Structure Determination of a Tetrahydro-β-carboline of Arthropod Origin: A Novel Alkaloid-Toxin Subclass from the Web of Spider *Nephila clavipes*

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The orb-web spiders are polyphagous animals in which the web plays a very important role in the capture of preys; oily droplets usually cover the capture-web of the spider *Nephila clavipes* and seem to be of great importance for prey capture. The knowledge of the chemical composition of these droplets is necessary to understand the function of this adhesive material in web mechanics and prey capture. A novel subclass of spider toxins, tetrahydro-β-carboline, was identified among the weaponry of compounds present inside of oily droplets. This type of alkaloid is not common among the natural compounds of spider toxins. Apparently, when the prey arthropods get caught by the spider web, their bodies are covered with many adhesive oily droplets, which disrupt delivering the tetrahydro-β-carboline to the direct contact with the prey integument. Toxicity assays demonstrated a potent lethal effect of the alkaloid toxin to the spider preys; topical applications of the tetrahydro-β-carboline at first caused clear signs of neurotoxicity, followed by the death of preys. The structure of the major component, a tetrahydro-β-carboline, among the alkaloid toxins was elucidated by means of UV spectrophotometry, ESI mass spectrometry, ^{1}H-NMR spectroscopy, and high-resolution mass spectrometry. The structure of the natural toxin was determined as 1-(2-guanidinoethyl)-1,2,3,4-tetrahydro-6-hydroxymethyl)-β-carboline; the investigation of the pharmacological properties and neurotoxic actions of this compound may be used in the future as reference for the development of new drugs to be applied at level of pest control in agriculture.

Introduction. – Studies of arthropod defensive chemistries continue to bring to light novel structures and unanticipated biosynthetic capabilities [1]. In the last decade, insecticide toxins from arthropod venoms have been the subjects of considerable emphasis [2]. Spider venoms generally are constituted of complex mixtures of biologically active substances [3], which may be grouped into three major classes of compounds according to their chemical nature: high-molecular-mass proteins ($M_r >$ 10 kDa), peptides (M_r 3–10 kDa), and low-molecular-mass compounds ($M_r < 1$ kDa) [4]. The latter class, in turn, can be subdivided into other chemical subclasses, according to the chemical nature of these toxins.

The subclass of the acylpolyamines, is well-characterized both in spiders and solitary wasp venoms [3][5–8]. Spider polyamines are formed by a hydrophobic moiety ((hydroxyphenyl)acetic acid, indole acetic acid, or (hydroxyindole) acetic acid) linked to a polyamine–amide backbone constituted of putrescyl or cadaveryl units, condensed to some amino acid residues such as glycine, alanine, arginine, and

asparagine. Currently, these toxins are classified into five sub-types (from A to E) according to their structural organization [9]. Although some polyamines can be toxic to vertebrates, by direct intracranial administration, they seem to possess essentially an insecticidal activity and are responsible for the fast insect prey paralysis observed during predation [4].

Two other organic compounds have been isolated from spider venoms although their biological function remains unknown: bis(agmatine)-oxalamide from the venom of *Plectreurys tristis* and HF-7, a gliconucleoside disulfate from the venom of *Hololena curta* [10].

Recently, three new tetrahydro-β-carbolines, another subclass of low-molecular-weight compounds, have been isolated from the venom of the social spider *Parawixia bistriata* [11]. These molecules are natural analogs of the trypargine, an alkaloid toxin isolated from the skin of the African frog *Kassina senegalensis* [12]. Trypargine is a tetrahydro-β-carboline derivative presenting a propyl-guanidine substituent connected to the chromophore group; this compound presents interesting biosynthetic and pharmacological features [13]. In the last 20 years, several tetrahydro-β-carboline were found in mammalian brain, urine, platelets, and other tissues, and have been studied by pharmacologists, who have indicated that these endogenously formed alkaloids act specially on various aspects of serotonergic neurotransmitter functions [13].

