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Research Article

CSTX-9, a toxic peptide from the spider *Cupiennius salei*: amino acid sequence, disulphide bridge pattern and comparison with other spider toxins containing the cystine knot structure

J. Schaller[a,*], U. Kämpfer[a], S. Schürch[a], L. Kuhn-Nentwig[b], S. Haeberli[b] and W. Nentwig[b]

[a] Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, 3012 Bern (Switzerland), Fax + 41 31 631 48 87, e-mail: johann.schaller@ibc.unibe.ch
[b] Zoological Institute, University of Bern, Baltzerstrasse 6, 3012 Bern (Switzerland)

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Abstract. CSTX-9 (68 residues, 7530.9 Da) is one of the most abundant toxic polypeptides in the venom of the wandering spider *Cupiennius salei*. The amino acid sequence was determined by Edman degradation using reduced and alkylated CSTX-9 and peptides generated by cleavages with endoproteinase Asp-N and trypsin, respectively. Sequence comparison with CSTX-1, the most abundant and the most toxic polypeptide in the crude spider venom, revealed a high degree of similarity (53% identity). By means of limited proteolysis with immobilised trypsin and RP-HPLC, the cystine-containing peptides of CSTX-9 were isolated and the disulphide bridges were assigned by amino acid analysis, Edman degradation and nanospray tandem mass spectrometry. The four disulphide bonds present in CSTX-9 are arranged in the following pattern: 1-4, 2-5, 3-8 and 6-7 (Cys_6-Cys_{21}, Cys_{13}-Cys_{30}, Cys_{20}-Cys_{48}, Cys_{32}-Cys_{46}). Sequence comparison of CSTX-1 with CSTX-9 clearly indicates the same disulphide bridge pattern, which is also found in other spider polypeptide toxins, e.g. agatoxins (ω-AGA-IVA, ω-AGA-IVB, μ-AGA-I and μ-AGA-VI) from *Agelenopsis aperta*, SNX-325 from *Segestria florentina* and curtatoxins (CT-I, CT-II and CT-III) from *Hololena curta*. CSTX-1/CSTX-9 belong to the family of ion channel toxins containing the inhibitor cystine knot structural motif. CSTX-9, lacking the lysine-rich C-terminal tail of CSTX-1, exhibits a ninefold lower toxicity to *Drosophila melanogaster* than CSTX-1. This is in accordance with previous observations of CSTX-2a and CSTX-2b, two truncated forms of CSTX-1 which, like CSTX-9, also lack the C-terminal lysine-rich tail.

Key words. *Cupiennius salei*; spider toxin; CSTX-9/CSTX-1; amino acid sequence; electrospray ionisation mass spectrometry; disulphide bridge; inhibitor cystine knot (ICK) structural motif.

Introduction

Recent structural studies of toxic polypeptides from spiders, scorpions, cone shells and snakes have considerably enlarged our knowledge concerning the relationship between structure and function of these important toxic compounds [for reviews see refs. 1–4 and references therein]. The elucidation of a series of three-dimensional structures and/or the disulphide bridge patterns in spider toxins [5–15] has provided evidence for common structural motifs and similarities in the disulphide pairing. An interesting peptide fold is the inhibitor cystine knot (ICK) structural motif characterised by a triple-stranded, antiparallel β sheet stabilised by a cystine knot containing three disulphide bridges [16, 17]. The ICK motif is found in ion channel-active polypeptide toxins from phylogen-

* Corresponding author.

etically very diverse sources such as spiders, scorpions, insects, snails, plants and fungi [16–21]. These small polypeptide toxins (4–10 kDa) with 6–14 Cys residues exhibit a more or less pronounced influence on cation channels, e.g. calcium, sodium and potassium channels.

During our studies on the venom of *Cupienpius salei,* we isolated 13 peptides with varying toxic effects from the multicomponent venom of *C. salei*. The amino acid sequence of CSTX-1 (74 residues), the peptide responsible for most of the toxicity of the crude venom, has two interesting and characteristic features: (i) a cystine knot with eight Cys residues in the N-terminal portion of the molecule and (ii) an extremely basic C-terminal tail containing seven lysines within the last ten residues [22]. Two truncated forms of CSTX-1, CSTX-2a (Ser_1–Arg_{61}) and CSTX-2b (Ser_1–Phe_{60}), both lacking the very basic C-terminal tail of CSTX-1, exhibit a 7- and 190-fold reduced activity, respectively, to *Drosophila melanogaster*, an indication of the direct involvement of the lysine-rich C-terminal tail of CSTX-1 in its toxicity [23].

