

## BotIT6: a potent depressant insect toxin from *Buthus occitanus tunetanus* venom

Thouraya Mejri<sup>a,\*</sup>, Lamia Borchani<sup>a</sup>, Najet Srairi-Abid<sup>a</sup>, Rym Benkhalifa<sup>a,b</sup>, Sandrine Cestele<sup>c</sup>, Imed Regaya<sup>a,c</sup>, Habib Karoui<sup>a</sup>, Marcel Pelhate<sup>b</sup>, Hervé Rochat<sup>c</sup>, Mohamed El Ayebe<sup>a</sup>

<sup>a</sup>Laboratoire des Venins et Toxines, Institut Pasteur de Tunis, BP 74-1002 Tunis, Tunisia

<sup>b</sup>Laboratoire de Neurophysiologie, UPRES EA 2647, 2, Bd Lavoisier, 49045 Angers Cedex 01, France

<sup>c</sup>Laboratoire d'Ingénierie des protéines, CNRS UMR 6560, Faculté de Médecine-Nord, Bd. Pierre Dramard, 13916 Marseille Cedex 20, France

Received 20 February 2002; accepted 10 August 2002

### Abstract

A new depressant insect toxin *Buthus occitanus tunetanus* insect-toxin 6 (BotIT6) was purified by high-performance liquid chromatography from *Buthus occitanus tunetanus* (Bot) venom. BotIT6 is very active against *Blattella germanica* (LD50 = 10 ng/100 mg body mass) thus being one of the most potent anti-insect toxin so far characterised. When compared to other insect toxin sequences, BotIT6 present high similarities with depressant insect toxins with an additional arginine residue at the C-terminus and a methionine at position 27. The calculated net charge of BotIT6 is positive (+3) whereas it is negative for classical depressant toxins: this might be associated with its high toxicity. Voltage current clamp studies show that BotIT6 is not a very potent depressant insect toxin despite its high toxicity in vivo. BotIT6 is able to fully inhibit the specific binding of <sup>125</sup>I AaHIT and <sup>125</sup>I-BotIT2 on *Periplaneta americana* synaptosomal membrane vesicles with high affinities. Despite its higher toxicity BotIT6 is a weaker competitor with <sup>125</sup>I AaHIT and <sup>125</sup>I BotIT2 as compared to the other β toxins.

Altogether, these results may suggest that BotIT6 probably defines a novel sub-group of depressant anti-insect toxins for which the receptor site can be overlapping, but not identical to that for classical depressant insect toxins.

© 2002 Elsevier Science Ltd. All rights reserved.

**Keywords:** Scorpion insect toxin; Depressant insect toxin; Sodium channel; Pharmacology; Electrophysiology

### 1. Introduction

Scorpions and their venoms have attracted the attention of investigators mainly because they are a hazard to human life and health. In nature, however, the encounters between humans and scorpions are coincidental. The stinging activity of scorpions in the field is mainly directed to arthropods and especially soft-bodied insects, which serve as their prey food.

**Abbreviations:** Bot, *Buthus occitanus tunetanus*; BotIT6, *Buthus occitanus tunetanus* insect-toxin 6; Aah, *Androctonus australis hector*.

\* Corresponding author. Tel.: +216-1-843-755; fax: +216-1-791-833.

**E-mail address:** mjthouraya@excite.com (T. Mejri), mjthouraya@yahoo.fr (T. Mejri).

Scorpions toxins can be broadly divided into those active on mammals or other vertebrates (Duval et al., 1989) but also molluscs (Pichon and Pelhate, 1984) and insects (Pelhate and Zlotkin, 1982; Eitan et al., 1990; Kopeyan et al., 1990; Borchani et al., 1997). Sodium channels serve as specific targets for these toxins (Catterall, 1992).

The toxins active on mammals have been divided into α- and β-type according to their pharmacological and electrophysiological effects (Jover et al., 1980; Couraud et al., 1982) α and α-like toxins (Rochoat et al., 1979; Catterall, 1984; Borchani et al., 1997) bind to receptor site three of the sodium channel in a potential-dependent manner. Their main effect is to slow down the inactivation of sodium channel. β Toxins bind to receptor site four and modify the activation of sodium channel (Cestèle and Catterall, 2000).

Based on binding and electrophysiological studies, toxins active on insects are classified into four categories: (i) Excitatory toxins, inducing a fast excitatory contraction paralysis in *Sarcophaga argyrostoma* fly larvae (Zlotkin et al., 1985) and repetitive firing in the motor nerves (Walther et al., 1976) by causing an increase of the peak sodium current and voltage-dependent slowing of sodium current inactivation. They bind to insect synaptosomal membranes independently of voltage (Gordon et al., 1984) and act on activation as mammal  $\beta$ -toxins (Walther et al., 1976; Pelhate and Zlotkin, 1981, 1982); (ii) Depressant toxins have dual functionality: a transient contraction paralysis followed by a flaccid paralysis (Zlotkin et al., 1991). Application of very long voltage pulses reveals that flaccid paralysis toxins must be considered as sodium channel openers rather than blockers (Benkhalifa et al., 1997a,b); (iii)  $\alpha$ -type toxins cause a delayed and sustained contraction paralysis in blowfly larvae 5 min post-injection (Eitan et al., 1990). These toxins do not compete with the excitatory toxins, slow down inactivation of sodium channel and bind in a potential-independent manner (Eitan et al., 1990; Kopeyan et al., 1993; Borchani et al., 1997); (iv) The fourth type consist of toxins that are potent to both mammals and insects (De Lima et al., 1986; Loret et al., 1991).

In addition, results obtained with BotIT2, from the venom of *Buthus occitanus tunetanus* (Bot), have to be considered. This toxin showed paralytic activity in insects (*B. germanica*) and was much less potent in mice. BotIT2 affects insect sodium channel activation but shows specific sequence and electrophysiological properties when compared to classic  $\alpha$ ,  $\beta$  and depressant insect toxins. BotIT2 specifically acts by inducing a new current with very slow activation/deactivation kinetics due to the transformation of normal fast channels into slow ones (Borchani et al., 1996; Cestèle et al., 1997).

