JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2008; **43**: 791–805
Published online 20 February 2008 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/jms.1377

JMS

Probing peptide libraries from *Conus achatinus* using mass spectrometry and cDNA sequencing: identification of δ and ω-conotoxins

Konkallu Hanumae Gowd,[1] Kalyan Kumar Dewan,[2] Prathima Iengar,[2] Kozhalmannom S. Krishnan[1,3] and Padmanabhan Balaram[2]*

[1] Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai 400005, India
[2] Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India
[3] National Centre for Biological Sciences, Bangalore 560065, India

Received 28 September 2007; Accepted 7 December 2007

The peptide library present in the venom of the piscivorous marine snail *Conus achatinus* has been probed using a combination of mass spectrometry and cDNA sequencing methods. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis, before and following global reduction/alkylation of peptide mixtures, permits the rapid classification of individual components on the basis of the number of disulfide bonds. Mass fingerprinting and the reverse phase HPLC retention times permit a further deconvolution of the library in terms of peptide size and hydrophobicity. Sequencing of cDNA derived using O-superfamily specific primers yielded five complete conotoxin precursor sequences, ranging in polypeptide length from 75–87 residues containing six Cys residues at the *C*-terminus. Sequence analysis permits classification of the five putative mature peptides (Ac 6.1 to Ac 6.5) as δ, ω, and ω-like conotoxins. The presence of these predicted peptides in crude venom was established by direct matrix assisted laser desorption ionization tandem mass spectrometry (MALDI-MS/MS) sequencing following trypsin digestion of the peptide mixture after global reduction/alkylation. The determination of partial peptide sequences and comparison with the predicted sequences resulted in the identification of four of the five predicted conotoxins. The characterization of posttranslationally modified analogs, which are hydroxylated at proline or amidated at the *C*-terminus is also demonstrated. Crude venom analysis should prove powerful in studying both inter- and intra-species variation in peptide libraries.

KEYWORDS: Conus peptide library; MALDI-MS; MALDI-MS/MS; cDNA library; RT-PCR

INTRODUCTION

Mass spectrometry offers a powerful and rapid way of probing the composition of peptide mixtures. While electrospray ionization methods generally require on-line liquid chromatographic separation, MALDI methods are robust when directly applied to a complex mixture.[1–4] In this report we demonstrate the utility of MALDI analysis in obtaining structural information directly from crude conus venom and in identifying peptide sequences deduced from sequencing of cDNA generated using superfamily specific primers. Mass spectrometric analysis of crude venom samples, together with chemical modification, provides a rapid approach to profiling heterogeneous mixtures of natural peptides.[5–10] Cone snail venom is a rich source of pharmacologically active peptides composed of both acyclic and disulfide bonded peptides. Conotoxins, a group of multiple disulfide-bonded peptides have been shown to be structurally diverse, with considerable affinity for a wide range of ion channel targets in the central nervous system.[11–15] Marine snails of genus *Conus* use these toxins to capture prey. The large number of cone snail species worldwide together with their antiquity and long evolutionary history suggest that their venom contains a rich diversity of biologically active peptides. As a part of a program to characterize the components of venom derived from marine snails found off the coast of India,[16–20] we have investigated the piscivorous snail *Conus achatinus*.[21,22] Relatively few piscivores have been reported from the Indian coast.

Two independent approaches have been used in analyzing the venom peptide libraries of *C. achatinus*. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) has been used to derive compositional information directly from crude venom, while cDNA library screening for specific families provides DNA sequences corresponding to conotoxin precursors. Crude venom samples have been probed by MALDI-MS using global reduction/alkylation procedures to identify multiple disulfide-bonded peptides. The presence of a large number of three

*Correspondence to: Padmanabhan Balaram, Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India.
E-mail: pb@mbu.iisc.ernet.in

disulfide-containing peptides has been established in the crude venom of *C. achatinus*. A cDNA library was constructed using primers designed against a highly conserved signal sequence of the O-superfamily,[23–27] which includes three well-characterized disulfide-containing conopeptides. The full-length cDNAs of five new O-superfamily conotoxins were cloned and sequenced using reverse transcriptase polymerase chain reaction (RT-PCR) and 3′-RACE (rapid amplification of cDNA ends). The sequences of putative mature toxins have been inferred by identifying potential sites of proteolytic cleavage. The MALDI-MS spectra of crude venom have been carefully examined for the presence of peptides whose masses match with that computed for putative sequences derived from cDNA. Hypothetical posttranslational modifications have also been factored into the search for mature peptides. A strategy to carry out global tryptic digestion of venom libraries has been developed in order to use *de novo* sequence determination of proteolytic peptides as a probe for the presence of mature toxins. Sequencing of proteolytic fragments by matrix assisted laser desorption ionization tandem mass spectrometry (MALDI-MS/MS) procedures permits the identification of venom components, which correspond to the peptide sequences derived from translating cDNA sequences. Identification of three putative δ-conotoxins and two ω-conotoxins are reported from cDNA analysis. Four of these are also shown to be expressed in the venom. The present study illustrates the potential of combined application of cDNA sequencing and MALDI-MS/MS in directly identifying peptide components of complex natural mixtures.

MATERIALS AND METHODS

Chemicals and reagents

Dithiothreitol (DTT), iodoacetamide, trifluroacetic acid (TFA), and dihydroxy-benzoic acid (DHB) were purchased from Sigma Chemicals Co. Acetonitrile and ammonium bicarbonate were from Ranbaxy and Nice Chemicals, respectively. Water was of Milli-Q grade. Sequencing grade trypsin was obtained from Promega. TLCK-treated chymotrypsin was from Sigma.

Sample collection

C. achatinus is a piscivorous cone snail found off the coast of Mumbai, Maharashtra, India. Snails were transported live in seawater to the laboratory and venom ducts were dissected along with the bulb. Venom duct was homogenized using 50% acetonitrile/water as solvent and the resulting mixture was subjected for centrifugation using Beckman centrifuge at a speed of 10 000 rpm. The clear supernatant containing the extracted peptide library was used for subsequent mass spectrometric analysis. A venom duct along with the bulb was frozen in liquid nitrogen and stored at −80 °C, which was further used for cDNA library construction.

Global reduction and alkylation

The venom was dissolved in 30 ml of 50 mM ammonium bicarbonate buffer (pH 8.0), 20 µl of DTT (125 mM) was added and incubated at 37 °C for 3 h. A measure of 20 µl of iodoacetamide (350 mM) in 50 mM ammonium bicarbonate was added and incubated at 37 °C in darkness for 1 h. The resulting mixture was subsequently analyzed by MALDI-MS. The mass spectrum of the reduced/alkylated crude venom was compared with the crude venom MS and peaks shifting by 116, 232, and 348 Da were assigned as one, two, and three disulfide containing peptides (mass matches ±1 Da), respectively. Peptides that do not shift in mass and yield a good distribution of fragment ions in MS/MS spectra are classified as linear sequences.

HPLC purification

Crude venom was concentrated by lyophilization and the peptide components fractionated over a C_{18} column (4.6 × 150 mm, 3.5 µm particle size) using ACN/H_2O/0.1% TFA on a HP 1100 series HPLC system. The flow rate was maintained at 0.5 ml min^{-1} following a linear gradient of 10–95% ACN over 85 min and the fractions were detected at 226 nm.

cDNA library construction and gene sequencing

Total RNA was extracted using the phenol-isothiocyanate method of Chomczynski and Sacchi.[28] cDNA was prepared from the total RNA with an oligo dT (18) primer and MuMLV reverse transcriptase (Fermentas) using a buffer provided by the manufacturer. For targeting the O-superfamily fraction of conotoxins in the cDNA, primers directed against the first five amino acids (following methionine) of the highly conserved signal sequence of this superfamily of conotoxins were designed using published sequences as a guide for the codon bias in *Conus*. A few degeneracies (shown below in bold face) were introduced where variations in amino acids were encountered and a 5′ Hind III restriction site (underlined) was introduced to facilitate subsequent subcloning. The primers designed to target O-superfamily conotoxins were the following: (a) forward: 5′ ATA AAG CTT AAA CTG ACG TG**Y** **R**TG **R**TG ATC G 3′; (b) reverse 5′ GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT 3′. As much as 2 µl of cDNA was used to seed a PCR using Taq polymerase (Bangalore Genei) in a 50 µl reaction (10 mM, Tris. Hcl, pH 9.0, 1.5 mM $MgCl_2$, 0.01% gelatin, 10 pmol of each primer, 0.8 mM dNTPs) and with temperature cycling parameters: denaturation 94 °C – 30 s, annealing 58 °C – 30 s, and extension 72 °C – 30 s. Final extension time at 72 °C was for 1 min. PCR amplification products, appearing at around the 500 bp region, were gel purified and ligated into a TA cloning vector (Bangalore Genei). The ligation mix was used to transform *Escherichia coli* JM109 cells and resulting recombinants were identified using blue white screening. Plasmids prepared from the white colonies were analyzed by using NcoI restriction sites flanking the insert on the vector. A subset of insert bearing plasmids were then sequenced using an automated ABI Prism sequencer (Model 3730).