Here, we report the determination of the amino acid sequence and the assignment of the disulphide pairing of CSTX-9 by Edman degradation, amino acid analysis and nanospray tandem mass spectrometry, the assignment of the disulphide bridge pattern in CSTX-1 by similarity, a comparison with other spider toxins and preliminary toxicological data.

Materials and methods

Materials

Trypsin (EC 3.4.21.4; immobilised on agarose beads, tosyl-L-Phe-chlormethylketone treated) was obtained from Sigma (St. Louis, Mo.) and endoproteinase Asp-N (EC 3.4.24.33; sequencing grade) from Roche Diagnostics (Rotkreuz, Switzerland). The reagents and solvents used for sequence analysis were purchased from Applied Biosystems (Foster City, Calif.). All other chemicals were of analytical grade either from Merck, Sigma, Fluka or Pierce.

Isolation of the toxins and bioassay

The spider venom was collected and CSTX-9 isolated as previously described [22]. The purity of the sample was verified by reverse-phase high-performance liquid chromatography (RP-HPLC), amino acid analysis, N-terminal sequence determination and electrospray ionisation mass spectromety (ESI-MS). The *D. melanogaster* bioassay was performed as described elsewhere [23]. Four different toxin concentrations were tested (20 flies for each concentration) and 20 flies were used as a control (injecting 0.05 µl insect Ringer).

Reduction and alkylation

One hundred micrograms CSTX-9 was reduced with dithiothreitol (tenfold molar excess over disulphides) in 0.1 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine-HCl for 1 h at 37 °C under nitrogen, and alkylated with iodoacetamide (fivefold molar excess over total thiols) for 1 h at 37 °C in the dark. Finally, 2-mercaptoethanol (tenfold molar excess over iodoacetamide) was added and the solution was acidified with trifluoroacetic acid prior to desalting by RP-HPLC.

Enzymatic cleavages

Fifteen micrograms reduced and alkylated CSTX-9 was digested with endoproteinase Asp-N in 50 µl 0.1 M NH_4HCO_3 buffer, pH 8.0 at an enzyme to substrate ratio of 1 : 100 (w/w) for 1 h at 37 °C under gentle shaking.

Ten micrograms native CSTX-9 was cleaved with immobilised trypsin (4.6 µl wet gel with 0.1 units trypsin) for 17 h at room temperature in 20 µl 0.1 M Tris-HCl buffer, pH 8.5, containing 1.0 mM iodoacetamide under gentle shaking. The suspension was centrifuged and the supernatant was recovered for separation by RP-HPLC.

RP-HPLC

The peptides resulting from the digestion with endoproteinase Asp-N were separated by RP-HPLC on a Nucleosil C18 column (2.0 × 125 mm, 12.5 nm, 5 µm; Macherey-Nagel, Oensingen, Switzerland) in a Jasco HPLC system (OmniLab, Mettmenstetten, Switzerland) using a combination of acetonitrile gradients in dilute trifluoroacetic acid (for details consult the legend to the chromatogram).

The tryptic peptides were separated by RP-HPLC on an Aquapore Butyl column (2.1 × 100 mm, wide-pore 30 nm, 7 µm; Applied Biosystems) in a Hewlett Packard 1090 liquid chromatograph (Hewlett Packard, Waldbronn, Germany) using an acetonitrile gradient in dilute trifluoroacetic acid (for details consult the legend to the chromatogram).

Amino acid analysis

Samples were hydrolysed in the gas phase with 6 M hydrochloric acid containing 0.1% (by volume) phenol for 24 h at 115 °C under N_2 vacuum according to Chang and Knecht [24]. The liberated amino acids were reacted with phenylisothiocyanate and the resulting phenylthiocarbamoyl amino acids were analysed by RP-HPLC on a Nova Pak ODS column (3.9 × 150 mm, 4 µm; Waters) in a Hewlett Packard liquid chromatograph 1090 with an automatic injection system according to Bidlingmeyer et al. [25]. Disulphide-containing peptides were identified by the presence of di-phenylthiohydantion-cystine (di-PTH-Cys).