The *Nephilinae* orb-web spiders are amazing predators, which use their orb-webs as part of the strategy for prey capture [14]. The web of *Nephila clavipes* generally is covered by adesive oily droplets, which contain different types of toxins directly involved with prey paralysis/killing without need for venom injection by the spider. These oily droplets contain small vesicles filled with solutions of low-molecular-weight organic compounds, which act as part of the cocktail of paralytic/killing arsenal of this spider. Most of the compounds already identified within these oily droplets are neurotransmitters, such as *N*-acetyltaurine, 4-aminobutyramide, glycine, betaine, choline, and putrescine [15]. Recently, we characterized an organometallic 1-(diazenylaryl)ethanol compound from the web of the spider *N. clavipes*, which presents a potent lethal action against the spiders's prey [16].

A novel insecticide compound has been now identified in very reduced concentrations from these oily droplets. It was isolated from the MeCN extracts of the web of the spider *N. clavipes* by chromatographic techniques, and a combination of spectroscopic methods led to its structure elucidation.

Results and Discussion. – The low-molecular-weight fraction from web of *N. clavipes* ($M_r < 3$ kDa) was initially fractionated by RP-HPLC (*C-18*). The chromatographic profile presented six distinct fractions (*Fig. 1*), which were submitted to insecticide bioassay; *Fraction 6* was among the most abundant, and the results have shown it to be responsible for honeybees deaths. This fraction has been rechromatographed under isocratic conditions at 26% (*v/v*) MeCN in H_2O (containing 0.1% TFA), resulting in five sub-fractions (*Fig. 2*). The sub-fraction *6.5* was the only one to present insecticide activity against honeybees, both when injected and topically applied on the insect's body. This fraction was submitted to UV spectrophotometry, mass spectrometry (ESI-MS and ESI-MS/MS) and high-resolution mass spectrometry and ^{1}H-NMR spectroscopy.

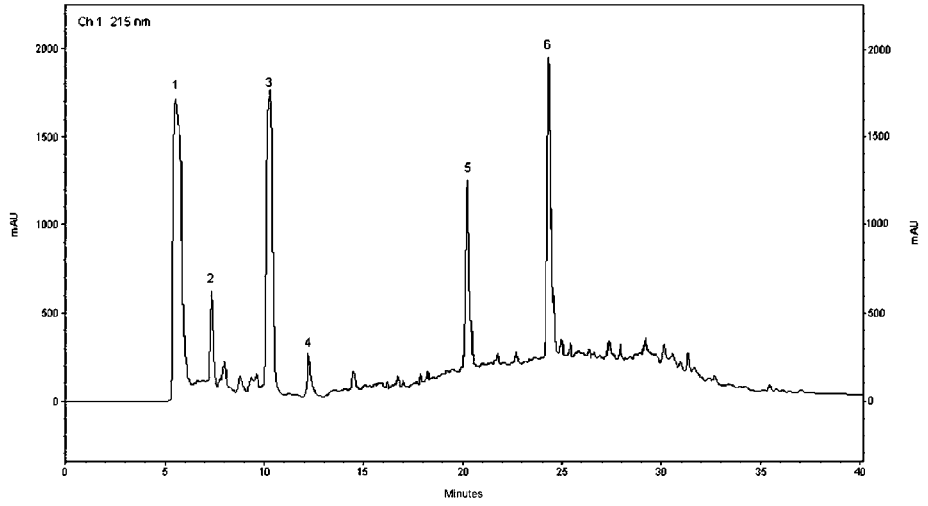


Fig. 1. *RP-HPLC Profile of the washed web extract of* Nephila clavipes *spider with a linear gradient from* 5–60% (*v*/*v*) *MeCN* (containing 0.1% (*v*/*v*) TFA) *in a semi-preparative column* (*ODS-80TM Shiseido* (10 × 250 mm)). The flow rate was 2.5 ml · min^{-1}, and the elution was monitored at 215 nm.

