Structure Determination of Hydroxytrypargine: A New Tetrahydro-β-Carboline Toxin from the Venom of the Spider *Parawixia bistriata*

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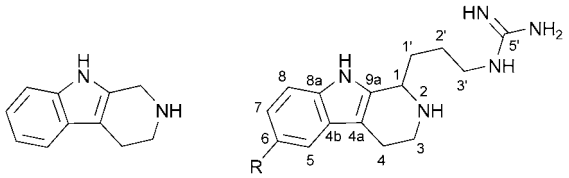
A new, highly active tetrahydro-β-carboline toxin from the spider *Parawixia bistriata*, the most-common species of social spider occurring in Brazil, was isolated. The new toxin was identified as 1,2,3,4-tetrahydro-6-hydroxy-β-carboline (= *N*-[3-(2,3,4,9-tetrahydro-6-hydroxy-1*H*-pyrido[3,4-*b*]indol-1-yl)propyl]guanidine; **3**). This type of alkaloid, not common among spider toxins, was found to be the most-potent constituent of the spider's chemical weaponry to kill prey. When *P. bistriata* catch arthropods in their web, they apparently attack their prey in groups of many individuals injecting their venoms. *In vivo* toxicity assays with **3** demonstrated a potent lethal effect to honeybees, giving rise to clear neurotoxic effects (paralysis) before death. The compound's toxicity (LD_{50} value) was determined to be *ca.* 8 ng/g of honeybee. The investigation of the pharmacological properties and neurotoxic actions of **3** may be used in the future for the development of new drugs to be applied for pest control in agriculture.

Introduction. – Among the aerial web-builder species, the social spiders are notorious animals due to their prey-capture behavior, which evolved in concert with the venom chemistry. Spiders have long been known to be among the major predators of insects, and generally it is thought they are polyphagous in their diet since most of them feed on a wide variety of preys. In the last decade, insecticide toxins from arthropod venoms have been the subjects of considerable emphasis in the literature. Web-building spiders evolved to use their web as part of their strategies for prey capture, especially the social spiders, which developed a prey-capture behavior based on massive attack by groups of many individuals, in concert with their venom chemistry [1].

Spider venoms generally contain a wide variety of components, presenting many pharmacological and neurotoxic activities [2]–[8]. *Parawixia bistriata* (Araneidae, Araneae) is a South American species of social web spider, very common in Brazil. It uses tetrahydro-β-carboline (=2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole; **1**) compounds as part of its chemical weaponry to kill/paralyze the prey arthropods [1]. β-Carbolines are natural analogues of trypargine (=1-(3-guanidinopropyl)-1,2,3,4-tetrahydro-β-carboline; **2**)[1]), an alkaloid previously isolated from the skin of the African frog *Kassina senegalensis* [9][10].

[1]) Systematic name: *N*-[3-(2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-1-yl)propyl]guanidine.

Here, we describe the structure elucidation and insecticidal activity of a novel compound named hydroxytrypargine (**3**), which was isolated from the MeCN/H_2O extract of the crude venom of the social web spider *P. bistriata*. The compound's structure was derived by means of the combined use of UV/VIS, ^{1}H- and ^{13}C-NMR spectroscopy, as well as ESI and high-resolution (HR) mass spectrometry.



1 **2** R = H
3 R = OH

Results and Discussion. – 1. *Isolation*. After extraction of the crude venom glands of *P. bistriata* with MeCN/H_2O 1:1 and centrifugation, the low-molecular-mass fraction (< 3 kDa) was subjected to reverse-phase (RP) HPLC. The resulting 14 distinct fractions (*Fig. 1*) were subjected to insecticide bioassay. The abundant *Fraction 7* was found to contain the most-active component of this venom, compound **3**, causing the death of honeybees.

