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A first exploration of the venom of the *Buthus occitanus* scorpion found in southern France

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Abstract

Even though *Buthus occitanus* scorpions are found throughout the Mediterranean region, a lack of distinctive characteristics has hampered their classification into different subspecies. Yet, stings from this particular scorpion family are reported each year to result in pain followed by various toxic symptoms. In order to determine the toxicity origin of the rare French *Buthus occitanus* Amoureux scorpion, we collected several specimens and studied their venom composition using a nano ultra high performance liquid chromatography and matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (nano UHPLC/MALDI-TOF-MS) automated workflow combined with an enzyme-linked immunosorbent assay (ELISA) approach. Moreover, we compared this dataset to that obtained from highly lethal *Androctonus australis* and *Androctonus mauretanicus* scorpions collected in North Africa. As a result, we found that the *Buthus occitanus* Amoureux venom is toxic to mice, an observation that is most likely caused by venom components that inhibit voltage-gated sodium channel inactivation. Moreover, we identified similarities in venom composition between *Buthus occitanus* scorpions living in the South of France and other Buthidae collected in Morocco and Algeria. As such, the results of this study should be taken into consideration when treating stings from the *Buthus occitanus* species living in the South of France.

Keywords

scorpion toxins; MALDI-TOF/MS; ionic channels; ELISA

1. Introduction

Of the 1900 different scorpion species identified worldwide so far, less than 20 of them constitute a health hazard to humans. Strikingly, almost all of the dangerous specimens

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belong to the Buthidae, a scorpion family stemming from the 'Old World'. For example, the genus *Androctonus* is highly lethal for mammals and is responsible for roughly 100 000 stings each year in North Africa and the Middle East, 1% of which results in death of the victim (Benguedda et al. 2002). Scorpion venoms are complex mixtures containing numerous neurotoxic polypeptides, which typically enhance nerve and muscle excitability. Although various families of scorpion toxins have been reported to target voltage-gated potassium (Kv) channels, voltage-gated calcium (Ca_v) channels, and chloride channels (Martin-Eauclaire and Couraud 1995; Possani et al. 1999), a large majority of peptide toxins is active on voltage-gated sodium (Na_v) channels, a family of transmembrane proteins that regulate cellular excitability (Catterall 2012).

The venoms from North African *Androctonus* scorpions primarily contain classical α -toxins, which target the domain IV voltage sensor within mammalian neuronal and muscle Na_v channel subtypes (Bosmans et al. 2008; Campos et al. 2008). Upon binding to their receptor site, these α -toxins are thought to prevent the domain IV voltage sensor from moving upward, thereby slowing down the fast inactivation process and provoke a persistent depolarization of cell membrane (Bosmans et al. 2008; Campos et al. 2008; Capes et al. 2013; Catterall 2012; Rogers et al. 1996; Sheets et al. 1999). In contrast, scorpions from the *Buthus* genus, such as *Buthus occitanus* in North Africa and the Middle East, *Buthus eupeus* in Russia, and *Buthus martensii* in China, are considered to be less dangerous for humans. The difference in overall toxicity may be related to the low amount of classical α -toxins in their venoms. Indeed, the majority of peptide toxins identified in *Buthus* venoms belong to the so-called α -like toxins, a group of peptides that target both mammals and insects and have been shown to be less lethal to mammals as opposed to classical α -toxins (Gordon et al. 1996). Moreover, their venoms contain a large amount of toxins that selectively influence ion channel function in insects, the principal prey of scorpions.

The habitat of *Buthus occitanus* scorpions spreads across North-Africa, Egypt, Ethiopia, and Somalia (Lourenço 2003), to the Southern part of Europe (Amoreux 1789). Even though toxic scorpions are a rare sight in Spain and the French Mediterranean coast, *Buthus occitanus* Amoreux is of medical importance since painful stings attributed to this scorpion are described each year. A previous investigation using nuclear 18S/ITS-1 DNA sequences and mitochondrial 16S and COI DNA sequences revealed a phylogenetic relationship between the *Buthus occitanus* populations across the Strait of Gibraltar (Gantenbein and Largiadèr 2003). Based on their results, the authors of this work concluded that the European clade was highly separated from North African clades and even split into three divergent subclades. Moreover, they reported that most of the detected mitochondrial DNA lineages, including the European lineages, were about three times older than the reopening of the Gibraltar Strait. As a result, they estimated an older separation time between the *Buthus occitanus* living in France and Spain. Despite their medical relevance, the biochemical, pharmacological, and immunological properties of the European *Buthus occitanus* venom have not been investigated; as for any European scorpion. Here, we analyse the venom composition of the rare French *Buthus occitanus* Amoreux. To this end, we collected several specimens in two different parts of the South of France (Provence-Alpes-Côte-d'Azur area): in the mounts around Orange city (Vaucluse County) and in Massif des Maures (Var County) and examined their individual venoms using nano ultra high performance liquid chromatography (UHPLC), matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), and enzyme-linked immunosorbent assay (ELISA) tests using specific antibodies raised against other Buthidae toxins (Martin-Eauclaire et al. 2013). Moreover, we compared venom components to those characterized using the same analytical methods in the venoms from different highly lethal Buthidae collected in Morocco (*Androctonus mauretanicus*) and in Algeria (*Androctonus australis*). Finally, we explored the biological activity of the *Buthus occitanus* venom in

