*Anal. Chem.* **2010,** *82,* 8313–8319

# Disulfide Bond Assignments by Mass Spectrometry of Native Natural Peptides: Cysteine Pairing in Disulfide Bonded Conotoxins

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**The critical, and often most difficult, step in structure elucidation of diverse classes of natural peptides is the determination of correct disulfide pairing between multiple cysteine residues. Here, we present a direct mass spectrometric analytical methodology for the determination of disulfide pairing. Protonated peptides, having multiple disulfide bonds, fragmented under collision induced dissociation (CID) conditions and preferentially cleave along the peptide backbone, with occasional disulfide fragmentation either by $C^{\beta}-S$ bond cleavage through $H^{\alpha}$ abstraction to yield dehydroalanine and cysteinepersulfide, or by S−S bond cleavage through $H^{\beta}$ abstraction to yield the thioaldehyde and cysteine. Further fragmentation of the initial set of product ions ($MS^n$) yields third and fourth generation fragment ions, permitting a distinction between the various possible disulfide bonded structures. This approach is illustrated by establishing cysteine pairing patterns in five conotoxins containing two disulfide bonds. The methodology is extended to the *Conus araneosus* peptides Ar1446 and Ar1430, two 14 residue sequences containing 3 disulfide bonds. A distinction between 15 possible disulfide pairing schemes becomes possible using direct mass spectral fragmentation of the native peptides together with fragmentation of enzymatically nicked peptides.**

Many naturally occurring peptides and proteins contain multiple disulfide bonds. Toxic venoms are rich sources of multiple disulfide bonded peptides, with cone snail venom being a particularly well studied example.[1−6] The determination of the correct disulfide pairing is generally more difficult than the determination of the polypeptide sequence. The number of possible isomers corresponding to different disulfide bonded pairing schemes is 3 for two disulfides, 15 for three disulfides, and 105 for four disulfide bonds. Unambiguous assignment of disulfide bonding patterns is, of course, possible if crystal structures are determined by X-ray diffraction.[7−9] However, this is not always possible because of the limitations in obtaining crystals of many classes of polypeptide toxins. The most widely used method has involved selective reduction and alkylation[10] of multiple disulfide bonded peptides. Careful choice of reducing agents, precise control of alkylation conditions, and the use of multiple reducing agents and various alkylating agents has proved successful in establishing the disulfide connectivity in a wide variety of peptide natural products, including several examples of conotoxins.[11−17] Chemical synthesis strategies using orthogonal protecting groups and stepwise formation of disulfide bonds, under conditions, which minimizes thiol disulfide interchange[18] have proved particularly valuable in confirming disulfide assignments.[19−26] NMR

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10.1021/ac101867e 
Published on Web 09/15/2010

methods, in which $^1H-^1H$ nuclear Overhauser effects (NOEs) are observed across the disulfide bridges, have also been used to determine the pairing between cysteine residues.[27−33] In relatively small polypeptides, which contain two or more disulfide bonds, such NOE assignments may prove misleading. A recent example of the correction of disulfide assignments in the peptide natural products neopetrosiamides, using selective chemical synthesis, is noteworthy.[34] A similar experience has also been documented in the case of hepcidin.[35] Recently, the use of selenocysteines to map the disulfide connectivity by determining the scalar coupling across the diselenide bridge, through $Se^{77}$ NMR spectroscopy, has been proposed to be an useful methodology to get rid of the "blind spot" of the conventional NOE method.[36]

During the course of studies, aimed at determining structures for conotoxins derived from cone snails, native to the coast of India, we were confronted with the problem of developing a relatively rapid method of establishing disulfide pairing schemes in conus peptides. Table S-1 in the Supporting Information provides a summary of the different disulfide pairings schemes in conotoxins, determined through various methods. Earlier studies have established that mass spectrometry in the negative ion mode can provide valuable information on the fragmentation pathway of disulfide bonded peptides.[37−42] Under these conditions, peptide bond fragmentation is inhibited, while cleavage at the disulfide bridge is facilitated by abstraction of the relatively acidic $C^{\alpha}-H$ and $C^{\beta}-H$ protons of cysteine residues. During the course of these investigations, we realized that useful structural information is readily derived upon fragmentation of the disulfide bonded peptide ions under conventional positive ion collision induced dissociation (CID) conditions, as also seen earlier.[43−50] In this report, we describe the establishment of disulfide pairing schemes in two and three disulfide containing peptides derived from cone snail venom.

## EXPERIMENTAL SECTION

**Methods and Materials.** Venom glands were isolated from the *Conus araneosus* and *Conus virgo* collected from the coastal area of India. Crude venoms were extracted from these isolated glands through batch extraction using $H_2O$/acetonitrile (ACN) (0.1% TFA) as the solvent system. Extracted crude venoms were lyophilized and redissolved in 1:1 $H_2O$/ACN (0.1% TFA). Peptide samples were purified from this crude on a RP C18 column coupled with an Agilent 1100 HPLC system, using $H_2O$/ACN (0.1% TFA). A linear gradient typically set from 5% ACN to 95% ACN, over a period of 45 min, was used.

