

Full Article

## Characterization of cDNA sequence encoding for a novel sodium channel $\alpha$ -toxin from the Iranian scorpion *Mesobuthus eupeus* venom glands

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### ABSTRACT

The venoms of Buthidae scorpions are known to contain basic, single-chain protein  $\alpha$ -toxins consisting of 60-70 amino acid residues that are tightly cross-linked by four disulfide bridges. Total RNA was extracted from the venom glands of scorpion *Mesobuthus eupeus* collected from the Khuzestan province of Iran and then cDNA was synthesized with the modified oligo (dT) primer and extracted total RNA as template. By applying the Semi-nested RT-PCR using homologous primers, the fragments of 273 bp was amplified and sequenced. The coding region encodes a polypeptide of 85 amino acid residues with a calculated molecular weight of 9.337 kDa and theoretical isoelectric point of 6.52. It was designated as MeNaTx $\alpha$ -4. This peptide with homology to the "Scorpion long chain toxin-like domain" is belongs to the Toxin-3 superfamily. Multiple alignment of the putative amino acid sequence of MeNaTx $\alpha$ -4 exhibited 95% sequence identity with lesser Asian scorpion *M. eupeus* venom sodium channel  $\alpha$ -toxin-3 but interestingly displayed only 63% identity with other  $\alpha$ -toxins from this species. The sequence was also identical to the counterpart sequences from *M. martensii* (92%) and *Buthus occitanus israelis* (80%). This high sequence similarity together with completely conserved in the positions of all 8 Cys indicated that MeNaTx $\alpha$ -4 is a new member of  $\alpha$ -toxin from the Iranian *M. eupeus* which may originate from a common ancestor.

**Keywords:**  $\alpha$ -toxin, Semi-nested RT-PCR, scorpion venom, *Mesobuthus eupeus*

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### INTRODUCTION

The venoms of Buthidae scorpions are known to contain basic, single-chain protein alpha-toxins that are tightly folded by four disulfide bridges (Martin-

Eauclaire & Couraud 1995). Scorpion venom constitutes a rich source of toxin peptides that are associated with the ion channels for sodium (Rochat *et al* 1979), potassium (Carbone *et al* 1982), chloride (Debin *et al* 1993) and calcium (Valdivia & Possani, 1998). They modify the opening or closing of the

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channels in nerve, muscle and heart cells, thus increasing the depolarization of the membrane and leading to the release of neurotransmitters (Catterall 1992). All known neurotoxins can be classified either as long-chain toxins (60-70 amino acid, 4 disulfide bridges) (Gordon *et al* 1998, Froy *et al* 1999), which target sodium channels, or short chain toxins (less than 40 amino acid residues, 3-4 disulfide bridges) which target potassium channels (Lebrun *et al* 1997). All these toxins have a common structural motif (Bontems *et al* 1991a), named CSH for cysteine-stabilised  $\alpha$ -helix. The long-chain neurotoxins derived from the venom of the Buthidae scorpions that are active on vertebrate sodium channels can be subdivided into insect-selective excitatory and depressant toxins ( $\alpha$ -toxins) based on their toxicity to insects and also the  $\alpha$ -like toxins which affect both mammals and insects (Gordon *et al* 2007).

The relative toxicity of the typical mammal  $\alpha$ -toxins such as AaHI (Martin *et al* 1984) and AaHII (Rochat *et al* 1972) were revealed according to electrophysiological studies. The sodium channels  $\alpha$ -toxins such as LqqIV, BotIII (Pelhate & Zlotkin, 1982, Cestele *et al* 1997a) prefer mammal and other  $\alpha$ -toxins such as LqhaIT, LqqIII and BotIV preferably affect insects (Eitan *et al* 1990, Kopeyan *et al* 1990, Cestele *et al* 1997b) has been characterized.

In this study, we report the characterization of a new member of  $\alpha$ -toxin-related gene from scorpion *Mesobuthus eupeus* of Khuzestan, Iran.

## MATERIALS AND METHODS

**Scorpion samples.** The Scorpion *Buthidae* family is widely distributed throughout Iran, with some of its species being clinically important in many Iranian provinces. *M. eupeus* from this family is the most abundant species in the Khuzestan province. The Scorpions were collected in the area of Khuzestan province, Iran and transported to the reference laboratory of the Razi Institute. They were sacrificed

two days after manual extraction of their venom to allow the toxin-producing cells of the venom glands to enter into a secretory phase. Twenty separated venom glands were used for total RNA extraction.