mice and on a neuronal Na_v channel isoform expressed in a heterologous system. As a result, we found that the venom from the *Buthus occitanus* scorpions collected in the Provence-Alpes-Côte-d'Azur area is unexpectedly similar to those from the lethal North African Buthidae. Even though we identified several K_v channel toxins, most of the proteins found belong to α -like toxins targeting Na_v channels. Surprisingly however, we uncovered the presence of a range of classical α -toxins as well, which may explain the toxicity of this venom towards humans.

2. Material and methods

2.1. Materials

Four specimens of *Buthus occitanus* were collected in different biotopes of the Provence-Alpes-Côte-d'Azur area: three in the Western part (Orange city, Vaucluse County) (Fig. 1A) and one other in the Easter part (Massif des Maures, Var County) (Fig. 1B), two places distant from each other by 200 km (125 mi). The venoms were individually obtained, by manual stimulations (one time) of the post-abdomen of the specimen collected in Massif des Maures and by electric stimulations (three times) for specimens collected near Orange city. Animals were thereafter released in their natural habitats.

Specific antisera were previously prepared in New-Zealand rabbits in the laboratory as described (Granier et al. 1989). We used sera against long-chain toxins, modulators of the Na_v channels: serum against the classical α -toxin AaH II (the reference for the structural and immunological group II and highly selective for the neuronal and muscular mammalian Na_v1.2 and Na_v1.4); serum against the contractile anti-insect toxin AaH IT1 from *Androctonus australis* Hector (selective for insect Na_v channels); serum against the α -like toxin Bot I from the Moroccan *Buthus occitanus tunetanus* (the reference for the structural and immunological group III, which is highly representative of the toxins found in *Buthus* venoms, active both on mammals and insects); serum against the classical β -toxin Css II from the Mexican scorpion *Centruroides suffusus suffusus* (selective for mammalian Na_v channels); serum against the β -like toxin Ts VII from the Brazilian scorpion *Tityus serrulatus* (showing an activity against mammals and insects). The sera against the potassium channels blockers Kaliotoxin (KTX) from *Androctonus mauretanicus* (with a particular specificity for K_v1.1 and K_v1.3 channels), and BmTX3 from *Buthus martenzii* (a K_v4.x blocker) were also used (Romi et al. 1993; Vacher and Martin-Eauclaire 2004).

Goat-anti-rabbit Fab and bovine serum albumin (BSA) were from Sigma. UV-grade acetonitrile was from Carlo-Erba and trifluoroacetic acid (TFA) was from Sigma-Aldrich. α -cyano-4-hydroxycinnamic acid and sinnapinic acid were from Bruker. All other reagents and solvents of analytical grade were obtained from commercial sources, and used without further purification unless otherwise stated.

2.2. Methods

2.2.1. Nano ultra high performance liquid chromatography (nano UHPLC) and conventional HPLC—The lyophilized crude venoms (50 to 100 μ g for venoms manually obtained and 300 to 2500 μ g for venoms obtained by electric stimulations) were first dissolved in solvent A (TFA 0.1% in water) in order to obtain a stock solution of 10 μ g/ml. This step was followed by a centrifugation at 13 000 g for 20 min to precipitate the non-soluble high molecular weight proteins, which are not toxic. The supernatant (100 ng of venom) was separated by nano UHPLC analyses using a Dionex UltiMate® 3000 RSLC nano system (Thermo) controlled by CHROMELEON®. Elution profile was monitored by absorbance readings at 214 nm. HPLC grade elution solvents were water plus 0.05% TFA (A) and 80/20% acetonitrile/water plus 0.04% TFA (B). Pre-column (200 μ m $\times$ 5 mm) and

column (100 μm $\times$ 250 mm) were PepSwift Monolithic Nano PS-DVB from Dionex. Running flow rate was 1.5 $\mu\text{L}/\text{min}$. The column pressure was 400 bars and its temperature kept at 60°C.

