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Rapid mass spectral identification of contryphans. Detection of characteristic peptide ions by fragmentation of intact disulfide-bonded peptides in crude venom

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The mass spectrometric cleavage of intact disulfide-bonded peptides in conus venom has been investigated. Contryphans containing a single disulfide bond are shown to fragment preferentially at X–Pro bonds, giving rise to linearized, unsymmetrical cystine peptides, which subsequently fragment by multiple pathways at the disulfide bridge. Cleavage at the disulfide bond can be initiated by initial loss of the $C^{\alpha}H$ or $C^{\beta}H$ proton, resulting in distinct product ions, with the subsequent loss of elemental sulfur, H_2S or H_2S_2. Contryphans from *Conus amadis*, *Conus loroisii*, and *Conus striatus* are presented as examples, in which detailed assignment of the product ions resulting from tandem mass spectrometric analysis of the intact disulfide is also accomplished. Characteristic fragments arising from conserved contryphan sequences can be used as diagnostic, permitting rapid identification of this class of peptides in crude venom. The observed fragment ions obtained for contryphans in diverse cone snail species are also compared. Copyright © 2007 John Wiley & Sons, Ltd.

Conus venom contains a mixture of linear and disulfide-bonded peptides. While the widely studied conotoxins contain multiple disulfide linkages, the venom also contains two major classes of peptides possessing a single disulfide bridge, conopressins and contryphans. The conopressins are vasopressin-like sequences,[1] while the contryphans are a unique class of heavily post-translationally modified peptides.[2–10] Contryphan sequences have been shown to contain D-amino acid residues, hydroxyproline, γ-carboxyglutamic acid and bromotryptophan, in addition to amidation at the C-terminus. While their precise biological targets remain to be definitively established, reports of their effects on diverse calcium channels and calcium-activated potassium channels have already appeared.[10,11]

As part of a program to screen conus venom peptide libraries for contryphans, we have developed a rapid mass spectrometric procedure, which is based on fragmentation of the intact disulfide peptides, under conditions of collision-induced dissociation (CID). The traditional approach to characterization of peptide disulfides involves reduction of the S–S bond, followed by alkylation, thus effectively linearizing the sequence. This procedure permits the interpretation of fragment ion spectra in terms of the standard modes of backbone cleavage, which have proved to be extremely effective in the mass spectrometric sequencing of peptides.[12] The gas-phase reactions which result in the further fragmentation of peptide ions containing intact disulfide bridges do not result in readily interpretable product ions. Relatively few studies address the issue of interpreting fragment ions derived from the cleavage of gas-phase ions possessing disulfide bridges. Bilusich and Bowie have reported the use of negative ion electrospray ionization mass spectrometry in identifying 'intermolecular' disulfide bridges in unsymmetrical, acyclic cysteine peptides.[13–16]

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EXPERIMENTAL

Cone snail samples were collected by Prof. K. S. Krishnan and collaborators from the southeastern coast of India. Crude venom samples were obtained by dissecting the venom duct and extracting the total peptide mixtures into alcohol solution. The extracts were subjected to screening by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

Reduction and alkylation of peptide mixtures

An aliquot of crude venom was taken in 20 μL 0.1 M NH_4HCO_3 buffer, pH 8.0. For the reduction, 200 mM stock dithiothreitol (DTT) was added to a final concentration of 8 mM and incubated at 37°C for 2 h. Iodoacetamide (IAM) stock solution was added to obtain a final concentration of 40 mM and the mixture was incubated at room temperature, in the dark, for 90 min. The reaction mixture was analyzed by

MALDI-MS. Peptide ions that correspond to the species with a single disulfide bridge were then chosen for MS/MS analysis. Purified natural contryphan samples were obtained as described previously.[10]

Mass spectrometry

The MALDI-MS data were acquired on an *Ultraflex* TOF/TOF spectrometer (Bruker Daltonics, Billericia, MA, USA and Bremen, Germany), equipped with 50 Hz pulsed nitrogen laser ($\lambda = 337$ nm), operated in positive ion reflectron mode using a 90-ns time delay, and a 25 kV accelerating voltage. The samples were prepared by mixing an equal amount of peptide (0.5 μL) with matrices dihydroxybenzoic acid/α-cyano-4-hydroxycinnamic acid saturated in 0.1% trifluoroacetic acid and acetonitrile (1:1). Masses below 500 *m/z* are not considered due to interference from the matrix.

Tandem mass (MALDI-MS/MS) data were acquired by selecting the precursor mass (±3 Da window), and fragments were generated in post-source decay mode with high laser intensity. An acquisition run was the sum of at least 50 series with 600 total added shots to obtain a high-quality mass spectrum. The data (MALDI-MS and MS/MS) were processed using Ultra Flex data analysis, version 3.2.