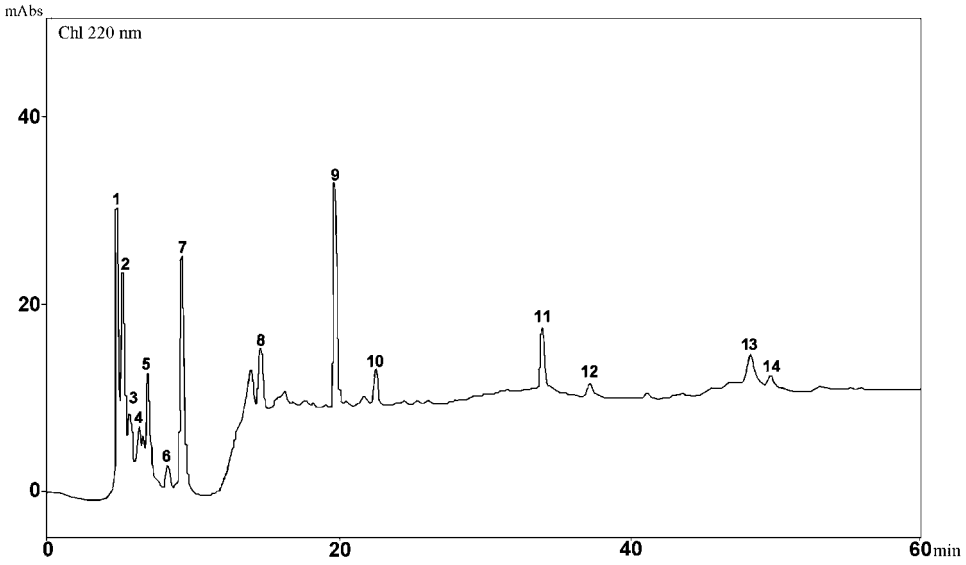


Fig. 1. *Reverse-phase HPLC profile of the crude venom of* P. bistriata. Conditions: semiprep. *ODS-80TM* column (10 × 250 mm, 5 μm; *Shiseido*), linear gradient from 5–60% MeCN in H_2O containing 0.1% (F_3CCO_2H), flow rate 2.5 ml/min, detection at 215 nm.

2. *Structure Elucidation*. The UV/VIS spectrum of **3** (*Fraction 7*) showed absorptions at λ_{max} (log ε) 224 (4.52), 275 (3.71), and 289 nm (3.42), which indicated the presence of an indole moiety, as deduced by comparison with UV-library data.

Electrospray-ionization mass-spectrometry (ESI-MS) experiments showed the $[M+1]^+$ ion at *m/z* 288. In the corresponding HR-MS, the $[M+1]^+$ signal was found at *m/z* 288.2 ($C_{15}H_{22}N_5O^+$; calc. 288.18244), giving rise to the molecular formula $C_{15}H_{21}N_5O$. The quasi-molecular ion was selected and re-subjected to ESI-MS/MS analysis under CID conditions. The corresponding MS/MS spectrum (*Fig. 2*) showed characteristic fragment ions at *m/z* 43.2, 57.8, 72.9, 85.3, 100.0, 170.3, 187.1, 201.5, 215.8, 228.0, 244.1, 270.8, and 287.0. The occurrence of sequential and successive alkyl fragmentation, probably influenced by the high basicity of the guanidino group, with fragments at *m/z* 43.2, 57.8, 72.9, 85.3, and 100.0, indicated a linear guanidinopropyl system. And the fragment-ion at *m/z* 270.8 indicated the loss of an OH group.

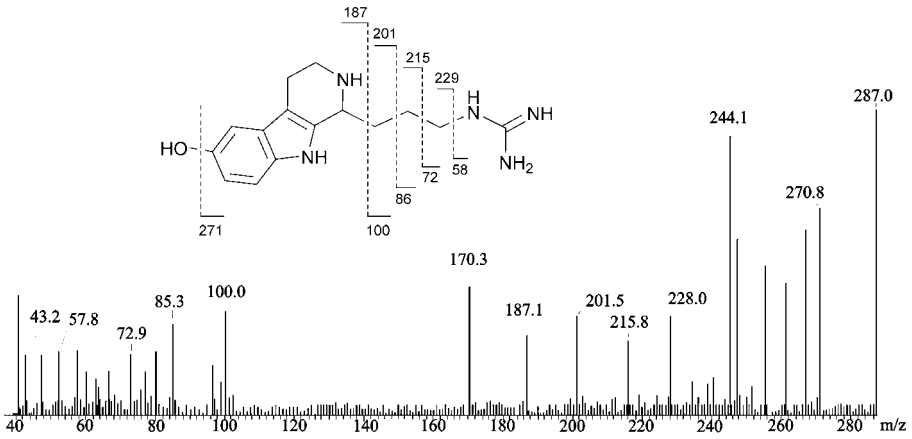


Fig. 2. *ESI-MS/MS spectrum of the [M + H]⁺ ion at* m/z *288 of the toxin* **3** (HPLC *Fraction 7*). Selected fragments are indicated in the chemical formula.