Sequence alignment and classification

Sequences derived from the plasmids were subjected to a search for open reading frames using an ORF search program (Biology Workbench, Computational Biology Group, NCSA,

DOI: 10.1002/jms

University of Illinois; *http://workbench.sdsc.edu*); the results in each case were inspected for the occurrence of the signature leader sequence of the O-superfamily conotoxins, MKLTV. To classify the conotoxin sequences, the programs BLAST, PSI-BLAST, and ClustalW[29–31] were used from the National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI) websites. Precursors as well as putative, mature conotoxin sequences were used as queries to carry out BLAST and PSI-BLAST searches. The UniProt database[32] was used in the searches.

MALDI–MS and screening for putative conotoxins in crude venom

The profiling of the crude venom from *C. achatinus* at individual level (a venom duct) has been achieved using MALDI-MS. MALDI spectra were collected using a Bruker Daltonics, *Ultraflex TOF/TOF* system, in reflectron positive ion mode, equipped with a nitrogen laser ($\lambda = 337$ nm). Mass spectra were acquired by averaging 10–250 unselected laser shots using a standard peptide mixture (consists of five components over the range of 1–3.1 kDa) for external calibration. The matrix used for positive ion mode detection was DHB in 50% acetonitrile containing 0.1% TFA. The Sample was prepared on a stainless steel target plate using the dried droplet technique.[33] For this, an aliquot of the sample solution (1 µl) and an equal aliquot of the matrix solution were mixed on the target and dried at room temperature in a gentle stream of air. In certain cases (if the droplet was not dried properly) the quality of the droplet was improved by carrying out an additional washing step using 0.1% TFA in water. A delayed extraction parameter was set to focus a range of m/z values (0.5–5.0 kDa), and the mass detector was used to reject ion signals below m/z 500. A measure of 10 µl of crude venom was incubated with 10 µl of 100 mM citric acid for 1 h and subjected to MS to reduce the contribution of sodium and potassium adducts. Clusters of closely related peaks separated by 16 Da correspond to hydroxylation of proline in the cDNA derived sequences. The putative sequences containing *C*-terminal glycine were assumed to undergo posttranslational modification to give the *C*-terminal amide,[34,35] with a corresponding difference between the observed and calculated masses ($\Delta M = 58$ Da, $-CO-NH-CH_2-COOH \rightarrow -CO-NH_2$).

Global trypsin digestion and MALDI-MS/MS

Global trypsin digestion of the reduced/alkylated crude venom was carried using sequencing grade trypsin at a concentration of 25 ng/µl of trypsin in 50 mM ammonium bicarbonate solution. A measure of 3 µl of trypsin was added to 30 µl of reduced/alkylated crude venom and the pH was adjusted to 8.0 using 100 mM NH_4HCO_3. The reaction was incubated at 37 °C and monitored at regular time intervals of 10 min over a period of 3 h using MALDI-MS. The observed tryptic fragments were compared with *in silico* trypsin fragments of the mature conotoxins predicted from the cDNA sequence. Theoretical tryptic digestion and masses of the resulting tryptic fragments were calculated manually and were also verified using PAWS software (ProteoMetrics). The tryptic fragments were further subjected to MALDI-MS/MS using the LIFT option of Bruker Daltonics, and fragmentation was induced by selecting a precursor ion of a particular m/z value (±5 Da). The precursor ion isolation and adequate fragmentation of particular tryptic fragments were achieved at different time intervals. Fragment ion assignments were carried out using the methodology developed by *Biemann*.[36]

Proteolytic digestion and MALDI-MS/MS

The purified HPLC fractions were subjected to reduction using DTT and alkylation by iodoacetamide. Reduced/alkylated peptide samples were passed through a Zip-tip (C18, Millipore) according to the protocol provided by the manufacturer. The purified samples were digested with sequencing grade trypsin or TLCK-treated chymotrypsin (Sigma). The concentration of the enzyme was 25 ng/µl and the reaction was incubated at 37 °C for 3 h. The digest was directly analyzed by MALDI-MS and proteolytic fragments were further subjected to MS/MS. Proteolytic fragments containing hydroxyproline can be easily inferred from mass difference. Further confirmation of the position of Hyp in the sequence is achieved by *de novo* sequencing.

RESULTS

The MALDI-MS spectrum of crude venom isolated from the tubular duct of *C. achatinus* (Fig. 1(a), upper panel) is indicative of the presence of several peptide components in the mass range of 1000–6000 Da. Disulfide bond reduction followed by subsequent alkylation with iodoacetamide was carried out on the crude venom. There is a mass increase of 116 Da for each disulfide bond present in the peptide. The presence of several peptides possessing three disulfide bonds ($\Delta M = 58 \times 6 = 348$ Da) is evident in Fig. 1(b) (right panel). Further analysis of the peptide library was carried out by determination of the molecular mass of venom components subjected to RP-HPLC (Fig. 2(a)). *C. achatinus* venom contains approximately 30 readily identifiable peptides. Figure 2(b) provides a summary of the deconvolution of the peptide library in terms of molecular mass, number of disulfide bonds, and peptide hydrophobicity, as reflected in RP-HPLC retention times. It is clear that *C. achatinus* venom is relatively rich in peptides containing three disulfide bridges. Previous reports on conotoxins suggest that three disulfide-bonded peptides belong to the O, M, A, and P superfamilies. Interestingly, the prominent MALDI peaks fell predominantly into two categories, three disulfide-bonded peptides and linear peptides lacking disulfides. Notably, five peptides containing three disulfide bonds were available for characterization after HPLC fractionation. These peptides fall into three classes based upon their masses and hydrophobicities (Fig. 2(b)).

In an independent approach, we probed a *C. achatinus* cDNA library with primers derived against O-superfamily conotoxins. Figure 3 lists five precursor polypeptide chain sequences of conotoxins derived from the corresponding cDNA sequences. The open reading frames correspond to polypeptides of length 75–87 residues. In all cases, six Cys residues lie toward the *C*-terminal end of the precursor polypeptide chain. Mature conotoxin sequences