RESULTS AND DISCUSSION

Figure 1 shows a MALDI mass spectrum of the crude venom sample from *Conus amadis*. The components with *m/z* >1500 correspond to multiply disulfide-bonded conotoxins. The *m/z* values 1823.8 (unpublished work) and 2766.8 correspond to three disulfide-containing peptides,[17,18] and are not considered in the subsequent analysis, which is restricted to peptides with a single disulfide bond. Peaks detected over a complete mass range, 500–3500 *m/z*, are shown as an inset. Two components with *m/z* values of 919.4 and 976.5 are observed in the mass range anticipated for contryphans. This mass difference suggests that the two peptides may be related by the presence or absence of a Gly residue ($\Delta M = 57$ Da). Reduction and alkylation with IAM results in an increase of mass by 116 Da, thus confirming that both the peaks correspond to singly disulfide-bonded species. It may be noted that the relative intensities of the reduced alkylated contryphans at *m/z* 1092.6 and 1035.6 are considerably different. The former contains a Gly residue at the N-terminus which is absent in the latter. Interestingly, such differences in ionization efficiencies for reduced and alkylated species differing in N-terminal Gly have been also noted in other cases.

The purpose of the present study was to develop a protocol which permits identification of contryphans in crude venom by directly selecting single disulfide-bonded peptide ions and subjecting them to further fragmentation. In order to establish the procedure a contryphan of known sequence, Am 975 ($[M+H]^+ = 976$ Da), was chosen. Figure 2 shows the MALDI-MS/MS spectra obtained for Am 975 (from crude venom), which had been subjected to reduction followed by alkylation with IAM, by selecting the peak at *m/z* 1092 ($M = 975 + 116$, $[M+H]^+ = 1092$ Da). Notably, the fragmentation pattern is dominated by intense peaks corresponding to fragmentation of X–Pro/Hyp bonds. The b_n and y_n ions which permit complete sequence determination are of relatively low intensity in crude venom. An almost identical

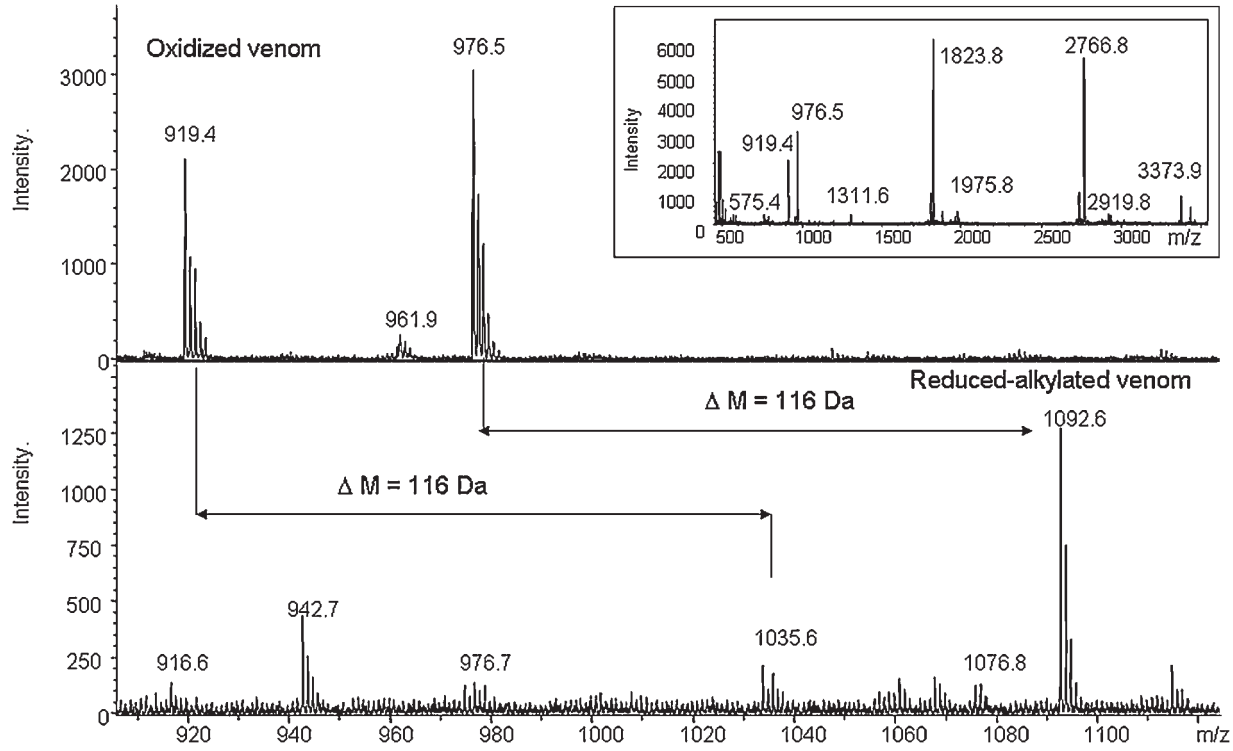

Figure 1. Comparison of oxidized and reduced-alkylated venom of *Conus amadis*. Inset: Crude venom (oxidized) of *Conus amadis* over the extended mass range up to 3500 *m/z*. The high mass peaks correspond to multiple disulfide conotoxins.