However, toxins active on insects previously purified from Bot do not explain the high toxicity of this venom against insects (0.05  $\mu$ g/100 mg body weight blowfly larvae) (Zlotkin et al., 1971). To explain this, we undertook the purification of all toxins active on insects present in this venom. In this study, we describe the purification and the pharmacological, electrophysiological as also biochemical characterisations of a new depressant toxin, called *Buthus occitanus tunetanus* insect-toxin 6 (BotIT6). This toxin shows a high level of toxicity on insects. Our results suggest that the BotIT6 receptor site is in close vicinity with the other scorpion toxins sites on the sodium channel.

## 2. Materials and methods

### 2.1. Scorpion venom

The scorpions Bot were collected from Beni Khdach (Tunisia). Crude venom was obtained by electrical stimu-

lation of scorpion in the Veterinarian Laboratory of Institut Pasteur of Tunis and kept frozen at  $-20^{\circ}\text{C}$  until used.

### 2.2. Purification procedure

Frozen venom was extracted with water and dialysed as described by Miranda et al. (1970). The dialysed water extract of the venom was subjected to three successive fractionation steps. The first one consisted of a gel filtration chromatography on Sephadex G-50 and the second one of a semi-preparative HPLC on a 10 mm  $\times$  250 mm column prepacked with ultrasphere octyl, 5  $\mu$ m (Beckman) as previously reported by Borchani et al. (1996, 1997). The third last step was carried out with an analytical C8 HPLC on a 4.6 mm  $\times$  250 mm column prepacked with ultrasphere octyl 5  $\mu$ m (Beckman). Buffer (A) was 0.1% trifluoroacetic acid (TFA) in water, buffer (B) was 0.1% TFA in acetonitrile. The column was equilibrated in 20% buffer B, then eluted with linear gradient of 20–40% buffer B over 60 min. The flow rate was 1 ml/min. The homogeneity of the toxin was assessed by the same column with a gradient of 20–30% solvent B over 60 min at a flow rate of 1 ml/min.

### 2.3. Toxicity test

Fractions derived from Bot G50 and purified toxin were tested on male *B. germanica* (100–120 mg body mass). Eight *Blattella* were used per dose. A volume of 0.5–2  $\mu$ l was injected into the abdominal segment. Toxicity was monitored by a lethality test after 24 h.

Toxicity on mammals was carried out with  $20 \pm 2$  g male C57/BL6 mice after intracerebro-ventricular (i.c.v) injection. Symptoms as well as death records were noticed as previously reported (Borchani et al., 1996).

### 2.4. ELISA characterisation

ELISA tests were used to check the cross-antigenicity of BotIT4, BotIT5, BotIT6, AaHIT and LqqIT2 using anti-AaHIT and anti-LqqIT2 rabbit sera. The wells of 96-well Nunc plates were coated with 100  $\mu$ l of 5  $\mu$ g/ml dilutions of BotIT4, BotIT5, BotIT6 or AaHIT in 0.1 M sodium carbonate, pH 9.6. The experimental procedures are the same as those reported by Borchani et al. (1996).

### 2.5. Sequence determination

Preparation of *S*-pyridylethylated protein (5 nmol) was performed after reduction of the disulfide bridges with dithioerythritol (Borchani et al., 1996). The reduced and alkylated protein was desalted on a Beckman HPLC system equipped with a C8 reverse-phase column (4.6 mm  $\times$  250 mm, 5  $\mu$ m). To establish the N-terminal sequence, the protein was subjected to Edman degradation in an Applied Biosystems 476A sequencer using the prescribed program cycles. For characterisation of

the C-terminal sequence, 1 nmol alkylated protein was chemically cleaved by the cyanogen bromide (CNBr) (70 mg CNBr per ml 70% formic acid) with an appropriate amount (10  $\mu$ l per 10  $\mu$ g of protein). Incubation was in the dark at room temperature for 24 h. Peptides generated from chemical cleavage were purified by microbore C18 reverse-phase column (1 mm  $\times$  10 cm, 7  $\mu$ m) on a ABI 172 HPLC system (Applied Biosystems Inc.). The elution gradient was 2–80% solvent B (70% acetonitrile/0.1% TFA) in solvent A (0.1% trifluoroacetic acid in water) over 60 min at a flow rate of 100  $\mu$ l/min.

## 2.6. Mass spectrometry

The sample was analysed on a Voyager DE RP MALDI-TOF mass spectrometer (Perceptive Biosystems, Inc., Framingham, MA). Analyses was done as previously reported (Srairi-Abid et al., 2000).

## 2.7. Sequence comparison

A search for proteins similar to BotIT6 was performed following BLAST analysis (Altschul et al., 1997) database search program. The amino acid sequence of BotIT6 was aligned with excitatory,  $\alpha$ -type and depressant toxins sequences using the CLUSTAL X program (Jeanmougin et al., 1998). The phylogeny analysis was visualised with the tree view program 1.5, to generate an unrooted tree.

## 2.8. Electrophysiological techniques

Current-clamp and voltage-clamp recordings were carried out on isolated giant axons from abdominal nerve cords of the adult male cockroach *Periplaneta americana* using the double oil-gap single fibre technique (Pichon and Boistel, 1967; Pelhate and Sattelle, 1982). All experiments were carried out at room temperature (18–20  $^{\circ}$ C). Normal