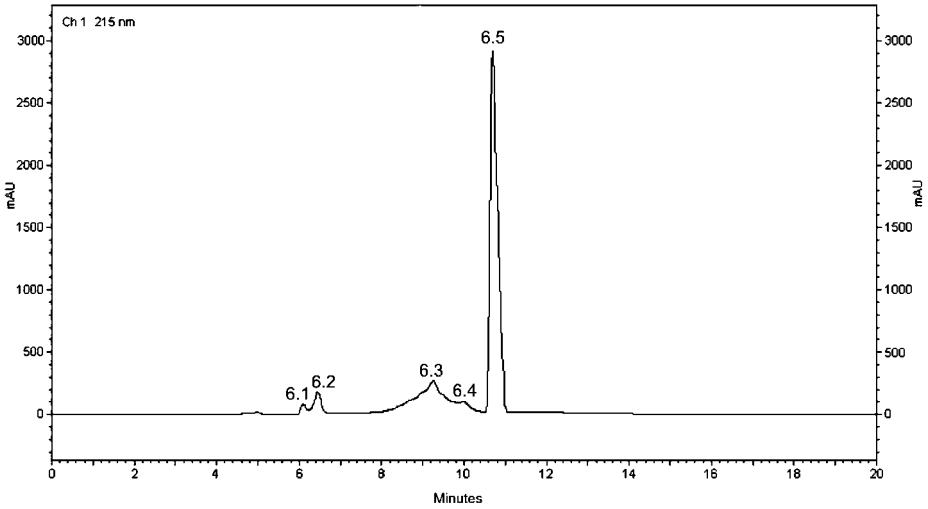


Fig. 2. *RP-HPLC Profile of the* Fraction 6 *under isocratic conditions, at 26%* (*v*/*v*) *MeCN* (containing 0.1% (*v*/*v*) TFA) *with a semi-preparative column* (*ODS-Shiseido* (10 × 250 mm)). The flow rate 1.0 ml · min^{-1}, and the elution was monitored at 215 nm.

The UV spectrum (log ε) of the *Fraction 6.5* (not shown) (*i.e.*, 224 (4.52), 275 (3.71), and 289 (3.42)), compared with UV library spectra indicated the presence of an indole chromophore.

This fraction has been submitted to mass spectrometry (ESI-MS and ESI-MS/MS) and ^{1}H-NMR spectroscopy. The monoisotopic molar mass of the insecticide compound

(*Fraction 6.5*) determined by ESI-MS analysis was 288 as $[M+H]^+$, protonated molecular ion.

The structure assignment started from the ^{1}H-NMR spectrum (*Fig. 3*). The spectrum showed one characteristic region of an aromatic moiety (6.5 to 8 ppm); in this region there are three different signals (*Fig. 3)*, two *doublets* and a *singlet*; this pattern is due to an aromatic 1,3,4-trisubstituted system with three different H-atoms [17]. The other signal was observed at 4.45 ppm and assigned to a CH_2 group bonded to the aromatic ring and to a OH group. The signals at 7.79 and 7.08 ppm appeared as a *doublet* (^3J(H,H) = 8.2 Hz *ortho*-coupling), and they were assigned to H^1 or H^2 (*cf. Fig. 3*). The *singlet* at 6.9 ppm was due to the H-atom H^4.

