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Highlights of Analytical Sciences in Switzerland

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Mass Spectrometric Characterization of Disulfide Bridges in Snake Venom Proteins

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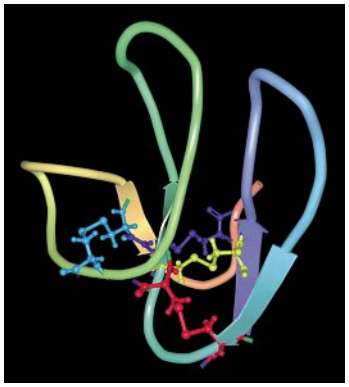
Venoms from snakes, spiders, scorpions *etc.* offer an Aladdin's cave of proteins and peptides for drug discovery. Within the venom of these species are numerous proteins and enzymes designed to kill or immobilise prey and aid digestion, including neurotoxins, cardiotoxins and muscarinic toxins. The individual proteins can be isolated (or produced recombinantly) and used for therapeutic effect.

Disulfide bridges are covalent bonds formed between the thiol groups of two cysteine residues. Many venom proteins contain a proportionally large number of such bridges, which play a fundamental role in defining their structure and specialized functionality. For many proteins, disulfide assignment can be achieved fairly simply by mass spectrometry (MS) analysis of bridged fragments following proteolytic digestion under non-reducing conditions. In venom proteins, elucidation of disulfide connectivities is often much more challenging due to the interwoven arrangement of bridges, which means that a simple proteolytic digest will not cut the protein in such a way that the connectivity can be determined unambiguously. Thus a variety of techniques are required to yield fragments that will reveal the disulfide connectivity.



3D model of the three-finger toxin, mambin. Disulfide bonds are shown in red, yellow, light blue and dark blue. Image of PDB ID: 2LA1 (C.H. Cheng *et al., Protein Sci.* **2012,** *21,* 1872) created using RCSB PDB Protein workshop (L. Moreland *et al., BMC Bioinformatics* **2005**, *6*, 1472).

At HES-SO Valais, recombinant snake venom proteins are being developed for use as new drugs. Part of the analytical strategy is to verify that disulfide bridges are correctly formed. To confirm their number, a high mass accuracy QTOF mass spectrometer is used to observe the mass difference of 1 Da between a disulfide bonded and free cysteine. The presence of disulfide isoforms (proteins with identical sequence but different disulfide connectivities) is determined *via* MS following their separation with RP-(U) HPLC. To assign bridges, proteins are digested using carefully selected combinations of proteolytic enzymes such as trypsin and pepsin to give proteolytic fragments which consist of several peptide chains linked by disulfide bridges. MS/MS with collision-induced dissociation is then used to cleave along the polypeptide backbone to give peptide fragments joined together by single disulfides, from which the bonding pattern can be deduced. This enables us to study challenging proteins containing interwoven disulfides and vicinal cysteines as are often an occurrence in venom proteins. **Verifying disulfide bonding in venom protein-based drugs is a key element in assuring Active Pharmaceutical Ingredient safety and efficacy.**

Artist's representation of mambaglin-1, a pain-relieving peptide from the venom of the black mamba snake. The peptide contains four disulfide bonds in the positions Cys1–3, 2–4, 5–6, and 7–8, as shown by the grey lines. Image reproduced with permission of John Wiley and Sons, from C. J. Craik, C. I. Schroeder, *Angew. Chem. Int. Ed.* **2013**, *52*, 3071.

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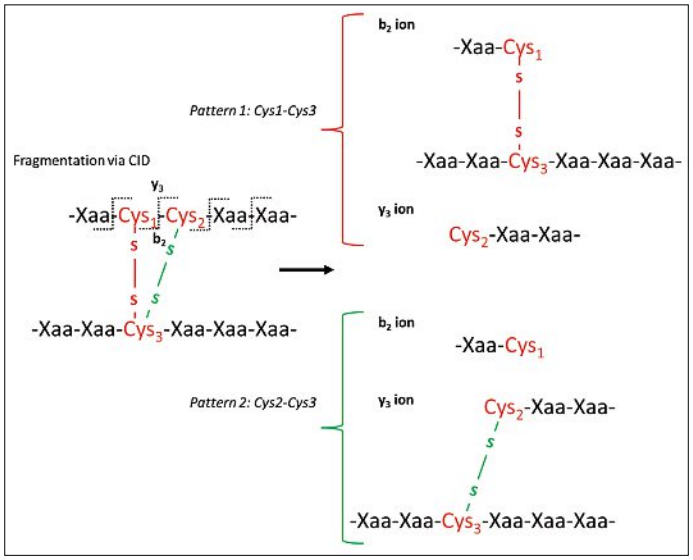

MS/MS disulfide connectivity determination strategy. Xaa represents any amino acid in a hypothetical protein, which has undergone enzymatic digestion to yield disulfide bridged chains with two possible disulfide connectivities: Cys1-Cys3 or Cys2-Cys3. In this case, b2 and y3 ions from fragmentation on the first chain elucidate the connectivity. Reproduced with permission of John Wiley and Sons, from M. S. Goyder, F. Rebeaud, M. E. Pfeifer, F. Kálmán, *Exp. Rev. Proteomics* **2013**, *10*, 489.

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