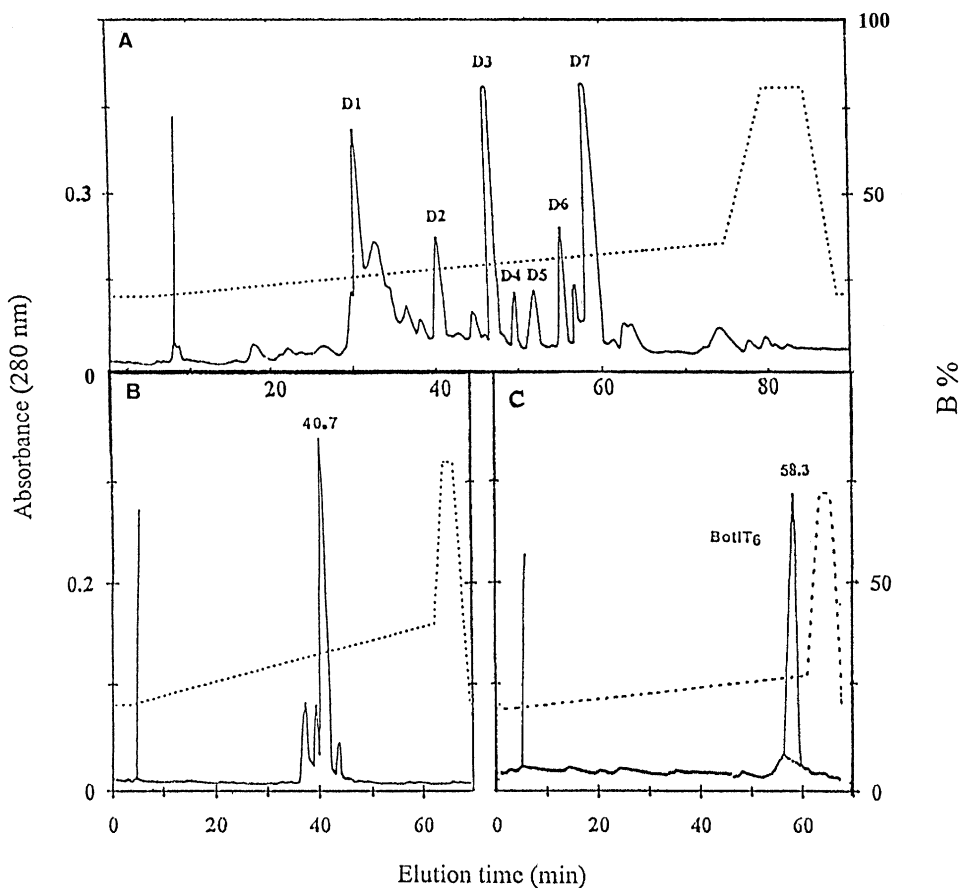


Fig. 1. Elution profiles of HPLC steps. (A) HPLC of the fraction (D) obtained from gel filtration and recycling on Sephadex G-50. The semi-preparative C8 HPLC (10 mm  $\times$  250 mm) was equilibrated with acetonitrile 20% in ammonium formate (12 mS, pH 2.75) then eluted with a linear gradient of acetonitrile 20–35% for 70 min at a flow rate of 3 ml/min. (B) HPLC chromatography of the semi-preparative fraction (D7) on a C8 column (4.6 mm  $\times$  250 mm) in a solvent system of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The gradient was 20–40% B in A in 60 min; the flow rate was 1 ml/min. (C) C8 HPLC of the 40.7 min, fraction collected in the preceding step. The column was equilibrated with 20% acetonitrile (0.1% trifluoroacetic acid) and eluted with a linear gradient of 20–30% acetonitrile for 60 min at a flow rate of 1 ml/min.

Table 1

Relative toxicity of HPLC semi-preparative fractions (D1–D7) derived from D (recycled G50 fraction) on C57/BL6 mouse and *B. germanica*

	Semi-preparative fractions						
	D1	D2	D3	D4	D5	D6	D7
Percentage of total load absorbance	25	8.5	19	2.5	3.5	8.5	29
LD 50 to C57/BL6 mouse ( $\mu\text{g}/20\text{ g}$ )	>2 $\mu\text{g}^a$	>2 $\mu\text{g}^a$	>2 $\mu\text{g}^a$	>2 $\mu\text{g}^a$	>2 $\mu\text{g}^a$	>2 $\mu\text{g}^a$	>2 $\mu\text{g}^a$
LD 50 to <i>B. germanica</i> (ng/100 mg)	216	133	160	5	25	15	10

<sup>a</sup> Non-active on mice until 2  $\mu\text{g}$ .

physiological saline had the following composition (in  $10^{-3}\text{ M}$ ): NaCl, 210;  $\text{CaCl}_2$ , 5.4; KCl, 3.1;  $\text{MgCl}_2$ , 5.2; HEPES buffer, 5; pH was maintained at 7.2. Potassium currents were blocked, when necessary by  $0.5 \times 10^{-3}\text{ M}$  3,4-diaminopyridine (3,4-DAP) (Sigma Chemical). Toxin was applied at  $1.4 \times 10^{-6}\text{ M}$  in saline.

### 2.9. Binding assays

Insect synaptosomal preparations were obtained from homogenates of nerve cords from the cockroach *P. americana* (De Lima et al., 1989).  $^{125}\text{I}$ -Iodination of the toxin AaHIT, purification and characterisation of the monoiodinated derivative were performed according to the method of De Lima et al. (1989). Increasing concentrations of BotIT4, BotIT5 and BotIT6 were tested for their ability to inhibit the binding of mono- $^{125}\text{I}$ -labelled AaHIT derivative

to insect synaptosomal fractions (De Lima et al., 1989). Data points correspond to the average value of duplicates.

## 3. Results

### 3.1. Purification of BotIT6

The five main fractions issued from recycling of the toxic fraction (Bot G50) on Sephadex G-50 equilibrated in 0.1 M ammonium acetate columns, differs in their toxicity towards mammals and insects (data not shown). The fourth fraction (D) toxic only to *B. germanica*, was further resolved in seven fractions (D1–D7) on semi-preparative C8 column (Fig. 1(A)). The most retarded fraction (D7) represents 29% of the fraction D and is among the most active against *B. germanica* (Table 1). Two analytical C8 HPLC steps were