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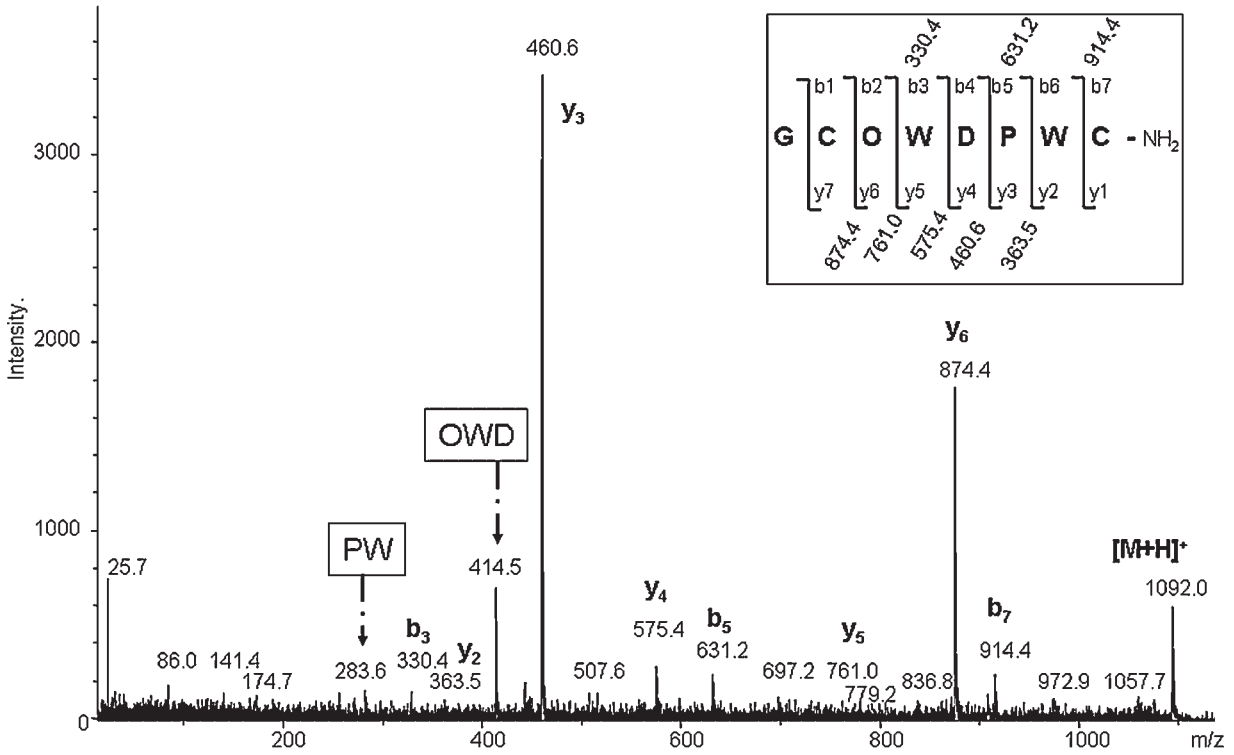

Figure 2. MALDI-MS/MS spectrum of reduced-alkylated Am 976 (*Conus amadis*).

fragmentation pattern on the linearized sequence can also be obtained by using purified Am 975 and selecting the peak at *m/z* 1092 following reduction and alkylation (data not shown).

Figure 3 shows the MALDI-MS/MS spectrum obtained from a purified sample of the intact disulfide-bonded peptide Am 975. An almost identical spectrum is obtained by the fragmentation of the *m/z* 976 ion in crude venom also (data not shown).

Most importantly, in both Figs. 2 and 3, the intense peak at *m/z* 414.5 and 414.6 may be assigned to the internal fragment ion OWD (O = 4-hydroxyproline, Hyp). Interestingly, in