Synthetic trypargine (**2**) was used as a reference for the NMR spectroscopic analysis of *Fraction 7*. While the ^{13}C-NMR spectrum of trypargine has been published [9][10], the corresponding ^{1}H-NMR data were missing. Thus, the 1D- and 2D-NMR spectra of synthetic trypargine (**2**) were recorded, and all H- and C-atoms were assigned (*Table*). The ^{1}H-NMRspectra of **2** and **3** showed distinct aromatic (6–8 ppm) and aliphatic regions (2–5 ppm). The latter was found to be very similar for both compounds[2]). Major differences, however, were observed in the aromatic region, with four distinct resonances for **2** at δ(H) 7.21 (*t*), 7.29 (*t*), 7.51 (*d*), and 7.62 (*d*) (*Fig. 3*), in contrast to three resonances for **3** at δ(H) 6.82 (*d*), 6.98 (s), and 7.33 (*d*) (not shown). The ^{1}H-NMR spectrum of trypargine, thus, indicated a 1,2-disubstituted aromatic moiety, while that of **3** was consistent with a 1,2,4-trisubstituted aromatic moiety. ^{1}H-NMR Spectral comparison of **3** with 5-hydroxyindole showed that the chemical shifts of the pertinent aromatic H-atoms were very similar in the aromatic region. Hence, the OH group in **3** had to be located in 6-position.

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[2]) The signals of the two ^{1}H-NMR spectra were not completely identical, though, in the aliphatic region, because different solvents were used (see *Table*).

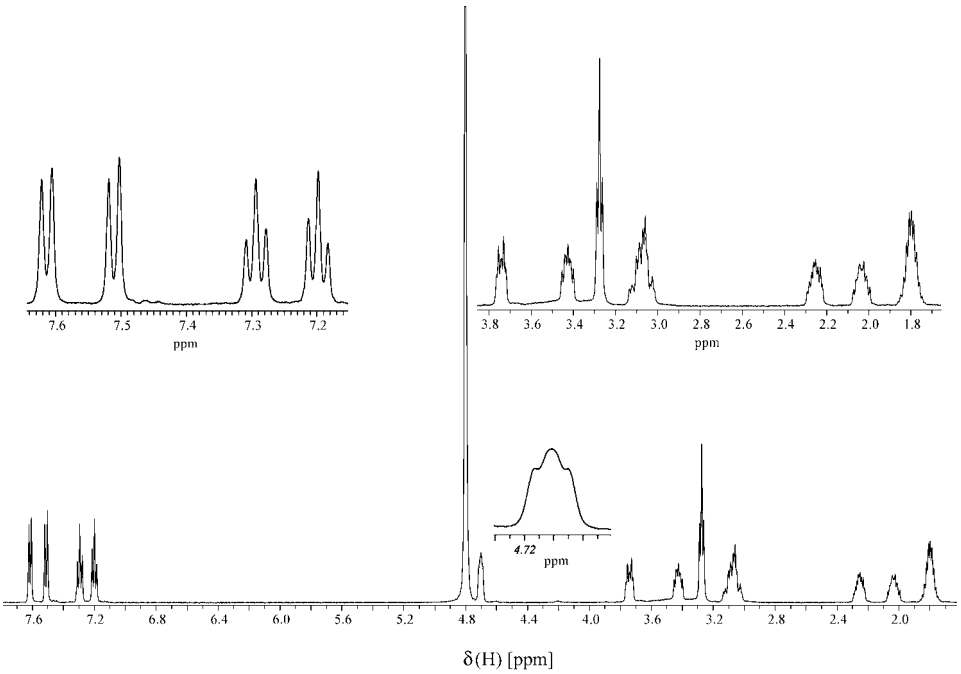

Fig. 3. *^{1}H-NMR Spectrum of trypargine* (**2**). At 500.13 MHz in D_2O. Insets: expanded regions at δ(H) 7.7 – 7.1; 3.8 – 1.7, and 4.73 – 4.69 ppm.

