁰

The identification of 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, which is used as a biochemical marker to identify the stimulated neurons.¹¹ The c-Fos protein is expressed by the proto-oncogene c-fos, which is an immediate expression gene (IEG). These IEGs are rapidly activated by neuronal cell stimuli, such as neurotransmitters and trophic factors, among others. The expression of these genes triggers the expression of other specific genes by intracellular secondary messengers, which in turn triggers biochemical events in the cell. Therefore, c-Fos protein expression is a useful tool for the analysis of neuronal activation, showing whether the compound under investigation presents some neuroactivity and the brain regions that are stimulated by the compound.^{12,13}

Consequently, we undertook a chemical study of the LMW compounds present in the venom of the social wasp *P. paulista*, which resulted in the isolation and structural elucidation of polybioside (**1**) by ¹H and ¹³C NMR, 2D NMR (gCOSY, gHSQC, and gHMBC), ESIMS, ESIMS/MS, and HRESI protocols. The ICV administration of polybioside (**1**) in rat brain and the immunohis-

Table 1. ¹H and ¹³C NMR Data of the α- and β-Anomers of Polybioside in D₂O

position	δC, mult.	position	δH (J in Hz)	HMBC ^a	COSY ^b
1	96.3, CH	1	4.52, d (7.9)		2
1'	92.4, CH	1'	5.12, d (3.4)		2'
2	74.5, CH	2	3.1, dd		
2'	73.1, CH	2'	3.39, dd		
3	71.9, CH	5	3.36, m		
3'	71.8, CH	3, 4, and 6	3.00–4.00, m		
4	69.9, CH	8	3.03, t (7.3)	10, 11	
4'	69.5, CH	9	3.22, t (7.3)	10	11
5	76.3, CH	10			
5'	76.1, CH	11	7.26, s	10, 12	12
6	61.1, CH ₂	12	8.54, s	10, 11	11
6'	60.9, CH ₂				
7	163.5, qC				
7'	163.2, qC				
8	38.3, CH ₂				
9	22.5, CH ₂				
10	128.7, qC				
11	117.3, CH				
12	134.2, CH				

^a HMBC correlations are from proton(s) stated to the indicated carbon. ^b In the COSY spectrum there is a weak long-range correlation between H9 and H11; the other hydrogens could not be correlated by this technique.

tochemistry labeling of CNS neurons for the detection of c-Fos protein were consistent with its neuroactive property.

Results and Discussion

The crude venom of the social wasp *P. paulista* was extracted with 50% (v/v) MeCN and fractionated by RP-HPLC using a gradient from 5% (v/v) to 60% (v/v) MeCN in a reversed-phase C18 column. Fifteen fractions were eluted (Figure S1a), collected, lyophilized, and ICV applied in rat brain. Fraction 1 caused convulsions and was submitted for refractionation by RP-HPLC under isocratic conditions with 4% (v/v) MeCN (containing 0.1% TFA). Two fractions eluted from the second chromatography, at 6.41 (fraction 1A) and 8.45 min (fraction 1B) (Figure S1b). The structural characterization of fraction 1A afforded compound **1**, while fraction 1B contained mostly histamine. Thus, the focus of the present investigation was concentrated on fraction 1A.

Compound **1** was isolated as a pale white, amorphous powder. Its molecular formula of C₁₂H₁₉N₃O₆ was determined by HRESIMS (*m/z* 302.1406 [M + H]⁺, calcd 302.1275), which indicated five degrees of unsaturation. The ¹³C NMR spectra in D₂O (Table 1) corroborated the assigned molecular formula.

The ¹H and ¹³C NMR data of compound **1** are shown in Table 1. The ¹H NMR spectrum showed signals in the region δ_H 3.0 to

* To whom correspondence should be addressed. E-mail: mspalma@rc.unesp.br. Phone: +55-19-35264163. Fax: +55-19-35264365.

[†] Institute of Biosciences, UNESP/Rio Claro.

[‡] Department of Chemistry, UNESP/Bauru.

[§] Chemistry Institute, UNICAMP, Campinas.