**Total RNA isolation and cDNA synthesis.** Scorpion glands kept at  $-70^{\circ}\text{C}$  were ground into fine powder under liquid nitrogen using mortar and pestle. Total RNA was extracted from ground venom glands and was dissolved in TRIPURE reagent (CinnaGen, Iran) according to the manufacturer's instruction. The RNA pellets were dissolved in water and used for cDNA synthesis immediately. For modifying the oligo(dT) primer, a linker containing two restriction endonucleases enzyme sites of *XbaI* and *XhoI* were added to the 5' ends of the primer. The sequence of the modified oligo (dT) primer was 5-GGGTCTAGAGCTCGAGTCAC(T)17. The two restriction sites were underlined. To synthesis the cDNA, 3  $\mu\text{g}$  of the extracted total RNA was incubated with 0.5  $\mu\text{g}$  of the modified Oligo(dT) primer at  $70^{\circ}\text{C}$ , for 10 min followed by a brief centrifugation. The reaction was chilled on ice for a few min and then added 1  $\mu\text{l}$  RNasin (CinnaGen, Iran), 1  $\mu\text{l}$  dNTP mixture (120 mM of each nucleotide), 2.5  $\mu\text{l}$  of 5 X enzyme buffer and 1  $\mu\text{l}$  (200 U) of Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (CinnaGen, Iran) in a total volume of 20  $\mu\text{l}$ . Negative control was performed without the reverse transcriptase enzyme. The reaction was incubated at  $42^{\circ}\text{C}$  for 1 h followed by a brief centrifugation and then inactivation of the enzyme by heating at  $100^{\circ}\text{C}$  for 10 min and immediately transferred on ice.

**Semi-nested RT-PCR.** The specific primers used for amplification of cDNA encoding the target genes were designed according to the sequence information from *Mesobuthus martensii* putative sodium channel toxin BmKT (AF370023) which was retrieved from the NCBI GenBank. The primers were BmKF 5'-cgcggatccTCCGTAAAACGGTTCAAATG (forward primer) and BmK R5'-cgcaagcttACCGCCATTGCATTTTCCT (reverse primer). These primers were designed based on a conserved

region close to the 5' and 3'-end of several genes for sodium channel  $\alpha$ -toxins. In order to facilitate the directional cloning in future study, *Bam*HI or *Hind*III were added to the 5' ends of the forward and reverse primers, respectively. The two restriction sites were in low cases and underlined. Since the cleavage sites were close to the ends of the primers, and some restriction enzymes have cut inefficiently at the end of the linear DNA molecules, additional bases (gcgc) were added to the 5' ends to ensure digestion of amplified fragments. This stage was followed by two-round Semi-nested PCR, using the diluted product of the first PCR as template for second PCR. In order to apply the second round of PCR, primer ModT-R (5'-CCCAGATCTCGAGCTCAGTG) was synthesized. The PCR was performed using a program of initial denaturation at 95°C (5 min), followed by 35 cycles of denaturation at 94°C (40 sec), annealing at 56°C (90 sec) and extension at 72°C (1 min), with a final extension at 72°C (10 min). The PCR conditions for both rounds were the same except for annealing in the second round which was 58°C. Amplicons were separated by 1% agarose gel electrophoresis and staining with ethidium bromide.

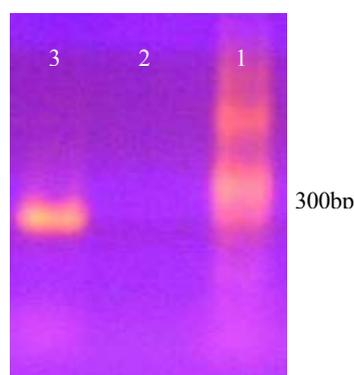
**DNA sequencing and data analyses.** PCR products of the appropriate size was excised from the gel, purified by an extraction kit (Qiagen, Iran) and sequenced from both ends using a dideoxy termination method in an automated DNA sequencer. Sequence similarity analysis against GenBank database entries was performed using BLAST at the NCBI website (<http://www.ncbi.nlm.nih.gov>). Primer sets were generated using Primer3 program ([http://biotools.umassmed.edu/bioapps/primer3\\_www.cgi](http://biotools.umassmed.edu/bioapps/primer3_www.cgi)). The CDD-Search software from the NCBI site was used to determine the conserved domains (Marchler-Bauer *et al* 2003). We used online tool software at the ExPASy website <http://expasy.org/tools> for nucleotide sequences to translate into the corresponding amino acids, and the predicted signal peptide sequence was identified using online tool software at the ExPASy website (<http://www.cbs.dtu.dk/services/SignalP/>). Multiple