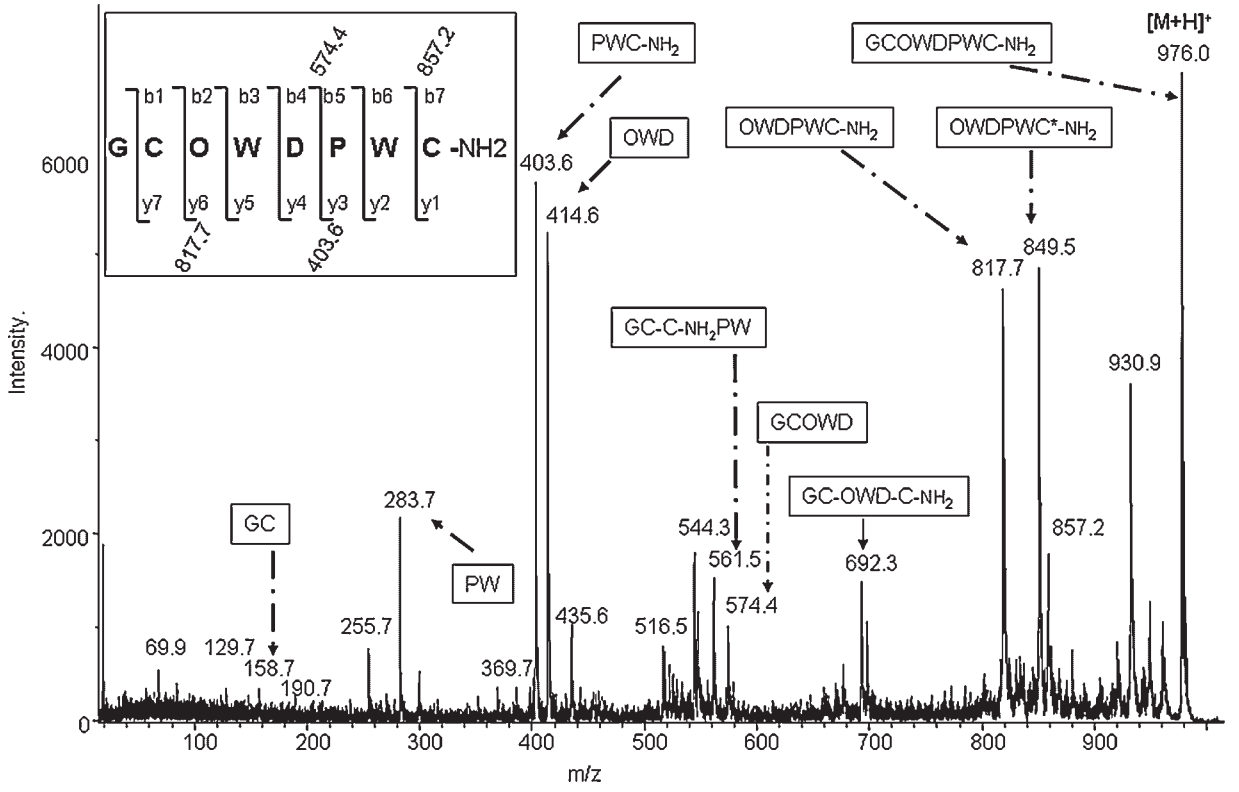

Figure 3. MALDI-MS/MS spectrum of purified, oxidized Am 975 (*Conus amadis*).

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Fig. 3, relatively intense y_6 and y_3 ions are also observed at m/z 817.6 and 403.6, respectively. Further, several y ions of low intensity are also detectable. The observed fragments may be rationalized by considering mechanisms in which the peptide backbone is selectively cleaved, resulting in unsymmetrical, acyclic cystine peptide ions. This species may also be viewed as two independent peptide chains linked in an intermolecular fashion by disulfide bridges. Subsequent fragmentation events involve gas-phase cleavages at the disulfide bridges.

Possible modes of fragmentation are illustrated in Fig. 4. In the peptide Am 975 preferential cleavage of the Cys2–Hyp3 bond results in a species containing two short peptide sequences linked by a disulfide bond. Alternative cleavage patterns which involve breakage of the labile tertiary amide bond between Asp5 and Pro6 are also possible, resulting in a different linearized, unsymmetrical cystine peptide (Fig. 4). Inspection of the MS/MS spectra in Fig. 3 reveals intense peaks at m/z values of 930.9, 849.5, 817.7, 414.6, 403.6 and 283.7. The assignment of the structures of these ions, and the mode by which they are formed, is indicated in Fig. 4. In cystine peptides, several possible modes of fragmentation are, in principle, feasible at the disulfide bridge. Prominent fragmentation pathways will involve the initial loss of the protons at $C^{\alpha}H$ (α-elimination) or $C^{\beta}H$ (β-elimination) at the Cys residue. Since asymmetrical cystine peptides are generated by the initial cleavage of an internal peptide bond, several possible fragment ions may also be anticipated. These are schematically illustrated in Fig. 5. It should be stressed that some of the observed fragment ions can be generated by different pathways from common or distinct precursor ions.

Abstraction of the C^{α} proton followed by cleavage of the C^{β}–S bond can give rise to two pairs of sequences containing dehydroalanine (Dha, ΔA, residue mass 69 Da) and R-S-SH species, which undergoes loss of elemental sulfur giving rise to cysteine (Cys)-containing peptides. Alternatively, H_2S loss by initial abstraction of the C^{β} proton can yield a modified cysteine residue containing a thioaldehyde (–CH=S), with a residue mass of 101 Da.[19,20] Notably, the fragmentation of the disulfide bond considered in Fig. 5 parallels the processes postulated in the degradation of protein disulfide bonds in solution, in the presence of alkali. Interestingly, the formation of elemental sulfur has been postulated during the alkaline digestion of lysozyme, but this finding has not yet been confirmed in other cases.[21]

Further fragmentation of the ions derived by cleavage of the covalent disulfide bridge can occur with the resultant formation of ions corresponding to the conventional a, b, c, x, y, z series of ions derived by backbone cleavages. The intense peak at m/z 849.5 corresponds to a hexapeptide fragment ion $O^{D}WDPWC^{\Delta}$-NH_2, containing the –S-SH moiety. Loss of sulfur yields the $O^{D}WDPWC^{*}$ sequence, with a mass of 817.7 Da (the symbols C^{Δ} and C^{*} are defined in the legend to

