

# Neurotoxic Peptides in the Multicomponent Venom of the Spider *Cupiennius Salei*

## Part II. Elucidation of the Disulphide-Bridge Pattern of the Neurotoxic Peptide CSTX-9 by Tandem Mass Spectrometry

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**Abstract:** The disulphide-bridge pattern of the CSTX-9 polypeptide present in the multicomponent venom of the spider *Cupiennius salei* was determined *de novo* by nano-electrospray tandem mass spectrometry. Cleavage of native CSTX-9 with immobilized trypsin resulted in four disulphide-linked peptides with a mass of 2953.43 Da, which were subjected to low-energy collision-induced dissociation (CID) in a hybrid quadrupole time-of-flight (QqTOF) mass spectrometer. The product ion spectra not only provided sequence information of the peptides, the occurrence of characteristic fragment ions generated by cleavage of the peptide bonds adjacent to the bridged cysteines also allowed the disulphide-bridge pattern to be identified unambiguously. CSTX-9 was found to be a member of the neurotoxic polypeptide family incorporating the inhibitor cystine knot (ICK) structural motif. The results demonstrate the potential of modern analytical instrumentation for elucidation of complex molecular structures.

**Keywords:** Collision-induced dissociation · CSTX-9 · *Cupiennius salei* · Disulphide-bridge pattern · Tandem mass spectrometry

### 1. Introduction

Within the last decade, mass spectrometry has undoubtedly established its position as an important tool in the field of bioanalysis. The introduction of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which for the first time allowed intact high-mass biomolecules to be ionized, has become a milestone in the development of biological mass spec-

trometry. Simultaneously, considerable progress has been made in designing high-performance mass analyzers suitable for interfacing with the new ionization techniques. As a result, highly versatile instruments with impressive specifications have become available. A high degree of automation enables high sample throughput, and data processing is readily performed with the help of sophisticated software tools and access to protein databases. As a consequence, protein identification by peptide mass mapping and MS/MS sequencing, using either single-stage or tandem mass spectrometers, has become routine and applications are found in many areas of life sciences. However, solving complex structural problems that reach beyond routine is still a challenge for the analytical chemist and the success of an approach often depends on the availability of high-performance analytical instrumentation.

### 2. Tandem Mass Spectrometry

Applications of mass spectrometry are not limited to the determination of molecular weight. Detailed structural information is obtained from tandem mass spectrometric (MS/MS) experiments using a combination of two mass analyzers with an intermediate collision cell. Within such an arrangement the first device acts as a mass filter for selection of a particular precursor ion, which is subsequently subjected to collision-induced dissociation (CID) in the collision cell, and the dissociation products are finally mass-analyzed in the second stage of mass spectrometry. Since the different steps of the experiment take place in sequentially coupled devices, this type of instrumentation is often referred to as tandem-in-space mass spectrometers, such as the triple-quadrupole instruments. Alternatively, similar experiments can be performed in an ion storage mass

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spectrometer, such as an ion trap or an ion cyclotron mass spectrometer, where the different steps are performed in a temporal sequence (tandem-in-time). An advantage of tandem mass spectrometry is its ability to produce a multitude of structurally characteristic fragment ions in a single experiment. Among the various instrument geometries available, the hybrid quadrupole time-of-flight (QqTOF) mass spectrometer is a relatively new design, which achieves outstanding instrumental performance in terms of mass resolving power and mass accuracy, due to the implementation of a time-of-flight (TOF) analyzer as the second stage of mass spectrometry. This type of analyzer has gained a reputation as a fast and highly accurate separation device for high-mass ions. Due to the need for pulsed ion injection, it is inherently well suited to be combined with a MALDI source, whereas excellent results are also obtained by orthogonal injection of ESI-generated ions, either from the ion source directly or from the preceding stage of mass spectrometry [1][2].

The amount of sample available from biological sources is often limited to minute quantities of a few picomoles or even less. This restriction is overcome by equipping the mass spectrometer with a miniaturized nano-electrospray ion source, enabling low flow rates on the order of a few tens of nanoliters per minute [3]. For example, about 5 pmol of sample dissolved in 2 ml of solvent were required for structural analysis of the CSTX-9 neurotoxic polypeptide of *C. salei*.

