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# Kbot55, purified from *Buthus occitanus tunetanus* venom, represents the first member of a novel α-KTx subfamily

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ABSTRACT

Kbot55 is a 39 amino acid peptide isolated from the venom of the Tunisian scorpion *Buthus occitanus tunetanus*. This peptide is cross-linked by 3 disulfide bridges and has a molecular mass of 4128.65 Da. Kbot55 is very low represented in the venom and thus represents a challenge for biochemical characterization. In this study, Kbot55 has been subjected to a screening on ion channels expressed in *Xenopus laevis* oocytes. It was found that Kbot55 targets voltage-gated potassium channels with high affinity. Kbot55 shows very low amino acid identity with other scorpion potassium toxins and therefore was considered a bona fide novel type of scorpion toxin. Sequence alignment analysis indicated that Kbot55 is the first representative of the new α-Ktx31 subfamily and therefore was classified as α-Ktx31.1.



## Introduction

Among ion channels, potassium channels play a major role in a variety of physiological processes such as cell excitability, release of neurotransmitters, and secretion of hormones, signal transduction, cell volume regulation, and neuronal integration [5]. In the mammalian genome more than 100 genes are encoding the pore forming α subunits and auxiliary β subunits of $K^+$ channels [13]. Together with these numerous genes, the presence of spliced variants, association with chaperone and scaffolding molecules and the formation of heteromultimeric channels contribute greatly to the diversity of the $K^+$ channel family [13]. This family has been divided into 15 subfamilies of which the voltage-gated potassium channels ($K_V$) represent one of these subfamilies [9,18,22,24]. Peptide toxins acting on potassium channels have been isolated from the venom of numerous animal species, such as marine cone snails, spiders, scorpions, sea anemones and snakes [6,17,19]. As highly specific probes, peptide toxins have proven themselves extremely useful to study ion channel structures and to investigate the involvement of these channels in certain channelopathies [3,15].

Scorpion venoms contain a diversity of toxins and peptides, which can be divided into disulfide-bridged and non-disulfide bridged peptides. The channel targeting scorpion peptides are usually classified under the disulfide-bridged group of scorpion peptides. The channel targeting peptides can be divided in 2 structurally distinct families, the long chain and short chain peptides. The short chain peptides are composed of 23–64 amino acids, cross linked with 3 disulfide bridges and a common structural cysteine-stabilized α/β motif (CSαβ). These toxins inhibit the potassium current by blocking or modulating potassium channels. The long chain peptides are 58–76 amino acids long. They are cross linked by 4 disulfide bridges and their structural signature is defined by the presence of a structural core with a βαββ topology [20]. These peptides exert their toxicity by modulating sodium channels.

The α-KTx family is the largest scorpion neurotoxin family and is divided into at least 30 subfamilies, defined according to the primary sequence alignments of the toxins [10]. Each member has diverse, specific blocking activities against voltage-gated ($K_V$) and/or calcium-activated ($K_{Ca}$) channels.

Scorpions of the Buthidae family are considered medically important because of the lethality and neurotoxic effects developed following upon envenomation [4]. In order to provide an efficient treatment against envenomation, there is a strong urge to fully

*Abbreviations:* Bot, *Buthus occitanus tunetanus* scorpion; BotG50, sephadex G-50 gel filtration toxic fraction of Bot venom; MALDI, matrix-assisted laser desorption ionization; i.c.v., intracerebroventricular.

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understand on a molecular level the exact targets of each venom component, and moreover, to characterize in detail the pharmacological effects induced by binding of individual components at their target.

In the present work, we isolated, identified and characterized a new peptide toxin from the venom of *Buthus occitanus tunetanus*. This toxin, Kbot55, shares less than 30% identity with existing α-KTx peptides and as such represents the first member of a novel subfamily of the α-KTx family. Kbot55 was found to potently inhibit several members of the *Shaker* voltage-gated potassium channel family.

## Experimental procedures

### *Scorpion venom*

*B. o. tunetanus* venom was provided in liquid state by electric stimulation of the post-abdomen of the scorpion, bred in captivity in Beni Khedach area (Tunisia). The pooled venom is kept frozen at −20 °C in its crude form until use. All reagents were purchased from Sigma–Aldrich® chemical company, except indicated otherwise.