5.5 (Figure S2), while the ^{13}C NMR spectrum showed corresponding signals in the region δ_{C} 60 to 100 (Figure S3), both with chemical shifts characteristic of carbohydrates, more specifically of glucose by comparison with data in the Biological Magnetic Resonance Data Bank (<http://www.bmrb.wisc.edu/metabolomics>). In the ^{13}C NMR spectrum (Figure S3), the signals were duplicated, indicating the presence of the α - and β -anomers of glucose. The two anomeric forms were also visible in the ^1H NMR spectrum (Figure S2) at δ 5.12 for H-1 with $^3J_{\text{H1H2}} = 3.4$ Hz for the α -anomer and δ 4.52 for the β -anomer with $^3J_{\text{H1'H2'}} = 7.9$ Hz. The ^1H NMR spectrum also showed the presence of two triplets at δ 3.03 and 3.22, both with coupling constants of $^3J_{\text{H8H9}} = 7.3$ Hz. These triplets were assigned to two linked CH_2 groups, which resonated in the ^{13}C NMR and DEPT spectra at δ_{C} 22.5 and 38.2. The ^{13}C NMR spectrum also displayed signals at δ_{C} 163.2 and 163.5, which were proposed to arise from an imino-ester group linked to the anomeric carbon of the glucose. These signals were absent in the DEPT spectrum (Figure S4), confirming that they originated from a quaternary carbon. The spectrum showed a resonance at δ_{C} 128.7, which was absent in the DEPT spectrum, suggesting the presence of a second quaternary carbon. The ^1H NMR spectrum additionally exhibited two singlets at δ_{H} 7.26 and 8.24, corresponding to two CH groups in the ^{13}C NMR spectrum at δ_{C} 117.3 and 134.2, respectively.

The gCOSY spectrum (Figure S5) revealed the ^1H – ^1H spin–spin coupling correlation of H-1 to H-2, H-1' to H-2', and H-11 to H-12. It was also possible to visualize a weak long-range coupling between H-9 and H-11 due to a resonant double bond between C-11 and C-10. The HSQC spectrum (Figure S6) showed correlations of H-1 to C-1, H-1' to C-1', H-8 to C-8, H-9 to C-9, H-11 to C-11, and H-12 to C-12. Expansion of the gHSQC spectrum (Figure S7) allowed the assignment of the chemical shift of H-5 (by correlation with C-5) and C-2 once the H-2 chemical shift was inferred by its correlation with H-1 through the COSY spectrum. Interpretation of the gHMBC spectrum (Figure S8) established the long-range correlation of C-11 to H-12 and H-8, C-12 to H-11, and quaternary carbon C-10 to H-8, H-9, H-11, and H-12 due to a double bond. These typical ^1H and ^{13}C chemical shifts and correlations strongly supported the presence of an imidazole ring in the structure. The carbons and hydrogens of glucose were difficult to correlate in the 2D spectra due to their close proximity with the diagonal correlation line.

The interpretation of the spectroscopic data indicated that this molecule was a glucoside of imidazolylpropanimide, corresponding to 3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl 3-(1H-imidazol-4-yl)propanimide, and was generically named polybioside (1) (Figure 1).

The ESIMS/MS spectrum of 1 revealed main fragment ions at m/z 286, 284, 275, 274, 268, 254, 250, 235, 221, 207, 190, 189, 183, 180, 173, 163, 147, 140, 138, 130, 122, 112, 110, 98, 95, and 81 as $[\text{M} + \text{H}]^+$ ions (Figure 2). A fragmentation scheme based on this spectrum is shown in Scheme 1. The m/z 284 and 266 ions were formed by the loss of one and two water molecules from the molecular ions $[\text{M} + \text{H} - 18]^+$ and $[\text{M} + \text{H} - 36]^+$, respectively (Scheme 1). Fragmentation of the methylene units and of the imine group resulted in the formation of two series of fragment ions, i.e., those having charge retention in the glucosyl side (m/z 235, 221, 207, 180, and 163) and their opposite fragments, i.e., those ions having a charge retention on the imidazole side (m/z 81, 95, and 122). Loss of the imidazolyl group (m/z 67) from the molecular ion resulted in the formation of the m/z 235 ion. The m/z 189 and 222 ions arose from fragmentations of the structure formed after the loss of one water molecule from the molecular ion, while m/z 127 and 248 ions originated from fragmentations of the structure resulting from the loss of two water molecules from polybioside. The m/z 275 ion apparently originated from fragmentation of the imidazole ring with subsequent formation of the azirine in a similar pattern of fragmentation and ring rearrangement to that previously