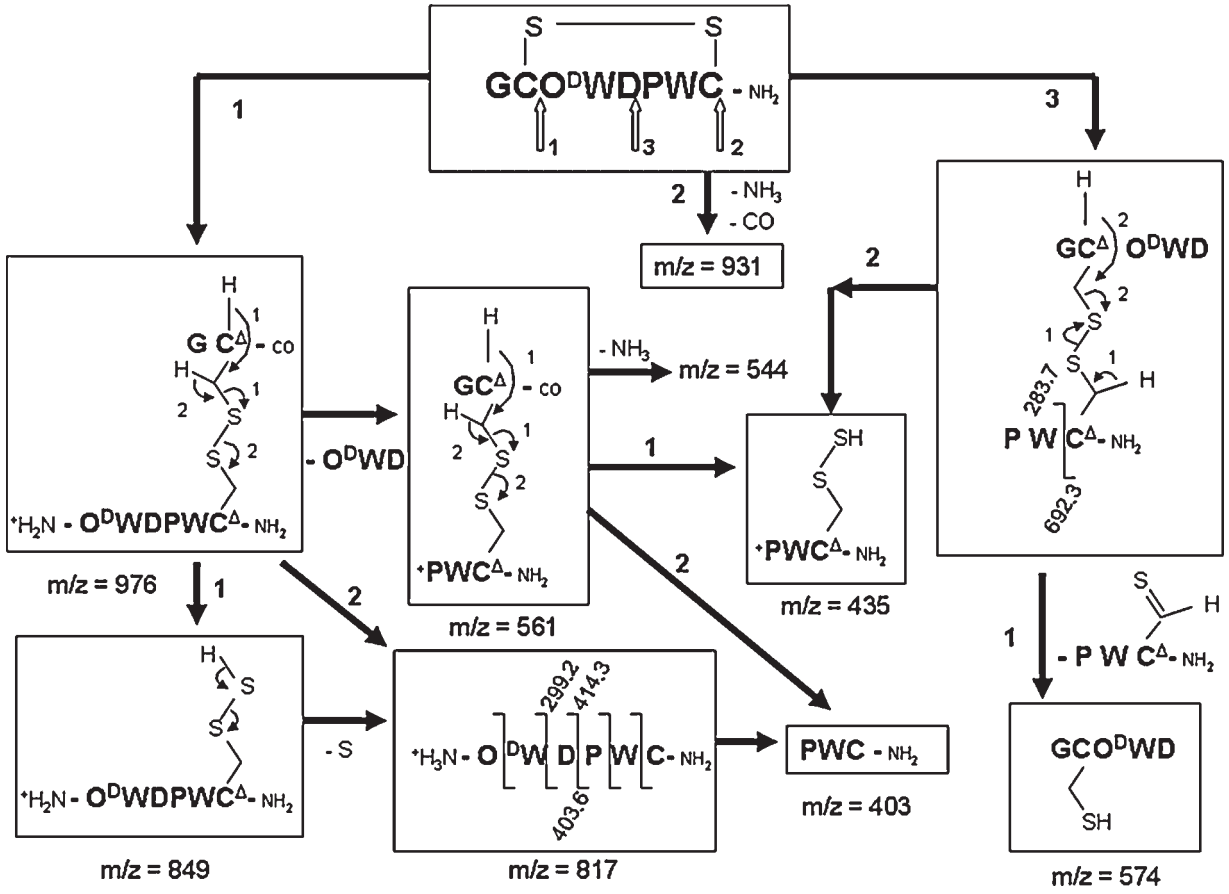

Figure 4. Mechanism of fragmentation of oxidized Am 975 (*Conus amadis*). C^{Δ} is the symbol used to denote the Cys residue bearing the modified side chain either as the persulfide or thioaldehyde.