### *Purification of Kbot55*

Purified Kbot55 was obtained from the scorpion venom *B. o. tunetanus* by gel filtration G50 followed by HPLC. Crude venom was dissolved in water and loaded on to a sephadex G50 column equilibrated with 0.1 M ammonium acetate pH 8.5. Different fractions were eluted and tested for their toxicity on mice. Only fraction (BotG50) showing a toxic activity [16] was then applied onto C8 semi preparative reversed-phase HPLC column (10 mm × 250 mm, 5 μm, Beckman Fullerton) equilibrated in 0.1% trifluoroacetic acid in water, at a flow rate of 1 ml/min. HPLC purification of the non-retained fraction was performed using an analytical C18 reversed-phase HPLC column (4.6 mm × 250 mm, 5 μm, Beckman). Elution was monitored at absorbance of 214 nm.

### *Mass spectrometry*

The peptide was then analyzed on a voyager de RP MALDI-TOF mass spectrometer (Perspective Biosystems, Inc., Framingham, MA). Sample was dissolved in CH3CN/H2O (30/70) with 0.3% trifluoroacetic acid to obtain a concentration of 1–10 pmol $\mu l^{-1}$. The matrix was prepared as follows: alpha-cyanohydroxycinnamic acid was dissolved in 50% CH3CN in 0.3% trifluoroacetic acid/H2O to obtain a saturated solution at 10 $\mu g\ \mu l^{-1}$. A 0.5 μl of peptide solution was placed on the sample plate, and 0.5 μl of the matrix solution was added. This mixture was allowed to dry. Mass spectra were recorded in linear mode, were externally calibrated with suitable standards and was analyzed by using the GRAMS/386 software

### *Amino acid sequence determination*

Reduction and alkylation of proteins, and sequence determination of native and S-alkylated peptides were performed as described in [21].

The automatic Edman degradation of 1 nmol of native Kbot55 was performed with a reproducible yield of 95% during the first 31 cycles. After these steps, the concentration of identified PTH became too low for reliable identification. Then, 1 nmol of S-alkylated-protein was used to complete the sequence determination of Kbot55 and to identify cystein positions.

### *Alignment*

The amino acid sequence of Kbot55 was compared with other sequences deposited in database of nrNCBI using FASTA and BLAST searches. Alignment was performed using CLUSTALW2.

### *Toxicity test*

Kbot55 was tested for *in vivo* toxicity on 20 ± 2 g male C57/BL/6 mice, by intracerebro-ventricular injection (i.c.v.), considered being the most sensitive route to mammals for scorpion toxins. Peptide was diluted in 0.1% (w/v) BSA and 5 μl of the solution containing increasing amounts of peptides (20, 40, 80, 100, 150, 200 nM) were injected in six mice for each concentration [7,23]. Experiments on mice were carried out in accordance with the European Community Council Directive (86/609/EEC) for experimental animal care, and all procedures met with the approval of the Institutional Research Board of the Pasteur Institute of Tunis.

### *Expression in Xenopus oocytes*

For the expression of the voltage-gated ion channels ($rK_v1.1$, $rK_v1.2$, $hK_v1.3$, $rK_v1.4$, $rK_v1.6$, *Shaker* IR, Nav1.4) in *Xenopus* oocytes, the linearized plasmids were transcribed using the T7 or SP6 mMESSAGE-mMACHINE transcription kit (Ambion, USA). The harvesting of stage V–VI oocytes from anaesthetized female *Xenopus laevis* frog was done as previously described [12]. Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng/nl using a micro-injector (Drummond Scientific, USA). The oocytes were incubated in ND96 solution containing (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 2 and HEPES, 5 (pH 7.4), supplemented with 50 mg/l gentamycin sulfate. The use of the frogs was in accordance with the license number LA1210239.

### *Electrophysiological recordings*

Two-electrode voltage-clamp recordings were performed at room temperature (18–22 °C) using a Geneclamp 500 amplifier (Molecular Devices, USA) controlled by a pClamp data acquisition system (Axon Instruments, USA). Whole cell currents from oocytes were recorded 1–4 days after injection. Bath solution composition was ND96. Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.7 and 1.5 MΩ. The elicited currents were filtered at 0.5 kHz and sampled at 2 kHz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a −P/4 protocol. $K_v1.1$–$K_v1.6$ and *Shaker* IR currents were evoked by 500 ms depolarizations to 0 mV followed by a 500 ms pulse to −50 mV, from a holding potential of −90 mV. In order to investigate the current–voltage relationship, current traces were evoked by 10 mV depolarization steps from a holding potential of −90 mV. All data represent at least 3 independent experiments ($n \geq 3$) and are presented as mean ± standard error.