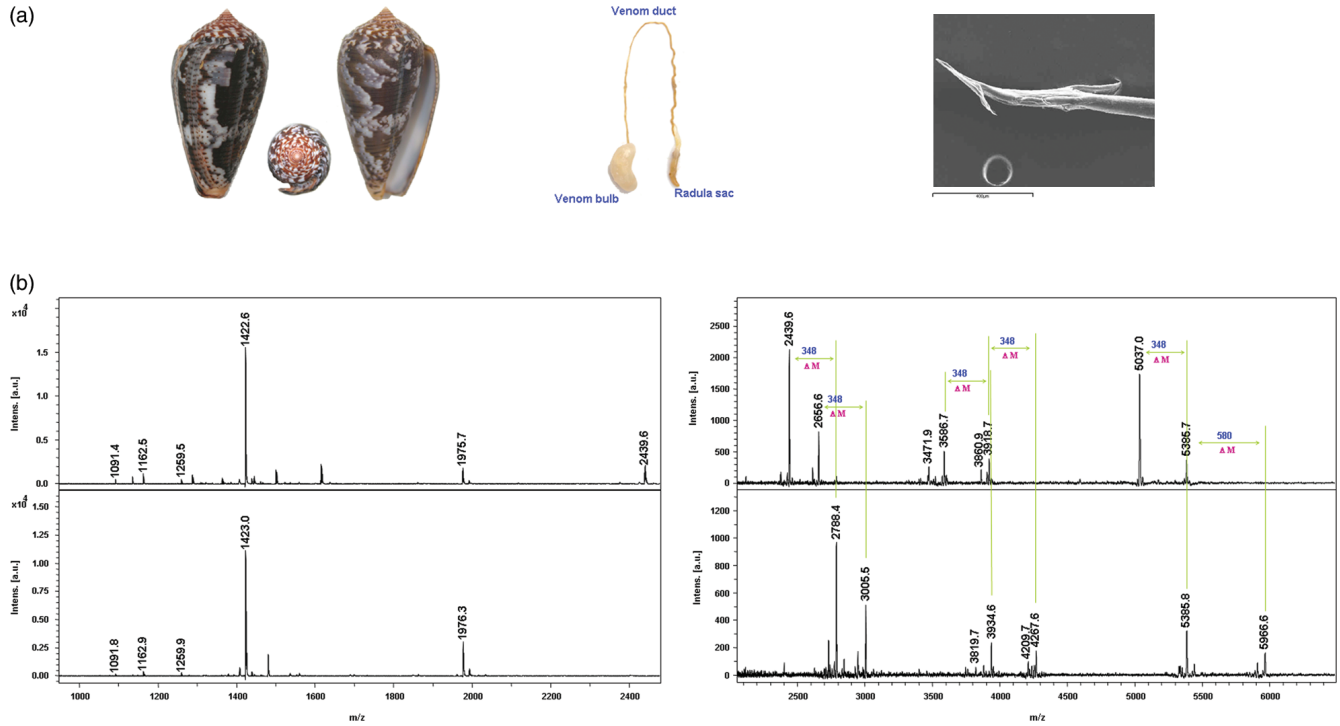

Figure 1. (a) A representative example of the shell of a *C. achatinus* specimen utilized in the present study, its venom apparatus, and a scanning electron micrograph of the radula tooth. (b) Global reduction/alkylation of crude venom. Top panel shows the MALDI-MS of the crude venom and bottom panel shows the MALDI-MS of reduced/alkylated crude venom. Two distinct *m/z* ranges are shown to illustrate the presence of linear (left panels) and disulfide-rich peptides (right panels), respectively.

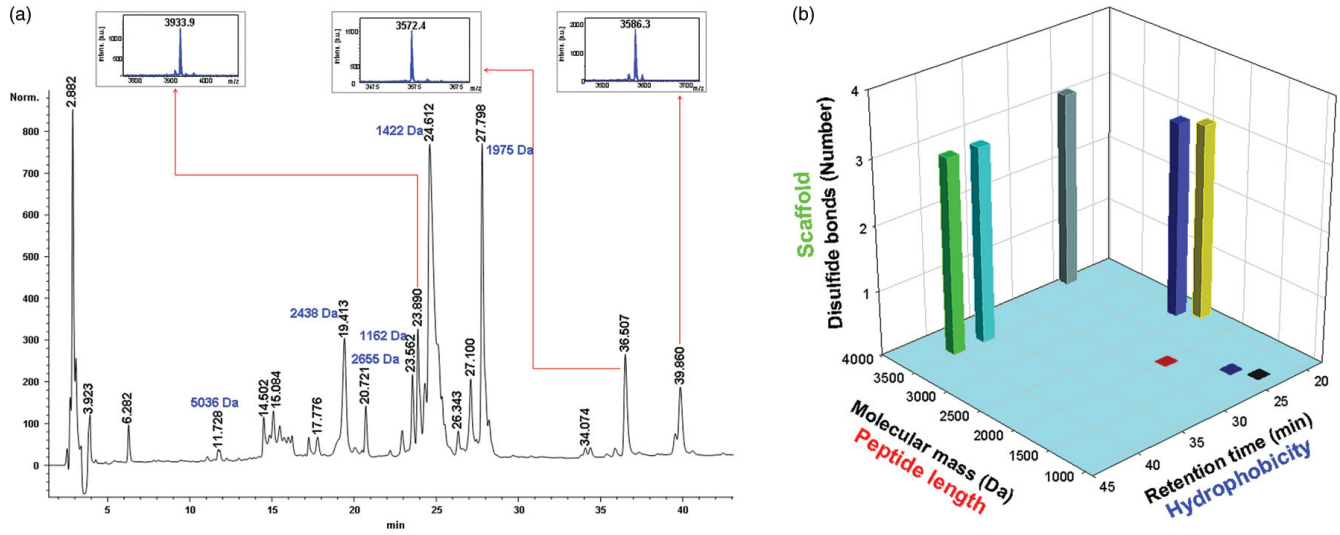

Figure 2. (a) RP-HPLC purification profile of *C.achatinus* crude venom. The molecular masses observed in the crude venom MALDI-MS are indicated above some fractions. Inset: MALDI-MS of peptides corresponding to the anticipated masses of the cDNA-derived sequences. (b) Graphical representation of the deconvoluted peptide library of *C. achatinus* in terms of mass, hydrophobicity, and number of disulfide bonds.

were predicted by identifying potential proteolytic cleavage sites and processing sites flanking the cysteine rich region. Sequences of five putative mature conotoxins are listed in Table 1. In the case of Ac 6.4, the presence of *C*-terminal glycine is suggestive of a probable posttranslational modification resulting in the formation of an amidated *C*-terminus cysteine residue. Armed with potential conotoxin sequences, we turned to the question of whether these peptides or posttranslationally modified derivatives were indeed present in the venom of *C. achatinus.*

In order to detect specific peptide components, we examined venom samples derived from individual cone snail venom ducts.[37] This procedure was followed, since the relative intensities of the mass spectral peaks corresponding to specific conotoxins differ between individuals of the same species. In Fig. 4 three distinct mass regions are highlighted corresponding to m/z values 3900–4000, 3500–3650, and 2500–3200 (inset). Figure 4(a), top panel) shows a distribution of species with the m/z value of 3902 corresponding to the anticipated $[M + H]^+$ ion from the peptide sequence

Figure 3. ClustalW alignments of five precursor sequences determined from *C. achatinus*. The putative preregion, proregion, and the mature toxin region have been demarcated by arrows. The length (number of residues) of the precursor and the number of hydrophobic and charged residues found within the mature regions of each conotoxin are also shown.

Table 1. Monoisotopic molecular masses of putative sequences derived from cDNA and the corresponding tryptic fragments

Id	Putative conotoxin sequences	Mass of peptide[a]	Expected tryptic fragments (reduced/alkylated peptide)	Observed tryptic fragments in MALDI-MS
Ac 6.1	DECFSPGTFCGIKPGLCCSAWCYSFFCLTLTF	3539.5	–	–
Ac 6.2	DECYPPGTFCGIKPGLCCSERCFPFVCLSLEF	3553.5	2501.9	–
			1417.5	1417.7
Ac 6.3	YECYSTGTFCGVNGGLCCSNLCLFFVCLFS	3243.3	–	–
Ac 6.4	CKGKGASCSRTMYNCCTGSCNRGKCG	2668.6	1522.4	1522.9
			636.2	–
Ac 6.5	ATDCIEAGNYCGPTVMKICCGFCSPFSKICMNYPQN	3901.6	1885.7	–
			1361.5	1361.0
			1038.4	1038.5

[a] Calculated mass of the putative sequence taking into consideration the disulfide bond formation by free cysteines.

Ac 6.5. Inspection of the spectrum readily reveals that the separations between isotopic clusters correspond to mass differences of 16 and 22 Da. In order to eliminate contributions from Na^+ ($\Delta M = +22$ Da) and K^+ ($\Delta M = +38$ Da) adducts; spectra were re-run by adding citric acid prior to spotting on the MALDI plate (Fig. 4(a), lower panel). Citric acid binds competently to Na^+ and K^+ adducts, thereby enhancing relative intensities of the $[M + H]^+$ species. It is clearly seen that the clusters corresponding to Na^+/K^+ adducts are dramatically suppressed. The spectrum at m/z 3902, 3918, and 3934 may be tentatively assigned to peptide Ac 6.5 and posttranslationally modified derivatives, which are hydroxylated at one or two proline residues; another possibility is the oxidation of one or two methionine residues. The first inference is based on the widespread occurrence of proline hydroxylation in *Conus* venom peptides. Similar results are shown in Fig. 4(b) for mass spectral clusters that may correspond to peptide Ac 6.2, which has an anticipated $[M + H]^+$ value of 3554. Figure 4(c) shows the m/z region where peptide ions with m/z value corresponding to the sequence Ac 6.4 are expected. Interestingly, peaks at m/z 2669.7 and 2611.7 match the values anticipated from peptides with *C*-terminus glycine and the posttranslationally modified sequence with an amidated Cys residue at the *C*-terminus, respectively.