Amino acid sequence analysis

N-terminal sequence analysis was carried out either in a Procise cLC 492 protein sequencer or in a pulsed liquid-phase sequencer 477A, both from Applied Biosystems. The released amino acids were analysed on-line by RP-HPLC. In the case of the disulphide-linked peptides, the di-PTH-Cys was detected in the corresponding cycle as a double peak in the vicinity of PTH-Tyr [26–28].

Mass spectrometry

The masses of the peptides [dissolved in acetonitrile/water (1:1, v/v) containing 0.5% formic acid] were determined by ESI-MS with a single-stage quadrupole instrument (VG Platform; Micromass, Manchester, UK). In addition, the final assignment of the disulphide bridges was achieved with a hybrid quadrupole time-of-flight mass spectrometer (QStar Pulsar, Applied Biosystems/Sciex, Concord, Canada) equipped with a nanospray ion source (Protana, Odense, Denmark). Samples were dissolved in methanol/water (1:1, v/v) containing 2% formic acid.

Results and Discussion

CSTX-9 was purified by a combination of gel filtration, ion exchange chromatography and RP-HPLC as described previously [22]. The yield of CSTX-9 was in the range of 1.4–8.2 µg/µl of fractionated venom (0.2–1.1 mM in the crude venom). The mass of 7529.75 ± 0.32 Da determined by ESI-MS is much smaller than the previously known value of 12.2 kDa obtained by SDS-PAGE (value of the non-reduced form). Reduced and alkylated CSTX-9 could be sequenced by Edman degradation up to position 47 (fig. 1). To obtain the rest of the sequence, reduced and alkylated CSTX-9 was cleaved with endoproteinase Asp-N and the peptides generated were separated by RP-HPLC and identified by ESI-MS (fig. 2a): Fraction N1, contained the peptide Lys_1–Asn_{15} (1895.75 ± 0.38 Da); fraction N2, Asp_{16}– Ala_{36} (2605.88 ± 0.06 Da), fraction N3, Lys_1–Ala_{36} (4482.88 ± 0.29 Da); fraction N4, Asp_{37}–Phe_{68} (3528.63 ± 0.24 Da) and fraction N5, contained Asp_{49}–Phe_{68} (2221.38 ± 0.07 Da). With the exception of the C-terminal amino acid, the complete sequence could be assigned with the peptides N4 and N5 (fig. 1). From the remaining mass difference and the missing amino acid in the amino acid composition, the C-terminal amino acid was deduced to be a Phe residue. Sequence comparison of CSTX-9 with CSTX-1 revealed an identity of 53% as well as identical positions of all eight Cys residues present in the N-terminal portion of the molecule (fig. 4).

To determine the disulphide bridges, native CSTX-9 was cleaved using immobilised trypsin to eliminate disulphide exchange during digestion, as in recent experiments with immobilised endoproteinase Glu-C by Dormady et al. [29]. In addition, iodoacetamide was used as a scavenger to alkylate any free thiol groups that may have been formed during the cleavage. The tryptic peptides were separated by RP-HPLC (fig. 2b) and each fraction was analysed by amino acid composition, sequence determination and ESI-MS measurements. The data are summarised in table 1. Fraction T8 contained the C-terminal tryptic tripeptide Gly_{66}–Phe_{68}, thus eliminating the last ambiguity in the sequence determination (fig. 1). For rea-

Table 1. Structural data of tryptic peptides from native CSTX-9 (cysteine residues in disulphide form).

HPLC peak number	Sequence data	Mass spectrometry		Disulphide bridges
		observed	calculated	
T2	$M^{28}K^{29}$ $G^{40}ATSER^{45}$ $H^{10}HEXTNDK^{17}$ $X^{30}K^{31}$	277.16 619.30 1229.75	277.15 619.29 1229.48	13-30 (C2-C5)
T3	$G^{24}LTK^{27}$	417.28	417.26	
T4	$H^{62}IIK^{65}$	509.28	509.33	
T8	$G^{66}LF^{68}$	335.23	335.18	
T9	$F^{56}GF^{58}$	369.21	369.17	
T10	$N^{5}XIPK^{9}$ $N^{19}XCK^{22}$ $C^{32}FTVADAK^{39}$ $C^{46}ACDSSLLQK^{55}$	2954.38	2955.44	6-21 (C1-C4) 20-48 (C3-C8) 32-46 (C6-C7)
T11	$N^{5}XIPK^{9}$ $N^{19}XCK^{22}$ $C^{32}FTVADA^{38}$ $C^{46}ACDSSLLQ^{54}$	2698.38	2699.09	6-21 (C1-C4) 20-48 (C3-C8) 32-46 (C6-C7)
T12	$F^{56}GFTGL^{61}$	640.33	640.32	

X, Cys residue expected from the sequence but not observed; C, di-PH T-Cys.