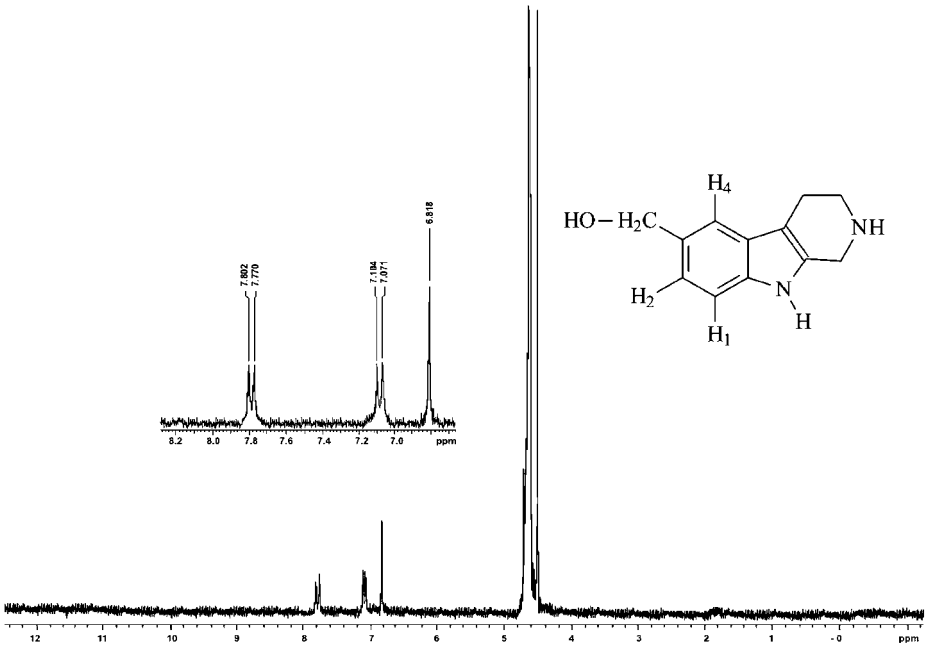


Fig. 3. *The ^{1}H-NMR spectrum of the insecticide toxin purified from the* Nephila clavipes *web in a concentration of 1 mg·cm^{-3} in D_2O* (*Fraction 6.5*). The spectrum was recorded at 499.88 MHz.

Thus, the results of UV spectrum and the ^{1}H-NMR analysis indicate the presence of 1,2,3,4-tetrahydro-β-carboline as the chromophore group of the compound present in the *Fraction 6.5*. On the basis of the known structures of the most tetrahydro-β-carboline compounds, further signals due to the presence of aliphatic H-atoms would be expected. The absence of these signals may be due to the fact that the spectrum was acquired with a very small amount of sample (*ca.* 500 μg). All signals due to these aliphatic H-atoms would split in more than six lines due to geminal and vicinal coupling constants; then, these signals appear at the base line, due to a low intensity of the signal for each H-atom. Thus, in *Fig. 3*, only the chromophore group is represented. The assignments of the aliphatic chain were performed through the interpretation of the fragmentation pattern of the ESI-MS/MS spectrum of the synthetic alkaloid toxin

(trypargine) compared to the fragmentation pattern of the natural toxin, as described below.

Proceeding with the structural assignment of the toxin, the precursor ion of *m*/*z* 288 as $[M+H]^+$ was selected and submitted to ESI-MS/MS analysis under CID conditions. The MS/MS spectrum of the natural compound presenting *m*/*z* 288 is shown in *Fig. 4*, which shows the characteristic fragment ions of the compound of *m*/*z* 288 ($[M+H]^+$) at *m*/*z* 43, 57, 71, 85, 98, 106, 113, 121, 128, 145, 189, 244, and 270.