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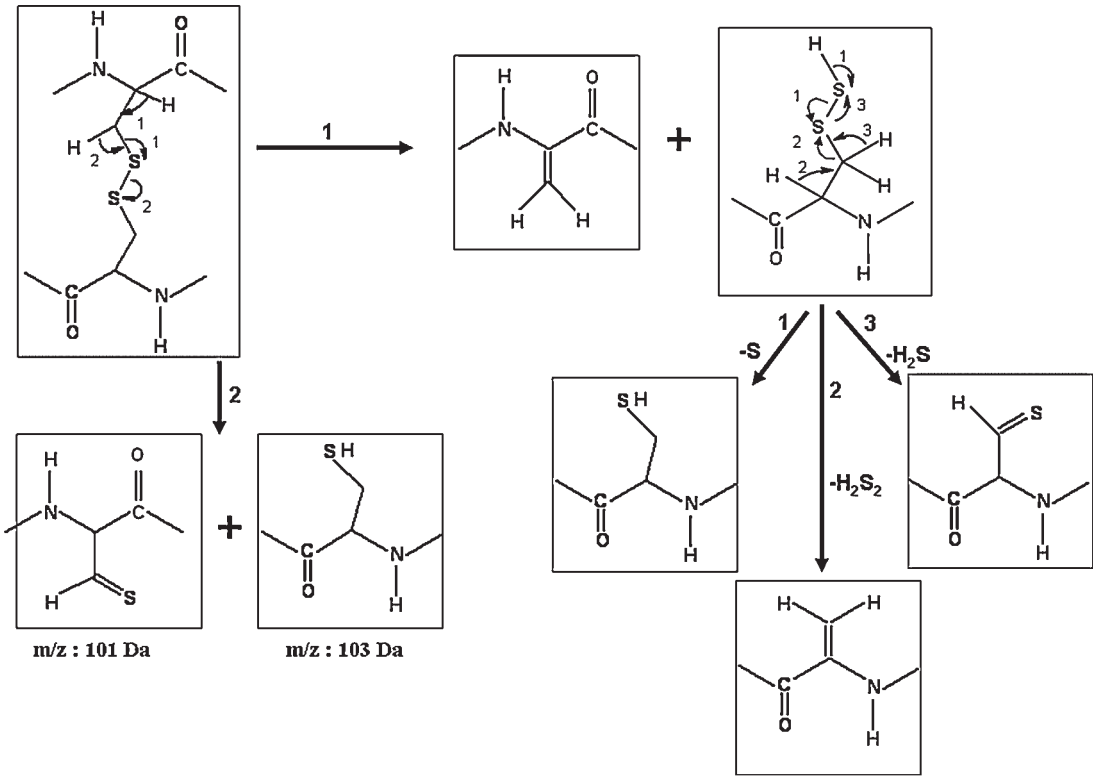

Figure 5. Possible modes of fragmentation at the disulfide bridge.

Table 1. Contryphans observed in different species

Source (*fh*)[a]	Mass	Sequence[b,c]	Diagnostic ions, *m/z*
Conus radiatus[2] (p)	990	GCO^{D}WEPWC*	PW, 284.0; PWC-NH_2, 404.0; PWΔA, 370.1; OWE,429.0; OWEPWC-NH_2, 832.1; GC-C-NH_2PW, 561.8
Conus textile[6] (m)	990	″	
Conus striatus[d] (p)	990	″	
Conus caracteristicus[d] (v)	990	″	
Conus leopardus[d] (v)	990	″	
Conus radiatus[2] (p)	933	CO^{D}WEPWC*	PW, 283.5; PWC-NH_2, 403.5; PWΔA, 369.6; OWE,428.3; OWEPWC-NH_2, 831.9
Conus striatus[d] (p)	933	″	
Conus radiatus[3] (p)	1070	GCO^{D}WEPWC*	
Conus purpurascens[4] (p)	975	GCO^{D}WDPWC*	PW, 283.7, PWC-NH_2, 403.6; PWΔA, 369.7; OWD, 414.6; OWDPWC-NH_2, 817.7; GC-C-NH_2PW, 561.5
Conus amadis[10,d] (m)	975	″	
Conus amadis[d] (m)	918	CO^{D}WDPWC*	PW, 283.8, PWC-NH_2, 403.8; PWΔA, 369.8; OWD, 414.3; OWDPWC-NH_2, 818.
Conus stercumascrun[4] (p)	989	GCO^{D}WQPWC*	
Conus textile[6] (m)	965	GCO^{D}WQPYC*	
Conus loroisii[10,d] (v)	959	GCP^{D}WDPWC*	PW, 283.9; PWC-NH_2, 403.3; PWΔA, 369.9; PWD, 398.8; PWDPWC-NH_2, 802; GC-C-NH_2PW = 561.6
Conus loroisii[d] (v)	902	CP^{D}WDPWC*	PW, 284.1; PWC-NH_2, 404.1; PWΔA, 370.1; PWD, 398.1; PWDPWC-NH_2, 802.5; C-C-NH_2PW, 504.1.
Conus ventricosus[7] (v)	1088	GDCP^{D}WKPWC*	
Conus marmoreus[8] (m)	1454	NγSγCP^{D}WHPWC*	
Conus purpurascens[4] (p)	888	GCV^{D}LLPWC	
Conus textile[6] (m)	880	CV^{D}LYPWC*	PW, 283.9; PWC-NH_2, 403.8; PWΔA, 370.0; VLY, 373; VLYPWC-NH_2, 773.9; C-C-NH_2PW = 504.1
Conus inscriptus[d] (v)	880	″	
Conus inscriptus[9,d] (v)	937	GCV^{D}LYPWC*	PW, 283.9; PWC-NH_2, 403.8; PWΔA, 370.0; VLY, 372.7; VLYPWC-NH_2, 773.7; GC-C-NH_2PW = 561.9
Conus zeylanicus[d] (v)	1187	VVGCO^{D}WQPWC*	PW, 283.8; PWC-NH_2, 403.2; PWΔA, 369.8; OWQ,427.6; OWQPWC-NH_2, 830.4; VVGC-C-NH_2PW, 759.6
Conus betulinus[d] (v)	1187	″	
Conus figulinus[d] (v)	1187	″	

[a] Feeding habit, *fh* (v: vermivorus, p: piscivorus, m: molluscivorus).
[b,] Hydroxyproline, O; amidated C-terminus, *; L-6 bromotryptophan, W; gamma-carboxyglutamic acid, γ.
[c] While the PWC fragment is largely conserved in contryphans, exceptions are the contryphan from *Conus textile*[6] and *Conus zeylanicus* (unpublished work) where W is replaced by Y. In these cases the diagnostic fragment ion PYC is observed at *m/z* 380.
[d] This work.