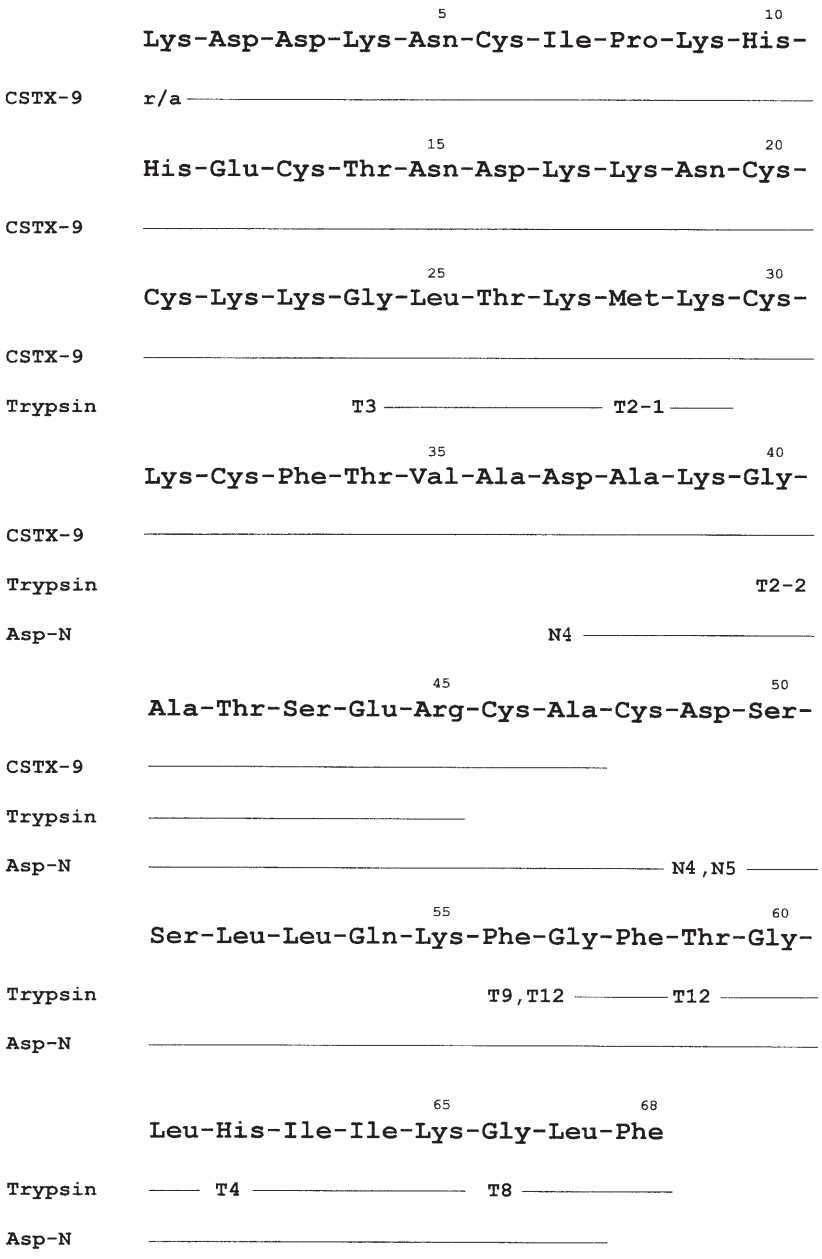

Figure 1. Amino acid sequence of CSTX-9. The sequence was determined by Edman degradation using reduced and alkylated CSTX-9 and peptides derived from cleavages with endoproteinase Asp-N and trypsin.