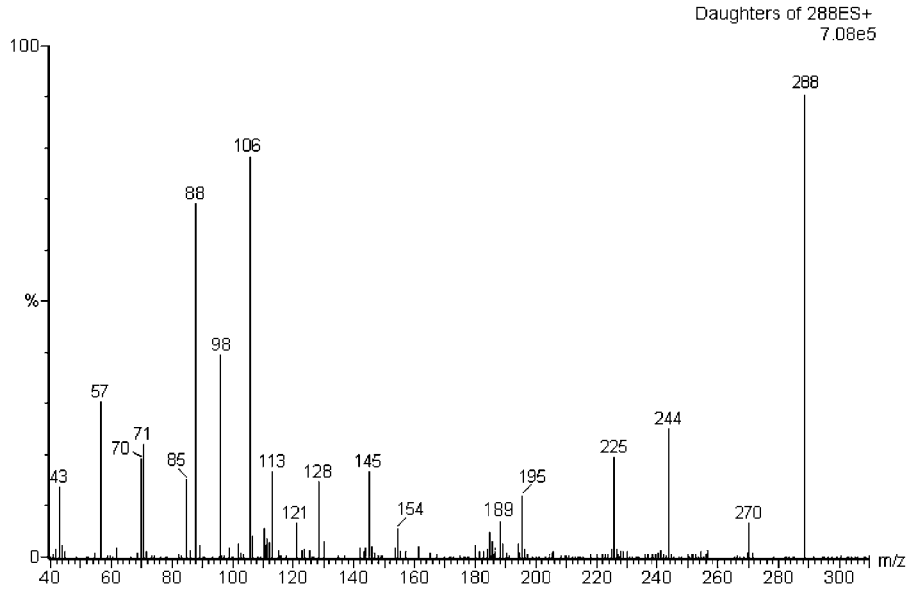
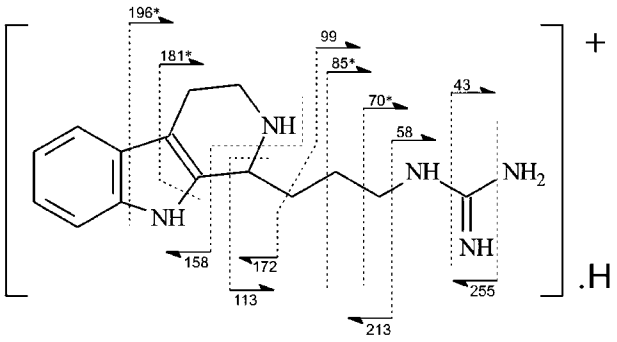


Fig. 4. *ESI-MS/MS* (ramp of energy collison from 10 to 40 eV) *of the [*M + *H]*$^+$ *ion* (*m*/*z* 288) *of the insecticide toxin* (*Fraction 6.5*) *purified from* Nephila clavipes *web*

Since the low abundance of the natural toxin did not permit us to obtain enough biological material to obtain reliable ^{1}H-NMR signals from the H-atoms of the substituents of the aromatic system, we decided to synthesize a standard tetrahydro-β-carboline compound, the trypargine toxin, to enable the interpretation of tandem mass spectrum of the natural toxin present in the *Fraction 6.5*. The spectrum for trypargine was obtained by using a ramp of energy collision from 10 to 40 eV, with the purpose to investigate the occurrence of internal fragmentation in the rings of the chromophore moiety and in the chain of alkyl substituents of the aromatic system. The MS/MS spectrum of the synthetic toxin presenting *m*/*z* 272 as $[M+H]^+$showed the characteristic fragmentions at *m*/*z:* 43, 58, 70, 85, 99, 113, 158, 172, 181, 196, 213, and 255 (not shown spectrum).

The interpretation of the pattern of fragmentation of the natural compound is represented in *Scheme 1.*

Scheme 1. *Interpretation of the Fragmentation Pattern of the ESI-MS/MS Spectrum of the Synthetic Trypargine.* The asterisks indicate the fragment ions, which suffered neutral rearrangements where the final value of *m/z* is one or two units of mass less than the expected hypothetical value.