Table. *One-Dimensional NMR Data of Compounds* **2** *and* **3**. At 500/100 MHz, resp.; $T = 300$ K; in D_2O for **2** or D_2O/CD_3CO_2D for **3**. Chemical shifts δ in ppm rel. to Me_4Si (= 0 ppm), J in Hz.

Position	^{1}H (**2**)	^{13}C (**2**)	^{1}H (**3**)
1	4.70 (*t*, *J* = 5.0)	53.3	4.68 (*t*, *J* = 5.1)
3	3.74 (*dt*, *J* = 12.6, 4.9)	41.9	3.72 (*dt*, *J* = 12.0, 4.5)
	3.42 (*ddd*, *J* = 12.6, 6.0, 4.9)		3.40 (*ddd*, *J* = 12.0, 6.5, 4.5)
4	3.08 (*m*)	18.2	2.97 (*m*)
4a	–	106.8	–
4b	–	126.1	–
5	7.51 (*d*, *J* = 8.1)	112.2	6.98 (*d*, *J* = 3.0)
6	7.29 (*t*, *J* = 8.1)	123.2	–
7	7.21 (*t*, *J* = 8.1)	120.3	6.82 (*dd*, *J* = 10.9, 3.0)
8	7.62 (*d*, *J* = 8.1)	118.8	7.32 (*d*, *J* = 10.9)
8a	–	136.8	–
9a	–	129.4	–
1′	2.03, 2.25 (2*m*)	29.1	2.17 (*m*)
2′	1.79 (*m*)	24.1	1.75 (*m*)
3′	3.27 (*t*, *J* = 6.75)	40.9	3.22 (*t*, *J* = 6.4)
5′	–	157.3	–

From these data, the structure of the major constituent of *Fraction 7* was identified as *N*-[3-(2,3,4,9-tetrahydro-6-hydroxy-1*H*-pyrido[3,4-*b*]indol-1-yl)propyl]guanidine or 1,2,3,4-tetrahydro-6-hydroxy-β-carboline (**3**).

3. *Insecticidal Activity.* The novel toxin **3** from *P. bistriata* was injected into the pronotum of honeybees at a dose of 37 ng/g bee. The insects became paralyzed after 5 min, and died after 1 h. The LD_{50} value for the paralytic effect of the toxin was determined to be 8 ± 2 ng/g of honeybee, which is significantly smaller than LD_{50} for the crude venom of the spiders to kill the honeybees (29 ng/g of honeybee) [1]. Compound **3**, actually, seems to be more lethal than most venoms from wandering spiders [11], being very toxic to the prey insects of *P. bistriata*. This is the first report of the occurrence of a β-carboline toxin component of an animal venom used as an insecticide.

Experimental Part

Spider Collection and Venom Purification. Parawixia bistriata specimens were collected in Rio Claro, SP, in the southeast of Brazil. Spiders were killed by freezing at $-20°$. The venom glands were removed with surgical micro-scissors, and the venom was extracted with MeCN/H_2O 1:1. The extract was centrifuged through *AMICON 3* spin filters (*Millipore*) at 8000 g during 15 min at 4°, and the low-molecular-mass (LMM) fraction (< 3 kDa) was collected, lyophilized, and stored at $-10°$. The LMM fraction was then dissolved in MeCN/H_2O (+0.1% TFA) 5:95, and fractionated by HPLC (*Shimadzu LC-10Advp*) equipped with a diode-array detector (*SPD-10Avp*), using a reverse-phase (RP C18) semi-prep. column (*ODS-80TM* (5 μm); 250 × 10 mm; *Shiseido*), eluting with a linear gradient from 5–60% MeCN in H_2O (containing 0.1% TFA) during 60 min at 30°, using a flow rate of 2.5 ml/min (UV detection at 215 nm, 254 nm, and 280 nm).

Mass Spectrometry. ESI Mass spectra were acquired on a triple quadrupole (*Quatro II*) mass spectrometer (*Micromass*, UK), equipped with a standard electrospray probe adjusted to *ca.* 5 μl/min. During all experiments, the source temp. was maintained at 80° (needle voltage 3.6 kV), applying a drying N_2 flow of 200 l/h and a nebulizer N_2 flow of 20 l/h. The mass spectrometer was calibrated with intact horse heart myoglobin and its typical cone-voltage induced fragments. The cone sample to skimmer lens voltage, controlling the ion transfer to the mass analyzer, was maintained at 30 V. About 50 pmol of each sample was injected into the electrospray transport solvent. The ESI spectra were obtained in the continuous acquisition mode, scanning from m/z 100–2000 at a scan time of 5 s.