Global reduction and alkylation of the crude *C. araneosus* venom was done by dissolving the crude venom in 100 mM $NH_4CO_3$ and then reducing it with 100 mM DTT solution for 2 h at room temperature followed by 45 min incubation with 50 mM iodoacetamide in the dark. The reduced and alkylated crude venom was then directly spotted onto a matrix-assisted laser desorption ionization (MALDI) target plate with α-cyano-4-hydroxycinnamic acid as the matrix.

TFA hydrolysis of the peptides Ar1446 and Ar1430, present in the same high-peformnace liquid chromatography (HPLC) fraction, was carried out by keeping the peptide in 75% TFA solution at 310 K for 2 days. The reaction mixture then spotted on a MALDI target plate with α-cyano-4-hydroxycinnamic acid as the matrix.

The mass spectrometers used in the present study are Bruker Ultraflex time-of-flight (TOF)/TOF MALDI MS and HCT-Ultra ETDII ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). For all the intact fragmentation and $MS^n$ experiments, purified HPLC fractions were injected directly into the ESI ion trap mass spectrometer using a syringe pump (Cole Parmer, Vernon Hills) at a flow rate of 120 $\mu$L/h, and He was used as the collision gas. The fragmentations were carried out inside the ion trap through the collision of He gas with the ions of interest, which were excited kinetically by an increased resonance amplitude of the dipolar field, with a value typically set between 1 and 3 V.


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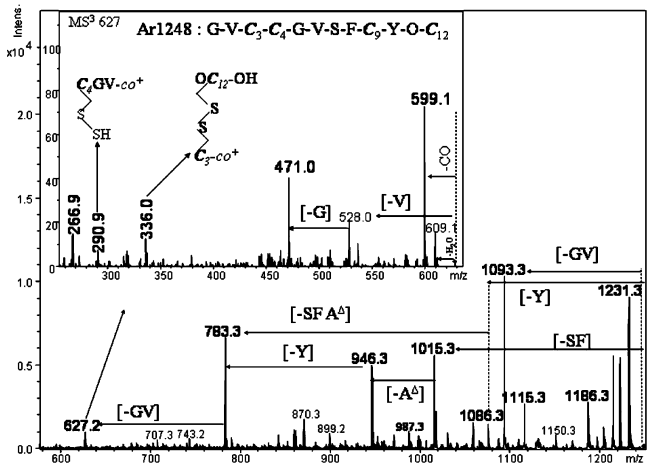


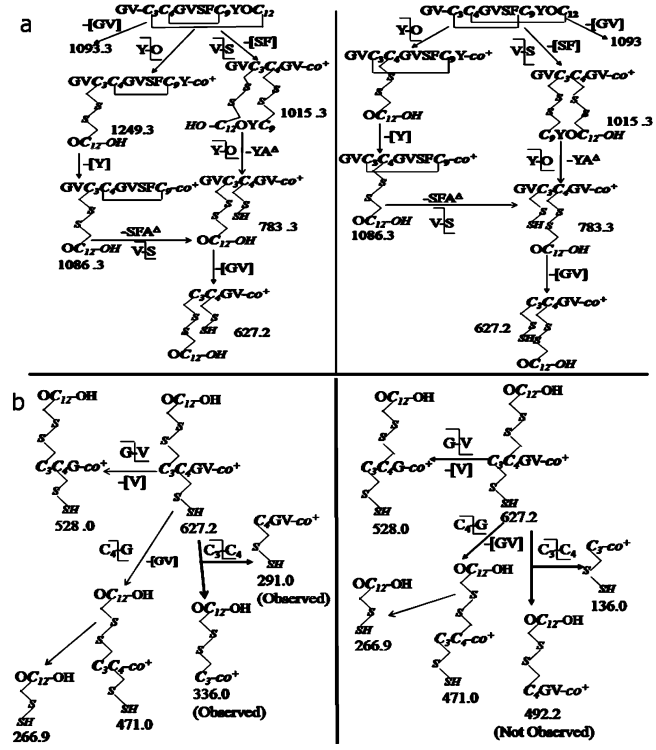

**Figure 1.** Positive ion CID $MS^2$ spectrum of the native disulfide bonded peptide Ar1248. Inset shows the $MS^3$ spectrum of the ion at *m*/*z* 627.2 showing the presence of the diagnostic ions at *m*/*z* 336.0 and 291.0.