In order to confirm the sequences of the peptides tentatively identified, tryptic digestion followed by mass spectral fragmentation was undertaken. Trypsin digestion was carried out on reduced/alkylated crude venom samples. Figure 5(a) and (b) illustrates the MALDI spectra of tryptic digests obtained of venom samples extracted from two individual snails. Table 1 lists the masses of the peptides, the expected tryptic fragments, and the observed tryptic fragments in the experimental digest. From Table 1 it is noted that tryptic fragments corresponding to the sequences of three of the five cDNA derived sequences are indeed observed (Ac 6.2, Ac 6.4, and Ac 6.5).

Figure 6(a) shows the MALDI-MS/MS spectrum arising by collision-induced dissociation (CID) of a peptide ion with m/z 1418. The relatively uniform distribution of fragment ions across the entire mass range permits assignment of a continuous series of b_n-ions, confirming that the precursor ion indeed corresponds to the anticipated sequence of the Ac 6.2 tryptic fragment. It is seen in Fig. 4(b) that the intensity of the $[M + H]^+$ species derived from Ac 6.2 is very weak compared to the isotopic clusters which show a mass increase of 16 and 32 Da (3570 and 3586 Da) which may be tentatively assigned as arising from posttranslational hydroxylation at proline residues. Ac 6.2 sequence has four proline residues and it was necessary to establish the site of hydroxylation. The HPLC profile of crude venom shown in Fig. 2(a) reveals a peak with a retention time of 39.8 min that yields an $[M + H]^+$ mass of 3586 Da, which matches that expected from Ac 6.2 with two Hyp residues. This peptide fraction was collected and subjected to reduction

DOI: 10.1002/jms

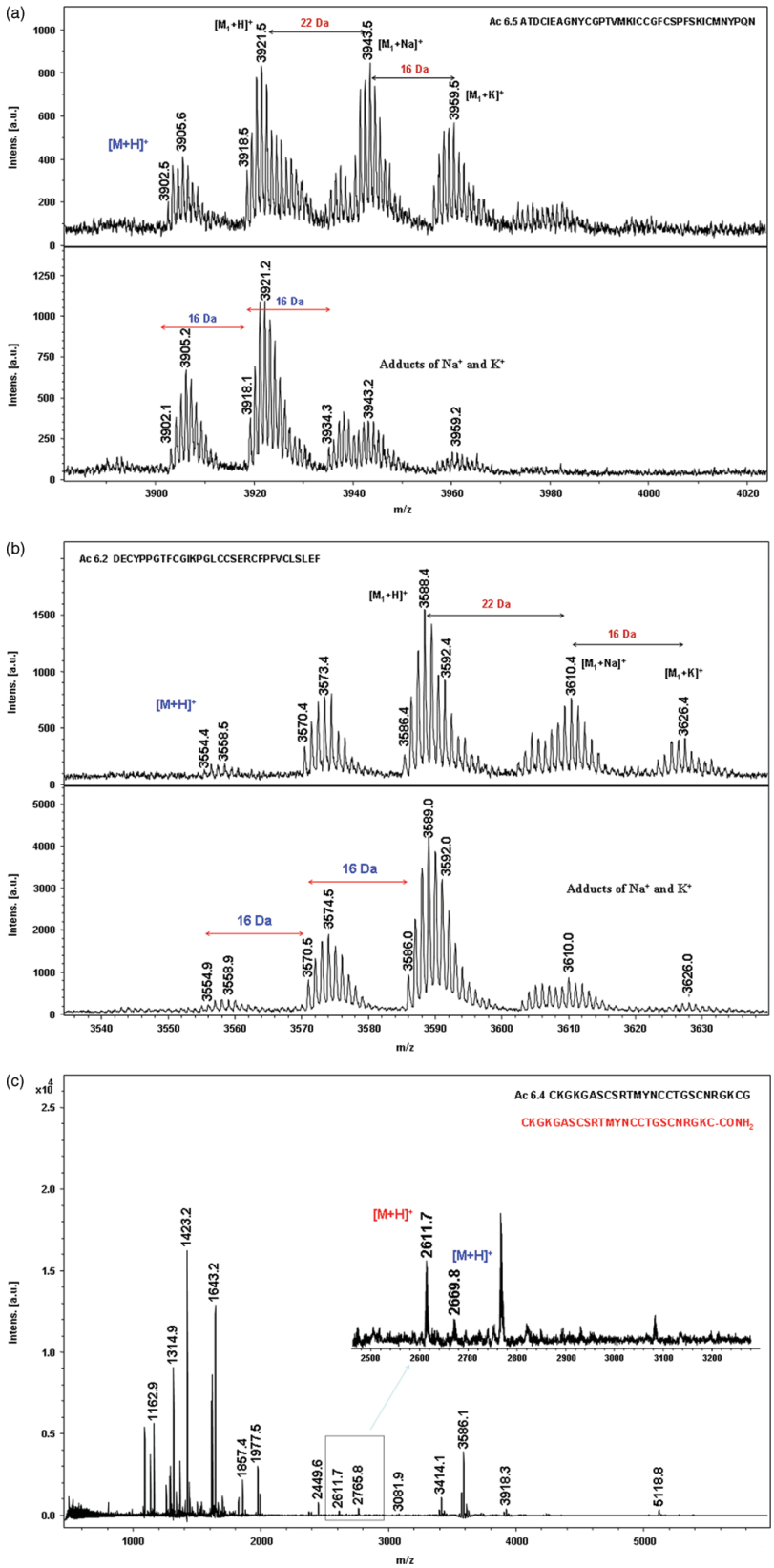

Figure 4. Screening for putative conotoxin sequences in the crude venom of individual *C. achatinus* using MALDI mass spectrometry. (a) MALDI-MS of crude venom indicates the presence of putative conotoxin derived from Ac 6.5 and the corresponding posttranslational modifications (hydroxylation of proline). (b) MALDI-MS of crude venom indicates the presence of putative conotoxin derived from Ac 6.2 and the corresponding posttranslational modifications (hydroxylation of proline). Top panel (a) and (b): crude venom spectrum and bottom panel (a) and (b): crude venom spectrum after incubation with citric acid. (c) MALDI-MS of crude venom indicates the presence of putative conotoxin derived from Ac 6.4 and the corresponding posttranslational modification (*C*-terminal amidation). Inset: Highlights the molecular mass of putative conotoxin and the corresponding *C*-terminally amidated peptide.