sons of completeness, the tryptic peptides without disulphide bridges were also included in the sequence diagram. According to the amino acid composition, disulphide-containing peptides were present in fractions T2, T10 and T11. Prior to identification, fraction T2 was rechromatographed isocratically to remove the tryptic peptides Met_{28}-Lys_{29} and Gly_{40}-Arg_{45} from the disulphide linked peptides His_{10}-Lys_{17} and Cys_{30}-Lys_{31} and the disulphide bridge Cys_{13}-Cys_{30} was assigned by Edman degradation and ESI-MS. T10 and T11 contained a cluster of three disulphide bridges. The release of di-PTH-Cys in the first cycle of the Edman degradation of T10 and T11 implies a disulphide bridge Cys_{32}-Cys_{46}. The release of di-PTH-Cys in cycle 3 is only indicative of the presence of disulphide bridges. However, the pairing of the other four Cys residues could not be assigned unambiguously by Edman degradation. Of the three theoretically possible combinations Cys_6-Cys_{21}/Cys_{20}-Cys_{48}, Cys_6-Cys_{20}/Cys_{21}-Cys_{48} and Cys_6-Cys_{48}/Cys_{20}- Cys_{21}, the last pair can be ruled out, since a hypothetical tryptic tetrapeptide Asn_{19}-Lys_{22} containing a rare but possible vicinal disulphide bridge (Cys_{20}-Cys_{21}) was never observed by RP-HPLC, ESI-MS or Edman degradation. To assign the two remaining disulphide bridges, fraction T10 (fig. 2b), containing the disulphide-linked

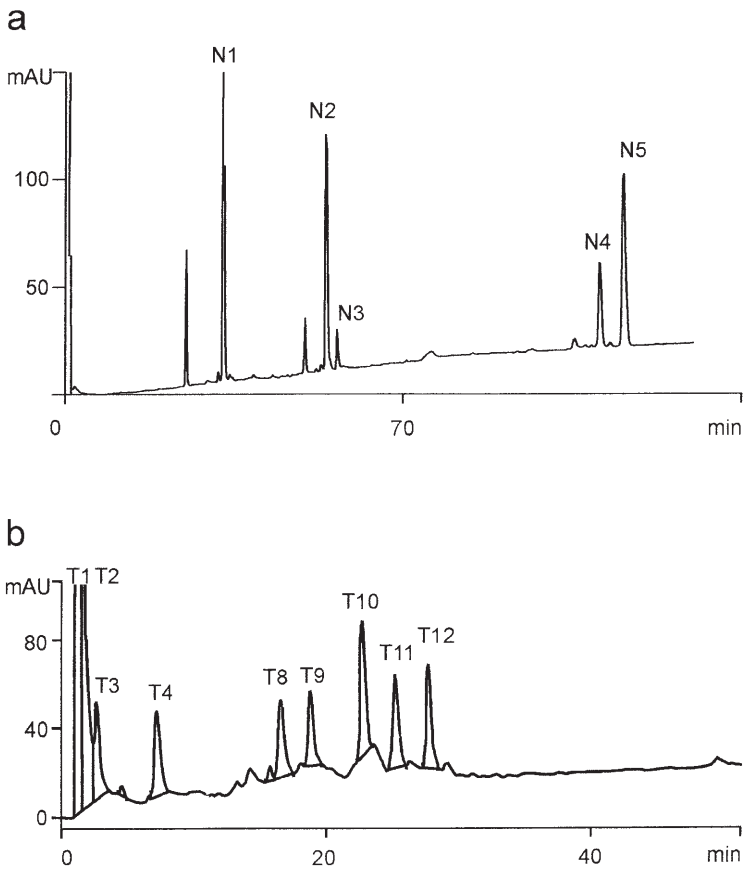

Figure 2. (*a*) RP-HPLC separation of the endoproteinase Asp-N digest of reduced and alkylated CSTX-9 on a Nucleosil C18 column (2.0 × 125 mm, 5 µm) applying the following gradients: 0–5 min, 0% B; 5–80 min, 0–30% B; 80–180 min, 30–50% B. Solution A: 0.1% (by volume) trifluoroacetic acid in distilled water. Solution B: 0.1% (by volume) trifluoroacetic acid in acetonitrile. Flow rate: 0.5 ml/min. All peaks were collected and the peptides identified by ESI-MS. Fractions N4 and N5 were further used for Edman degradation. (*b*) RP-HPLC separation of the tryptic digest of CSTX-9 on an Aquapore Butyl column (2.1 × 100 mm, 7 µm) applying a linear acetonitrile gradient from 0% B to 60% B in 60 min. Solution A: 0.1% (by volume) trifluoroacetic acid in distilled water. Solution B: 0.1% (by volume) trifluoroacetic acid and 80% (by volume) acetonitrile in distilled water. Flow rate: 0.3 ml/min. All peaks were collected and the peptides identified by a combination of ESI-MS, amino acid analysis and Edman degradation (see table 1). The Cys-containing fractions T2, T10 and T11 were used for the assignment of the disulphide bridges. Prior to the identification, T2 was rechromatographed isocratically (chromatogram not shown).