## RESULTS AND DISCUSSION

Figure S-1 in the Supporting Information shows the HPLC chromatogram of crude venom extracted from *C. araneosus*. Each HPLC peak is marked with the molecular mass of the major components and the number of disulfide bonds present in that molecule. The MALDI spectra of five *C. araneosus* peptides used in this study are also shown. In the seven peptides listed below, which were chosen for further structural characterization, peptide sequences were readily determined by mass spectrometry under electrospray ionization-mass spectrometry (ESI-MS) conditions using an ion trap for CID/electron transfer dissociation (ETD) fragmentation. *De novo* sequencing can be carried out relatively rapidly for sequences of this size when b and/or y or c and/or z ions are observed across the entire sequence. Several successful examples of sequencing of these classes of molecules have been reported earlier.[51,52] Annotated mass spectral data used for *de novo* sequencing in the conus peptide Ar1248 and Ar1232 are provided as Supporting Information (Figures S-2−S-5). The sequences of peptide Ar1311, Vi1360, Vi1358, Ar1446, and Ar1430 have been previously reported.[17,53,54] We have used a nomenclature in which a two letter abbreviation denotes the conus species (Ar, *Conus araneosus*; Vi, *Conus virgo*), and the number corresponds to the measured mass of the peptide molecule. The determined sequences are given: (1) Ar1248, GVCCGVSFCYOC; (2) Ar1232, GVCCGVSFCYPC; (3) Ar1311, RCCGYKMCHOC; (4) Vi1360, ZCCPTMPECCRI*; (5) Vi1358, ZCCITIPECCRI*; (6) Ar1446, CCRLACGLGCHOCC*; (7) Ar1430, CCRLACGLGCHPCC* (* = amidated C-terminus, Z = pyroglutamic acid, O = hydroxyproline (Hyp)).

**Determination of Disulfide Pairing.** *Ar1248 and Ar1232.* Figure 1 shows the distribution of product ions upon fragmenting

**Scheme 1. (a) Probable Structures of the Product Ions, Observed in the CID $MS^2$ Spectrum of Ar1248, For the Two Possible Disulfide Bonded Isomers and (b) Diagnostic $MS^3$ Fragment Ions for Two Possible Structures of the Ion at *m*/*z* 627.2[a]**




[a] Cysteine residues are shown in bold and italic. The disulfide bridges are shown along with the $\beta$-methylene groups.

the singly charged ion at *m*/*z* 1249.3. Several intense product ions are observable, suggesting that peptide bond cleavage results in the formation of species which retain disulfide bonds. Scheme 1a rationalizes the formation of several product ions invoking preferential cleavages at the $Tyr_{10}$-$Hyp_{11}$ tertiary amide bond and the $Val_6-Ser_7$ secondary amide bond. Inspection of Scheme 1a reveals that both disulfide pairing patterns considered would be consistent with the observed set of product ions. The third possible disulfide pairing scheme $Cys_3-Cys_4$ and $Cys_9-Cys_{12}$ is not considered because of the absence of the b and y fragment ions, which must arise due to cleavages occurring at the GVSF segment and the presence of the ions at *m*/*z* 1015.3 and 783.3 that appear as a consequence of internal loss of [SF] and [$SFA^{\Delta}$] segment, through $Val_6$-$Ser_7$ peptide bond cleavage. The peak at *m*/*z* 1093.3 corresponds to loss of the N-terminus Gly-Val (G-V) dipeptide fragment. The ion at *m*/*z* 1086.3 can arise by cleavage of the $Tyr_{10}$-$Hyp_{11}$ tertiary amide bond, followed by the loss of terminal [Tyr]. The preferential cleavage of the Xxx-Pro/Hyp bond under CID conditions is well established.[55] The other prominent ions at *m*/*z* 1015.3, 783.3, and 627.2 can be rationalized by initial fragmentation of the $Val_6$-$Ser_7$ peptide bond. It may be noted that these observed ions do not provide an unambiguous distinction between the two possible disulfide bonding patterns. The insets to Figure 1 illustrate the product ions formed by further fragmentation ($MS^3$) of the species at *m*/*z* 627.2. Scheme 1b provides a summary of the anticipated cleavages and expected product ions. It should be noted that while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the product ions at $m/z$ 528 and 471, occurring through G−V and $C_4$−G bond cleavages, are the same for both the probable structures, the ions generated through $C_3$−$C_4$ bond cleavage are distinctly different. Importantly, fragmentation of $m/z$ 627.2 yields product ions at $m/z$ 291 and 336, while the ion at $m/z$ 492, diagnostic for the alternative disulfide pairing, is not observed. These results establish the conotoxin Ar1248 has the disulfide pairing scheme $C_3$−$C_{12}$ and $C_4$−$C_9$. The peptide Ar1232 is very closely related in sequence to Ar1248, differing only at position 11 (Pro/Hyp). The corresponding mass spectrometric experiments are summarized in Figures S-6−S-8 in the Supporting Information. In this case, the ion at $m/z$ 611.2, which contains Pro in place of Hyp, is further fragmented to yield the diagnostic ion at $m/z$ 320. Here the $m/z$ 291 ion is very weak in intensity. The $C_3$−$C_{12}$ and $C_4$−$C_9$ disulfide bonding pattern is consistent with these observations. The diagnostic ion at $m/z$ 476 expected for the alternate disulfide pairing is not observed.