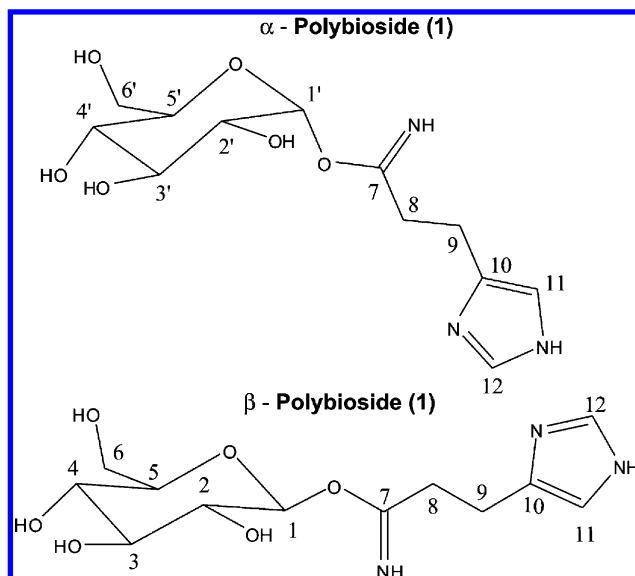


Figure 1. α - and β -Anomers of polybioside.

reported for 1-methylimidazole.¹⁴ The fragmentation of the azirine cation from the m/z 275 ion led to the formation of the m/z 234 ion; the m/z 113 ion is typical of a structure bearing the azirine group. The loss of $-\text{NH}_2$ from polybioside, followed by hydrogen rearrangement at positions 7 and 8, resulted in the formation of the m/z 286 fragment ion (Scheme 1); the m/z 106, 254, and 268 fragment ions are characteristic of this structure. The pattern of fragmentation proposed in Scheme 1 fits well with the chemical structure proposed for polybioside (1) in Figure 1, corroborating the structural elucidation made by NMR analysis.

Mapping the Neuronal Activity of Polybioside. Polybioside (1) was ICV administered to male Wistar rats, and the c-Fos-immunoreactive neurons were counted in all active brain regions. Examination of the four coronal sections sliced from the rat brains revealed that 12 brain regions expressed the c-Fos protein; therefore, all of these regions were mapped (Figure S9 and S10), and the c-Fos-immunoreactive neurons were counted (Table 2). The comparison between the counting of polybioside-treated and saline-treated neurons revealed that 11 brain areas were stimulated by polybioside (piriform cortex, lateral septal nucleus dorsal, anterior hypothalamic area, paraventricular nucleus of hypothalamus, dorsomedial nucleus of hypothalamus, hippocampus, reuniens nucleus, rhomboid nucleus, central medial nucleus, dorsal intermediate nucleus, and paraventricular nucleus of thalamus). Only in the central amygdaloid nucleus the number of c-Fos-ir cells was not statically significant. The widespread activation of c-Fos by polybioside in several 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, as previously reported for the spatiotemporal spreading of Fos induction by different chemical/physical stimuli.^{15,16}

Different brain regions present different propensities to generate epileptiform activity in the presence of convulsant stimuli.¹⁷ The piriform cortex and the hippocampus have strong tendencies to generate epileptiform events;^{17,18} the piriform cortex has a propensity to generate spontaneous interictal spikes, which in turn may result in epileptic events.¹⁹ Additionally, the hippocampus is known to play a role in convulsions by recruiting NMDA receptors after the excessive firing of neurons, helping to maintain and prolong seizures.¹⁷ It is interesting to note that the piriform cortex and the hippocampus are among the most intensively labeled regions of c-Fos expression in the rat brain after treatment with polybioside (Table 2). Thus, it may be speculated that these regions may be the primary target of polybioside action, which, upon activation,