For MS/MS, the following typical conditions were used: capillary voltage 3 kV, cone voltage 30 V, collision-gas pressure 3.5×10^{-3} mbar, desolvation gas temp. 80°. In these experiments, Q_1 was used to select the parent ion and was not scanned. The ion of interest was individually selected in Q_1, and structurally characterized by collision-induced dissociation (CID). It was subjected to *ca.* 25-eV collision energy at 5×10^{-3} mbar collision-gas pressure (Ar) in Q_2. The CID fragments were analyzed by scanning Q_3.

NMR Experiments. The ^{1}H- and ^{13}C-NMR and 2D gCOSY, gHSQC, and gHMBC spectra for trypargine (**2**) were recorded on a *Bruker DRX-500* spectrometer (2.5-mm gradient probe) operating at 500.13 (^{1}H) and 125.78 (^{13}C) MHz. Measurements were carried out at a probe temp. of 300 K, with solns. of *ca.* 5 mg/ml D_2O. The 1D-NMR spectra were recorded under standard conditions [12]. 2D-NMR spectra were recorded with standard *Bruker* pulse programs. For ^{1}H,^{1}H gCOSY, typical conditions were 16 transients, accumulated into 2-k data points with 128 experiments and a sweep width of *ca.* 5000 Hz; the FID was zero filled to 2-k data points in F_2 and F_1. ^{1}H,^{13}C-gHSQC and gHMBC spectra were acquired applying 32 transients, accumulated into 1-k data points with 256 experiments; the FID were zero filled to 2-k data points in F_2 and F_1. For the natural insecticide **3** (*Fraction 7*), the ^{1}H-NMR spectrum was acquired with less than 1 mg of material in D_2O containing one drop of CD_3CO_2D.

Trypargine (= N-*[3-(2,3,4,9-Tetrahydro-1*H*-pyrido[3,4-*b*]indol-1-yl)propyl]guanidine*; **2**). This compound was prepared from 2,3,4,9-tetrahydro-3-(methoxycarbonyl)-2-(phenylmethyl)-1*H*-pyrido[3,4-*b*]indole-1-propanoic acid, prepared by asymmetric Pictet–Spengler reaction, as described in [10]. The hydrochloride of **2** was identified through comparison of physico-chemical properties with literature data [10]. M.p. 211–213°. $[\alpha]_D = +37$ (MeOH). MS: 272 ($[M+H]^+$).

1,2,3,4-Tetrahydro-6-hydroxy-β-carboline (= N-*[3-(2,3,4,9-Tetrahydro-6-hydroxy-1*H*-pyrido[3,4-*b*]indol-1-yl)propyl]guanidine*; **3**). UV/VIS (MeOH): 224 (4.52), 275 (3.71), 289 (3.42). ^{1}H-NMR: see *Table.* ESI-MS ($C_{15}H_{21}N_5O$): 288 ($[M+1]^+$); for fragmentation, see *Fig. 2.* HR-MS: 288.2 ($[M+1]^+$, $C_{15}H_{22}N_5O^+$; calc. 288.18244).

Insecticidal Activity. Different doses of **3** (from 2–100 ng/mg of insect) were injected with a micro-syringe in a final volume of 1 µl into the pronotum of honey bees (Africanized *Apis mellifera*). The compound was also assayed in acetone soln., applying 2 µl onto the thorax of the honeybees. The insects were maintained within a *Petri* dish for 4 h in the presence of candy (food) and a H_2O supply, and, during this period, the toxicity effects and/or lethal action of the new toxin were observed, both for the internal and topical applications. As controls, physiological solution was injected into the pronotum, or acetone alone was applied on the thorax of the insects. The LD_{50} value of **3** was determined by varying the concentration of the toxin ($n = 5$ for each concentration). The number of dead or paralyzed insects was determined after 4 h of toxin action. Toxicity levels were calculated according to the *Probit* method [13].

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