*Ar1311.* The distribution of the cysteine residues in this peptide sequence, determined by mass spectrometry, matches that of Ar1248 and Ar1232. In this case, the presence of a trypsin cleavage site, within the sequence, permits direct elimination of the disulfide bond pairing scheme $C_2$−$C_3$ and $C_8$−$C_{11}$. Incubation with trypsin yields the doubly charged species with $m/z$ 587.5, which may be assigned to a product ion in which the N-terminus [Arg] residue is cleaved and the $Lys_6$-$Met_7$ bond is nicked, resulting in an overall decrease of mass by 138 Da, as shown in Figure S-9 in the Supporting Information. Figure S-10 in the Supporting Information shows the distribution of product ions upon fragmenting the $[M + H]^+$ at $m/z$ 1312. As in the preceding cases, the observed ions at $m/z$ 1183.8, 1052.7, 1027.7, and 1020.7 are readily rationalized as shown in Figure S-11 in the Supporting Information. Both possible disulfide pairing patterns are consistent with the observed pattern of product ions. The ion at $m/z$ 974.8 arises by loss of $Hyp_{10}$−$Dha_{11}$ (O−$A^{\Delta}$; dehydroalanine, Dha, $A^{\Delta}$) fragment from a species with anticipated mass as 1175.1, which is of very low intensity in the spectrum. The $m/z$ 974.8 ion corresponds to a structure in which one of the disulfide bridges has been cleaved with the formation of two independent fragments, one of which contains the O−$A^{\Delta}$ residue and the other the Cys−persulfide residue. Figure S-10 in the Supporting Information shows further $MS^3$ fragmentation of the $m/z$ 974.8 ion. The persulfide nature of this ion is further confirmed by loss of 66 Da (− $H_2S_2$) resulting in the formation of the ion at $m/z$ 908.8. The product ion at $m/z$ 818.7 corresponds to loss of the N-terminus [Arg] residue. The ions at $m/z$ 957.8, 929.8, and 896.8 are readily explained by invoking losses of $NH_3$, CO, and the fragmentation of the guanidine side chain of [Arg] from the parent ion. The ion at $m/z$ 846.8 corresponds to loss of a [Lys] residue. This can be formally rationalized by the conversion of the acylium ion structure to a neutral oxazolone and the protonation of the $Lys_6$-$Met_7$ peptide bond, followed by cleavage, within the framework of the intact disulfide bonded ring. Subsequent loss of a [Lys] residue then yields the ion at $m/z$ 846.8. Both disulfide bonding schemes are still indistinguishable at this point. Another round of mass spectrometric fragmentation of the ion at 818.7 provides the product ion distribution shown in the inset of Figure S-10 in the Supporting Information. The observed ion at $m/z$ 683.6 can be rationalized by a loss of the fragment $Cys_2$−S−SH (Cys−persulfide, 135 Da). This is possible only from the ion with the disulfide pairing scheme $C_2$−$C_{11}$ and $C_3$−$C_8$. Subsequent loss of CO yields $m/z$ 655.4. These results support the disulfide pairing scheme $C_2$−$C_{11}$and $C_3$−$C_8$. This pairing scheme is analogous to that established above for Ar1248 and Ar1232, which belongs to the chi/lamda ($\chi/\lambda$) class of conotoxins having a loop within a loop disulfide arrangement.[23,24] A very high sequence similarity of these three peptides with the other $\chi/\lambda$ conotoxins strongly supports the mass spectrometrically determined disulfide connectivity. In order to test our methodology using intact disulfide fragmentation, we turn to two peptides from *C. virgo* (Vi1360 and Vi1358) for which the alternative pairing scheme "interlocked loops" have been previously determined by selective reduction and alkylation.[17]

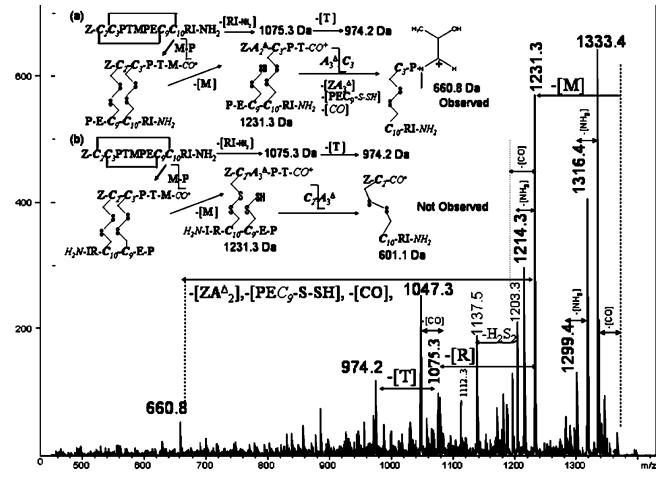



**Figure 2.** Positive ion CID $MS^2$ spectrum of native Vi1360. The scheme shows the probable structures and $m/z$ of the product ions that can arise through the same fragmentation route for the two probable structures. Note that the ion at $m/z$ 660.8, corresponding to the structure (a) is observed, whereas the diagnostic ion for the alternative structure (b) at $m/z$ 601.1 is not observed.