peptides Asn_5-Lys_9, Asn_{19}-Lys_{22}, Cys_{32}-Lys_{39} and Cys_{46}-Lys_{55}, was analysed by nanospray tandem mass spectrometry. The $[M + 4H]^{4+}$ ion (m/z 739.34) was selected as the precursor for subsequent collision-induced dissociation. Elucidation of the disulphide bridge pattern was based on fragment ions generated by cleavages at the N-terminal side of residues Cys_{21} and Cys_{48} (fig. 3), respectively. Cleavage at Cys_{48} resulted in the loss of the disulphide linked peptides Cys_{32}-Lys_{39} and Cys_{46}-Ala_{47}, thus assigning the Cys_{32}-Cys_{46} disulphide bridge in accordance with the data obtained by Edman degradation. The complementary fragment containing the disulphide-linked peptides Asn_5-Lys_9, Asn_{19}-Lys_{22} and Cys_{48}-Lys_{55} was observed as the y^{3+} ion at m/z 643.63 (fig. 3). Cleavage between residues Cys_{20} and Cys_{21} yielded a doubly charged y-type fragment ion (m/z 411.21) containing the disulphide-linked peptides Asn_5-Lys_9 and Cys_{21}-Lys_{22}, thus identifying the disulphide bridge Cys_6-Cys_{21}. The remaining fourth disulphide bridge, Cys_{20}-Cys_{48}, is contained in the disulphide- linked peptides Asn_{19}-Cys_{20} and Cys_{48}-Lys_{55}. In conclusion, the four disulphide bonds present in CSTX-9 are arranged in the following pattern (fig. 4):

1-4, 2-5, 3-8 and 6-7

(Cys_6-Cys_{21}, Cys_{13}-Cys_{30}, Cys_{20}-Cys_{48}, Cys_{32}-Cys_{46}).Besides the high degree of sequence similarity between CSTX-9 and CSTX-1 (53% identity), all eight Cys residues are in identical positions in each toxin; therefore, CSTX-9 and CSTX-1 most likely share the same disulphide bridge pattern as indicated in figure 4. Lys_{31} in CSTX-9, located between Cys_{30} and Cys_{32}, is replaced by a Gln residue in the CSTX-1 sequence, thus preventing a cleavage by trypsin between the two Cys residues. The cleavage of native CSTX-1 with immobilised trypsin yielded only one fraction containing all disulphide-linked peptides, namely Ser_1-Lys_5, His_6-Lys_{13}, His_{14}-Arg_{18},

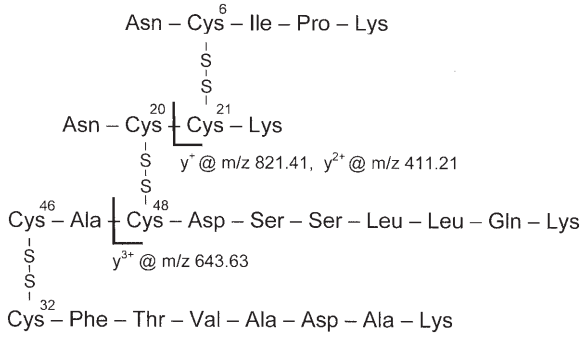

Figure 3. Elucidation of the disulphide bridge pattern by ESI-MS/MS. Nanospray tandem mass spectrometry of fraction T10 (fig. 2b). The precursor ion $[M + 4H]^{4+}$ (m/z 739.34) of the disulphide linked tryptic peptides Asn_5-Lys_9, Asn_{19}-Lys_{22}, Cys_{32}-Lys_{39} and Cys_{46}-Lys_{55} was analysed by collision-induced dissociation tandem mass spectrometry on a hybrid quadrupole time-of-flight mass spectrometer (QStar Pulsar; Applied Biosystems/Sciex). Characteristic fragment ions defining the disulphide bridge pattern were generated by cleavage of the peptide bonds Cys_{20}-Cys_{21} and Ala_{47}-Cys_{48}.