Conventional HPLC was conducted as follows: the most abundant venom (2.5 mg, coming from a scorpion collected near Orange city, sample 2) was first dissolved in cold MilliQ-water (250 μL), and centrifuged at 13 000 g for 20 min at 4°C. Part of this material (0.5 mg) was loaded onto a reverse-phase HPLC column (100 RP-18, 4 $\times$ 250 mm, Lichrospher, 5 μm , Merck, Germany) using a Dionex UltiMate 3000 HPLC Basic systems (Thermo). HPLC grade elution solvents were water plus 0.1% TFA (A) and acetonitrile plus 0.1% TFA (B). The elution (0.5–1 ml/min) was monitored by absorbance at both 230 nm and 280 nm, simultaneously.

2.2.2. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)—

MALDI-TOF analyses were performed on a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer operated in the positive linear mode using FLEXCONTROL. For 3D analysis WARP-LC 1.0 was used. All experimental data reported in this study are average molecular masses. Direct on-line spotting of the column effluent on a Bruker MTP 384 polished steel plate was performed using a LC Packing Probot and $\mu\text{CARRIER}$ 2.0. The sampling rate was 3 spots/min with the adding of 0.5 μL *per* spot of a filtered and twice-diluted saturated α -cyano-4-hydroxycinnamic acid (CHCA) solution in 50/50 (v/v) acetonitrile/water plus 0.1% TFA. MALDI-TOF spectra (m/z range: 800 to 16 000) were automatically recorded with the AUTOEXECUTE fuzzy logic feedback control system. Each spectrum was the accumulation of 1000 laser shots (S/N threshold 10) in 50 shot-steps across each spot. Laser intensity varied between 40% and 80% at 200 Hz and the measuring raster was hexagonal. External multipoint calibrations were made using peptide calibration standard II from Bruker. Spectra were batch processed with FLEXANALYSIS 2.4 and exported in an Excel workbook for each MALDI plate acquisition process. For smoothing and base line subtraction, the algorithms selected were Stavitzky Golay and Convex Hull, respectively. The peak detection algorithm was Apex using thresholds as follows: S/N 5, relative intensity 1, maximal number of peaks 20 and peak width 5 m/z . Within the workbook, each individual worksheet contains all the m/z peaks detected for one spot. A specially designed Excel macro, PMF Merger v1.00, assembled the data and removed redundancy as a fully automated process (Martin-Eauclaire et al. 2013). All user-defined parameters (m/z error in ppm, time start of the spotting in min, time between each spot in min, minimum intensity threshold in a.u., mass aggregation threshold in ppm, time aggregation threshold in min) were entered at the beginning of the Excel macro in a graphical interface. If needed, there was a possibility to overcome the presence of ion adducts by increasing appropriately the m/z error threshold of the merge step or the minimum intensity filter using the PMF Merger v1.1 user interface, knowing that this might also result in loss of relevant information.

2.2.3. Enzyme-linked immunosorbent assay (ELISA)—

The nano UHPLC effluent was automatically and directly dropped every 20 second (*i.e.* 0.5 μL by well) from the tip of the column capillary in a Falcon Microtest™ 96-Well ELISA Plate, each well already containing 50 μL of 50 mM carbonate/bicarbonate buffer, pH 9.6. The plate was shaken overnight at 4°C to achieve the antigen coating process. Then, the plate was saturated for 1h with a blocking solution (100 μL by well of 5% dried milk in PBS, 0.05% Tween 20, pH 7.4). After an extensive washing (3 times 300 μL by well of 0.1% dried milk in PBS, 0.05% Tween 20, pH 7.4), the binding of the toxin-specific serum (100 μL by well of a 1/10 000 dilution) was allowed for 90 min at room temperature. After washing (same as above), an alkaline phosphatase-coupled goat anti-rabbit Fab (100 μL by well of a 1/40 000 dilution) was added. After 90 min at room temperature and washing (same as above), the 4-nitrophenyl phosphate substrate from Merck was added (300 μL by well of a 3 mg/mL

solution in 10% diethanolamine buffer, 0.5 mM MgCl₂, pH 9.8). After the colour had developed (between 15 and 60 min), the absorbance was measured at the wavelength 405 nm using a Labsystems iEMS Reader MF.