*Vi1360/Vi1358.* Figure 2 shows the product ion distribution obtained upon fragmenting the parent ion at $m/z$ 1361.4. The scheme presented shows that the ion at $m/z$ 1231.3 can be rationalized as arising from the cleavage of one of the two disulfide bridges followed by the cleavage of the $Met_6$−$Pro_7$ peptide bond and subsequent loss of the $Met_6$ residue. This process can be realized from either of the two disulfide pairing schemes. The distinction between the two structures becomes possible by the observation of the ion at $m/z$ 660.8. While, readily explainable cleavages and neutral losses allow the $m/z$ 660.8 species to be obtained from the pairing scheme $C_2$−$C_9$ and $C_3$−$C_{10}$ (interlocked loops), the alternative pairing scheme does not provide a facile interpretation for formation of this ion. Indeed the pairing scheme $C_2$−$C_{10}$ and $C_3$−$C_9$ might be expected to give an ion at $m/z$ 601.8, which is not observed. Thus fragmentation of the intact peptide disulfide yields the "interlocked loops" pairing scheme, fully consistent with the previous study.[17] Similar results were obtained for Vi1358; the data are shown in Figures S-12 and S-13 in the Supporting Information.

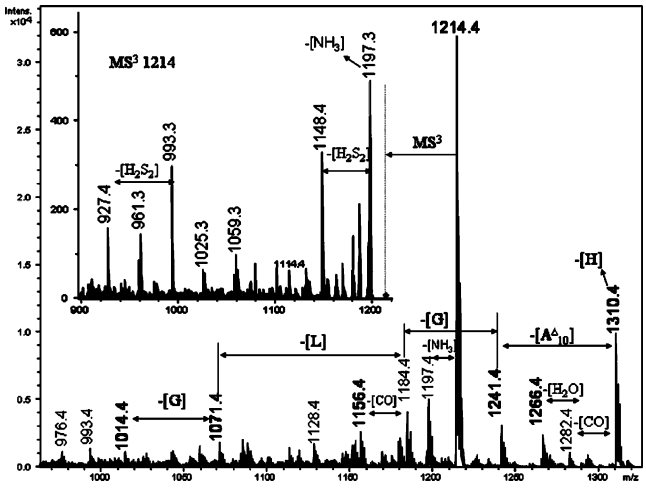
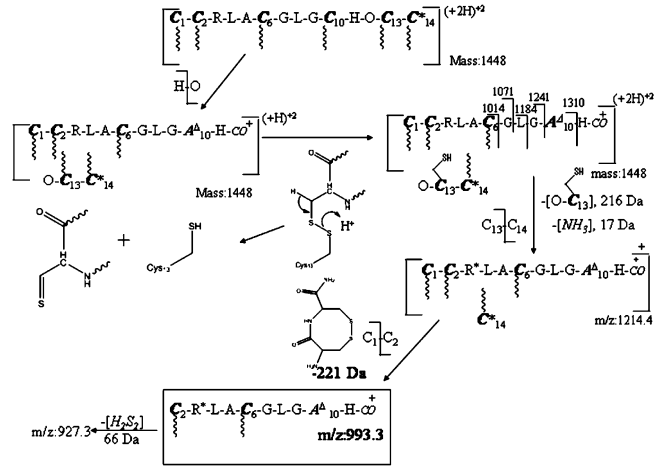



**Figure 3.** Positive ion CID $MS^2$ spectrum of disulfide bonded native Ar1446. Inset shows the CID $MS^3$ spectrum of the ion at *m/z* 1214.4. A characteristic 66 Da ($H_2S_2$) loss from both $MS^3$ parent ion 1214.4 and the corresponding product ion at *m/z* 993.3 strongly suggests the persulfide nature of these ions.

**Scheme 2. Probable Assignments of the Product Ions Observed in Figure 3**[a]




[a] A loss of 221 Da upon further fragmenting the product ion at *m/z* 1214.4, to yield an ion at *m/z* 993.3, readily establishes the $C_1-C_{14}$ connection. Cysteine with the symbol Cys with a squiggly line denotes that either they are disulfide bonded with one of the probable Cys residues or in the form of a cysteine persulfide or dehydroalanine.