DOI: 10.1002/jms

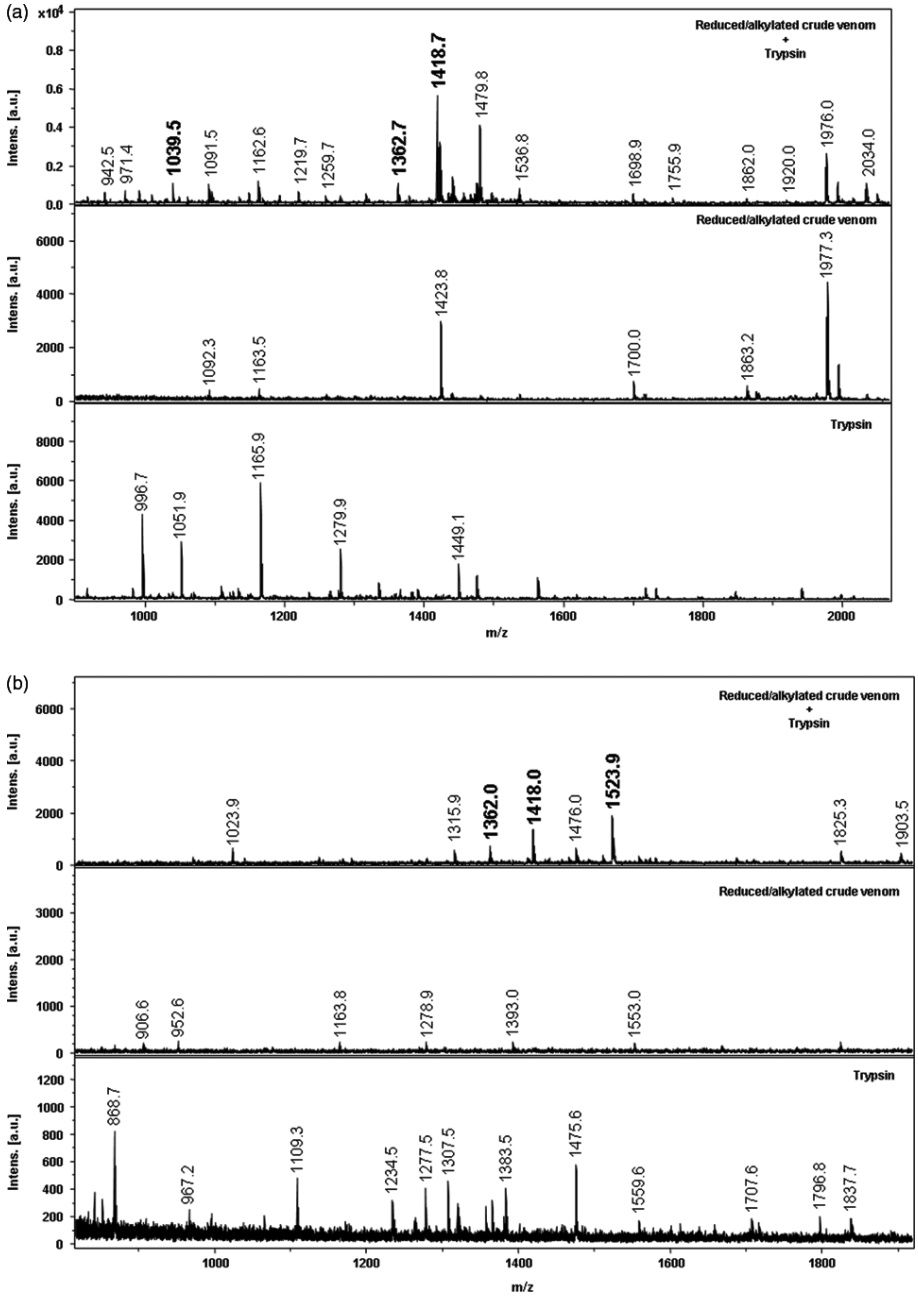

Figure 5. Characterization of the putative conotoxins in the crude venoms of two individual specimens of *C. achatinus* using global trypsin digestion and MALDI-MS. (a) MALDI-MS of the tryptic digest of reduced/alkylated crude venom. Highlighted masses correspond to the predicted tryptic fragments of Ac 6.2 and Ac 6.5. (b) MALDI-MS of the tryptic digest of reduced/alkylated crude venom. Highlighted masses correspond to the predicted tryptic fragments of Ac 6.2, Ac 6.4, and Ac 6.5. Mass spectra of the tryptic digest of reduced/alkylated crude venom (top panels), reduced/alkylated crude venom (middle panels), and controlled trypsin (lower panels) are also displayed.

of disulfide bonds followed by alkylation and subsequent digestion with trypsin. MALDI analysis of the digest yielded a peptide with m/z 2536, which corresponds to residues 1–21 (2502 Da) of the proposed sequence, with two hydroxylated proline residues. MALDI-MS/MS analysis (Fig. 7(a)) reveals the presence of a y_8 ion (OGLCCSER) at m/z 994, suggesting that P-14 is hydroxylated. In order to identify the second site of hydroxylation, the parent peptide fraction after disulfide reduction/alkylation were subjected to digestion with chymotrypsin. MALDI analysis revealed the peptide at m/z 1102, corresponding to residues 1–9 of the proposed sequence. Mass spectral fragmentation of this ion (Fig. 7(b)) revealed a b_5 ion at m/z 665 and a y_5 ion at m/z 534 clearly establishing that the site of hydroxylation is P-6.

Figure 6(b) shows the MALDI-MS/MS spectrum derived by fragmentation of peptide ion at m/z 1523.6, which is expected to correspond to a part of the peptide Ac 6.4. In this case, the intensities of the fragment ions are considerably weaker. However, a complete series of y-ions can be identified, confirming that the tryptic fragment sequence is indeed that anticipated from Ac 6.4. In this sequence, the only post-translational modification is the amidation of the *C*-terminus.

DOI: 10.1002/jms

JMS

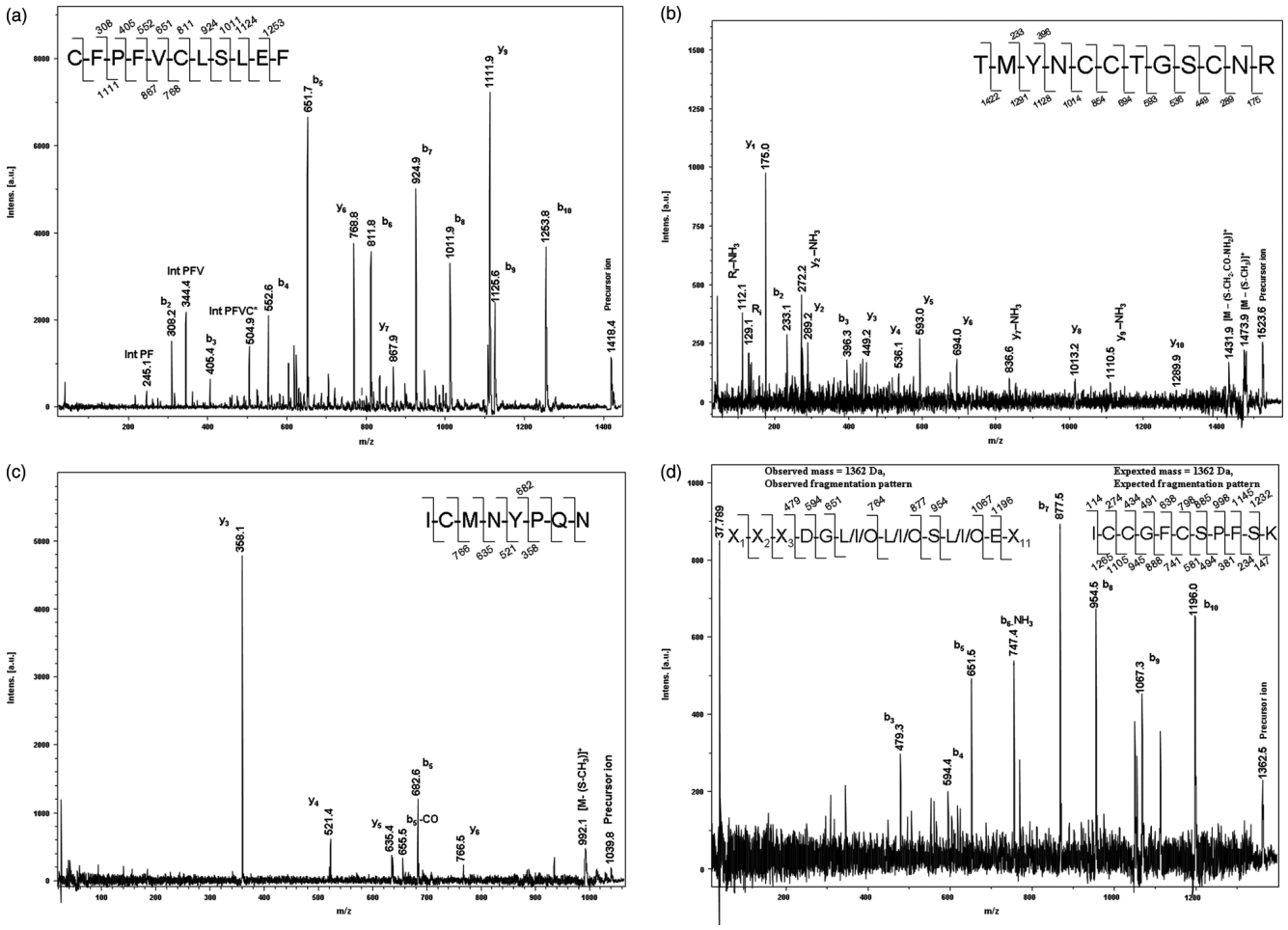

Figure 6. MALDI-MS/MS of the tryptic fragments derived from the global trypsin digestion of reduced/alkylated crude venom. (a) MALDI-MS/MS of the tryptic fragment with *m/z* value 1418 and the derived sequence indeed corresponds to the *C*-terminal fragment of the putative conotoxin Ac 6.2. (b) MALDI-MS/MS of the tryptic fragment with *m/z* value 1523 and the derived sequence indeed corresponds to the internal fragment of the putative conotoxin Ac 6.4. (c) MALDI-MS/MS of the tryptic fragment with *m/z* value 1039 and the derived sequence indeed corresponds to the *C*-terminal fragment of the putative conotoxin Ac 6.5. (d) MALDI-MS/MS of the tryptic fragment with *m/z* value 1362 and the derived sequence does not correspond to the putative conotoxin Ac 6.5. Inset (a), (b), (c) and (d): mass spectrometrically derived sequence with suitable b- and y-ions.