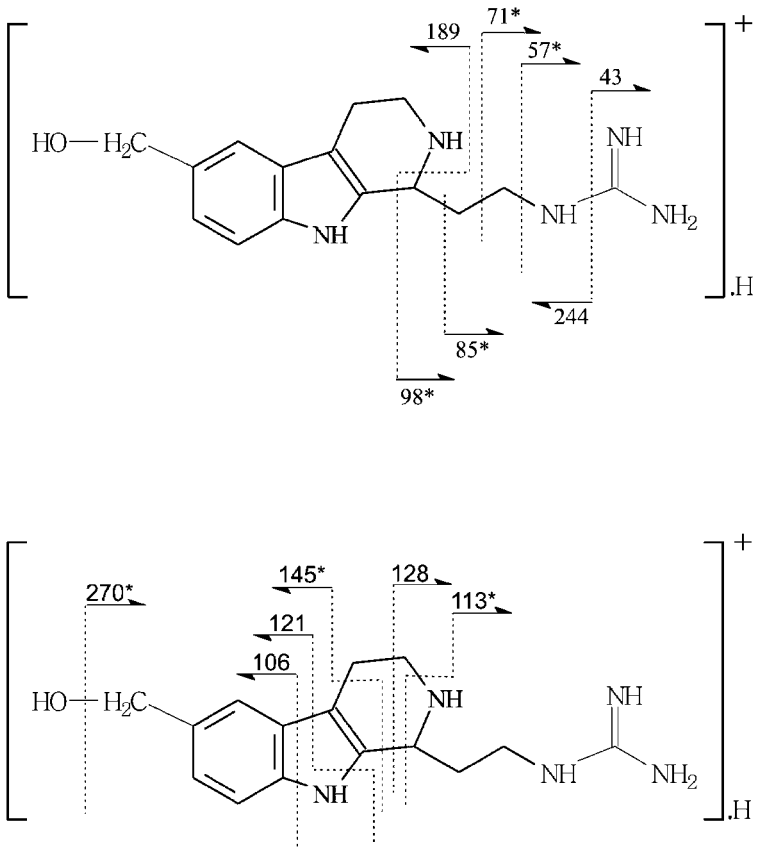
Although the chemical structures of the tetrahydro-β-carboline compounds are relatively simple, sometimes the fragmentation patterns of these compounds in mass spectrometry are complex, due the frequent neutral H rearrangements that occur in the regions located between the chromophore and the guanidine group of the alkyl substituent chain. Some *m/z* values were assigned with asterisks to indicate the fragment ions that suffered neutral H rearrangements where the final value of *m/z* is one or two units of mass less than expected hypothetical value (*Schemes 1* and *2*).

At first, a comparison between the ESI-MS/MS spectra of natural and synthetic compounds reveals a high similarity, suggesting a high level of structural similarity between the natural and the synthetic alkaloid toxins. The fragmentation pattern of the synthetic trypargine (*Scheme 1*) shows some characteristics that must be emphasized: *i*) the occurrence of sequential and successive fragmentation in the alkyl chain located between chromophore and guanidine group (probably influenced by the strong basicity of the guanidine group); thus, the fragment ions of *m/z* 43, 58, 70, 85, and 99 observed in the MS/MS spectrum of the synthetic trypargin, presenting charge retention in the alkyl-guanidine chain as substituent of the tetrahydro-β-carboline chromophore (*Scheme 1*) are useful to assign the chemical structure of the alkyl substituent as a propyl-guanidino; *ii*) the fragment ion of *m/z* 113 (*Scheme 1*) may be used to assign the connectivity between the propyl-guanidine substituent and the chromophore moiety for the synthetic trypargine.

Another characteristic of the fragmentation pattern of the synthetic trypargine (*Scheme 1*) is the fragmentation of the chromophore tetrahydro-β-carboline both in the non-aromatic six-membered ring (revealed by the fragment ions of *m/z* 113 and 158) and in the five-membered ring, despite its aromaticity (characterized by the presence of the fragment ions of *m/z* 181 and 196).

By analogy with the pattern of fragmentation of synthetic trypargine, the fragment ions of *m/z* 43, 57, 70/71, and 85 in the spectrum of the natural alkaloid toxin suggest that the natural toxin has an ethyl-guanidino as alkyl substituent of the chromophore group; the fragment ion of *m/z* 98 may be used to assign the connectivity between the ethyl-guanidine substituent and the chromophore moiety. The general pattern of fragmentation of the chromophore observed for the natural toxin is quite similar to that

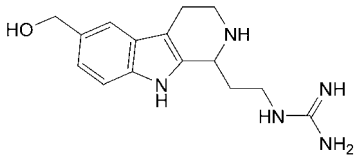
Scheme 2. *Interpretation of the Fragmentation Pattern of the ESI-MS/MS Spectrum of the Toxin Purified from* N. clavipes *Web* (*Fraction 6.5*). The asterisks indicate the fragment ions, which suffered neutral rearrangements where the final value of *m/z* is one or two units of mass less than expected hypothetical value.