sequence alignments were obtained by using the CLUSTAL\_W program (Thompson *et al* 1994) and the resulting alignment was highlighted using the BOXSHADE software ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). Phylogenetic tree was constructed by using online tool software ([http://www.phylogeny.fr/version2.cgi/simple\\_phylogeny.cgi](http://www.phylogeny.fr/version2.cgi/simple_phylogeny.cgi)).

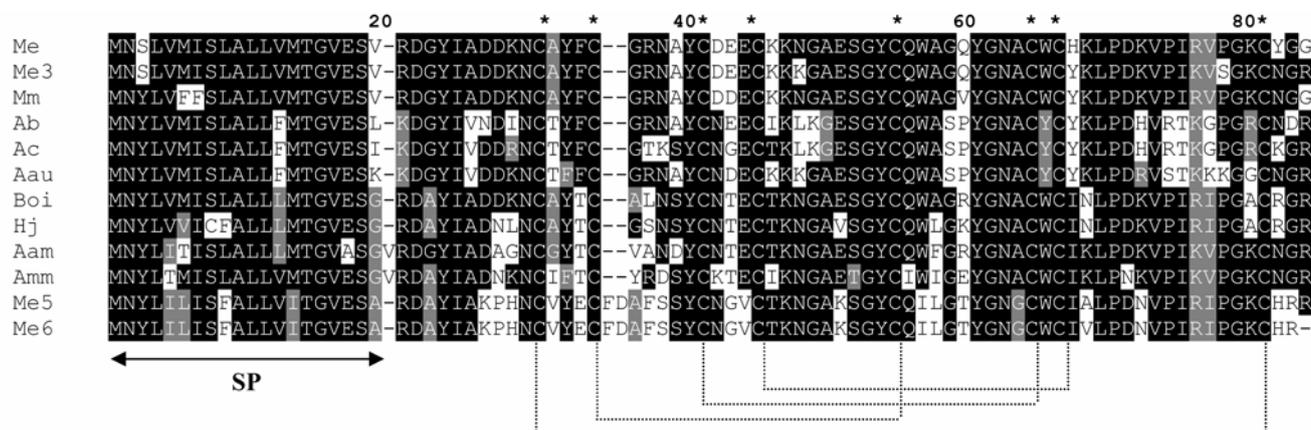
## RESULTS

### Amplification and sequencing of MeNaTx $\alpha$ -4.

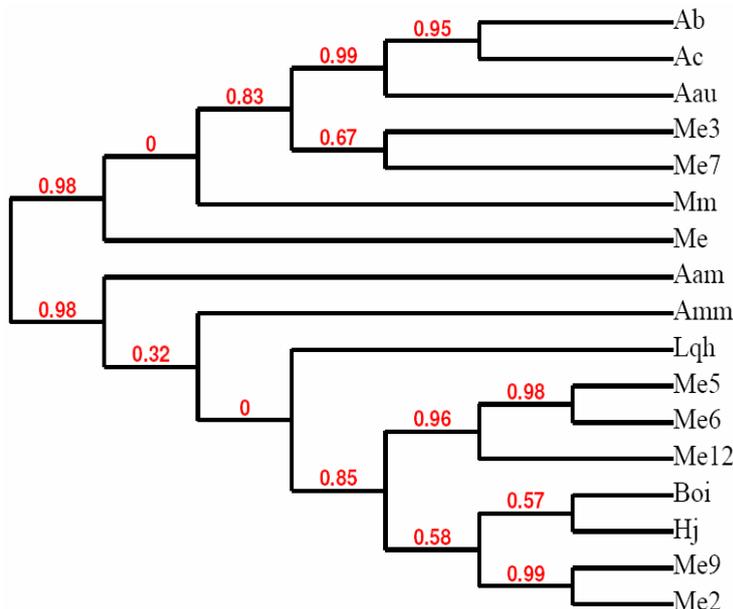
Starting with 10 fresh venom glands giving 0.5 g of tissue, 4  $\mu$ g of total RNA were obtained. The  $\alpha$ -toxin cDNA from *M. eupeus* was amplified by Semi-nested RT-PCR method using homologous primers designed to anneal to conserved sequences in the 5' and 3'-UTR (un-translated region) of various scorpion venom derived  $\alpha$ -toxins. To amplify the mRNAs using Semi-nested RT-PCR, cDNA was synthesized by using the modified oligo (dT). This technique allowed the amplification of the target gene in a two-round PCR method. The first round of PCR was performed using ModT-R and BMK-F primer, and the synthesized cDNA as template. The second round of PCR was performed using BMK-F and BMK-R primers, and one-tenth dilution of the first round of PCR as templates (Figure 1).