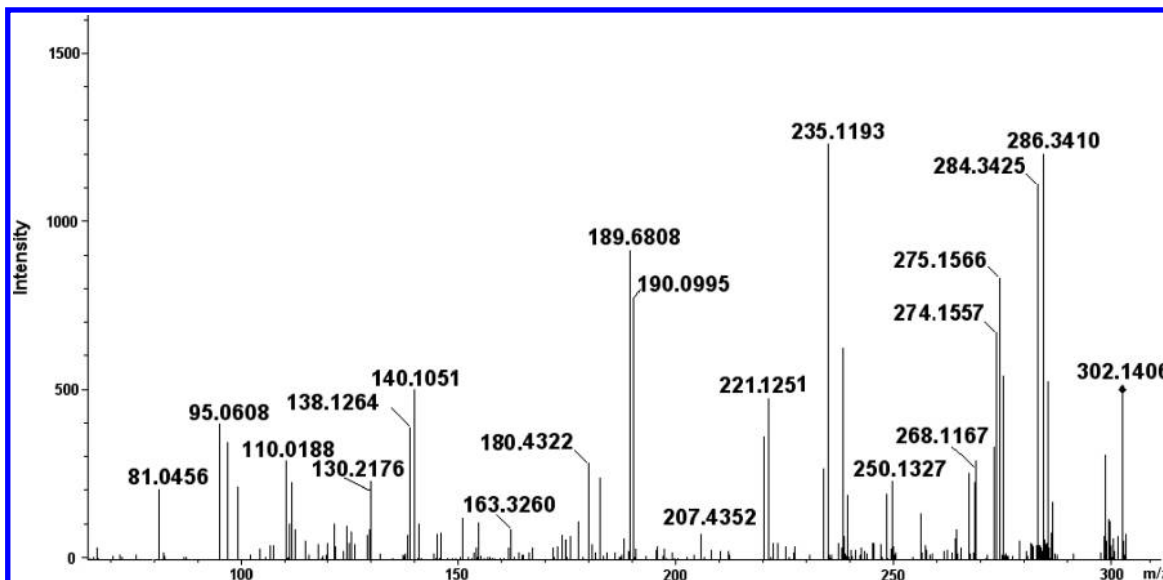
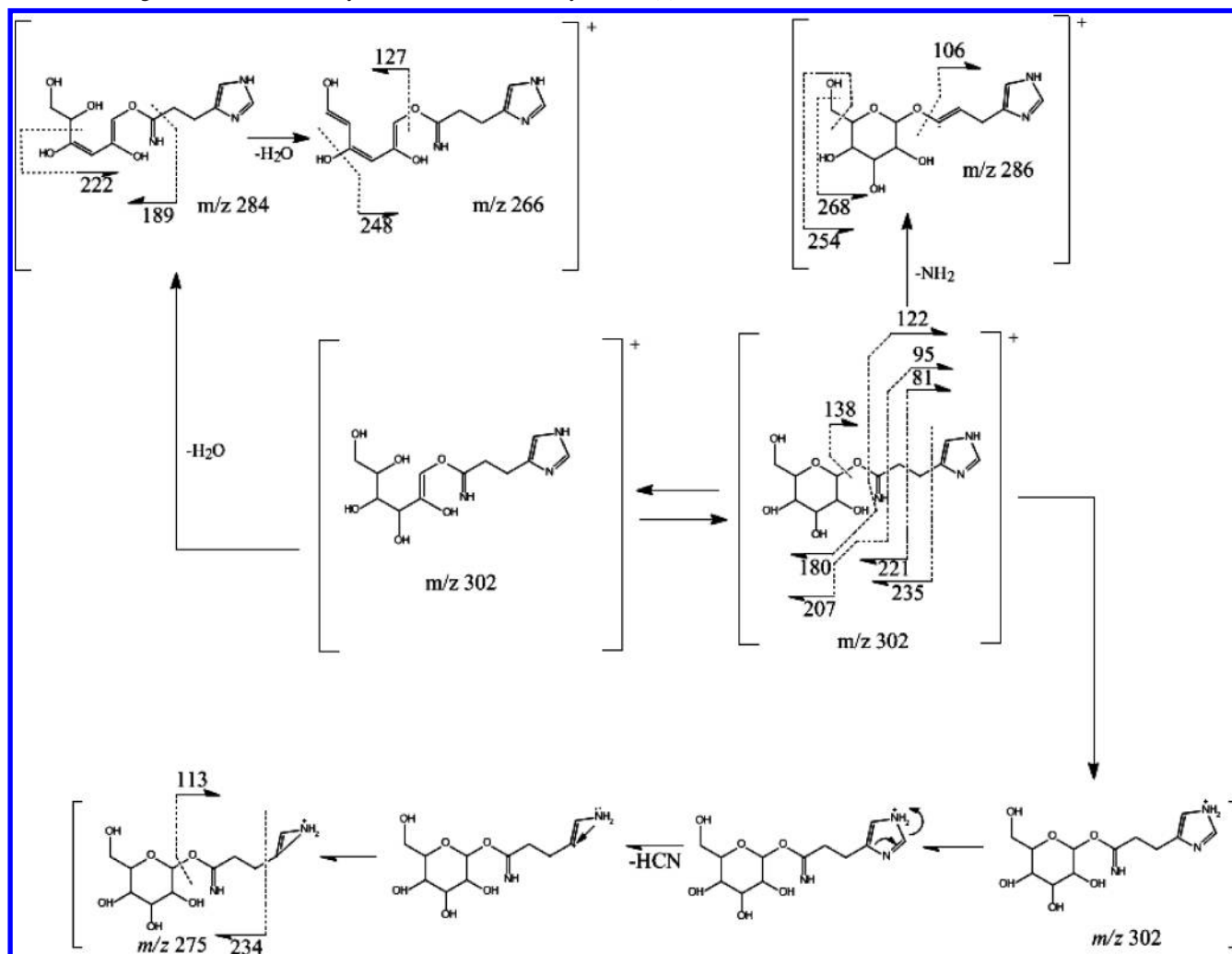