2.2.4. Bioassay—Lethality tests were performed in male Swiss mice (n = 48) weighting 18 ± 3 g by subcutaneous (s.c.) or intracerebroventricular (i.c.v.) injections as described (Gordon et al. 1996; Romi et al. 1993). Experiments were carried out in accordance with the European Communities Council Directive 2010/63/EU.

2.2.5. Electrophysiology—rNa_v1.2a cRNA was synthesized using T7 polymerase (mMessage mMachine kit; Life Technologies) after linearizing the DNA with appropriate restriction enzymes. Next, rNa_v1.2a was expressed in *Xenopus* oocytes and studied after 1 day incubation after cRNA injection (incubated at 17 °C in 96 mM NaCl, 2 mM KCl, 5 mM HEPES, 1 mM MgCl₂, 1.8 mM CaCl₂, and 50 µg/ml gentamycin, pH 7.6 with NaOH) using the two-electrode voltage clamp recording technique (OC-725C; Warner Instruments) with a 150 µL recording chamber. Data were filtered at 4 kHz and digitized at 20 kHz using pClamp10 software (Molecular Devices). Microelectrode resistances were 0.5–1 MΩ when filled with 3 M KCl. The external recording solution contained 100 mM NaCl, 5 mM HEPES, 1 mM MgCl₂, and 1.8 mM CaCl₂, pH 7.6 with NaOH. All experiments were performed at room temperature (<22 °C). Na_v channel currents were isolated using tetrodotoxin (Alomone Labs). Venom fraction was kept at –20 °C and aliquots were dissolved in appropriate solutions containing 0.1% BSA. Voltage–activation relationships were obtained by measuring steady-state currents and calculating conductance, and a single Boltzmann function was fitted to the data according to: $I/I_{\max} = (1 + e^{-zF(V - V_{1/2})/RT})^{-1}$, where $I/I_{\max}$ is the normalized tail current amplitude, z is the equivalent charge, $V_{1/2}$ is the half-activation voltage, F is Faraday's constant, R is the gas constant, and T is temperature in Kelvin (293 K) as described previously (Bosmans et al. 2008). After the addition of the venom fraction to the recording chamber, the equilibration between the venom and the channel was monitored using weak depolarisations elicited at 5s intervals. Offline data analysis was performed using Clampfit10 (Molecular Devices) and Origin 8 (OriginLab).

3. Results and discussion

3.1. Toxins purification and identification

The four collected venoms were individually submitted to a reversed-phase nano UHPLC step (100 ng each) in order to separate their components. Base on their chromatographic patterns, among the three venoms collected near Orange city one (sample 1, Fig. 2A) was rather different from the two others. Those were much more similar among themselves (sample 2, Fig. 2B; sample 3, Supplementary data 1). The chromatographic pattern of the venom of the specimen collected in Massif des Maures is reported in Fig. 2C and it shows noticeable differences with the venoms from Orange origin. Chiefly, the venom complexity is much higher and the relative intensities among peaks equally distributed over the whole chromatogram.

The compounds contained in the eluted fractions were further analysed using automated off-line MALDI/TOF-MS, which revealed masses ranging mainly from about 2500 to 8000 Da, as previously observed in toxic fractions for other Buthidae venoms (Batista et al. 2007; Martin-Eauclaire et al. 2013; Oukkache et al. 2008; Pimenta et al. 2001; Valdez-Velázquez et al. 2013). The MS raw data corresponding to Fig 2A, B and C and Supplementary data 1 can be found in Supplementary data 2 to 5, respectively. Table 1 reports masses found in the sample 2 *Buthus occitanus* venom collected near Orange, which are also included at least twice in all the other venoms studied, as well as ELISA cross-reactivity and toxicity in mice.