In the case of Ac 6.5, tryptic fragments with m/z value 1039 and 1362 correspond to the anticipated sequence (Table 1). Figure 6(c) and (d) shows the MALDI-MS/MS spectra obtained by fragmentation of these two precursor ions. The peptide with m/z 1039.8 indeed yields a series of y-ions, which match the anticipated m/z values. The intense peak at m/z 358 is consistent with preferential cleavage at the X-Pro bond. Notably, the fragmentation pattern obtained in Fig. 6(d) from the precursor ion m/z 1362.5 does not match that anticipated for sequence Ac 6.5 and may arise from a tryptic fragment from an entirely different peptide present in the venom sample. This observation points to the danger of using only tryptic peptide masses without further fragmentation in any proteomic approach for identification of peptides and proteins. Inspection of the crude venom spectra shown in Fig. 4(a) reveals that in Ac 6.5 also peaks with isotopic clusters corresponding to mass difference of 16 and 32 Da are observed (3918 and 3934 Da) which may be tentatively assigned as arising from posttranslational hydroxylation at proline residues. Ac 6.5 contains three proline residues at positions 13, 25, and 34. The mass of peptide fraction isolated by RP-HPLC, 3934 Da (Fig. 2(a)), is consistent with the Ac 6.5 sequence in which two proline residues are hydroxylated. Disulfide reduction/alkylation followed by trypsin cleavage yielded peptide fragments with m/z 1379 (residues 18–28) and m/z 1903 (residues 1–17). Mass spectral fragmentation of the ion at m/z 1379.1 peak yielded an intense y_4 ion at m/z 494 (Fig. 8(a)) corresponding to the peptide ion (OFSK), with the proline residue hydroxylated. Similarly, fragments of the peptide ion with m/z 1903.2 (ATDCIEAGNYCGPTVMK) yielded a series of y_n ions beginning with fragments arising from y_5 ion at m/z 591 (Fig. 8(b)), which corresponds to hydroxylation at P-25 residue (OTVMK). Thus P-13 and P-25 are identified as the sites of hydroxylation. The absence of hydroxylation at position P-34 is confirmed by the observation of ion at m/z 1039, which corresponds to the peptide (ICMNYPQN) in the tryptic digest of the reduced/alkylated peptide eluting at the retention time 23.8 min.

Of the five cDNA-derived putative conotoxin sequences, three peptides are posttranslationally modified derivatives

DOI: 10.1002/jms

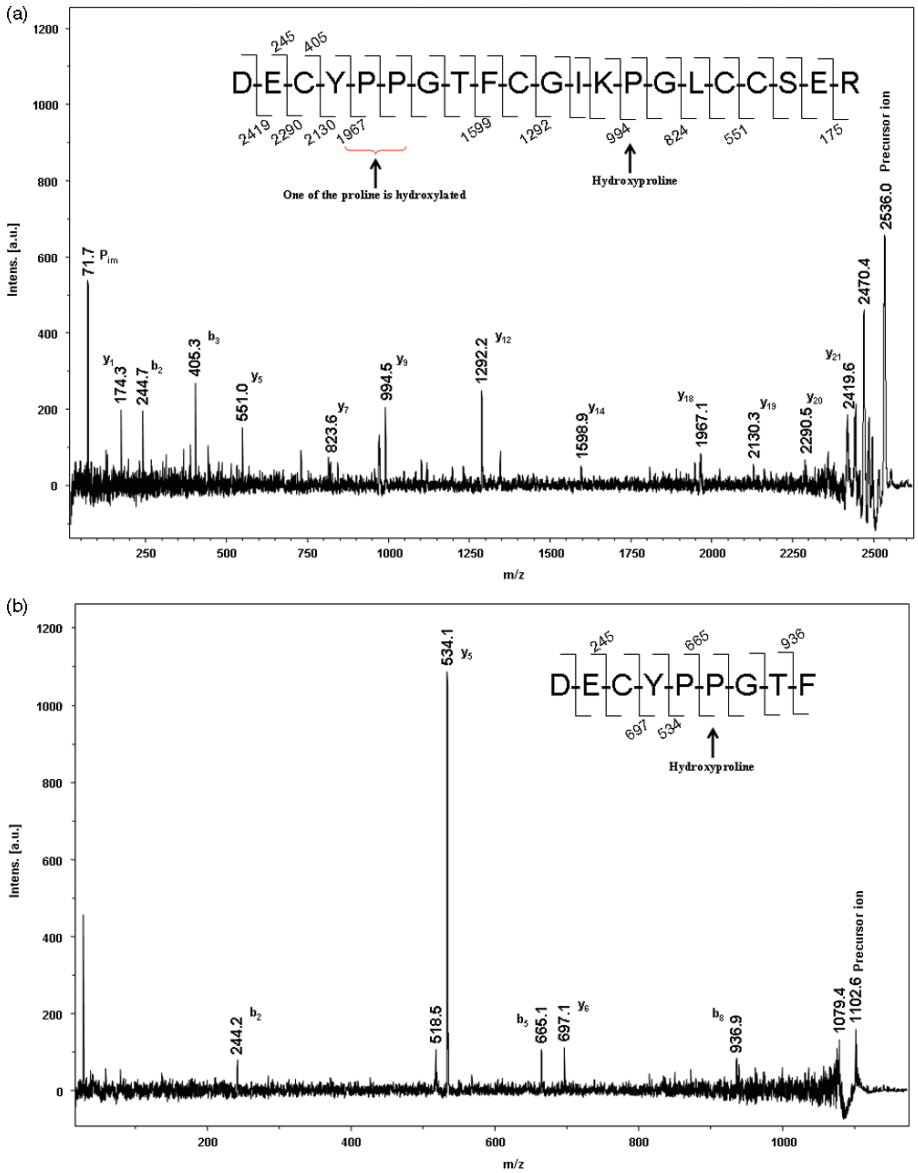

Figure 7. Establishing the presence of hydroxyproline in Ac 6.2 by enzymatic digestion and subsequent mass spectrometric fragmentation of the peptide eluting at retention time 39.86 min. (a) MALDI-MS/MS of the tryptic fragment with *m/z* 2536 and (b) MALDI-MS/MS of the chymotryptic fragment with *m/z* 1102. Inset (a) and (b): Mass spectrometrically derived sequence with suitable b- and y-ions; the position of hydroxylation of proline is indicated by an arrow.

of Ac 6.2, Ac 6.4, Ac 6.5 and have been identified in crude venom from *C. achatinus*. Examination of the sequences in Table 1 reveals that the sequences Ac 6.1 and Ac 6.3 do not contain any potential sites for cleavage by trypsin. Ac 6.1 does contain a lysine residue, but this is followed by a proline residue. Lys-Pro and Arg-Pro sequences are known to be resistant to trypsin cleavage.