## AMINO ACID SEQUENCES

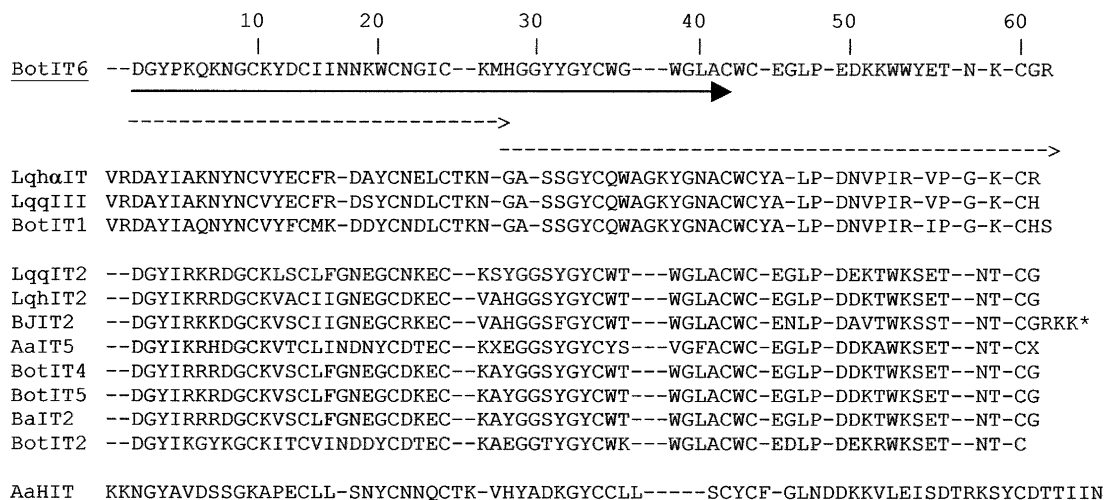


Fig. 2. Amino acid sequence of BotIT6 and its comparison with those of others insect-selective toxins derived from Buthidae scorpions. The arrow ( $\rightarrow$ ) represents the N-terminal sequence of S-alkylated BotIT6, the discontinuous arrows (--->) represents sequences of CNBr derived and isolated peptides. Amino acid sequences were aligned for maximum similarity with the aid of the CLUSTAL X program (Jeanmougin et al., 1998).

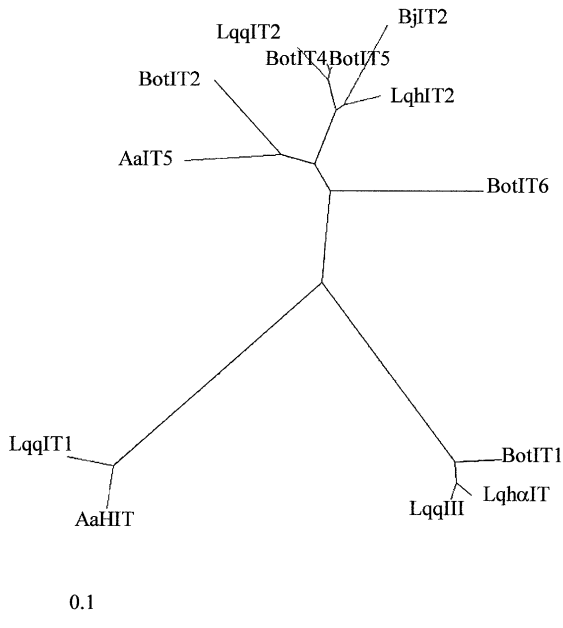


Fig. 3. Evolutionary tree of some scorpion insect toxins including BotIT6 constructed from sequence homology and gap events. Aah, *Androctonus australis Hector* (Zlotkin et al., 1971; Nakagawa et al., 1997); Bot, *Buthus occitanus tunetanus* (Borchani et al., 1996, 1997); Lqq, *Leiurus quinquestriatus quinquestriatus* (Zlotkin et al., 1985; Kopeyan et al., 1990, 1993); Lqh, *Leiurus quinquestriatus hebraeus* (Zlotkin et al., 1985; Eitan et al., 1990); Bj, *Buthotus judaicus* (Lester et al., 1982).

needed to purify to homogeneity a sharp symmetric peak designated BotIT6 (Fig. 1(B) and (C)).

### 3.2. Biological activity of BotIT6

BotIT6 induced on *B. germanica* a flaccid paralysing effect with a high toxicity. Its LD<sub>50</sub> was of 10 ng/100 mg body mass. BotIT6 LD<sub>50</sub> is 10 times lower than that of BotIT4/BotIT5 (110 ng/100 mg body mass) and three times lower than that of AaHIT (26 ng/100 mg body mass), known as the most potent anti-insect toxin. When injected intracerebro-ventricularly to C57/BL6 mice, BotIT6 had no effect, even with concentrations as high as 3 μg/20 g mouse.

### 3.3. Primary structure determination

The first 42 N-terminal amino acid sequence was determined by automatic Edman degradation of 1 nmol of reduced and alkylated BotIT6 (Fig. 2). The examination of this sequence allowed us to locate the unique methionine residue present in BotIT6 (as determined by amino acid analysis). We took advantage of this result to cleave specifically the reduced and alkylated BotIT6 into two peptides (Fig. 2), separated by reverse phase HPLC on C18 column. The alignment of the two-peptide sequences, allowed the complete primary structure of BotIT6 to be established (Fig. 2). The experimentally determined molecular mass of the native BotIT6 was 7260.84 Da (data not shown). Within experimental accuracy, this mass measurement is in good correlation with the sequence of

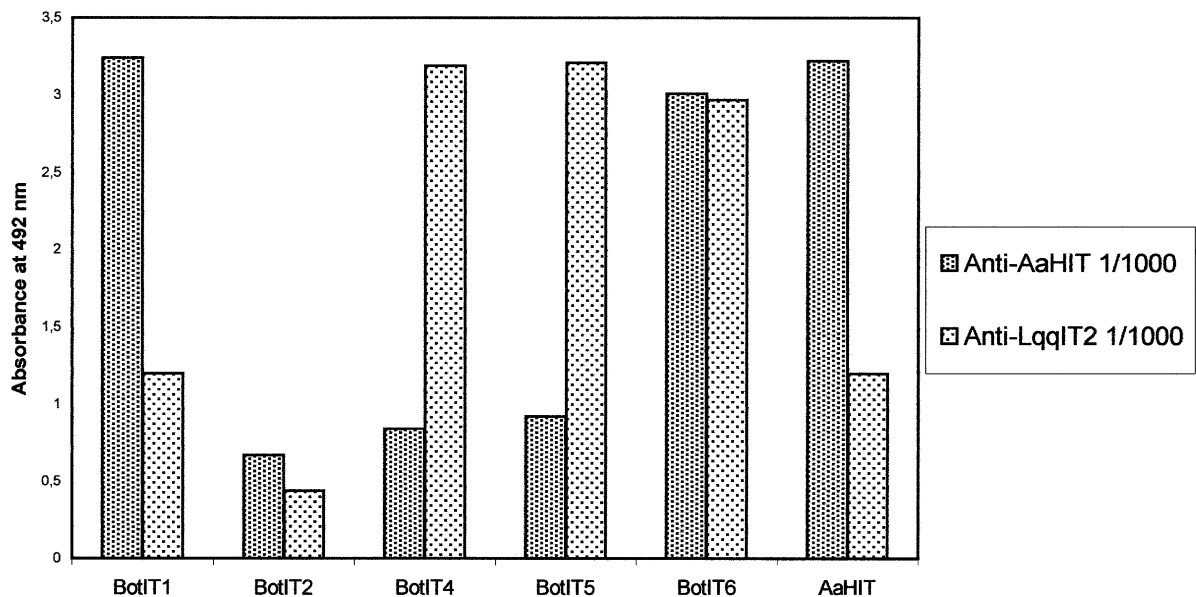


Fig. 4. Binding of anti-AaHIT and anti-LqqIT2 on BotIT1, BotIT2, BotIT4, BotIT5, BotIT6 and AaHIT at 5 μg/ml adsorbed on plates. Elisa tests were conducted as described in Section 2.