**Figure 1.** Agarose gel electrophoresis of RT-PCR products from  $\alpha$ -toxin gene isolated from the Iranian scorpion *M. eupeus*. Lane 1; the negative control (water), lane 2; the second round of semi-nested RT-PCR amplification product, and lane 3 is 50 bp DNA size marker. Each lane was loaded with 8  $\mu$ l of the total reaction.

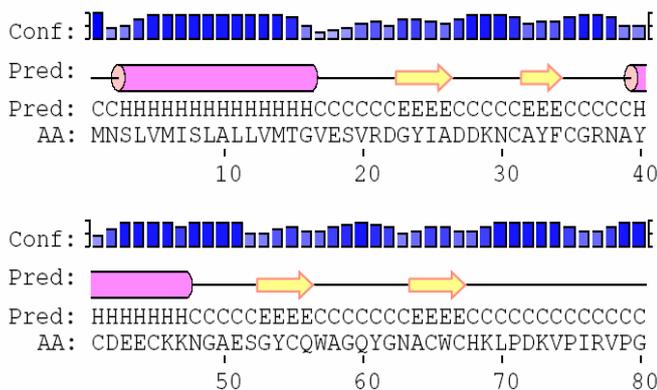


**Figure 2.** Multiple sequence alignment of MeNaTx $\alpha$ -4 and other scorpion  $\alpha$ -toxins. The amino acid sequence of MeNaTx $\alpha$ -4 was aligned with venom sodium channel toxin 3 (ABR21041), 5 (ADU05410) and 6 (ABR21068) from species of lesser Asian scorpion *M. eupeus* and also with  $\alpha$ -neurotoxin Tx15 *Mesobuthus martensii* (Q9GNG8),  $\alpha$ -toxin Tx405 *Buthus occitanus israelis* (ACJ23096), neurotoxin 8 precursor *Androctonus bicolor* (ADE42758), neurotoxin 2 precursor *Androctonus crassicauda* (ADE42760), Toxin AahP1005 *Androctonus australis* (Q9BLM4),  $\alpha$ -insect toxin BjaIT *Hottentotta judaicus* (Q56TT9),  $\alpha$ -neurotoxin 2 *Androctonus amoreuxi* (Q86SE0) and  $\alpha$ -neurotoxin 8-related gene product *Androctonus mauretanicus mauretanicus* (Q2YHM1). The amino acids are denoted by one-letter symbols. Shading indicates identity (black) or conservative substitutions (grey) relative to MeNaTx $\alpha$ -4. Gaps represented by dashes were introduced to maximize the alignment. Disulfide bridges are indicated at the bottom by dashed lines and marked by stars above the sequence. The signal peptide (SP) is underlined by a double arrows bar.