*Ar1446 and Ar1430.* In order to test whether the methodology of fragmenting intact disulfides to determine cysteine pairing schemes could be extended to more complex conus peptides, we turn to analysis of two peptides Ar1446 and Ar1430, which are 14 residue peptides containing six cysteines and three disulfide bonds. The primary sequence determination has been previously reported.[54] Figure 3 shows the fragmentation pattern obtained from the parent doubly charged ion at *m/z* 724. The doubly charged species $[M + 2H]^{2+}$ was chosen for fragmentation because of the low intensity of the singly charged molecule $[M + H]^+$. The ESI-MS of Ar1446 shows the following intensity distribution $[M + 2H]^{2+} > [M + 3H]^{3+} > [M + H]^+$, a feature consistent with the presence of two basic residues. It is seen from Figure 3 that the most intense fragment ions are observed at *m/z* 1310.4 and 1214.4. The ion at *m/z* 1310.4 may be assigned to a species in which the ($H^+$ + [Histidine]) residue [− 138 Da] is lost from the parent doubly protonated species, with the nominal mass of 1448 Da. This can arise from selective fragmentation of the $His_{11}-Hyp_{12}$ [$H_{11}-O_{12}$] peptide bond, followed by loss of the [His] residue. The peak at *m/z* 1241.4 arises by the fragmentation of the S−S bond involving $Cys_{10}$, as a result of $\beta$ elimination, which converts this residue to dehydroalanine (Dha, $A^{\Delta}$, residue mass 69 Da), followed by the loss of this residue (Scheme 2). Subsequent cleavages leading to losses of Gly (*m/z* 1184.4), Leu (*m/z* 1071.4), and Gly (*m/z* 1014.4) confirm this assignment. The most intense product ion at *m/z* 1214.4 arises from a loss of 234 Da from the parent doubly charged ion with mass 1448 Da. The singly charged nature of the ion at *m/z* 1214.4 is evident from the isotopic multiplet pattern. Interestingly, fragmentation with the doubly charged ion of peptide Ar1430, which differs from Ar1446 by having a proline (Pro, residue mass 97 Da) at position 11 in place of hydroxyproline (Hyp, residue mass 113 Da), also yields the product ion at *m/z* 1214.4 (Figure S-14 in the Supporting Information). Notably, the product ion at *m/z* 1310.4 in the $MS^2$ spectrum of Ar1446 appears with a mass difference of 16 Da at *m/z* 1294.4 in the $MS^2$ spectrum of Ar1430, followed by a trail of fragmentation products resulting in subsequent losses of $A^{\Delta}_{10}$, Gly, Leu, and Gly. From these results it is clear that the *m/z* 1214.4 species does not contain residue 12(Hyp/Pro). The mass difference of 234 Da may be rationalized by invoking disulfide bond cleavage of $Cys_{13}$, in which abstraction of the $H^{\beta}$ proton from the partner Cys residue results in S−S bond cleavage, forming a Cys residue at position 13 and Cys-thioaldehyde in the position of the partner Cys residue. Subsequent elimination of the $Hyp_{12}-Cys_{13}$ diketopiperazine in its protonated form (217 Da) followed by a loss of $NH_3$ from the side chain of Arg (17 Da) would result in the ion at *m/z* 1214.4 (Scheme 2). The inset of Figure 3 shows a further fragmentation ($MS^3$) of *m/z* 1214.4. While the anticipated neutral losses of $NH_3$ (*m/z* 1197.3) and $H_2S_2$ (*m/z* 1148.4) are observed, the product ion at *m/z* 993.3 is important in disulfide assignment. This species corresponds to a neutral loss of 221 Da from the parent ion at *m/z* 1214.4. As shown in the Scheme 2, this neutral loss can be rationalized by the $C_1-C_2$ peptide bond cleavage that eliminates $Cys_1$ (mass 102 Da) along with its disulfide bonded partner C-terminal amidated $Cys_{14}$ [102 + 16 (amidated C-terminus, $CONH_2$) + 1 (N-terminus hydrogen) = 119 Da], through a cyclic eight-membered peptide disulfide formed between $Cys_1$ and $Cys_{14}$. Such cyclic disulfides have been characterized in both peptides and proteins[56−59] and may be readily formed in the gas phase by intramolecular cyclization, involving peptide bond formation between the liberated amino terminus of $Cys_{14}$ and the carbonyl group of $Cys_1$.[60,61] These results establish that Ar1446 and Ar1430 possess the $Cys_1-Cys_{14}$ disulfide bond.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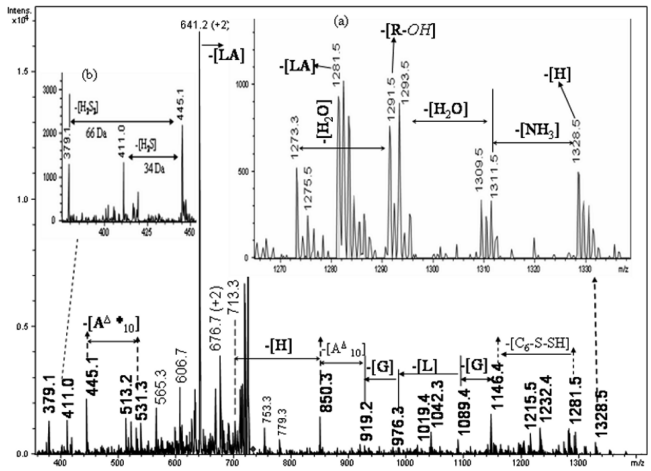
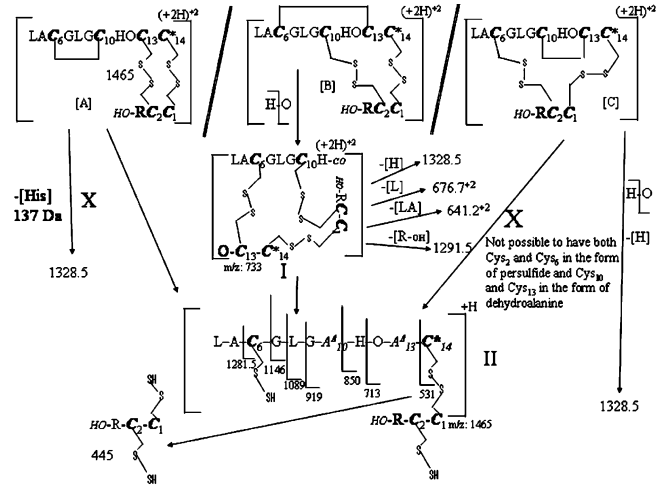