observed for the synthetic trypargine, except that the MS/MS spectrum of the natural compound is richer in fragment ions (characterized by the *m/z* values 106, 113, 121, 128, and 145). Probably, this is occurring due to the presence of a second substituent in the aromatic moiety of the natural toxin, already identified in the ^{1}H-NMR analysis (*Fig. 3*) as 3-(hydroxymethyl) group; the fragment ion at *m/z* 270, presenting charge retention in the guanidine side, corroborates the presence of a OH group in this substituent (*Scheme 2*). The fragment ion at *m/z* 106.0 was interpreted as resulting from the fragmentation of the five-membered ring, which apparently may be followed by the dehydration of the alkyl-substituted aromatic six-membered ring (*m/z* 106), resulting in the formation of the fragment ion of *m/z* 88.0 (not shown in *Scheme 2*).

The next step of the structure elucidation was to acquire a high-resolution mass spectrum for the natural toxin, which revealed the molecular formula for this compound as $C_{15}H_{21}N_5O$ that presents a molecular mass of 287.1746 Da and protonated molecular mass of 288.1824 Da. Therefore, the insecticide toxin found in the *Fraction*

6.5 corresponds to 1-(2-guanidinoethyl)-1,2,3,4-tetrahydro-3-(hydroxymethyl)-β-carboline, and its structure is shown below.



This natural compound was topically applied on the dorsal part of the thorax of honeybees, and it was observed that the insects became paralyzed after 8 min, remaining under this condition during many hours. The ED_{50} value was determined for the paralytic effect of toxin as 31 ± 4 ng/g of honeybee; this value is similar to the ED_{50} value observed for the crude venom of the spider *N. clavipes* to paralyze the honeybees (37 ng/g) [18]. However, when this compound was injected in the pronotum of honeybees, it caused death of the insects after 1 h; thus, the LD_{50} value determined for the alkaloid web toxin is 9 ng/g of honeybees, which seems to be more lethal than the most of crude venom from the wandering spiders [18]. These results indicate that the tetrahydro-β-carboline toxin from the oily droplets of the webs of *N. clavipes* is very toxic to the prey insects of this spider and certainly must contribute to the toxicity of the spider web to the prey capture.

Conclusions. – The results indicate $C_{15}H_{21}N_5O$ as the molecular formula for the new toxin, corresponding to the compound 1-(2-guanidinoethyl)-1,2,3,4-tetrahydro-3-(hydroxymethyl)-β-carboline, which seems to be a potent paralytic/lethal toxin, applied by the spider within the oily droplets, which, in turn, is then spread over the capture web, to ensure the prey capture even without the need for venom injection. Recently we characterized another web toxin corresponding to [1-(3-diazenylphenyl)ethanol]iron, which is also a potent insecticidal toxin [17]. The synergistic actions between the organometallic natural toxin and the new alkylindole alkaloid toxin make the adhesive oily droplets from the web of *N. clavipes* a very efficient instrument of prey capture.

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Experimental Part

Sample Preparation: Webs of *N. clavipes* spider were collected from a wooded area in Rio Claro-SP, Southeast of Brazil. The web toxins were extracted from the webs by soaking *ca.* 5 g of webs into 30 ml of MeCN (*Riedel*)/H_2O 1:1 (containing 0.1% (*v/v*) CF_3COOH (TFA, *Aldrich*)) during 4 h at r.t. The resulting extract was centrifuged at 13,000 rpm/min during 60 min. The washed-web-extract (WWE) was then lyophilized, and the volume was concentrated to 3 ml. The concentrated WWE was filtered through *Microcon 3* (*Amicon*, Beverley, MA) to eliminate materials over 3 kDa. The filtrate was fractionated in a HPLC system (*SHIMADZU*, model *LC-10Advp*) equipped with a diode-array detector (*SHIMADZU*, model *SPD-10Avp*), using a reversed-phase semi-prep. column *ODS-80TM* (*SHISEIDO*; 250 × 10 mm). Elution was carried out in a linear gradient from 5 to 60% (*v/v*) MeCN in H_2O (containing 0.1% TFA) during 40 min at 30°. The UV absorbance was monitored at 215, 254, and 280 nm, at a flow rate of 2.5 ml · min^{-1}.