## Results

### *Purification of Kbot55*

After sephadex G50 chromatography of the *B. o. tunetanus*, each fraction obtained was fractionated by a C8 semi preparative reversed-phase HPLC (Fig. 1A). Fraction B2 were pooled and applied to an analytical C18 reverse-phase HPLC column using an isocratic gradient (12% in 10 min; 12–35% in 60 min; 35–95% in 10 min). B'2 was eluted at 33, 7 min. Fractions were collected and injected into C18 reverse-phase HPLC column using a more resolute gradient (5–35% in 70 min) (Fig. 1B). Kbot55 was eluted at 55 min and



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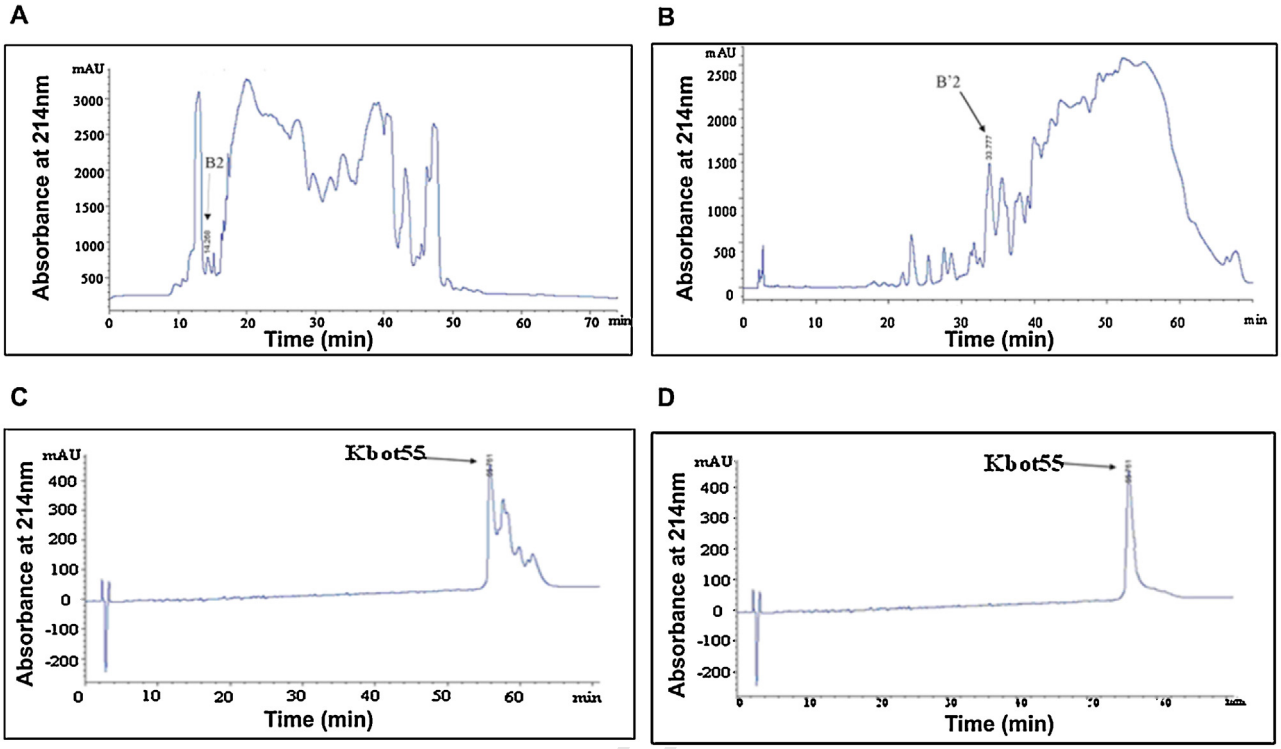


**Fig. 1.** Purification of Kbot55 from the venom scorpion *Buthus occitanus tunetanus*. (A) A purification of BotG50 on semi preparative C8 reverse phase HPLC column. (B) Separation of fraction B2 on C18-RP-HPLC, B'2 was eluted at 33 min. (C) Kbot55 was purified from the fraction B'2 by C18-RP-HPLC. HPLC and eluted at 55 min. (D) Final purification step to ensure the purity of Kbot55.