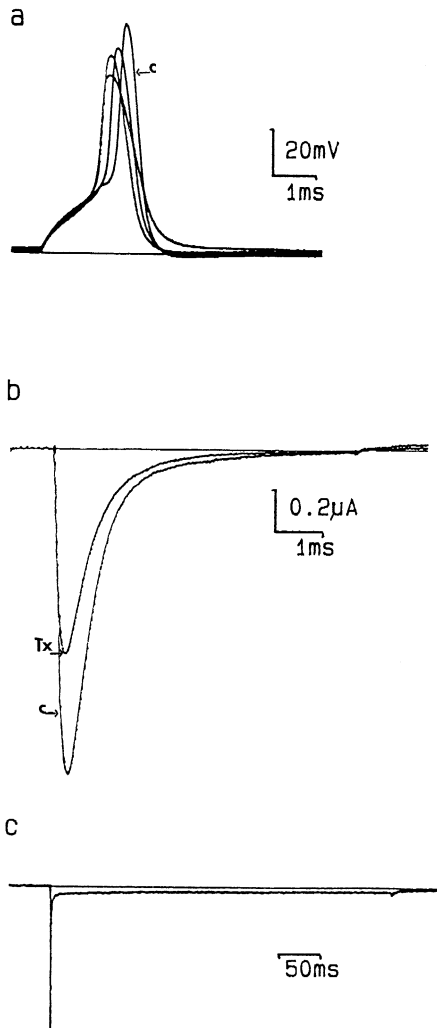


Fig. 5. Effects of BotIT6 on the cockroach axonal potentials and sodium current. (a) Evoked AP before and 5 min after BotIT6 application at  $1.4 \times 10^{-6}$  M concentration. (b) and (c) Transmembrane sodium current recorded in *P. americana* giant axon. The inset (b) shows superimposed sodium currents recorded under voltage short (5 ms) pulses to  $-10$  mV from a HP of  $-60$  mV, before (c) and after application (Tx) of BotIT6 ( $1.4 \times 10^{-6}$  M). The inset (c) shows the sodium current under BotIT6 and 350 ms voltage pulses.

BotIT6 which gives a sequence-derived calculated  $(M + H)^+$  molecular mass of 7258.28 Da.

The alignment of BotIT6 amino acid sequence with other toxins active on insects is shown in Fig. 2. BotIT6 is 58–66% identical to depressant insect toxins. The degree of similarity with  $\alpha$ -type and excitatory toxins is much lower: 24–34%.

### 3.4. Evolutionary data

A classification taking into account sequence homologies and gaps made it possible to separate scorpion toxins

active on insects into groups corresponding to specific pharmacological properties (Fig. 3). The first group corresponded to insect toxins inducing a contractive paralysis in fly larvae (AaHIT and LqqIT1). The second one is represented by the depressant insect toxins (AaIT5, BotIT2, LqqIT2, BotIT4, BotIT5, BjtIT2 and LqhIT2) and includes BotIT6. The third group corresponded to  $\alpha$ -type toxins (BotIT1, Lqh $\alpha$ IT and LqqIII).

Immunological experiments showed that BotIT6 was the only tested to react with both Anti-AaHIT and Anti-LqqIT2 (Fig. 4). These results may be in agreement with phylogenetic data (Fig. 3), since BotIT6 is in a central position in between excitatory and depressant toxins. From an evolutionary point of view BotIT6 might be considered as an ancestral depressant toxin.

### 3.5. Electrophysiological studies

The electrophysiological properties of BotIT6 were tested on giant axons dissected from abdominal nerve cords of the adult male cockroach *P. americana*.

In current-clamp conditions, evoked action potentials (AP), by short current pulses of 10 nA amplitude and 0.5 ms duration, were affected by BotIT6 ( $1.4 \mu\text{M}$ ) application. This toxin slightly depolarises the axonal membrane (1–2 mV) and decreases the initial AP amplitude of about 22% (Fig. 5(a)). Under BotIT6, a post-depolarisation phase is also observed. Nevertheless, no complete block of the AP and no repetitive activity are noticed as observed for depressant toxins.

In voltage-clamp and in control, sodium inward current, recorded during 5 ms voltage pulses to  $-10$  mV from a holding potential (HP) equal to  $-60$  mV, is transient and becomes totally blocked within 2–3 ms (Fig. 5(b)). After 10 min of BotIT6 perfusion, a decrease of the peak inward sodium current of about 38% is observed (Fig. 5(b)). This decrease is not associated with a maintained late inward current as described with 'classical' depressant toxins. However, when very long voltage pulses (350 ms) are applied, an inward late sodium current develops after the transient phase and shows a slow deactivation decay (Fig. 5(c)).

These results indicate a slowing of the sodium current activation induced by BotIT6 application. Although it is not very potent, this toxin presents flaccid paralysis toxin properties.