The peak containing the alkaloid toxin (*Fraction 6*) was refractionated by using a RP-HPLC system under isocratic conditions at 26% (*v/v*) MeCN (containing 0.1% (*v/v*) TFA) in a semi-prep. column (*ODS-Shiseido* (10×250 mm)) with a flow rate of 1.0 ml $\cdot$ min^{-1}, and the elution was monitored at 215 nm.

Trypargine Synthesis. Trypargine was synthesized from 2-benzyl-1,2,3,4-tetrahydro-3-(methoxycarbonyl)-9*H*-pyrido[3,4-*b*]indole-1-propanoic acid, prepared by asymmetric *Pictet–Spengler* reaction of *N*(b)-benzyl-D-tryptophan methyl ester , as described in [13]. The synthetic toxin in its HCl form was identified by some physico-chemical properties (m.p. 211–213°; $[\alpha]_D = +37$ (MeOH); MS: 272 ($[M+H]^+$) by comparison with the data from a previous publication [13].

NMR Analysis. The ^{1}H-NMR spectrum was recorded at 25° on a *Varian INOVA 500* spectrometer, operating at 499.88 MHz for ^{1}H. Spectrum was obtained of *ca.* 1 mg $\cdot$ cm^{-3} soln. in D_2O, which was used as D lock and reference for spectrum. The signal for remaining H_2O was partially suppressed applying presaturation sequence [19].

ESI-MS Analysis. Samples were dissolved in 50% (*v/v*) MeCN (containing 0.1% (*v/v*) TFA) and analyzed in a triple quadrupole mass spectrometer (*Micromass* (*Altrinchan*), model *QUATRO II*), equipped with a standard electrospray probe. During all experiments, the source temp. was maintained at 80° and the needle voltage at 3.6 kV, applying a drying gas flow (N_2) of 200 l $\cdot$ h^{-1} and a nebulizer gas flow (N_2) of 20 l $\cdot$ h^{-1}. The mass spectrometer was calibrated with a standard mixture of NaI and CsI from *m/z* 22.98 to 772.46. The cone sample to skimmer lens voltage controlling the ion transfer to mass analyzer was maintained at 30 V. About 50 pmol of each sample was injected into electrospray transport solvent by using a microsyringe (50 µl) coupled to a micro infusion pump at a flow rate of 5 µl $\cdot$ min^{-1}. The ESI spectra were obtained in the continuous acquisition mode by scanning from *m/z* 100 to 1000 at scan time of 7 s. A high-resolution ($R = 10{,}000$) mass spectrum was acquired with a *Q-TOF* mass spectrometer (*Micromass*), and the molecular formula was obtained by using elemental composition (*MassLynx*) software. The conditions of analysis were the same as described above.

ESI-MS/MS Experiment. 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 a ramp of collision energy from 10 to 40 eV and 5×10^{-3} mbar collision gas pressure (Ar) in Q_2. The CID fragments were analyzed by scanning Q_3.

Insecticide Activity. Different doses of the natural tetrahydro-β-carboline (from 2 to 100 ng $\cdot$ mg^{-1} of insect) were injected in a final volume of 2 µl into the pronotum of honeybees (Africanized *Apis mellifera*) by using a *Hamilton* microsyringe (10 µl). The compound was also assayed by dissolving it into acetone and topically applying 2 µl onto the thorax of the honeybees. The insects were maintained within a *Petri* dish up to 24 h in the presence of candy (food) and water supply. During this time, the toxicity effects and/or the lethal action of the new toxin were observed, both for the internal and topical applications. By injecting physiological solns. into insect pronotum or applying acetone alone on honeybee thorax, control experiments were performed. The LD_{50} or ED_{50} values for each case were determined by using different concentrations of the toxin and compared against a control ($N = 5$ per concentration). The number of dead or paralyzed insects was determined after 24-h toxin application. Toxicity levels were calculated according to *Probit* method [20] and expressed as lethality (LD_{50}) or paralysis (ED_{50}).

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