**Figure3.** Phylogenetic tree of MeNaTx $\alpha$ -4 (Me) and other scorpion counterpart sequences. Phylogeny was reconstructed based on the regions encoding the mature toxin including venom sodium channel toxin 2 (Me2), 3 (Me3), 5 (Me5) and 6 (Me6), 7 (Me7), 9 (Me9) and 12 (Me12) from species of lesser Asian scorpion *M. eupeus* and also with  $\alpha$ -neurotoxin Tx15 *Mesobuthus martensii* (Mm),  $\alpha$ -toxin Tx405 *Buthus occitanus israelis* (Boi), neurotoxin 8 precursor *Androctonus bicolor* (Ab), neurotoxin 2 precursor *Androctonus crassicauda* (Ac), Toxin AahP1005 *Androctonus australis* (Aau),  $\alpha$ -insect toxin BjaIT *Hottentotta judaicus* (Hj),  $\alpha$ -neurotoxin 2 *Androctonus amoreuxi* (Aam) and  $\alpha$ -neurotoxin 8-related gene product *Androctonus mauretanicus mauretanicus* (Q2YHM1) (Amm), and also Lqh4 (Lqh) from *Leiurus quinquestriatus hebraeus* were used. Numbers in nodes indicate the correlation coefficients between groups.

The cDNA contained of 273 bp that the coding region was 255 bp encoding a polypeptide of 85 amino acid residues with a calculated molecular weight of 9.337 kDa and theoretical isoelectric point of 6.52. It was designated as MeNaTx $\alpha$ -4. The open reading frame of this sequence possesses high similarity to  $\alpha$ -toxins and is closely related to sodium channel toxin-3 from lesser Asian scorpion *M. eupeus* (ABR21041) (Figure 2). The initiation codon was assigned by homology to known neurotoxin sequences (Figure 2). We assumed that the first ATG serves as the translation start codon, following a hydrophobic region which is typical for a signal peptide. Amino acid residues 1-19 was identified a typical secretory signal peptide with a cleavage site predicted to lie between residues 19 and 20 (Nielsen *et al* 1997) and are presumably removed by signal peptidase in the endoplasmic reticulum.



**Figure 4.** Schematic representation for the secondary structure of MeNaTx $\alpha$ -4. The amino acid sequences predicted to form amphipathic  $\alpha$ -helix (H),  $\beta$ -sheets (E) and random coils (C) are indicated.

Their consensus sequences were MN-L-ALL-TGV-S. It was assumed that the Valine at position 20 represent the start of the mature protein (Figure 2). Therefore, The cDNA encoding a putative precursor peptide with two segments in the following sequence: a signal peptide of 19 amino acid residues, a mature 66 residues. All the signal peptides showed a high degree of homology (Figure 2). Domain analysis of MeNaTx $\alpha$ -4 using the CDD-search (Conserved Domain Database) showed e-value of 1.04e-18 with conserved domain of

"Scorpion long chain toxin-like domain" (pfam00537) between amino acid residues 21 to 74. This family domain contains both neurotoxins and plant defensins. The scorpion neurotoxin binds to sodium channels and inhibits the activation mechanisms of the channels, thereby blocking neuronal transmission.

**Multiple alignment and sequence analysis of MeNaTx $\alpha$ -4.** The amino acid sequence of MeNaTx $\alpha$ -4 was aligned with venom sodium channel toxin-3 (ABR21041), 5 (ADU05410), 6 (ABR21068) from species of lesser Asian scorpion *M. eupeus* and also with  $\alpha$ -neurotoxin Tx15 *Mesobuthus martensii* (Q9GNG8),  $\alpha$ -toxin Tx405 *Buthus occitanus israelis* (ACJ23096), neurotoxin 8 precursor *Androctonus bicolor* (ADE42758), neurotoxin 2 precursor *Androctonus crassicauda* (ADE42760), Toxin AahP1005 *Androctonus australis* (Q9BLM4),  $\alpha$ -insect toxin BjaIT *Hottentotta judaicus* (Q56TT9),  $\alpha$ -neurotoxin 2 *Androctonus amoreuxi* (Q86SE0) and  $\alpha$ -Neurotoxin 8-related gene product *Androctonus mauretanicus mauretanicus* (Q2YHM1) as shown in Figure 2. It is noteworthy that the amino acid sequence presented in Figure 2 represents a putative translation rather than the final secreted toxin. Multiple alignment of MeNaTx $\alpha$ -4 with those  $\alpha$ -neurotoxins using the CLUSTAL\_W program revealed a high degree of primary structural similarity. The putative amino acid sequence of the Iranian scorpion *M. eupeus* (MeNaTx $\alpha$ -4) exhibited high sequence identity with venom sodium channel toxin-3 (ABR21041) (95%), 1 (ADT82850) (92%) and 7 (ADF49570) (91%) from the lesser Asian scorpion *M. eupeus* but interestingly displayed only 63-73% identity with venom sodium channel toxin-2 (ADF49574), 5 (ADU05410), 6 (ABR21068), 9 (ADF49575) and 12 (ADT82854) from this species. MeNaTx $\alpha$ -4 exhibited 92% identity with  $\alpha$ -neurotoxin Tx15 *M. martensii*, 80% with  $\alpha$ -toxin Tx405 *Buthus occitanus israelis*, but only displayed between 72-76% identity with  $\alpha$ -neurotoxins from other scorpions such as: *A. bicolor*, *A. australis*, *A. amoreuxi*, *H. judaicus* and *A. m. mauretanicus*. Moreover, the homology is higher at nucleotide level