**Table 1**
Toxicity test.

| Kbot55 concentration (ng/mouse) | Time (h) | Mice (death/total) |
|---|---|---|
| 20 | 24 | 0/6 |
| 40 | 24 | 0/6 |
| 80 | 24 | 2/6 |
| 100 | 24 | 3/6 |
| 150 | 24 | 5/6 |
| 200 | 24 | 6/6 |

Increasing concentration of Kbot55 was performed by i.c.v. injection routes and symptoms of toxicity and the number of death mice was determined.

therefore assigned as such. An analytical HPLC run of Kbot55 showed a single symmetric peak (Fig. 1C and D).

### *Sequence determination of Kbot55*

Edman degradation of 2 nmol of S-pyridylethylated peptides led to the identification of the complete amino acid sequence of the peptide. Kbot55 is a peptide composed of 38 amino acid residues containing six cysteine residues. The experimental molecular mass of native Kbot55 (4128.65 Da) obtained by MALDI is nearly identical with the average theoretical molecular mass calculated for the fully oxidized form of Kbot55 (4128.6 Da) (data not shown).

### *In vivo toxicity of Kbot55*

Different concentrations of Kbot55 were injected into mice by the i.c.v. It seems that Kbot55 toxicity effect was dose-dependent and the maximum of death was reached at a concentration of 200 ng (Table 1 and Fig. 2B). In addition, a rapid paralysis in the lower half of the body was observed few minutes after the injection and after 10 min the animals died. The median lethal dose ($LD_{50}$) which

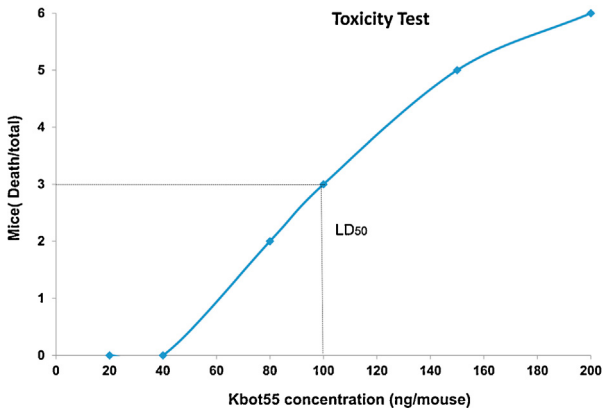


**Fig. 2.** Toxicity test. From the results illustrated in Table 1, the curve of correlation between the number of dead mice and the concentration of Kbot55 injected was made. The LD50 was calculated and it is corresponding to 100 ng.

matches to the dose required to kill half the members of a tested population is below 100 ng.

### *Electrophysiological recordings*

At a concentration of 1 μM, Kbot55 was investigated for its activity against 7 different voltage-gated ion channels (Fig. 3). Kbot55 was tested against 6 $K_v$ channels and 1 Nav channel. It was observed that Kbot55 could completely inhibit the potassium current through $K_v1.2$ and *Shaker* IR channels. The same concentration inhibited $K_v1.3$ channels with 83 ± 3%. $K_v1.1$ and $K_v1.6$ channels are less sensitive to Kbot55 since only 32 ± 4% and 21 ± 3% respectively of the currents were inhibited. No activity was seen on $K_v1.4$ and Nav1.4 channels.



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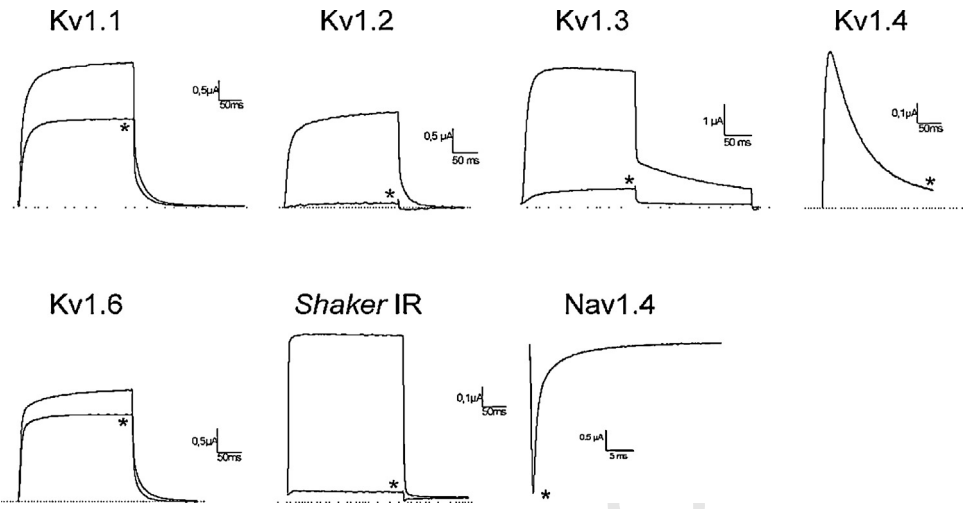