### 3. The Disulphide-Bridge Pattern of CSTX-9

Identification of the disulphide-bridge patterns of polypeptides and proteins is commonly performed by a combination of partial reduction/alkylation, enzymatic degradation and subsequent analysis of the peptides by either NMR, Edman sequencing or peptide mass mapping [4–9]. However, these methods may fail in the case of highly knotted structures with cystines arranged in close proximity, most likely due to either steric hindrance that prevents immediate alkylation of reduced disulphide-bridges and promotes disulphide bridge exchange (disulphide-scrambling) [10], or the generation of non-characteristic peptides upon degradation.

The neurotoxic polypeptide CSTX-9 (68 residues, the sequence is given in the

preceding article) was obtained from the crude venom of *C. salei* by a combination of gel filtration, cation-exchange chromatography, and reversed-phase HPLC [11]. Cleavage of native CSTX-9 with immobilized trypsin and separation by reversed-phase HPLC yielded two cystine-containing fractions. Edman degradation of the bridged peptides His<sub>10</sub>–Lys<sub>17</sub>, Cys<sub>30</sub>–Lys<sub>31</sub> of fraction 1 revealed a first disulphide-bridge between Cys<sub>13</sub> and Cys<sub>30</sub>, and analysis of the disulphide-linked peptide cluster Asn<sub>6</sub>–Lys<sub>9</sub>, Asn<sub>19</sub>–Lys<sub>22</sub>, Cys<sub>32</sub>–Lys<sub>39</sub>, and Cys<sub>46</sub>–Lys<sub>55</sub> of fraction 2 (Fig. 1) gave evidence of the presence of a further disulphide-bridge Cys<sub>32</sub>–Cys<sub>46</sub>. However, assignment of the remaining two linkages by Edman degradation failed, since the two cystines were both released within the same cycle of degradation, allowing no differentiation between the possible combinations Cys<sub>6</sub>–Cys<sub>20</sub> / Cys<sub>21</sub>–Cys<sub>48</sub> and Cys<sub>6</sub>–Cys<sub>21</sub> / Cys<sub>20</sub>–Cys<sub>48</sub> [12]. Thus, we focused on an alternative approach based on tandem mass spectrometry for further elucidation of the disulphide-bridge pattern.

The [M+4H]<sup>4+</sup> (m/z 739.62) and [M+5H]<sup>5+</sup> (m/z 591.69) ions of the disulphide-linked peptides of fraction 2 were selected as the precursors for CID experiments. The internal energy deposited into the precursor ions upon collisional activation induced extensive dissociation, yielding a large number of structurally valuable fragment ions. As demonstrated by the product ion spectrum in Fig. 2, the complete amino acid sequences ranging from the bridged cysteines to the C-termini of the peptides (Ile<sub>7</sub>–Lys<sub>9</sub>, Phe<sub>33</sub>–Lys<sub>39</sub>, and Asp<sub>49</sub>–Lys<sub>55</sub>) were readily identified. Sequence assignment is based on the detection of the series of singly charged y-type ions, which all have a

common starting point at m/z 147.11, indicating the presence of the C-terminal lysines as a result of tryptic digest. The complementary b-type ions of the individual peptides were not detected, since they are all disulphide-linked.

The quality and range of information obtained from MS/MS data strongly depend on the performance of the second stage of mass spectrometry. Dissociation of multiply charged precursor ions may result in fragment ions of different charge states folding into a narrow m/z region, and the probability of overlapping peaks is greatly increased when more complex precursor ion structures, such as the cross-linked peptides of tryptically processed CSTX-9, are subjected to CID. Additionally, a considerable number of internal fragment ions and secondary dissociation products may complicate spectral interpretation. Thus, a second stage of mass spectrometry providing high mass resolving power and mass accuracy is an indispensable prerequisite for unambiguous peak assignment.

The fact that disulphide bonds are not preferentially cleaved upon low-energy CID conditions [13][14] provides an opportunity for direct elucidation of the bridge pattern. The key information was obtained from fragment ions generated by cleavage of the peptide bonds adjacent to the bridged cysteines. The cleavage sites along with the corresponding fragment ion peaks are shown in Fig. 3. Dissociation of the peptide bonds adjacent to Ala<sub>47</sub> and Cys<sub>48</sub> resulted in the triply charged y-type fragment ions with m/z 667.30 (fragment A) and m/z 643.63 (fragment B), respectively, clearly indicating the presence of a Cys<sub>32</sub>–Cys<sub>46</sub> disulphide-bridge. Confirmation was obtained from the complementary fragment (C), which was detected as a b<sup>+</sup>-ion with