Cys_{26}-Arg_{41} and Cys_{42}-Arg_{46}, as shown by amino acid analysis, Edman degradation and ESI-MS. The arrangement of the disulphide bridges in CSTX-1 was assigned by comparison with the experimentally determined pattern of CSTX-9 (fig. 4).

The disulphide bridge pattern 1-4, 2-5, 3-8 and 6-7 is also found in other spider toxins, e.g. ω-agatoxins (ω-AGA-IVA and ω-AGA-IVB) and μ-agatoxins (μ-AGA-I and μ-AGA-VI) from *Agelenopsis aperta* [30, 31], SNX-325 from *Segestria florentina* [8], and curtatoxins (CT-I, CT-II and CT-III) from *Hololena curta* [32] (fig. 5). Although the sequence similarity between CSTX-9/CSTX-1 and other spider toxins with four disulphide bridges is very low (fig. 5), the disulphide bridge pattern is obviously identical. Many other spider toxins like agelenin [10] and huwentoxin [11] have only three disulphide bridges arranged in a 1-4, 2-5 and 3-6 pattern, according to the well-known consensus sequence of the ICK structural motif: $\mathbf{CX_{3-7}CX_{3-8}CX_{0-7}CX_{1-4}CX_{4-13}C}$ (X can be any amino acid, including Cys). The additional fourth disulphide bridge (C_6-C_7) present in CSTX-1/CSTX-9 can be thought of as an insertion between bridge 3-6 of the ICK structural motif, as has already been demonstrated for ω-AGA-IVA/ω-AGA-IVB and μ-AGA-I/μ-AGA-IV [16, 17]. The length of the inserted sequence between the C_3–C_6 disulphide bridge in the ICK structural motif is quite variable: 9 residues for CT-I/CT-II/CT-III and μ-AGA-I/μ-AGA-IV/μ-AGA-VI, 10 residues for ω-AGA-IVA/ω-AGA-IVB, 17 residues for CSTX-1 and CSTX-9 and 20 residues for SNX-325. Assuming that the model of the inserted disulphide bridge C_6-C_7 of the spider toxins containing four disulphide bridges is correct, and to satisfy all known examples, the ICK structural motif has to be modified into the following consensus sequence: $\mathbf{CX_{3-7}CX_{3-10}CX_{0-7}CX_{1-8}CX_{4-20}C}$. The longer sequences between C_2-C_3 and C_4-C_5 imply extended loops between these Cys residues. The insertion of an additional disulphide bridge with a variable sequence length in between (from 9 amino acids in CT-I to 20 residues in SNX-325) is suggestive of an extended loop structure.