**Fig. 3.** Electrophysiological characterization of Kbot55 on $K_v$ channel expressed in *X. laevis* oocytes. Representative whole-cell current traces in control and peptide conditions are shown. The dotted line indicates the zero-current level. Asterisks mark steady-state current traces after application of 1 μM Kbot55. Traces shown are representative traces of at least three independent experiments ($n \geq 3$).

## Discussion

The electrophysiological experiments show that Kbot55 is a potent $K_v$ inhibitor, capable of interacting with several different subtypes of the $K_v1$ subfamily. Unfortunately, no in depth structure–function characterization of Kbot55 could be performed due to peptide scarcity. The last decade, great progress has been made in recombinant and synthetic production of peptide toxins. As such, future studies, using recombinant or synthetic Kbot55, could be conducted in order to obtain an in depth-structure function analysis of this interesting peptide. Since Kbot55 is only present in low amounts in the venom, it is very difficult to obtain large amounts of pure peptide. Nevertheless, the obtained electrophysiological results showing $K_v$ channel inhibition do provide an explanation for the observed *in vivo* toxicity in mice (Fig. 4).

A general nomenclature for scorpion toxins actives on potassium channels was suggested by Tytgat et al. [22]. The basis of this unified nomenclature is the similarity between the primary structures of those toxins. When this nomenclature was proposed, the number of known scorpion toxins was 49 distributed over 12 subfamilies. Since then, this number has increased significantly as well as the number of subfamilies. Nowadays, there are more than 200 different scorpion toxins specific for potassium channels. They are divided in over 30 subfamilies. In this way, Kbot55 was compared with the first member of each α-KTx subfamily described so far. The identities fell lower than 30% as illustrated in Fig. According to the published criteria and to the best of our knowledge, Kbot55 is the first member of the new α-KTx31 subfamily and is therefore classified as α-KTx31.1.

Recently, it was shown that a toxin signature sequence can be assigned to α-KTxs. This signature sequence comprises eight

```
                                                                    Id(%)
Kbot55          ---AGSMDSCSETGVCMKACSERIRQV-ENDNKCPAGE--------CICTT------- 100
alpha-KTx13.1   --------AC---GSCRKKCK--------GSGKCINGR--------CKCY-------- 29
alpha-KTx26.1   --NFKVEGACS--KPCRKYCIDK-G---ARNGKCINGR--------CHCYY------- 28
alpha-KTx23.1   ----AAAISCVGSPECPPKCRAQ-G---CKNGKCMNRK--------CKCYYC------ 28
alpha-KTx4.1    ---VFINAKCRGSPECLPKCKEAIG---KAAGKCMNGK--------CKCYP------- 26
alpha-KTx15.1   --QNETNKKCQG-GSCASVCRRVIG---VAAGKCINGR--------CVCYP------- 26
alpha-KTx29.1   ----EGDCPISEAIKCVEKCK-------EKVEVCEPGV--------CKCSG------- 26
alpha-KTx2.1    ---TIINVKCTSPKQCSKPCKELYG--SSAGAKCMNGK--------CKCYNN------ 25
alpha-KTx8.1    ---------VSCEDCPEHCST----Q-KAQAKCDNDK--------CVCEPI------ 25
alpha-KTx3.1    --GVEINVKCSGSPQCLKPCKDA-G---MRFGKCMNRK--------CHCTPK------ 24
alpha-KTx10.1   -------AVCVY-RTCDKDCKRR----GYRSGKCINNA--------CKCYPYGK---- 24
alpha-KTx12.1   WCSTCLDLACGASRECYDPCFKAFG---RAHGKCMNNK--------CRCYT------- 24
alpha-KTx16.1   --DLIDVKCISSQECWIACKKVTG---RFEGKCQNRQ--------CRCY-------- 24
alpha-KTx28.1   --------ACVTHEDCTLLCY-------DTIGTCVDGK--------CKCM-------- 24
alpha-KTx9.1    ----------VGCEECPMHCKG----K-NAKPTCDDGV--------CNCN-V------ 23
alpha-KTx21.1   ----GKFGKCKP-NICAKTCQTEK---GKGMGYCNK-TE--------CVCSEW------ 23
alpha-KTx30.1   ----EDKLKCTKTDDCAKYCSQFT--D-VHP-ACLGGY--------CECLRWEGGISS 22
alpha-KTx18.1   ---TGPQTTCQA-AMCEAGCKGL----GKSMESCQG-DT--------CKCKA------- 21
alpha-KTx19.1   -------AACYS-SDCRVKCVAM----GFSSGKCINSK--------CKCYK------- 21
alpha-KTx5.1    -------AFCNL-RMCQLSCRSL----GLL-GKCIGDK--------CECVKH------ 20
alpha-KTx6.1    ------LVKCRGTSDCGRPCQQQTG---CPNSKCINRM--------CKCYGC------ 20
alpha-KTx7.1    ------TISCTNPKQCYPHCKKETG---YPNAKCMNRK--------CKCFGR------ 20
alpha-KTx11.1   --DEEPKESCSD-EMCVIYCKGE----EYSTGVCDGPQK-------CKCSD------- 20
alpha-KTx20.1   ---G-----CTP-EYCSMWCKVK-----VSQNYCVKN---------CKCPGR------ 20
alpha-KTx1.1    ---QFTNVSCTTSKECWSVCQRLHN---TSRGKCMNKK--------CRCYS------- 18
alpha-KTx17.1   ------QTQCQSVRDCQQYC--------LTPDRCSYGT--------CYC--------- 18
alpha-KTx14.1   ---TPFAIKCATDADCSRKCP--------GNPSCRNGF--------CACT-------- 13
alpha-KTx22.1   EVDGRTATFCTQ-SICEESCKRQ-----NKNGRCVIEAEGSLIYHLCKCY-------- 14
```