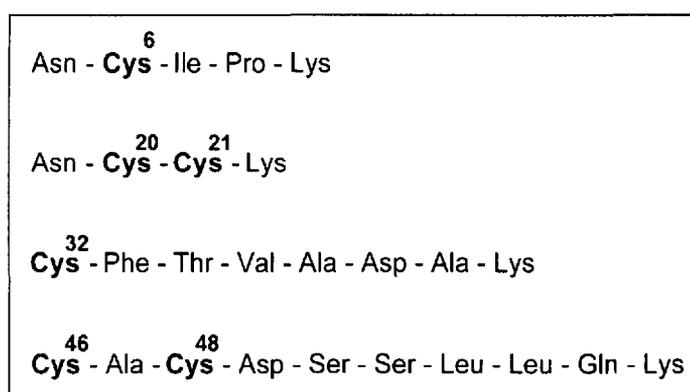


Fig. 1: Amino acid sequences of the four disulphide-linked peptides obtained by degradation of the CSTX-9 polypeptide with immobilized trypsin (fraction 2).

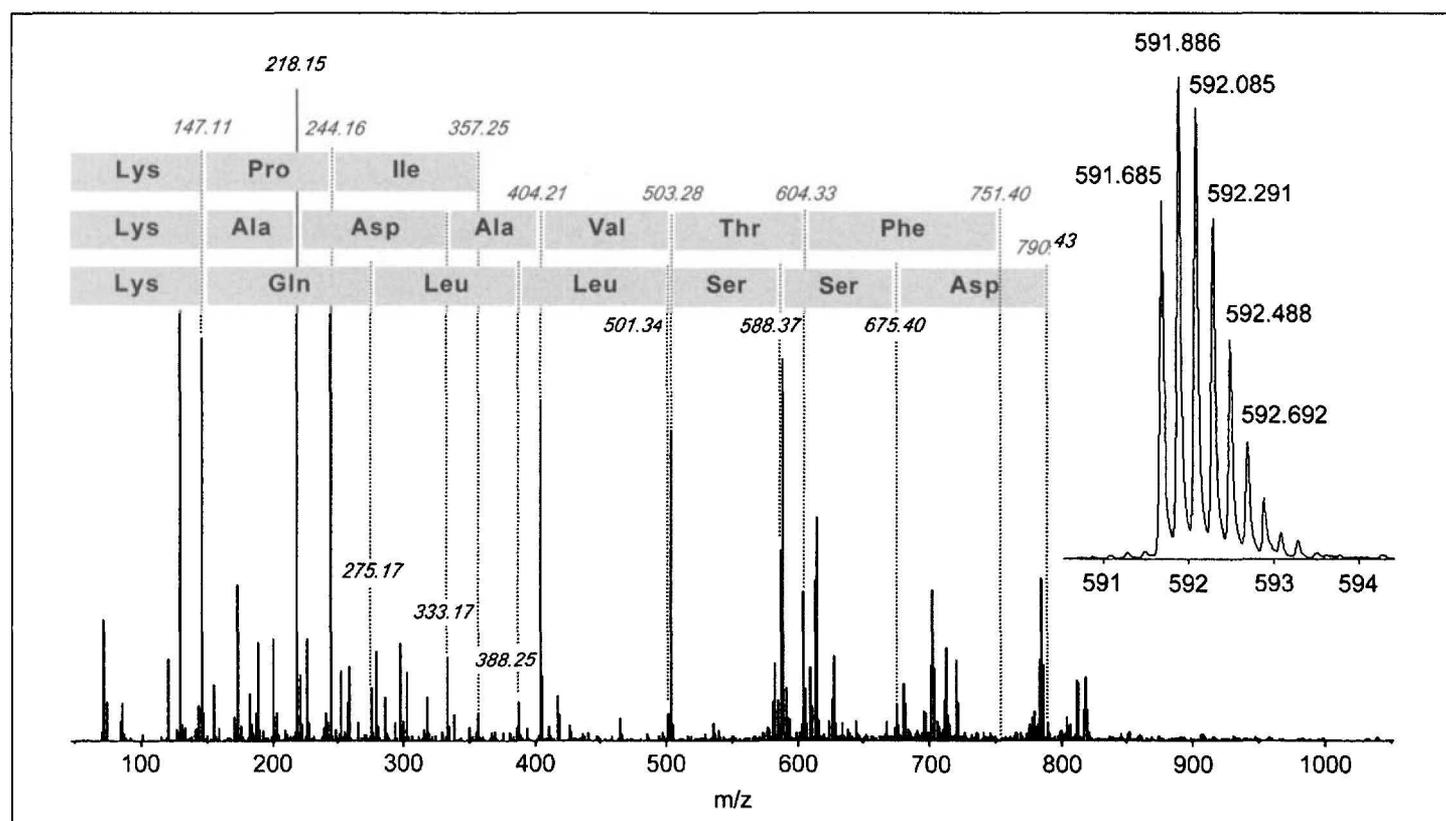


Fig. 2. Product ion spectrum of the disulphide-linked peptides. The inset shows the  $[M+5H]^{5+}$  precursor ion with  $m/z$  591.69, which was subjected to CID in the QqTOF mass spectrometer. The amino acid sequences of the individual peptides are reflected by the abundant  $y^+$ -ions. Experiments were performed on an Applied Biosystems / MDS Sciex QSTAR Pulsar QqTOF mass spectrometer (Sciex, Concord, Canada), equipped with a nano-electrospray ion source (Protana, Odense, Denmark). The HPLC-purified sample was dissolved in 70% methanol / 5% formic acid, resulting in a final concentration of 2.5 pmol/ $\mu$ l. About 2  $\mu$ l of the sample solution were used for analysis. Analysis was performed in the positive-ion mode with a potential of 800 V applied to the nanospray needle. The collision energy was set to 30 eV and nitrogen was used as the collision gas. The time-of-flight analyzer was tuned for an average mass resolving power of 12,000 (FWHM) and calibrated externally prior to analysis. The Applied Biosystems Analyst QS software package was used for data processing.

$m/z$  1026.43. The key fragments distinguishing the remaining two combinations  $Cys_6-Cys_{20}/Cys_{21}-Cys_{48}$  and  $Cys_6-Cys_{21}/Cys_{20}-Cys_{48}$  were generated by cleavage of the N-terminal peptide bond of  $Cys_{21}$ . Detection of a singly and a doubly charged  $y$ -type ion with  $m/z$  821.41 and  $m/z$  411.21 (fragment D), respectively, revealed the presence of a  $Cys_6-Cys_{21}$  disulphide-bridge. This linkage was confirmed by the complementary quadruply protonated  $b$ -type ion with  $m/z$  534.23 (fragment E), and no evidence for the presence of a fragment containing a  $Cys_6-Cys_{20}$  disulphide-bridge was found. Fragment ions exclusively containing the third disulphide-bridge, which by exclusion must be  $Cys_{20}-Cys_{48}$ , were not observed. Each of the two peptides connected by this internal disulphide-bridge is further disulphide-bridged to another peptide, requiring the dissociation of two bonds in order to isolate the single  $Cys_{20}-Cys_{48}$  disulphide-bridge. No evidence showing a different bridge pattern was observed. Thus, disulphide-

scrambling potentially occurring upon sample preparation or analysis can be excluded.

The disulphide-bridge pattern identified by tandem mass spectrometry ( $Cys_6-Cys_{21}$ ,  $Cys_{20}-Cys_{48}$ ,  $Cys_{32}-Cys_{46}$ ) and Edman sequencing ( $Cys_{13}-Cys_{30}$ ) was found to be identical with the inhibitor cystine knot (ICK) structural motif inherent in various ion channel blocking toxins, isolated from the venoms of a number of spiders and cone snails [15–17].

#### 4. Conclusions

The identification of the disulphide-bridge pattern of CSTX-9 demonstrates that tandem mass spectrometry represents a valuable alternative to traditional methods for elucidation of complex molecular structures. The modern high-performance instrumentation greatly increases the potential of this technique, and a wide range of information can be

obtained from a single experiment using minute amounts of sample. Sample preparation is essentially reduced to a purification step, thus avoiding the risk of potential structural alterations (e.g. disulphide-scrambling). However, efficient CID is limited to structures of a few thousand mass units and proteolytic degradation of larger molecules has to be performed prior to analysis.