The crude venom HPLC profile present in Fig. 2(a) shows a prominent component at a retention time of 36.5 min, which yields an $[M + H]^+$ mass of 3572.4 Da. The observed mass is consistent with a posttranslationally modified derivative of Ac 6.1 in which both proline residues are hydroxylated. This HPLC fraction was collected and a concentrated peptide solution was used for disulfide reduction/alkylation. The reduced/alkylated peptide yields a mass 3920.3, which matches the anticipated value for modified Ac 6.1 (Fig. 9(a)). The chymotrypsin digestion yields a fragment with an *m/z* value of 1424.9, which corresponds the fragment CGIKPGLCCSAW derived by cleavage at the *C*-terminal end of the flanking aromatic residue in modified Ac 6.1. Mass spectral fragmentation of this precursor ion yields a series of b-ions (Fig. 9(b)), which are consistent with the anticipated sequence. Indeed the mass difference between b_4 and b_5 ions is in accordance with the presence of Hyp at position 5 of the chymotryptic fragment. Despite the poor signal/noise ratio for some of the b-ion peaks, the presence of an intense y_8-ion at *m/z* 966 strongly suggests that this chymotryptic fragment is indeed derived from modified Ac 6.1.

DISCUSSION

Functional assignment based on cDNA sequences

The family assignment of the three classes of cDNA was made by using the five mature sequences to conduct independent

DOI: 10.1002/jms

JMS

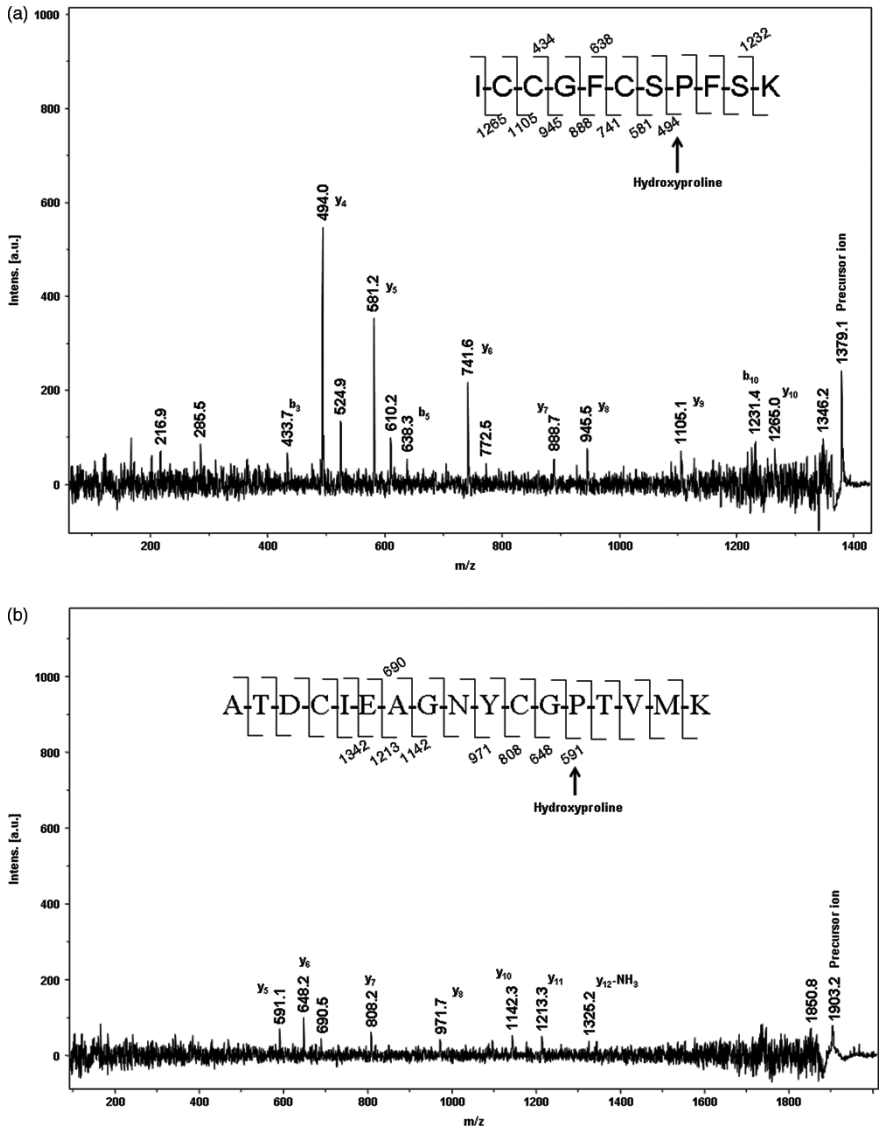

Figure 8. Establishing the presence of hydroxyproline in Ac 6.5 by enzymatic digestion and subsequent mass spectrometric fragmentation of the peptide eluting at retention time 23.8 min. (a) MALDI-MS/MS of the tryptic fragment with *m/z* 1379 and (b) MALDI-MS/MS of the chymotryptic fragment with *m/z* 1903. Inset (a) and (b): Mass spectrometrically derived sequence with suitable b- and y-ions; the position of hydroxylation of proline is indicated by an arrow.

searches of short, similar proteins in the sequence database at the NCBI. The highest scoring BLASTp sequence recovered for each conotoxin is shown in Table 2. Sequences Ac 6.1, Ac 6.2, and Ac 6.3 align with δ-conotoxins,[11,38] while sequences Ac 6.4 and Ac 6.5 align with ω- and ω-type conotoxins,[11,39] respectively. In all the cases, the length of the sequences and the number of hydrophobic and charged residues between the *C. achatinus* sequences and those recovered from the database match very closely. Based upon these alignments and similarities in the nature of the constituent residues, the sequences Ac 6.1, Ac 6.2, Ac 6.3 have been classified as δ-conotoxins, while sequences Ac 6.4, Ac 6.5 are classified as ω- and ω-type conotoxins, respectively. All the best aligning hits to the *C. achatinus* sequences also belong to piscivorous species of cone snails.

Ac 6.1, 6.2, 6.3

δ-conotoxins target voltage-sensitive Na channels (NaV), and inhibit the fast inactivation of Na^+ currents that restore the resting potential in neuronal membranes following a spike of depolarization.[40,41] The precise inhibitory binding site of the peptide on the inactivation domain of the ion-channel has not been defined though the effect of this inhibition is a protracted state of activation for a conducting nerve resulting in what would be equivalent to an electric shock.[42] The δ-conotoxins are therefore considered to be a group of peptides in the piscivorous cone snail venom responsible for the instantaneous stunning of their prey and are members of what has been termed the 'lightning strike' cabal.[11,38] *Bulaj et al.* (2001) have compared δ-conotoxins from nine species of piscivorous cone snails. Their analysis has shown that the residues G7, F9, G11, I12, G15, L16, S19 are conserved across all the piscivorous species of cone and conclude that these residues are functionally important in piscivorous δ-conotoxins. These residues are also conserved in the three δ-sequences from *C. achatinus*, (besides isoleucine at position 12 in Ac 6.3 that has undergone a conservative mutation to valine). An alignment of the conotoxins described by *Bulaj*