than that at amino acid level. As expected, all the  $\alpha$ -toxins which have been compared contain 8 Cys residues cross-linked by four disulfide bridges (C31-82, C35-55, C41-65 and C45-67), and the Cys positions are highly conserved. A region almost totally conserved in the core of MeNaTx $\alpha$ -4 includes the sequence 'Leu-Pro-Asp' between amino acid residues 70-72 is recognizable. This position was mentioned as a possible evolutionary link between long- and short-chain scorpion toxins (Ceard *et al* 2001).

The regions encoding the mature toxin depicted in Figure 2 in addition to 5 sequences including venom sodium channel toxin-2 (ADF49574), 5 (ADU05410), 7 (ADF49570), 9 (ADF49575) and 12 (ADT82854) from Lesser Asian scorpion *M. eupeus* and also Lqh4 (P83644) from *Leiurus quinquestriatus hebraeus* were aligned and then used to construct a phylogenetic tree (Figure 3). Those sequences fell into two well-defined groups which are phylogenetically distant despite their primary sequence similarity. The tree shows that the venom sodium channel toxin-2, 5, 6, 9 and 12 relating to lesser Asian scorpion *M. eupeus* were placed, as expected, in the same group. However, MeNaTx $\alpha$ -4 from the Iranian scorpion *M. eupeus* and venom sodium channel toxin-3 and 7 from lesser Asian scorpion *M. eupeus* accompanied with other  $\alpha$ -toxins from *Mesobuthus martensii* (Mm), *Androctonus australis* (Aau), Ab (*Androctonus bicolor*) and Ac (*Androctonus crassicauda*) were more closely related to each other as the second group.

## DISCUSSION

In this study, a new member of  $\alpha$ -toxin from the Iranian scorpion *M. eupeus* belongs to the long chain scorpion Toxin-3 superfamily was identified. In accordance with all known toxins in this family, MeNaTx $\alpha$ -4 is sodium channel inhibitor with four conserved disulfide bridges. We have aligned MeNaTx $\alpha$ -4 with the amino acid sequences of 16 long-chain scorpion  $\alpha$ -toxins retrieved from the NCBI database. As described in Figure 2 comparison of

MeNaTx $\alpha$ -4 with the GenBank database revealed that this sequence is highly homologous with  $\alpha$ -toxin of other scorpions. It is suggested that  $\alpha$ -toxin is belongs to the Scorpion *Buthidae* family and  $\alpha$ -toxins are from the common progenitor. MeNaTx $\alpha$ -4 and seven  $\alpha$ -toxin homologues (sodium channel  $\alpha$ -toxin-2, 3, 5, 6, 7, 9 and 12) isolated from lesser Asian scorpion *M. eupeus* reported previously in the NCBI database, constitute a large family of toxins. As expected, they are highly conserved in the positions of the 8 Cys residues, amino acid length, and shared 63-95% homology among them. The conserved positions of 8 Cys residues which are well conserved among them are responsible for the tight packing of the molecule. It was suggested that the C31-C82 disulfide bridge put together the N- and C-terminal regions. As indicated in Figure 4, the toxin core sequence share a similar scaffold contained a CSH motif: a  $\alpha$ -helix linked to 4  $\beta$ -sheets by three other disulfide bridges present in all scorpion  $\alpha$ -toxins (Bontems *et al* 1991b). These factors clearly indicated that MeNaTx $\alpha$ -4 gene is a new member of  $\alpha$ -toxin from the Iranian scorpion *M. eupeus* which may originate from the duplication of a common ancestor gene.

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