The results presented here demonstrate that tandem mass spectrometry and Edman sequencing are by no means competing techniques. These two techniques can be considered as complementary tools, each providing particular advantages. Edman sequencing is a straightforward and efficient approach for the determination of amino acid sequences of large polypeptides and proteins, whereas tandem mass spectrometry exhibits its ultimate potential for the elucidation of structural details. If both techniques are available to the researcher, bioanalytical problems can be solved most accurately and efficiently.

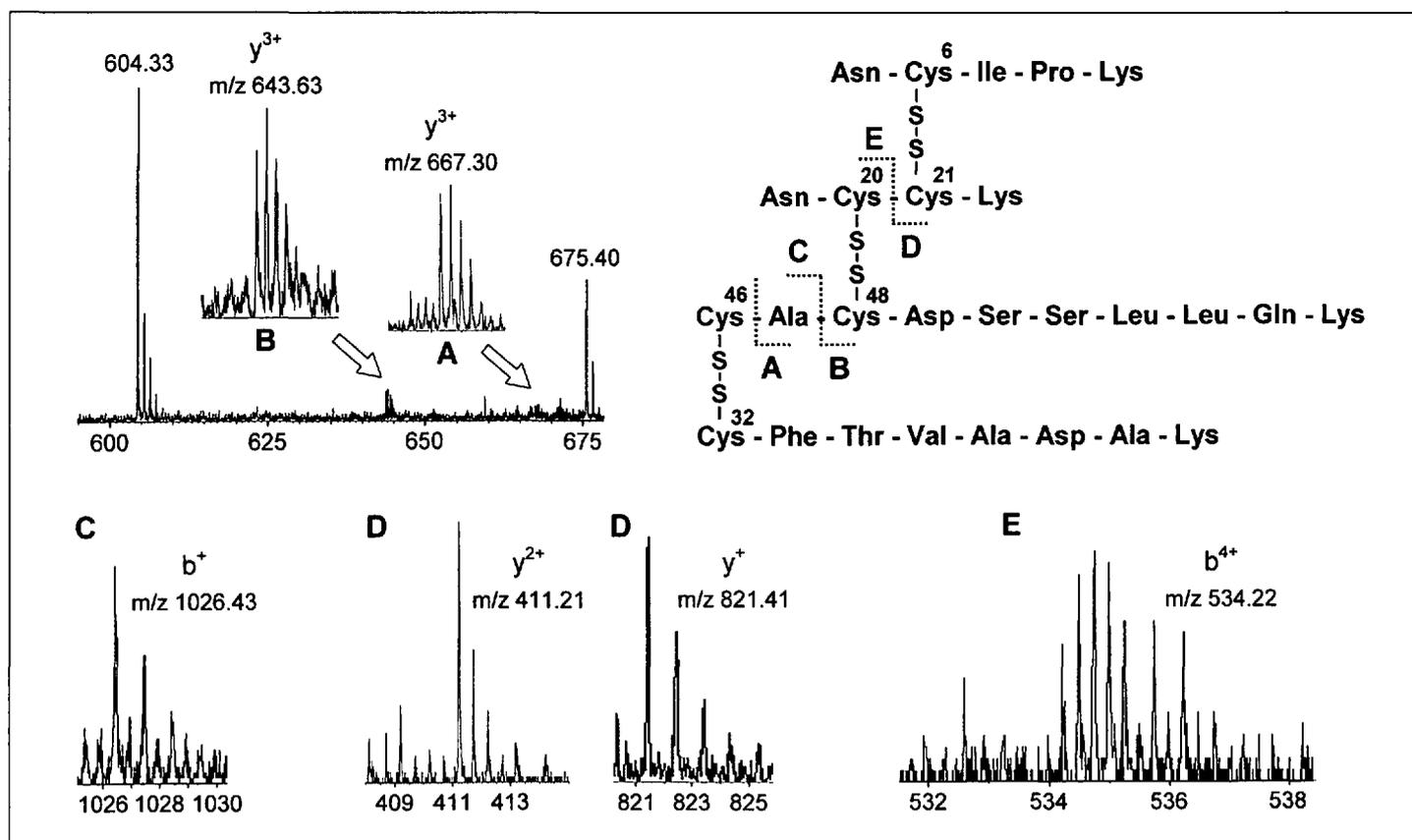


Fig. 3. Disulphide-bridge pattern of the cross-linked peptides. Enlargements show the peaks of characteristic fragment ions generated by cleavage of the peptide bonds adjacent to the bridged cysteines, which the identification of the disulphide-bridge pattern is based on.

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