Figure 2. HRESIMS/MS spectrum of polybioside (1).

Scheme 1. Fragmentation Obtained by HRESIMS/MS for Polybioside (1)



may generate epileptic events that propagate through the brain to several other regions.

The toxicity of polybioside (1) to rats was not evaluated due to the limited amounts of material. However, animal behavior under the effect of polybioside was observed after ICV and peripheral application of the compound. The rats showed convulsions 5 min

after the ICV application (10 ng kg^{-1}) of polybioside, characterized by tonic-clonic crises, which lasted up to 10 min. The animals' fur looked bristled with partially diffuse piloerection localized around the neck and on the head. During the subsequent 20 min, the eyelids appeared partially closed with porphyrin accumulation around the eyes. After 30 min, the animals lost sensor motor reflexes, as

Table 2. Number of c-Fos-Immunoreactive Neurons in Different Brain Regions of Wistar Rats, Responding to the Presence of Polybioside (**1**) (0.1 $\mu\text{g}/\mu\text{L}$), in Comparison with the Control (0.9% (w/v) saline solution)^a

brain area	polybioside	saline (control)
piriform cortex	154 \pm 5*	7 \pm 3
lateral septal nucleus dorsal	102 \pm 7*	5 \pm 3
anterior hypothalamic area	124 \pm 7*	40 \pm 5
paraventricular nucleus of hypothalamus	79 \pm 7*	9 \pm 3
dorsomedial hypothalamic nucleus	14 \pm 2*	2 \pm 1
hippocampus	83 \pm 6*	3 \pm 2
reuniens nucleus	18 \pm 3*	2 \pm 1
rhomboid nucleus	10 \pm 2*	2 \pm 1
central medial nucleus	13 \pm 3*	2 \pm 1
dorsal intermediate nucleus	13 \pm 2*	2 \pm 1
paraventricular nucleus of thalamus	39 \pm 3*	3 \pm 2
central amygdaloid nucleus	7 \pm 4	3 \pm 2

^a The c-Fos-immunoreactive cells were counted on fixed 1 mm² areas defined as follows: 0.2 mm² (piriform cortex, lateral septal nucleus dorsal, paraventricular nucleus of hypothalamus, dorsomedial hypothalamic nucleus, reuniens nucleus, central medial nucleus, and dorsal intermediate nucleus) or 1 mm² (for the anterior hypothalamic area, hippocampus, paraventricular nucleus of thalamus, and central amygdaloid nucleus). The attributed values are the mean (\pm SD) of the c-Fos-positive cells ($n = 3$); (*) represents the significant counting values in relation to the respective controls for $p < 0.05$ (Student's t -test).