3.2. Immunological characterisation

3.2.1. Na_v channel toxins—The ability of the HPLC fractions to cross-react with highly specific antisera previously raised against different purified toxins was further evaluated using ELISA tests. We first focused on molecules similar to α -toxins purified from the venom of North African Buthidae scorpions, mainly because of their abundant presence in these venoms and their ability to modulate mammalian Na_v channel function. Due to their structural and antigenic polymorphism, the α -toxins in Old World scorpions have been classified into four structural and immunological groups (Granier et al. 1989). Typically, *Buthus* venoms contain mainly α -like toxins, as identified in the venom of *Buthus occitanus tunetanus* or *mardochei* from Morocco (Vargas et al. 1987), *Buthus martenzii* (Goudet et al. 2002; Liu et al. 2011) and *Buthus eupeus* (Volkova et al. 1985) from Asia. α -like toxins differ from classical α -toxins in that they exhibit a lower specificity for mammalian Na_v channels but are able to interact with Na_v channels present in the insect nervous system. Therefore, we first employed a serum raised against the α -like toxin Bot I, an abundant toxin from the *Buthus occitanus tunetanus* venom and a reference for the structural and immunological Group III (Granier et al. 1989; Martin and Rochat 1984). This large group contains almost all the α -like toxins purified so far from *Buthus* venoms and the serum is also able to recognize the insect-specific α -insect toxins such as Lqh α IT from *Leiurus quinquestriatus hebraeus* (Gordon et al. 2007). Our screening indicates that numerous components within all of the *Buthus occitanus* venoms submitted to ELISA tests react positively with the Bot I antiserum. After MALDI/TOF/MS analysis, the average masses determined for these components were between 6926 and 7400 Da (from 31.15 to 36.15 min). However, we did not find an exact mass corresponding to Bot I or to any isoforms of its immunological group (as Bot II) previously isolated from the *Buthus occitanus tunetanus* venoms from animal collected in Safi, Morocco or in Mecheria, Algeria (Martin and Rochat 1984). Yet, Bot I was also never found in the venoms from other Moroccan *Buthus* subspecies as *Buthus occitanus mardochei* (Vargas et al. 1987) or *Buthus occitanus* Paris (Martin-Eauclaire, unpublished data). This is in good agreement with the phylogenetic results from Gantenbein and Largiadèr, which revealed that the European nuclear 18S/TTS-1 DNA sequences and mitochondrial 16S and COI DNA sequences samples were highly separated from North African samples, the DNA sequences from Europe and Tunisia being also separated from the Moroccan sequences (Gantenbein and Largiadèr 2003). Altogether, our results confirm the high polymorphism in toxins found in different venoms from the same scorpion subspecies residing in a particular geographical area. Moreover, the data reported here underline that the *Buthus occitanus* venoms mainly consist of a large amount of α -like toxins and α -insect toxins, both belonging to the Bot I structural and immunological group III (Gordon et al. 2003; Gordon et al. 1996; Goudet et al. 2002; Martin and Rochat 1984; Vargas et al. 1987; Volkova et al. 1985).

In addition to α -like toxins, two structural homologous to the classic α -toxin AaH II from *Androctonus australis* Hector were identified previously in the North African *Buthus occitanus tunetanus* scorpion venom (Martin and Rochat 1984; Sampieri et al. 1987). AaH II is the reference peptide for the structural and immunological group II and its homologues can be detected using its highly specific anti-serum. AaH II itself has been the target of intensive immunological and pharmacological studies because this toxin constitutes 40% to 80% of the total lethality of the *Androctonus australis* venom towards mammals. Neutralizing AaH II is crucial to improve serotherapy against *Androctonus* stings in North Africa. After elution of our *Buthus occitanus* venoms, a substantial positive reaction was obtained against the anti-AaH II serum with peptidic fraction having a mass of 7304 Da. A more precise ELISA answer was obtained with the UHPLC fraction in which a peptide with a mass of 7465 Da was found.