### 3.6. Binding assays

Results of competition experiments (Fig. 6) indicate that BotIT6 is able to fully inhibit the specific binding of  $^{125}\text{I}$ -BotIT2 on *P. americana* synaptosomal membrane vesicles. The concentration of BotIT6 giving half effect ( $K_{0.5}$ ) is  $2.69 \pm 0.70$  nM. Data presented in Table 2 reveal that the specific binding of  $^{125}\text{I}$ -BotIT2 to cockroach neuronal membranes is completely inhibited by AaHIT, BotIT4 and

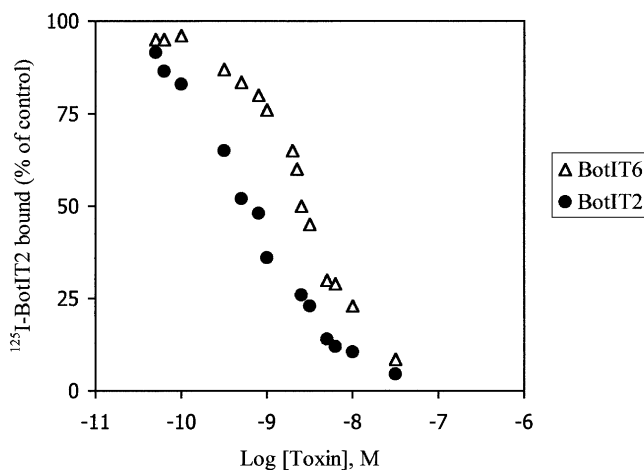


Fig. 6. Competitive displacement of the specific binding of:  $^{125}\text{I}$  BotIT2 by BotIT2 and BotIT6. The concentration of native toxins inhibiting 50% of the binding of radiolabeled ligand is  $0.74 \pm 0.29$  nM for BotIT2 (●) and  $2.69 \pm 0.7$  nM for BotIT6 (Δ).

BotIT5. BotIT6 shows a lower inhibiting effect (five times less) than AaHIT and (10 times less) than BotIT4 and BotIT5.

BotIT6 also, inhibited the binding of  $^{125}\text{I}$ -AaHIT to the same synaptosomal preparation, with a half effect ( $K_{0.5}$ ) of  $9.03 \pm 0.9$  nM (data not shown). Compared to BotIT4 and BotIT5, BotIT6 presents a lower inhibiting effect than the two others depressant toxins (Table 2). BotIT1 was found quite less potent to inhibit both  $^{125}\text{I}$ -AaHIT and  $^{125}\text{I}$ -BotIT2.

#### 4. Discussion

Zlotkin and coworkers have reported that the venom of Bot is among the most potent of 16 tested venoms to blowfly larvae (Zlotkin et al., 1971). Toxins active on insects so far purified from this venom (BotIT1, BotIT2, BotIT4 and BotIT5) do not explain its high toxicity against insects (Borchani et al., 1996, 1997). The explanation might be the existence in this venom of other toxins more active on

insects than those previously purified or the fact that a cooperativity between them (Herrman et al., 1995).

The first hypothesis led us to carry out a large screening of the venom of Bot which leads to the purification of a new insect toxin: BotIT6, which appears to be the most potent toxin active on insect so far characterised.

Its amino acid sequence is similar to those of known depressant insect toxins, such as BotIT4, BotIT5, BotIT2, LqIT2, LqIT2, BJT2, and AaIT5 (Fig. 2). The main differences between BotIT6 sequence and those of other flaccid toxins are residues 4 (P/I); 19 (K/E or N); 23 (G/K or T); 24 (I/E); 49 (E/D); 52 (K/T or A); 59 (K/T) and the additional arginine residue at the C-terminus. The presence of a proline at position 4 may confer for BotIT6 a unique and characteristic secondary structure at its N-terminal part. Other particular characteristic of BotIT6 compared to other toxins active on insect is the basic character of its 19–24 region, whereas in depressant toxins the similar region is generally acid except for BJT2 which is neutral. Moreover, the C-terminus is also more basic because of the presence of the K52, 59 and the R62. As demonstrated previously, residues belonging to the C-terminal sequences are often involved in toxin-receptor interactions (Kharrat et al., 1989; Loret et al., 1990). On the other hand, basic toxins are more potent than acidic ones (Li et al., 1996). Considering the fact that scorpion toxins bind to ion channels via regions of positive surface potential (Selisko et al., 1996), we have demonstrated for BotIT6, that the whole charge is positive (+3), whereas it is negative for BotIT2 (−3), BotIT4/BotIT5 (−2). It may be noticed also that the toxicity increases with the total positive charge of these toxins. Thus, BotIT2, which has the lowest charge, is the less potent ( $\text{LD}_{50} = 135$  ng) among the four toxins belonging to the same group, purified in our laboratory. For BotIT4/BotIT5,  $\text{LD}_{50}$  is 110 ng, whereas it is 10 ng for BotIT6. So the positive charge patches of BotIT6, might favour its recognition by the sodium channel (Li et al., 1996).

Table 2

Concentrations of different toxins inhibiting 50% of the specific binding of  $^{125}\text{I}$ -AaHIT and  $^{125}\text{I}$ -BotIT2 to their specific sites on *P. americana* synaptosomal membrane vesicles

Ligands	$K_{0.5}$ (nM)	
	$^{125}\text{I}$ -AaHIT	$^{125}\text{I}$ -BotIT2
AaHIT	$0.80 \pm 0.2$	$0.59 \pm 0.20$
BotIT2	$3.90 \pm 0.9$	$0.74 \pm 0.29$
BotIT6	$9.03 \pm 0.9$	$2.69 \pm 0.70$
BotIT4	$2.04 \pm 0.9$	$0.25 \pm 0.08$
BotIT5	$3.03 \pm 0.2$	$0.23 \pm 0.02$
BotIT1	$\gg 1000$	$< 1000$

Like other depressant toxins, Bot IT6 inhibits specifically the binding of  $^{125}\text{I}$ -AaHIT and  $^{125}\text{I}$ -BotIT2 on *P. americana* synaptosomal membranes. The competitive interaction between these three toxins is a consequence of a partial overlap of their points of attachment to the external segments of the insect sodium channels, indicating that these three toxins have closely localised if not identical binding sites (Cestèle et al., 1997).

On the other hand, in contrast with the toxicity data, BotIT6 has a lower inhibiting effect (Table 2) and is less potent on the *P. americana* giant axon than other depressant toxins. These observations may suggest that the receptor site occupied by BotIT6 is only partially identical to the one of the other depressant toxins and/or that the sodium channel isoform recognised by BotIT6 in the toxicity test is not the same than in the pharmacological test.