estimated by the righting reflexes and the wire suspension test. The observed effects were transient, and the rats recovered fully after 80 min. In order to evaluate whether polybioside crosses the blood–brain barrier, compound **1** was peripherally administered to the animals (100 ng.kg⁻¹). The same clinical signs as reported above were observed, although they appeared in a milder form. These results suggested that polybioside might cross the blood–brain barrier. The natural abundance of this compound in *P. paulista* venom is about 0.5 $\mu\text{g}/\text{insect}$; thus, an adult man (about 70 kg) would require about 14 stings from the *P. paulista* wasp in order to become envenomated at a dose of 100 ng.kg⁻¹, which would lead to clinical signs similar to those described above. This type of incident is relatively common and could explain some of the convulsive effects reported after this type of accident.

Experimental Section

General Experimental Procedures. The NMR spectra were recorded at 25 °C on a Bruker DRX 500 operating at 500.11 MHz for ¹H and 125.08 MHz for ¹³C. Spectra were obtained for about 3 mg of compound in 0.7 mL of solution in D₂O, which was used as a D lock. The samples were filtered in order to obtain better digital resolution. The reference used was TMS for ¹H and ¹³C. The H₂O signal was partially suppressed by applying a presaturation sequence. ¹H, ¹³C, DEPT, and two-dimensional gCOSY, gHSQC, and gHMBC spectra were obtained.

The MS analyses were performed in a triple quadrupole mass spectrometer (Micromass, model Quattro II). The instrument was equipped with a standard electrospray probe (ESI-Micromass, Altrincham). The samples were injected into the electrospray transport solvent system using a microsyringe (500 μL) coupled to a microinfusion pump (KD Scientific) at a flow rate of 4 $\mu\text{L}/\text{min}$. The mass spectrometer was calibrated with a NaI/CsI mixture and the typical cone-voltage peaks to operate at a resolution of 4000 units. The samples were dissolved in 50% (v/v) MeCN [containing 0.1% (v/v) formic acid] for analysis by positive electrospray ionization (ESI+) using typical conditions: a capillary voltage of 3.5 kV, a cone voltage of 30 V, a desolvation gas temperature of 80 °C, a flow of nebulizer gas (nitrogen) of about 15 L/h, and a drying gas (nitrogen) flow of about 200 L/h. The spectra were obtained in the continuous acquisition mode scanning from m/z 50 to 1000 for a scan time of 5 s. The acquisition and treatment of data were performed with MassLynx software.

The HRMS analyses were carried out in an ultratOF ESI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA) coupled to a microinfusion pump operating at a flow rate of 150 $\mu\text{L}/\text{h}$ using 50%

(v/v) MeCN as the solvent. The instrument was externally and internally calibrated using a 10 mg/mL Na⁺-TFA solution and setting the instrument with the following parameters: end plate, 3500 V; capillary, 4000 V; capillary exit, 310 V; skimmer 1, 50 V; skimmer 2, 25 V.

HPLC separations were performed on a LC-10AD (Shimadzu) apparatus equipped with a Nucleosil C18 column (Shiseido) under reversed-phase conditions.

Biological Material. The *Polybia paulista* wasps (Hymenoptera, Vespidae) were collected on the campus of UNESP at Rio Claro-SP, southeast Brazil, (S22°23'42.9" S; 047°32'32.5" W). The wasps were immediately frozen after collection and stored at -20 °C until use. A voucher specimen was deposited in the Hymenoptera Collection of the IBRC-UNESP under the code Polpa 1.01.14.

Adult male Wistar rats used in the bioassays were obtained from the Central Bioterium of UNESP and kept in the bioterium of IBRC-UNESP/Rio Claro, SP. The animals (weighing 250–300 g) were housed two per cage at 21 °C \pm 2 in a light–dark cycle (12 h) controlled room for one week prior to experimentation to allow them to acclimate. Food and water were freely available. All experiments were carried out in accordance with the guidelines of the Institutional Committee for Research and Animal Care of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.²⁰