**Figure 4.** Positive ion CID $MS^2$ spectrum of the trypsin nicked disulfide bonded Ar1446 with mass of 1464 Da. Insets shows the region of the spectrum from (a) *m/z* 1266−1338 and (b) *m/z* 375−450.

Three possible pairing schemes may now be considered for the remaining four Cys residues. $C_2-C_6/C_{10}-C_{13}$, $C_2-C_{13}/C_6-C_{10}$, and $C_2-C_{10}/C_6-C_{13}$. In order to distinguish these possibilities, we examine a trypsin nicked species in which the $Arg_3-Leu_4$ peptide bond is cleaved. The product of this cleavage has a mass of 1464 Da ($[M + 2H]^{2+} = 733$). Figure 4 shows the fragmentation of the doubly charged species of *m/z* 733. In the sequence of Ar1446, the $His_{11}-Hyp_{12}$ bond is expected to be the most fragile under CID MS/MS conditions. Indeed, from Figure 3 it is evident that formation of the ion *m/z* 1310.4, from the doubly charged parent ion, corresponds to the cleavage of the $His_{11}-Hyp_{12}$ peptide bond followed by loss of the [His] residue. The observation of the product ion at *m/z* 1328.5, in the $MS^2$ spectrum of the trypsin nicked peptide, is consistent with a loss of [His] residue. This loss of only the $[His]_{11}$ residue from the tryptic product to yield the ion at *m/z* 1328.5 is not feasible from the $Cys_1-Cys_{14}$, $Cys_2-Cys_{13}$, and $Cys_6-Cys_{10}$ disulfide pairing scheme, since $His_{11}-Hyp_{12}$ bond cleavage would be expected to give rise to two smaller peptides. Further support for eliminating $Cys_2-Cys_{13}$ and $Cys_6-Cys_{10}$ as possible disulfide pairs is obtained by subjecting the tryptic cleaved product to a cleavage by trifluoroaceticacid (TFA). Xxx-Pro/Hyp peptide bonds are extremely susceptible to TFA hydrolysis,[62] a feature that is also observed in this case by the presence of a hydrolysis product with a mass of 1482 Da (Figure S-15 in the Supporting Information). If the $Cys_6-Cys_{10}$ disulfide bond is present, then the hydrolysis reaction on the trypsin nicked peptide should yield two product ions with mass 770 and 712 Da.

The spectrum shows the presence of an ion at *m/z* 1483.4 and complete absence of any product ions in the *m/z* region 700−800. Clearly, the absence of the $Cys_6-Cys_{10}$ disulfide link is established. At this point, a distinction needs to be made between two possible pairing schemes, $C_2-C_6/C_{10}-C_{13}$ and $C_2-C_{10}/C_6-C_{13}$. A distinction between these two possibilities can be made by the product ions obtained upon the cleavage of the trypsin nicked product at *m/z* 733 ($[M + 2H]^{2+}$). Scheme 3 rationalizes the observed distribution of product ions. The trypsin nicked product can undergo mass spectral cleavage of the labile $His_{11}-Hyp_{12}$ bond to yield the

**Scheme 3. Anticipated Product Ions from Three Probable Disulfide Bonded Structures of Trypsin Nicked Ar1446 (Mass 1464)**[a]