Next, we tested whether our venoms react to specific sera raised against β -toxins from 'New-World' scorpion species, *i.e.* toxin II (Css II) from the Mexican scorpion *Centruroides suffusus suffusus*, and TsVII (also called Ts γ or Ts1) from the Brazilian scorpion *Tityus serrulatus*. From a mechanistic point of view, β -toxins are thought to trap and stabilize one or more Na $_v$ channel voltage-sensor in their outward, activated position, thereby enhancing channel activation in response to subsequent depolarization and negatively shifting the voltage dependence of activation (Bosmans et al. 2008; Cestèle et al. 1998; Cestèle et al. 2006; Pedraza Escalona and Possani 2013). After extensive testing, it was clear that none of the proteins found in the HPLC fractions tested here using anti-Css II and anti-TsVII sera share any common epitope with β -toxins and that no immunological relation exists between these different families. However, Old World scorpion venoms contain a small amount of β -type peptides with no demonstrable toxic activity when inoculated into mammals by subcutaneous injection. This rather new protein family of the Birtoxins-like is still poorly studied (Abbas et al. 2011; Caliskan et al. 2006). Moreover, another family of β -type toxins from Old World scorpion venoms active on both mammals and insect has been described (Gordon et al. 2003; Gordon et al. 2007; Loret et al. 1991). Since no serum is available against these toxins, we cannot test their presence in the venoms studied in this work. However, these high MW toxins are very hydrophobic (up to three tryptophans and eight tyrosines in the same sequence) and usually elute at the end of the chromatogram (high acetonitrile concentration). As such, molecules with masses 7751 Da and 7812 Da, which gave no ELISA answer against any serum tested, may correspond to these β -type peptides. Finally, we tested whether a serum against AaH IT, a β -toxin only lethal to insects (Darbon et al. 1982), recognized toxins within our *Buthus occitanus* venoms. As a result, we identified at least one anti-insect toxin that is antigenically related to AaH IT1. This peptide elute after 34 minutes from the column under the conditions used, have a mass of approximately 7984 Da and was found in the venoms collected near Orange city. This mass is close by that of AaH IT (7824 Da) and AaH IT2 (7869 Da) from *Androctonus australis*.

3.2.2. Kv channel toxins—In addition to long-chain peptides such as α -toxins which constitute about 10% of the detected masses, we found that 19% of the masses are situated around 2000 Da, 57 % over 3000 Da and only 14% over 4000 Da. It is worth mentioning that a peptide with a molecular weight of 3178 Da gave a high intensity peak in MS. A toxin with an identical mass, P01, is thought to be a ligand for the small conductance Ca $^{2+}$ activated K $^{+}$ channels, and was first purified from the venom of the Moroccan scorpion *Androctonus mauretanicus*. A similar peptide was also purified from the venoms of two other North African scorpion species, *Androctonus australis* from Algeria and Tunisia, and *Buthus occitanus tunetanus* from Egypt (Zerrouk et al. 1996). Unfortunately, no specific antibodies for this family (α -KTx8) are available to verify the identity of this toxin on our *Buthus occitanus* venoms.

Anti-KTX serum, which targets K $_v$ channel pore blockers (Romi et al. 1993), was also used to screen the fractions eluted by nano UHPLC (Table 1). At least one member of the α -KTx3 toxin family, active against members of the K $_v$ 1.x potassium channel family, was detected at 18.82 min using our ELISA test. Moreover, a mass of 4149 Da is in accordance with that of KTX (4150 Da) purified from *Androctonus mauretanicus* venom. Similar to this venom, KTX was also found in its deoxidised form (4182 Da, 20.15 min) (Oukkache et al. 2008).

The last serum tested was that against BmTX3, a short peptide toxin isolated from *Buthus martensi*, able to selectively block A-type K $^{+}$ currents in cerebellum granular cells or cultured striatum neurons. The distribution of binding sites in rat brain and its electrophysiological characteristics suggest that BmTX3 specifically binds to the K $_v$ 4.x subfamily of K $^{+}$ channels (Vacher and Martin-Eauclaire 2004). A strong positive response

was observed from 25.48 to 27.48 min (Table 1), which resulted in the mass determination of 3777 Da, displaying a very high MS intensity. This mass likely corresponds to a BmTX3 analogue in its Z-form, *i.e.* with its N-terminal amino acid under the form of a pyroglutamic acid (Vacher and Martin-Eauclaire 2004). A second mass at 3793 Da was also found, referring probably to a N-terminal glutamine (called N-form because the N-terminal is in the native state and available for Edman degradation) as previously observed be present concomitantly in venoms (Martin-Eauclaire and Bougis 2012).

3.3. Toxicity assays

We evaluated overall venom toxicity by s.c. injection in mice. To this end, we used the most abundant venom sample available (2.5 mg, sample 2 collected near Orange city). Similar to the North African *Androctonus australis* and *Leiurus quinquestriatus* scorpions, a low median lethal dose (LD₅₀) of 6 µg *per* mouse was found, suggesting that the tested venom is indeed lethal for small mammals. It is worth mentioning that the *Buthus occitanus* venom belongs to only one specimen and previous reports demonstrate that large differences in toxicity and composition can arise between individual venoms. Due to the low amount of material resulting from UHPLC fractionation, it was experimentally impossible to obtain mouse toxicity data of the individual venom components (*i.e.* 0.5 µL by well collected from the tip of the column capillary). In order to further the biological characterization of individual fractions, conventional HPLC was used to fractionate sample 2 (500 µg). Fractions of interest were first analysed using ELISA and mass spectrometry in order to verify that masses similar to those detected using nano UHPLC were injected into mice. Several fractions tested by i.c.v. injection induced moderate convulsions or other toxic signs (paralysis, twitching, sweating, tachycardia) in mice lasting 10 min to 1 h (Table 1). Only two fractions, immunoreactive to the anti-Bot I serum, were lethal. The most lethal fraction promoting death in less than 45 min and having a mass of 7465 Da was also highly positive for the anti-AaH II serum. Other fractions giving a strong positive reaction against the anti-Bot I serum were able to induce toxicity in mice with envenomation signs corresponding to those of α-like toxins.