Extraction and Isolation. The venom was obtained by dissection of the individuals with microscissors; the venom glands were removed and washed with 5% (v/v) MeCN in deionized H₂O for 15 min at 4 °C to extract the low molecular weight compounds. The extract was centrifuged at 8000g for 15 min at 4 °C; the supernatant was collected, lyophilized, and maintained at -20 °C until use. The dried venom extract was solubilized in 5% (v/v) MeCN at a concentration of 100 mg/mL and fractionated using RP-HPLC on a Nucleosil-C18 (10 \times 250 nm, 5 μm) column at a flow rate of 2 mL/min under a gradient from 5 to 60% (v/v) MeCN (containing 0.1% TFA) for 45 min. The elution was monitored at 215 nm, and each eluted peak was manually collected into 2 mL glass vials. Fraction 1 was subjected to RP-HPLC using the same column under isocratic elution with 2% (v/v) MeCN (containing 0.1% TFA) at a flow rate of 700 $\mu\text{L}/\text{min}$ for 30 min. The homogeneity of the preparation was checked by ESIMS analysis. A total of 3 mg of compound **1** was isolated.

Polybioside (1): white, amorphous powder; UV (MeOH) λ_{max} (log ϵ) 210, 310, 323 nm; ¹H NMR (500 MHz, DMSO-*d*₆) data, see Table 1; ¹³C NMR (125 MHz, DMSO-*d*₆) data, see Table 1; HRESIMS (m/z 302.1406 [M + H]⁺, calcd 301.1275); ESIMS/MS m/z 81.0, 95.0, 110.0, 130.2, 138.1, 163.3, 183.4, 189.6, 190.0, 207.4, 221.1, 235.1, 250.1, 268.1, 275.1, 284.3, 286.3, and 302.1.

Evaluation of Polybioside Action in the CNS of Rats.

ICV Sample Application and Immunohistochemical Assay. The guide cannula was implanted in the lateral ventricle (AP = -0.4; ML = -1.4; DV = -3.4) under anesthetic 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 polybioside (**1**). The animals 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 minimize stress. Polybioside was solubilized in 10 μL of saline (0.9% w/v) and ICV injected at a concentration of 1 ng/ μL . The control group ($n = 3$) received only vehicle injection (saline: 0.9% w/v) for comparison with the effects of polybioside administered ICV in vehicle. After the two hours necessary for an effective c-Fos induction, the animals were anaesthetized with a lethal dose of the same anesthetic 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, postfixed for four hours 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.¹⁹ One series of each brain slice was submitted for immunohistochemistry 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) 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 Laboratories, 1:1000) and for one additional hour in avidin–biotin complex (Vector, 1:500). Next, the sections were incubated in diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and 0.01% (v/v) hydrogen peroxide dissolved in KPBS. The reaction was terminated after 2–3 min with repeated rinses in KPBS.^{21–23} 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), the Nissl method of counterstaining with thionin was used.²⁴

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, color balance, and brightness were adjusted, and images were combined in plates using Corel Draw 11 software.

Venous Catheterization. For intravenous administration of polybioside, the rats were anesthetized with chloral hydrate (7%, 350 mg/kg, ip) and submitted for venous catheterization. A Silastic catheter containing heparinized saline (10 U/mL of pyrogen-free saline, Sigma, St. Louis, MO) was inserted into the femoral vein and sutured in place. The free end of the catheter was passed under the skin of the back, exteriorized between the scapulae, and plugged with a sterile wire stylet. A week later, polybioside (100 ng kg⁻¹) was intravenously applied.

Statistical Analysis. For quantitative analysis of c-Fos-ir cells, three representative slices of each brain region expressing c-Fos protein were chosen for each rat. All of the areas expressing c-Fos 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 mm² for the piriform cortex, 0.5 mm² for the lateral septal nucleus dorsal, paraventricular nucleus of the hypothalamus, dorsomedial hypothalamic nucleus, reuniens nucleus, central medial nucleus, and dorsal intermediate nucleus, and 1 mm² for the paraventricular thalamic nucleus and the prelimbic cortex. The statistical analyses were performed using SigmaStat software and the Student's *t*-test for comparisons between groups (*p* < 0.05).

Acknowledgment. The authors thank Prof. Dr. Norberto P. Lopes for the HRESIMS analyses. This research was supported by grants from FAPESP (BIOprospecTA Proc. 04/07942-2, 06/57122-6), CNPq (472870/2004-1), and INCT-Imunologia. M.S.P. is a researcher for the Brazilian Council for Scientific and Technological Development (CNPq).