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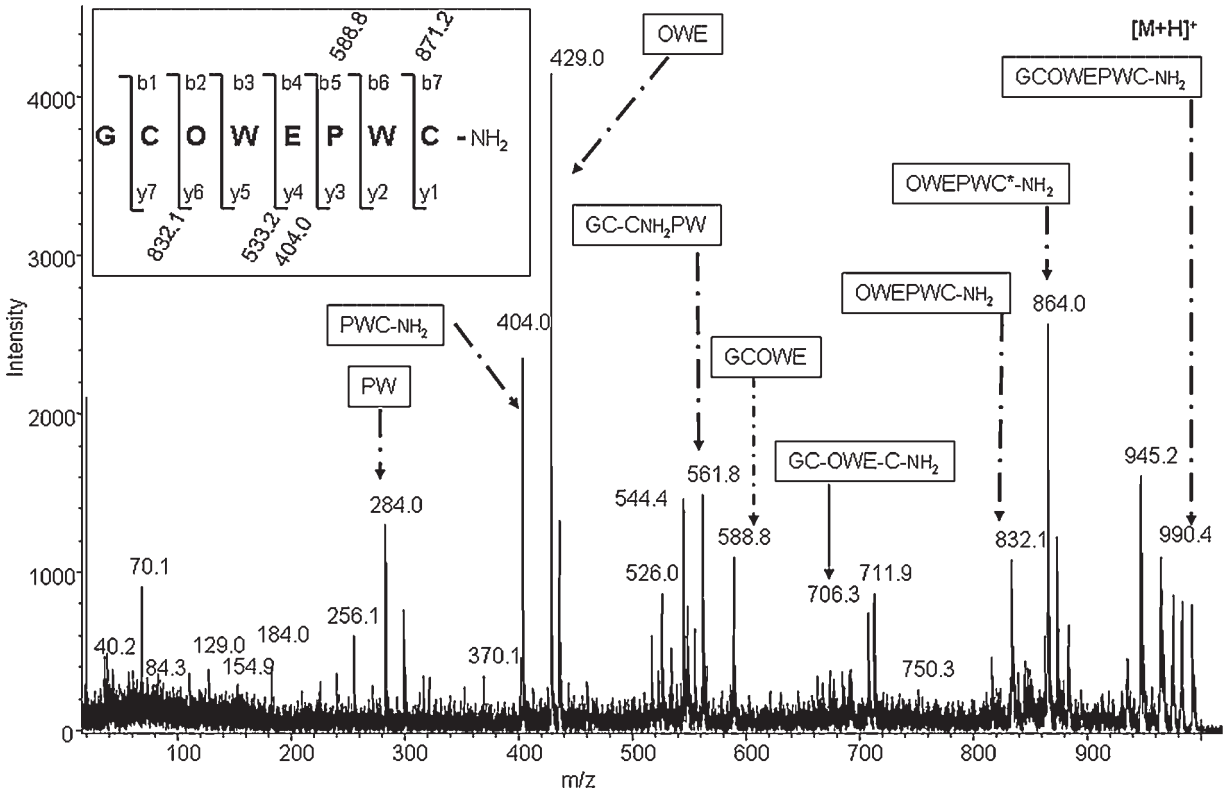

Figure 6. MALDI-MS/MS spectrum of oxidized St 990 from the crude venom of *Conus striatus*.

Fig. 4 and Table 1, footnote, respectively). The intense peaks at *m*/*z* 403.6 (PWC*) and 414.6 (O^{D}WD) formally correspond to the b_3 and y_3 fragment ions derived from the linear hexapeptide with mass 817.7 Da (Fig. 3). The relatively intense peak at *m*/*z* 283.7 is assigned to the dipeptide ion PW, which can be formally considered as a b_2 ion arising from species containing the PWC sequence, which were in turn derived by cleavage of the DP bond in Am 975. The intense