Next, we examined the activity of the toxic fraction containing the mass 7465 Da on sodium currents generated by the neuronal rNa_v1.2a channels heterologously expressed in *Xenopus laevis* oocytes using the two-electrode voltage clamp technique. As is typically seen with α-toxins, a concentration of 0.5 µM inhibits fast inactivation of the channel resulting in a persistent current at the end of a 50 ms test pulse (Fig. 3A). Boltzmann fits of the deduced conductance-voltage curves reveal a hyperpolarizing shift in channel activation ($V_{1/2}$ from -41 ± 1 mV (control) to -47 ± 1 mV (venom fraction)) whereas channel availability (or steady-state inactivation) is not significantly altered ($V_{1/2}$ from -50 ± 1 mV (control) to -51 ± 1 mV (venom fraction)) (Fig. 3B). Altogether, our toxicity assays are in concordance with the presence of α-toxins in the venom of *Buthus occitanus* Amoreux that may underlie toxicity symptoms when humans are stung by this scorpion.

4. Conclusions

To our knowledge, this is the first report on the venom composition of the European scorpion *Buthus occitanus* Amoreux. Although envenomation cases are rare, they can be severe and therefore warrant our investigation. A complicating factor for our work is that the *Buthus occitanus* Amoreux is rare and therefore protected by the French government. Despite limited availability, we were able to demonstrate that *Buthus occitanus* Amoreux venom is homologous to the highly lethal North African Buthidae venoms. Moreover, we found that a low amount of crude venom can be lethal to mice and that individual venom fractions can induce moderate convulsions, paralysis, twitching, sweating, and tachycardia. Electrophysiological experiments on heterologously expressed Na_v channels suggest that the

underlying mechanism of these symptoms is most likely the result of toxins delaying Na_v channel fast inactivation. Based on our results using MS, immunological ELISA experiments, and biological toxicity assays, it is clear that multiple compounds from the French *Buthus occitanus* venoms are related to the α -toxins found in Old World scorpions. Moreover, we identified K_v channel-targeting toxins that are also present in North African *Androctonus* and *Buthus* species. Finally, we observed no cross-reaction with serum raised against β -type toxins, thereby distancing the *Buthus occitanus* Amoreux scorpion from its New World brethren. Typically, the immunological classification of scorpion toxins is superimposed on the structural and pharmacological characteristics, primarily because particular epitopes are implicated in both immunological cross reactivity and mode of action (Bouhaouala-Zahar et al. 1996). As such, we propose that the toxins from the *Buthus occitanus* venoms of French origin studied here are closely related in sequence to those present in *Buthus* species from North Africa and Asia, taking in mind that identical MS data may also result from a few amino acid changes or extensive amino acid replacements. These findings should be considered when treating stings from the *Buthus occitanus* Amoreux scorpion

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

There are 5 supplementary data files with this manuscript.

Highlights

- We characterized the venom of the French scorpion *Buthus occitanus* Amoureux.
- We used a nano UHPLC/MALDI-TOF-MS automated workflow, toxin specific ELISA and electrophysiology.
- We identified similarities with other lethal Buthidae from North Africa.

Fig 1. Pictures of the specimens of *Buthus occitanus* studied. A) Animal collected near Orange city (Vaucluse County; Photo credit: S. Diochot) and B) in Massif des Maures (Var County; Photo credit Thierry: Hupin).

Fig 2.