Supporting Information Available: ¹H and ¹³C NMR spectra, ESIMS and MS/MS spectra, and the schemes and photomicrographs

of neuroactivity assays with compound **1**. This information is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

- (1) Palma, M. S. In *Handbook of Biologically Active Peptides*; Kastin A. J., Ed.; Academic Press: Burlington, 2006; Chapter 56, pp 389–396.
- (2) Means, E. D.; Barron, K. D.; Van Dyne, B. J. *Neurology* **1973**, *23*, 881–890.
- (3) Light, W. C.; Reisman, R. E.; Shimizu, M.; Arbesmann, C. E. *J. Allergy Clin. Immunol.* **1977**, *59*, 391–397.
- (4) Gale, A. N. *Br. Med. J.* **1982**, *284*, 20–21.
- (5) Behan, P. O.; Behan, W. M. H. *Br. Med. J.* **1982**, *284*, 504–505.
- (6) Mortari, M. R.; Cunha, A. O. S.; Oliveira, L.; Gelfuso, E. A.; Vieira, E. B.; Santos, W. F. *J. Biol. Sci.* **2005**, *5*, 449–454.
- (7) Owen, M. D. *Toxicol.* **1979**, *17*, 519–523.
- (8) Nakajima, T. In *Venoms of Hymenoptera*; Piek T., Ed.; Academic Press: London, 1986; Chapter 6, pp 307–327.
- (9) Hisada, M.; Satake, H.; Masuda, K.; Aoyama, M.; Murata, K.; Shinada, T.; Iwashita, T.; Ohfun, Y.; Nakajima, T. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 1048–1054.
- (10) Kobayashi, K.; McBurney, R. N.; Goldin, S. M. In *New Drugs from Natural Sources*; Coombes, J. D., Ed.; Information Press: Oxford, 1992; Part 3, pp 143–165.
- (11) Morgan, J. I.; Curran, T. *Annu. Rev. Neurosci.* **1991**, *14*, 421–451.
- (12) de Oliveira, L. C.; Sales, A. J.; Del Bel, E. A.; Silveira, M. C. L.; Guimarães, F. S. *Brain Res. Bull.* **2001**, *55*, 747–754.
- (13) Herrera, D. G.; Robertson, H. A. *Prog. Neurobiol.* **1996**, *50*, 83–107.
- (14) Mamer, O. A.; Lesimple, A. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1771–1774.
- (15) McIntosh, A. R.; Rajah, M. N.; Lobaugh, N. J. *J. Neurosci.* **2003**, *23*, 6520–6528.
- (16) Tchêlingèrian, J. J.; Le Saux, F.; Pouzet, B.; Jacque, C. *Neurosci. Lett.* **1997**, *226*, 175–178.
- (17) Mirzajafi-Zadeh, J.; Fathollahi, Y.; Zeraati, M.; Namvar, S. *Can. J. Neurol. Sci.* **2008**, *35*, 226–231.
- (18) Stone, T. W. *Neuropharmacology*; W. H. Freeman and Co. Limited: New York, 1995; Chapter 4, pp 57–60.
- (19) Rigas, P.; Castro-Alamancos, M. A. *Neuroscience* **2004**, *124*, 953–961.
- (20) National Academy of Sciences. *Guide for the Care and Use of Laboratory Animals*; Institute of Laboratory Animal Resources; Commission on Life Sciences; National Research Council, National Academy Press: Washington, D.C., 1996 (available on line at <http://www.nap.edu/readingroom/books/labrats/>).
- (21) Sita, L. V.; Elias, C. F.; Bittencourt, J. C. *Brain Res.* **2003**, *970*, 232–237.
- (22) Gerfen, C. R.; Sawchenko, P. E. *Brain Res.* **1984**, *290*, 219–238.
- (23) Shu, S.; Ju, G.; Fan, L. *Neurosci. Lett.* **1988**, *85*, 169–171.
- (24) Windle, W. F.; Rhines, R.; Rankin, J. A. *Stain Technol.* **1943**, *18*, 77–86.

NP900424T