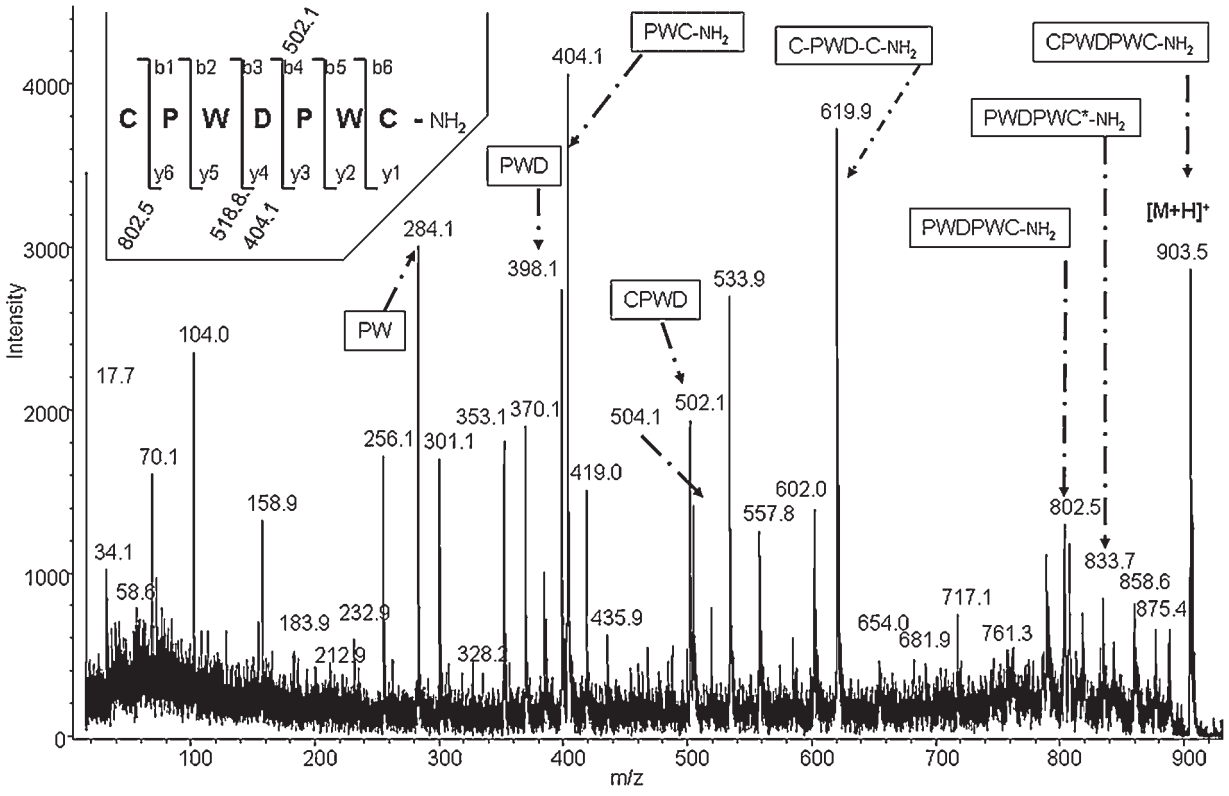

Figure 7. MALDI-MS/MS spectrum of oxidized Lo 902 from the crude venom of *Conus loroisii*.

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peak at *m/z* 931.9 arises from the loss of CO and NH_3 from the parent peptide ion. The assignment of the weak peaks observed at *m/z* 692.3, 574.4, 561.5, 544.3, 435.6 and 369.7 are also indicated. The ion of *m/z* 369.7 arises from the species with a dehydroalanine residue.

Figures 6 and 7 show the representative MS/MS fragmentation patterns obtained for contryphans present in the crude venoms from *Conus striatus* (St 990) and *Conus loroissi* (Lo 902). The PWC fragment which is conserved in a majority of the contryphans is readily identified by peaks at *m/z* 404.0 and 404.1 in both the spectra. The assignments of key ions which permit establishment of the sequence are indicated. In addition, the following product ions merit mention. The peak at *m/z* 533.9 in the spectrum of Lo 902 may be assigned to the C^{Δ}PWD sequence containing the S-SH group. The ion at *m/z* 370.1 corresponds to the sequence PWΔA-NH_2 (ΔA = dehydroalanine). This is a common ion observed in the contryphans. Interestingly, in Lo 902 the relatively prominent ion observed at *m/z* 104 may be assigned to the Cys H^+ species, which is obtained following the preferential cleavage of the Cys–Pro bond. Inspection of the *Conus striatus* St 990 spectrum shows the absence of this peak. Since contryphan sequences often differ by the presence or absence of an N-terminal Gly residue, the peak at *m/z* 104 may serve as a diagnostic feature for the absence of Gly residues. The sequences obtained by the interpretation of intact disulfide MS/MS spectra have been confirmed by MS/MS analysis of the corresponding acyclic, reduced and alkylated peptides.

Table 1 summarizes the fragmentation data obtained for contryphans present in the crude venom of diverse species. An interesting feature of the contryphans is that the same peptide is found in different cone snail species. For example, the contryphan with the sequence $GCO^{D}WEPWC^{*}$ (990 Da) has been identified in as many as five different species, encompassing piscivorus, molluscivorus and vermivorus snails. The biological implication of the conservation of contryphan sequences is not clear. The results of the present study provide an interpretation of fragment ions observed upon mass spectral fragmentation of contryphan ions obtained during MALDI-MS analysis of crude venom samples.

Rapid assignment of sequence becomes possible if three to four key fragment ions are identified. The presence of multiple proline residues in the contryphan sequences facilitates the preferential cleavage at X–Pro bonds, giving rise to linearized sequences which then fragment in multiple ways at the disulfide bridge. For cyclic peptide disulfides which do not possess internal proline residues, the fragmentation patterns may be more difficult to interpret with fewer intense ions being observed.

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