Representative chromatograms of collected *Buthus occitanus* venoms. A) Venom sample 1 (100 ng) and B) venom sample 2 (100 ng) obtained under electric stimulation of the post-abdomen of scorpions collected near Orange city (Vaucluse County). The chromatogram of the venom sample 3 is very similar to that of sample 2 and it is not shown. C) Venom sample (100 ng) obtained by manual stimulation from a single animal collected in Massif des Maures (Var County). Inset: relative proportion of the mass-classes detected. Solvents were water plus 0.05% TFA (A) and 80/20 (v/v) acetonitrile/water plus 0.04% TFA (B). The experimental conditions used were: elution gradient 4–50% of B; Dionex PepSwift monolithic column, 200 μm i.d. $\times$ 250 mm equipped with a trap column 200 μm i.d. $\times$ 5 mm; flow rate 1.5 $\mu\text{L}/\text{min}$; UV recording at the wavelength of 214 nm.

Fig 3.

Electrophysiological characterization. A) $rNa_v1.2a$ current at 0mV before (black) and after (red) addition of the venom fraction (0.5 μ M) to the recording chamber. Oocyte was held at -90 mV and depolarized to test pulse voltage for 50ms. Current amplitude is shown on vertical axis, time scale is shown on horizontal axis. B) Normalized steady-state inactivation (I/I_{max} : open circles) and conductance (G)-voltage (V) curves (filled circles) before (black) and after (red) venom fraction addition. G - V curves were deduced from current-voltage relationships obtained by depolarizing $rNa_v1.2a$ -expressing oocytes in 5mV steps from a holding potential of -90 mV over a wide voltage range. The obtained fit values using the Boltzmann function for $V_{1/2}$ are -41 ± 1 mV (control) and -47 ± 1 mV (venom fraction). Slope coefficients are 6.3 ± 0.6 and 5.2 ± 0.6 , respectively. Steady-state inactivation or channel availability curves were obtained by a standard double pulse protocol while holding the oocyte at -100 mV. The obtained fit values for $V_{1/2}$ are -50 ± 1 mV (control) and -51 ± 1 mV (venom fraction). Slope coefficients are 6.0 ± 0.2 and 7.8 ± 0.6 , respectively. $n = 3$ for all experiments and data are shown as mean $\pm$ SEM.

Table 1

Masses found in the sample 2 *Buthus occinatus* venom collected near Orange, which are also included at least twice in all the other venoms studied (a.u. arbitrary unit, m/z , mass-to-charge ratio; SN, signal-to-noise ratio, i.c.v. intracerebroventricular injection, n.d. not determined).

Buthus occinatus Orange (sample 2, Fig. 2B)			TOXIN ?	ELISA			TOXICITY
m/z [M+H] ⁺	Intens. (a.u.)	SN	Time (min)	Anti-KTX	Anti-BmTx3	Anti-Bot I	Anti-AaH IT1
3564	1796	274	18.82				i.c.v.
3599	1214	185	18.82				
4148	1182	224	18.82		/++++		n.d.
4121	36896	9354	20.15				
4183	5017	1272	20.15	KTX1 dioxide	/+++		
3549	29929	6830	22.48				
3666	852	180	23.48				
3731	15533	4599	23.48	Z-AmTX3-like	/++		
3746	796	236	23.48	N-AmTX3-like	/++		Epilepsy crisis
4821	6486	1106	24.15				
5115	50523	8616	24.15				
5099	34099	7369	24.82				
3716	89906	32684	25.48				
3768	944	272	25.15				
3777	15811	5748	25.48	Z-AmTX3-like	/+++		
3793	1654	346	27.82	N-AmTX3-like	/+++		
3731	18010	3772	27.82				
2025	1323	85	28.48				
3889	8210	761	29.15				
3635	47249	6193	29.48				
3811	13987	2442	30.82				
7305	31732	5135	31.15			/+++	Toxic signs
6948	18925	4110	31.82				
7040	3351	291	32.15				
7102	54259	8847	34.15	Bot I-like	/++++		Lethal at 24 h

Buthus occitanus Orange (sample 2, Fig. 2B)				TOXIN ?	ELISA			TOXICITY	
m/z [M+H] ⁺	Intens. (a.u.)	SN	Time (min)	Anti-KTX	Anti-BmTx3	Anti-Bot I	Anti-AaH II	Anti-AaH ITI	i.c.v.
7984	1301	273	34.82	AaH IT-like			Toxic signs		
7422	737	155	34.82				Toxic signs		
7465	41212	4834	34.48	AaHII-like			Lethal at 45 min		
7205	926	195	35.15				Toxic signs		
7409	1427	301	35.15						
7160	7339	694	36.15				Toxic signs		
7513	1969	177	39.48						
7751	4599	412	39.48				Prostration		