**Fig. 4.** Sequence alignment. Alignment of Kbot55 with the first member of every alpha-KTx subfamily described up to date. Cysteine residues are highlighted in red. The numbers next to each toxin's name represent the percentage of identity (Id) with Kbot55.

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structurally and functionally important residues conserved across the family, in which six cysteines are involved in three disulfide bridges and two amino acids (Lys and Asn) in a four-residue long motif around the fourth cysteine (Lys-Cys4-Xaa-Asn) (Xaa, any amino acid) are key functional residues of α-KTxs [25]. Mutations at these two sites (Lys27 and Asn30) had the largest destabilizing effects on binding of agitoxin2 (AgTx2), an α-KTx isolated from the venom of the scorpion *Leiurus quinquestriatus hebraeus*, to the *Shaker* $K^+$ channel in *Drosophila* [8,14]. This is consistent with a toxin-channel complex model derived from solid-state nuclear magnetic resonance (NMR) studies where the side chains of Asn30 on the toxin kaliotoxin (KTX) and Asp64 on the pore helix of one chain of KcsA-$K_v$1.3 (structurally equivalent to Asp431 of *Drosophila melanogaster Shaker* $K^+$ channel or Asp361 of rat $K_v$1.1) are predicted to form hydrogen bonds, whereas side chains of Lys27 directly enter into the pore region to contact the backbone carbonyls of Tyr78 on the channel filter (structurally equivalent to Tyr445 of *D. melanogaster Shaker* $K^+$ channel or Tyr375 of rat $K_v$1.1) [11]. The functional importance of these two residues was also identified in a recent crystal structure of a $K_v$ channel in complex with an α-KTx (charybdotoxin) though in this complex the location of the Asn slightly differs from the NMR-based complex model [2,11,25]. It is interesting to note that the hyper-conserved and functionally crucial Lys27 is indeed present in Kbot55. However, Kbot55 sequence displays at position 30 an Ala instead of an Asn. It thus can be assumed that Kbot55 forms a less stable interaction with the $K_v$ channel due to the lack of stabilizing hydrogen bonds otherwise formed between Asn30 of the toxin and Asp residues within the channel filter. In future structure–function studies it would be interesting to mutate Ala30 of Kbot55 into an Asn residue and compare the activity of this mutant with the wild type Kbot55.

*B. o. tunetanus* is considered to be a medically important scorpion. This species contributes significantly to scorpion envenomation cases, especially in northern Africa [1]. Therefore, it is important to characterize the active compound constituting its venom. This is the first step in a better understanding of which compounds are responsible for which effects upon envenomation and furthermore, it can provide essential information on how to treat patients after being stung by this scorpion.

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