The use of known selective sodium channel neurotoxins and studies of their different pharmacological behaviours towards their receptor sites in sodium channels, may contribute to the elucidation of the structural basis for their selectivity and their structure–function relationships. In fact, toxins active on insects presents a variable specificity against different species of insects. The comparison of the primary structures of toxins active on insects does not demonstrate any evident correlation between the primary structure and the specificity of these proteins. Thus, the use of a toxin such as BotIT6 engineered by peptide synthesis or direct mutagenesis, combined with toxicity and pharmacological studies, should allow us to better understand the specificity of a given toxin to a given species of insect.

The lack of AaHIT toxicity in mammals and its potent activity on insects have been used to amplify the natural insecticide ability of baculoviruses (Maeda et al., 1991; McCutchen et al., 1991; Stewart et al., 1991). Despite, the insertion of the AaHIT coding gene, which notably improved the speed of killing of insects infected by the baculovirus, more investigation is necessary before recombinant baculoviruses can be considered a good alternate or a complement to chemical insecticides in the biological struggle against harmful insects and particularly against lepidopterans which constitute their natural target.

Our results concerning the activity of BotIT6 and those obtained by Nakagawa and collaborators concerning the high toxicity of AaIT5 to lepidopterans (such as *Heliothis virescens*) (Nakagawa et al., 1997) lead us to think that the depressant insect toxins, are the more suitable toxins to be used for the construction of new recombinant baculoviruses.

## Acknowledgements

We gratefully acknowledge Pr. Koussay Dellagi, Head of Pasteur Institute of Tunis for his constant encouragement. We would like also to thank Dr Zakaria Ben Lasfar and his collaborators (Veterinary Laboratory, Pasteur Institute of

Tunis) for providing the venom. This research was supported in part by funds from Cooperation inter Universitaire Franco-Tunisienne CMCU 10908/10911.

## References

- Altshul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402.
- Benkhalifa, R., Stankiewicz, M., Pelhate, M., Serrano-Hernandez, S.E., Possani, L.D., Hinkel, H., Mebs, D., 1997a. Action of Babycurus-toxin 1 from the east African scorpion *Babycurus centrurimorphus* on the isolated cockroach giant axon. *Toxicon* 35 (7), 1069–1080.
- Benkhalifa, R., Stankiewicz, M., Lapied, B., Turkov, M., Zilberberg, N., Gurevitz, M., Pelhate, M., 1997b. Refined electrophysiological analysis suggests that a depressant toxin is a sodium channel opener rather than a blocker. *Life Sci.* 61 (8), 819–830.
- Borchani, L., Mansuelle, P., Stankiewicz, M., Grolleau, F., Cestèle, S., Karoui, H., Lapied, B., Rochat, H., Pelhate, M., El Ayeb, M., 1996. A new scorpion toxin paralytic to insects that affect  $\text{Na}^+$  channel activation purification, structure, antigenicity and mode of action. *Eur. J. Biochem.* 241, 525–532.
- Borchani, L., Stankiewicz, M., Kopeyan, C., Mansuelle, P., Kharrat, R., Cestèle, S., Karoui, H., Rochat, H., Pelhate, M., El Ayeb, M., 1997. Purification, structure and activity of three insect toxins from *Buthus occitanus tunetanus* venom. *Toxicon* 35, 365–382.
- Catterall, W.A., 1984. The molecular basis excitability. *Science* 223, 653–661.
- Catterall, W.A., 1992. Cellular and molecular biology of voltage-gated sodium channels. *Physiol. Rev.* 72, S15–S48.
- Cestèle, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 82 (9–10), 883–892.
- Cestèle, S., Kopeyan, C., Oughideni, R., Mansuelle, P., Granier, C., Rochat, H., 1997. Biochemical and pharmacological characterization of a depressant insect toxin from the venom of the scorpion *Buthacus arenicola*. *Eur. J. Biochem.* 243, 93–99.
- Couraud, F., Jover, E., Dubois, J.M., Rochat, H., 1982. Two types of scorpion toxin receptor sites, one related to the activation, the other to the inactivation of the action potential sodium channel. *Toxicon* 20, 9–13.
- De Lima, M.E., Martin, M.F., Diniz, C.R., Rochat, H., 1986. *Tityus serrulatus* toxin TsVII bears pharmacological properties of both  $\beta$ -toxin and insect toxin from scorpion venom. *Biochem. Biophys. Res. Commun.* 139, 296–302.
- De Lima, M.E., Martin-Eauclaire, M.F., Hue, B., Loret, E., Diniz, C.R., Rochat, H., 1989. On the binding of two scorpion toxins to the central nervous system of the cockroach *Periplaneta americana*. *Insect Biochem.* 19, 413–422.
- Duval, A., Malécot, C.O., Pelhate, M., Rochat, H., 1989. Changes in  $\text{Na}^+$  channel properties of frog and rat skeletal muscles induced by the AaH II toxin from the scorpion *Androctonus australis*. *Pflügers Arch.* 415, 361–371.
- Eitan, M., Fowler, E., Herrmann, R., Duval, A., Pelhate, M., Zlotkin, E., 1990. A scorpion venom neurotoxin paralytic to insects that affects sodium current inactivation: purification,