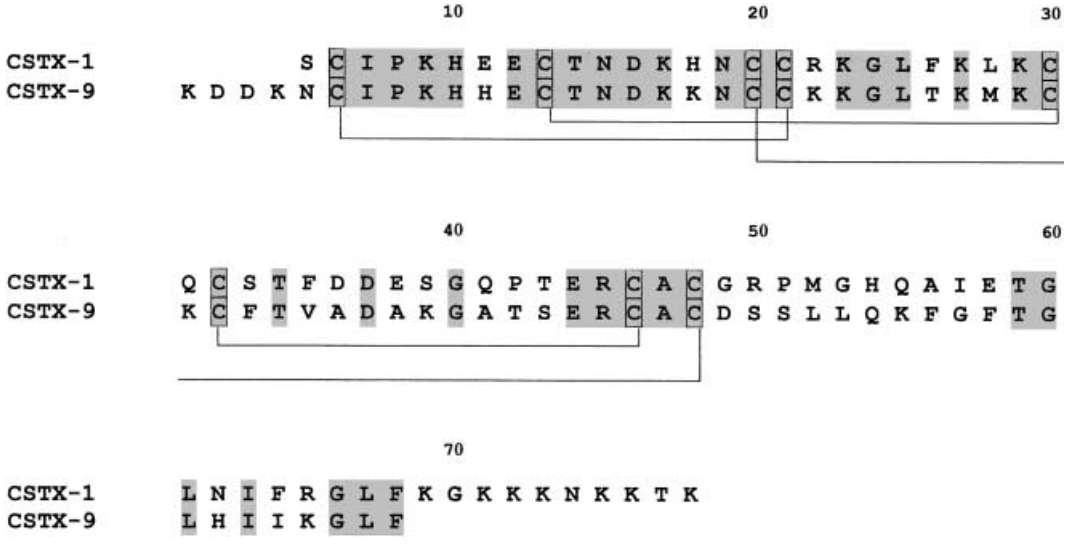

Figure 4. Sequence comparison of CSTX-1 and CSTX-9 and schematic representation of the disulphide bonding pattern. Identical amino acids are shaded in grey. Disulphide bridges are represented by lines and the corresponding cysteine residues are within boxes. The disulphide bridges were determined by a combination of Edman degradation and ESI-MS of the corresponding disulphide-linked tryptic peptides. The sequence of CSTX-1 is from Kuhn-Nentwig et al. [22].

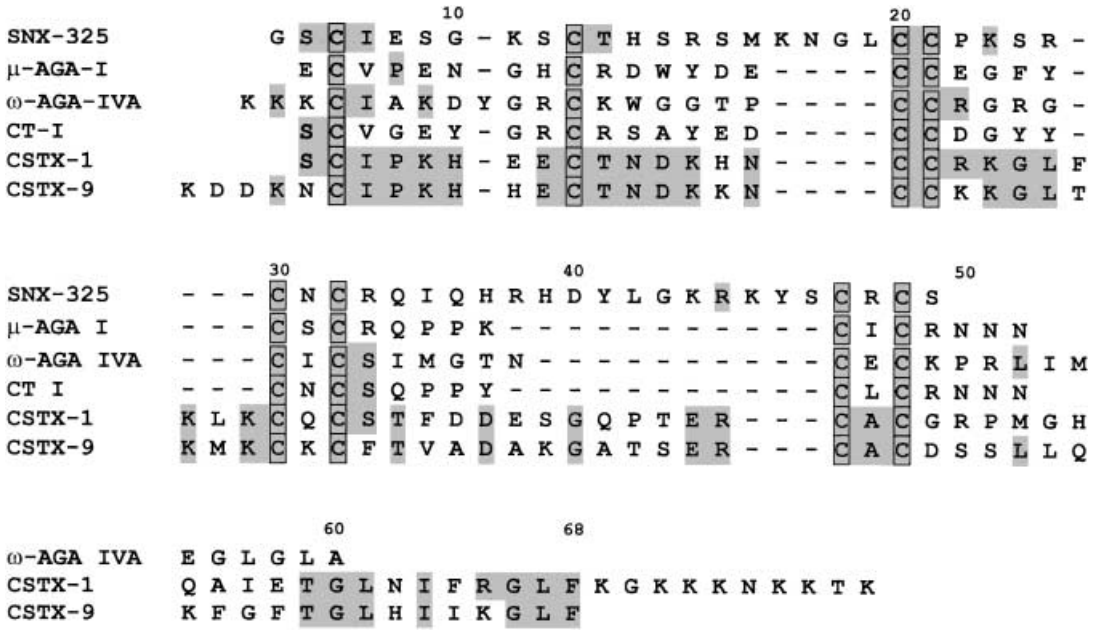

Figure 5. Comparison of a selection of spider toxins with the same disulphide bridge pattern C1-C4, C2-C5, C3-C8 and C6-C7. Identical amino acids with the toxins CSTX-1/CSTX-9 are shaded in grey. The Cys residues of the pattern are shaded in grey and within boxes. CSTX-1/CSTX-9: *Cupiennius salei*; agatoxins μ-AGA-I/ω-AGA-IVA: *Agelenopsis aperta* [30, 31]; SNX-325: *Segestria florentina* [8]; curtatoxin CT-I: *Hololena curta* [32].

CSTX-9 lacks the very basic, lysine-rich, ten-residue long C-terminal tail of CSTX-1 (fig. 4). Testing CSTX-9 in the *D. melanogaster* bioassay, the LD_{50} was 3.12 pmol/mg fly (95% confidence limits: 2.66–3.60), thus exhibiting a ninefold lower toxicity than CSTX-1. This observation is in accordance with previous data for two truncated forms of CSTX-1, CSTX-2a (Ser_1–Arg_{61}) and CSTX-2b (Ser_1–Phe_{60}), both lacking the very basic C-terminal tail of CSTX-1. They exhibit a 7- and 190-fold reduced activity, respectively, to *D. melanogaster*, an indication of direct involvement of the lysine-rich C-terminal tail of CSTX-1 in its toxicity [23].

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