[a] Note that the ion at *m/z* 1328.5, resulting from the internal loss of [His] from the doubly charged parent ion (*m/z* 733.3), eliminates the possibility of structure A. The presence of Cys−persulfides at positions 6 and 2 and the dehydroalanine residues ($A^{\Delta}$) at positions 10 and 13 differentiate structure B from structure C.

cyclic species **I**. This species yields *m/z* 1465 for $[M + H]^+$ and 733 for $[M + 2H]^{2+}$. Loss of the protonated [His] residues can give rise to a singly charged species with *m/z* 1328.5 (Figure 4). The inset to Figure 4 provides an assignment for the product ions in the region *m/z* 1250−1350. It is clearly seen that the residues which lie external to the trisdisulfide macrocycle are readily lost, i.e., [Arg−OH], [His], and the dipeptide [Leu-Ala]. The most prominent ion that is observed corresponds to *m/z* 641.2, which is a doubly charged species, obtained by loss of the dipeptide [Leu-Ala] fragment from a doubly charged precursor. Loss of only the [Leu] residue yields the doubly charged ion at *m/z* 676.7. Interestingly, inspection of the product ion distribution in Figure 4 reveals several singly charged fragments. These may be rationalized as arising from linear precursors obtained by fragmentation of disulfide bridges involving $Cys_2$, $Cys_6$, $Cys_{10}$, and $Cys_{13}$. This species **II**, shown in Scheme 3, possess dehydroalanine ($A^{\Delta}$) residues at positions 10 and 13 and cysteine persulfide at positions 2 and 6. The product ions observed at *m/z* 531.3, 713.3, 850.3, 919.2, 976.3, 1089.4, and 1146.4 now correspond to the backbone cleavages along the sequence. The ion at *m/z* 445.1 is obtained by cleavage of the $Cys_1-Cys_{14}$ disulfide bridge through $\beta$-elimination from $Cys_{14}$. The doubly charged ion at *m/z* 641.2 and 676.7 can arise from both the precursors **I** and **II**. The presence of cysteine persulfide at positions 2 and 6 and dehydroalanine at positions 10 and 13 can be accommodated only with the disulfide pairing scheme $Cys_2-Cys_{10}$ and $Cys_6-Cys_{13}$. Thus we conclude that Ar1446 has the disulfide bonding scheme $Cys_1-Cys_{14}$, $Cys_2-Cys_{10}$, and $Cys_6-Cys_{13}$. The related peptide Ar1430, which contains proline at position 11, possesses an identical disulfide bonding scheme. This determined disulfide connectivity has also been previously reported in previously known M2 superfamily conotoxins.[14]

## CONCLUSIONS

The examples of two and three disulfide bonded conotoxins presented above establish that mass spectral fragmentation of intact

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disulfides can provide a means of establishing the cysteine pairing schemes. A question that may arise is whether the generation of reactive thiol species can lead to disulfide scrambling in the gas phase. If such scrambling occurs, product ions must be observed which are diagnostic of the presence of dehydroalanine ($A^{\Delta}$), cysteine persulfide (Cys−S−SH), cysteine-thioaldehyde, and cysteine at specific positions along the sequence. It is these residues which are generated by fragmentation of the disulfide bridge by the initial abstraction of the $C^{\alpha}H$ or $C^{\beta}H$ proton followed by the cleavage of the disulfide bond. In the present study, we have not observed key product ions which provide evidence for disulfide scrambling. Under the conditions of CID of positive ions, disulfide cleavage may be facilitated by proximal residues. In the case of peptide Ar1446 and Ar1430, the [His] residue at position 11 may facilitate cleavage of the disulfide of $Cys_{10}$. Interestingly, gas phase cyclization of acylium/protonated oxazolone ions has been shown to facilitate sequence scrambling for protonated peptide ions.[60,61] It is likely that under mass spectrometric conditions, used in present study, thiol disulfide interchange processes are not favored in the gas phase, although such reactions are extremely facile in aqueous solution at alkaline pH.[63−65]

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## ACKNOWLEDGMENT

We thank Prof. K. S. Krishnan for initiating the project on Indian marine snails and for facilitating the collection of marine organisms and conus venoms used in this present study. K.G. acknowledges the Council of Scientific and Industrial Research (CSIR), India, for the award of a senior research fellowship. This research was supported by a grant of the Department of Biotechnology (DBT), Government of India, and the mass spectrometric facility was supported by a program run by the Department of Biotechnology (DBT), Government of India.

## SUPPORTING INFORMATION AVAILABLE

Figures S-1−S-6, S-9, S-10, S-12, S-14, and S-15, mass spectrometric data; Supporting Figures S-7, S-8, S-1,1 and S-13, schemes rationalizing observed product ions; Supporting Table S-1, summary of the disulfide connectivity of different conotoxins obtained by various techniques. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review July 13, 2010. Accepted August 31, 2010.

AC101867E