- primary structure and mode of action. *Biochemistry* 29, 5941–5947.
- Gordon, D., Jover, E., Couraud, F., Zlotkin, E., 1984. The binding of the insect selective neurotoxin (AaIT) from scorpion venom to locust synaptosomal membranes. *Biochim. Biophys. Acta* 778, 349–358.
- Herrman, R., Moskowitz, H., Zlotkin, E., Hammock, B.D., 1995. Positive cooperativity among insecticidal scorpion neurotoxins. *Toxicon* 33, 1099–1102.
- Jeanmougin, F., Gouy, M., Higgins, D.G., Gibson, T.J., Thompson, J.D., 1998. Multiple sequence alignment with Clustal X. *TIBS* 23, 403–405.
- Jover, E., Couraud, F., Rochat, H., 1980. Two types of neurotoxins characterized by their binding to two separate receptor sites on rat brain synaptosomes. *Biochem. Biophys. Res. Commun.* 95, 1607–1614.
- Kharrat, R., Darbon, H., Rochat, H., Granier, C., 1989. Structure–activity relationships of scorpion  $\alpha$ -toxins. Multiple residues contribute to the interaction with receptors. *Eur. J. Biochem.* 181, 381–390.
- Kopeyan, C., Mansuelle, P., Sampieri, F., Brando, T., Bahraoui, E.M., Rochat, H., Granier, C., 1990. Primary structure of the scorpion anti-insect toxins isolated from the venom of *Leiurus quinquestriatus quinquestriatus*. *FEBS Lett.* 261, 423–426.
- Kopeyan, C., Mansuelle, P., Martin-Eauclaire, M.F., Rochat, H., Miranda, F., 1993. Characterization of toxin III of the scorpion *Leiurus quinquestriatus quinquestriatus*: a new type of  $\alpha$  toxin highly toxic to mammals and insects. *Nat Toxins* 1, 308–312.
- Lester, D., Lazarovicy, P., Pelhate, M., Zlotkin, E., 1982. Purification, characterization and action of two insect toxins from the venom of the scorpion *Buthus judaicus*. *Biochem. Biophys. Acta* 701, 370–381.
- Li, H.M., Wang, D.C., Zeng, Z.H., Jin, L., Hu, R.Q., 1996. Crystal structure of an acidic neurotoxin from scorpion *Buthus Martenzii Krasch* at 1.85 Å resolution. *J. Mol. Biol.* 261, 415–431.
- Loret, E.P., Mansuelle, P., Rochat, H., Granier, C., 1990. Neurotoxins active on insect: amino acid sequences, chemical modifications, and secondary structure estimation by circular dichroism of toxins from the scorpion *Androctonus australis Hector*. *Biochemistry* 29, 1492–1501.
- Loret, E.P., Martin-Eauclaire, M.F., Mansuelle, P., Sampieri, F., Granier, C., Rochat, H., 1991. An anti-insect toxin purified from the scorpion *Androctonus australis Hector* also acts on the  $\alpha$ - and  $\beta$ -sites of the mammalian sodium channel: sequence and circular dichroism study. *Biochemistry* 30, 633–640.
- Maeda, S., Volrath, S.L., Hanzlik, T.N., Harper, S.A., Majima, K., Maddox, D., Hammock, B.D., Fowler, E., 1991. Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. *Virology* 184, 777–780.
- Mc Cutchen, B.F., Choudary, P.V., Crenshaw, R., Maddox, D., Kamita, S.G., Palekar, N., Volrath, S., Foxler, E., Hammock, B.D., Maeda, S., 1991. Development of a recombinant baculovirus expressing an insect-selective neurotoxin: potential for pest control. *Biotechnology* 9, 848–852.
- Miranda, F., Kopeyan, C., Rochat, H., Rochat, C., Lissitzky, S., 1970. Purification of animal neurotoxins. Isolation and characterization of eleven neurotoxins from the venoms of the scorpion *Androctonus australis Hector*, *Buthus occitanus tunetanus*, and *Leiurus quinquestriatus quinquestriatus*. *Eur. J. Biochem.* 16, 514–523.
- Nakagawa, Y., Moo Lee, Y., Lehmborg, E., Herrmann, R., Moskowitz, H., Jones, A.D., Hammock, B.D., 1997. Anti-insect toxin 5 (AaIT5) from *Androctonus australis*. *Eur. J. Biochem.* 246, 496–501.
- Pelhate, M., Zlotkin, E., 1981. Voltage dependent slowing of the turn off of  $\text{Na}^+$  current in the cockroach giant axon induced by the scorpion venom insect toxin. *J. Physiol. (Lond.)* 319, 30P–31P.
- Pelhate, M., Sattelle, D.B., 1982. Pharmacological properties of insect axons. *J. Insect Physiol.* 28, 889–903.
- Pelhate, M., Zlotkin, E., 1982. Action of insect toxin and other toxins derived from the venom of the scorpion *Androctonus australis* in isolated axons of the cockroach *Periplaneta americana*. *J. Exp. Biol.* 97, 67–77.
- Pichon, Y., Boistel, J., 1967. Current–voltage relations in the isolated giant axon of the cockroach under voltage-clamp conditions. *J. Exp. Biol.* 47, 343–355.
- Pichon, Y., Pelhate, M., 1984. Effects of toxin I from *Androctonus australis Hector* on sodium currents in giant axons of *Logilo forbesi*. *J. Physiol. (Paris)* 79, 318–326.
- Rochat, H., Bernard, P., Couraud, F., 1979. Scorpion toxins: chemistry and mode of action. In: Ceccarelli, B., Clementi, F. (Eds.), *Advances in Cytopharmacology*, vol. 3. Raven Press, New York, pp. 325–334.
- Selisko, B., Garcia, C., Becerril, B., Delpierre, M., Possani, L.D., 1996. An insect-specific toxin from *Centruroides nixius Hoffmann* cDNA, primary structure, three-dimensional model and electrostatic surface potentials in comparison with other toxin variants. *Eur. J. Biochem.* 242, 235–242.
- Srairi-Abid, N., Mansuelle, P., Mejri, T., Karoui, H., Rochat, H., Sampieri, F., El Ayeb, M., 2000. Purification, characterization and molecular modelling of two toxin-like proteins from the *Androctonus australis hector* scorpion venom. *Eur. J. Biochem.* 267, 5614–5620.
- Stewart, L.M.D., Hirst, M., Ferber, M.L., Merryweather, A.T., Cayley, P.J., Possee, R.D., 1991. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature* 352, 85–88.
- Walther, C., Zlotkin, E., Rathmayer, W., 1976. Action of different toxins from scorpion *Androctonus australis* on locust nerve-muscle preparation. *J. Insect Physiol.* 22, 1187–1194.
- Zlotkin, E., Frankei, G., Miranda, F., Lissitzky, S., 1971. The effect of scorpion venom on blowfly larvae: a new method for the evaluation of scorpion venom potency. *Toxicon* 9, 9–13.
- Zlotkin, E., Kadouri, D., Gordon, D., Pelhate, M., Martin, M.F., Rochat, H., 1985. An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch. Biochem. Biophys.* 240, 877–887.
- Zlotkin, E., Eitan, M., Bindokas, V.I., Adams, M.E., Moyer, M., Burckhart, W., Fowler, E., 1991. Functional duality and structural uniqueness of depressant insect-selective neurotoxins. *Biochemistry* 30, 4814–4820.