DOI: 10.1002/jms

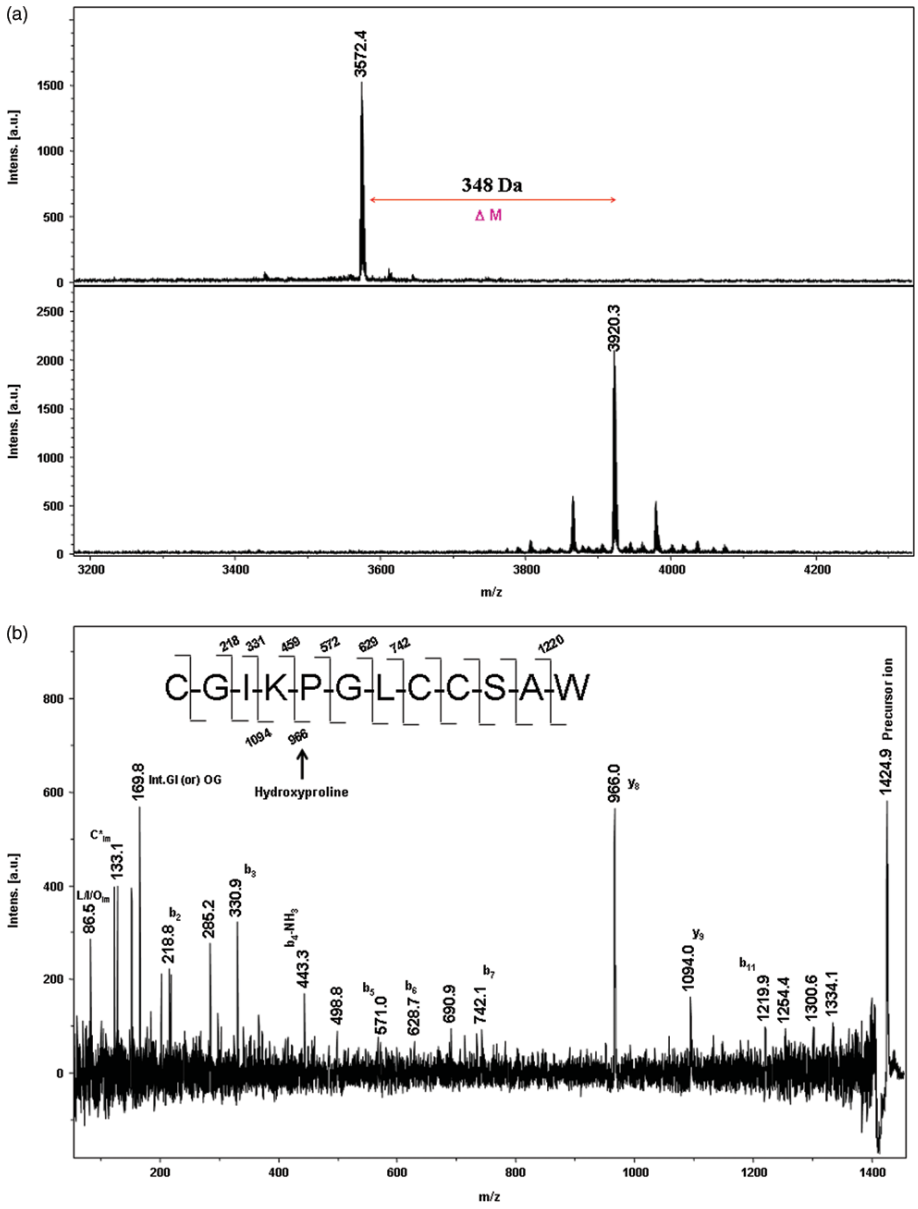

Figure 9. Posttranslationally modified Ac 6.1 in the venom of *C.achatinus.* (a) MALDI-MS of the reduced/alkylated peptide eluting at the retention time 36.5 min. Top panel: Intact peptide; bottom panel: Reduced/alkylated peptide. (b) MALDI-MS/MS of the chymotryptic fragment with *m/z* 1424 and the derived sequence indeed corresponds to the internal fragment of Ac 6.1. Inset: Mass spectrometrically derived sequence with suitable b- and y-ions; the position of hydroxylation of proline is indicated by an arrow.

et al. (2001) and the three δ-conotoxins from *C. achatinus* are shown in Table 3. The latter sequences merge imperceptibly with the δ-conotoxins from other species and, in fact, show greater segmental similarities to other conotoxins than to each other. Ac 6.3 is relatively dissimilar to Ac 6.1 and Ac 6.2 and is nearly identical in sequence to the conotoxin CnVIA from *C. consors*. Given the close similarities of Ac 6.1, Ac 6.2, and Ac 6.3 to other δ-conotoxins, it is expected that the natural targets of the three *C. achatinus* conotoxins are the voltage gated sodium channels. The fact that δ-conotoxins are among the more functionally relevant conotoxins for the piscivorous cone snails could probably explain why three of the five conotoxin transcript sequences that were randomly obtained from the O-superfamily of *C. achatinus* belong to this group. δ-Conotoxins are regarded as unusually hydrophobic peptides, with over a third of the residues in the mature peptide being hydrophobic. The hydrophobicity of these peptides appears to be an important feature for their function.[11] δ-Conotoxin SVIE from *Conus striatus* has been reported to interact with a conserved hydrophobic triad (YFV) in the voltage sensor (domain-4) of the Na^+ channel through hydrophobic interactions.[43]

Ac 6.4

ω-Conotoxins target voltage-sensitive presynaptic Ca^{2+} (CaV) channels and have consistently been found within the venom of piscivorous cone snails. These conotoxins have been shown to target only neuronal subtypes of the CaV channels and not those of the smooth, the skeletal or cardiac muscles.[39] Some of the better known conotoxins, GVIA and MVIIC, block different subtypes of presynaptic voltage-gated

DOI: 10.1002/jms

Table 2. Family identification of the mature *C. achatinus* sequences

Id	Sequence	Number of residues	E-value[a]	Family	Possible target
Ac 6.1	DECFSPGTFCGIKPGLCCSAWCYSFFCLTLTF	32	6.2	δ-conotoxin	Voltage-gated
δ-NgVIA	SKCFSPGTFCGIKPGLCCSVRCFSLFCISFE-	31			Na^+ channels
Ac 6.2	DECYPPGTFCGIKPGLCCSERCFPFVCLSLEF	32	8e-04	δ-conotoxin	"
δ-PVIA	EACYAPGTFCGIKPGLCCSEFCLPGVCFGG–	30			
Ac 6.3	YECYSTGTFCGVNGGLCCSNLCLFFVCLFS–	30	4e-09	δ-conotoxin	"
δ-CnVIA	YECYSTGTFCGINGGLCCSNLCLFFVCLTFS-	31			
Ac 6.4	CKGKGASCSRTMYNCCTGSCNRGKCG	26	0.002	ω-conotoxin	Voltage-gated
ω-MVIIb	CKGKGASCHRTSYDCCTGSCNRGKC*	25			Ca^{2+} channels
Ac 6.5	ATDCIEAGNYCGPTVMKICCGFCSPFSKICMNYPQN	36	9e-10	ω-like conotoxin	Voltage-gated
ω-SO4	ATDCIEAGNYCGPTVMKICCGFCSPYSKICMNYPKN	36			Ca^{2+} channels

[a] The reported E-values were derived by the BLAST analysis against the nonredundant database at http://www.ncbi.nlm.nih.gov. on 26th November 2007.

Table 3. δ- and ω-conotoxins of piscivorous Conus Sp. Alignments of the *C. achatinus* mature δ- and ω-conotoxins Ac 6.1, 6.2, 6.3, and 6.4 (boldface) with the sequences from other δ- and ω-conotoxins isolated from piscivorous snails

Species	Toxin ID	Sequence
δ-Conotoxins		
C. achatinus	**Ac 6.1**	DE C FSPGTF C GIKPGL CC SAW C YSFF–C LTLTF
C. nigropunctatus	NGVIA	SK C FSPGTF C GIKPGL CC SVR C FSLF–C IS FE
C. achatinus	**Ac 6.2**	DE C YPPGTF C GIKPGL CC SER C FPFV–C LSLEF
C. magus	MVIC	DE C YPPGTF C GIKPGL CC SAI C LSFV–C ISFDF
C. ermineus	EVIA	EA C YPPGTF C GIRPGL CC SEL C LPAV–C VG
C. magus	MVID	EA C YNAGTF C GIKPGL CC SAI C LSF-VW C ISFDF
C. magus	MVIB	EA C YNAGSF C GIHPGL CC SEF C IL – W C ITFVDS
C. magus	MVIA	DG C YNAGTF C GIRPGL CC SEF C FL – W C TTFVDS
C. purpurascens	PVIA	EA C YAPGTF C GIKPGL CC SEF C LPG-V- C FG
C. stercusmuscarum	SmVIA	DG C SSGGTF C GIRPGL CC SEF C FL – W C IT ID
C. striatus	SVIE	DG C SSGGTF C GIHPGL CC SEF C FL – W C ITFID
C. aurisiacus	AVIA	DG C SNAGAF C GIHPGL CC SEI C T – VW C T
C. catus	CVIE	YG C SNAGAF C GIHPGL CC SEI C L – VW C T
C. achatinus	**Ac 6.3**	YE C YSTGTF C GVNGGL CC SNL C LFFV–C LFS
C. consors	CnVIA	YE C YSTGTF C GINGGL CC SNL C LFFV–C TFS
ω-Conotoxins		
C. striatus	AF146346	C KLKGQS C RKTSYD CC SGS C GRSGK C G
C. striatus	AF174244	C KAAGKS C SRIAYN CC TGS C RSGK- C G
C. striatus	AF174245	C KAAGKP C SRIAYN CC TGS C RSGK- C G
C. catus	AF174218	C KGKGAS C RRTSYG CC TGS C RSGR- C G
C. catus	AF174227	C KSTGAS C RRTPYD CC TGS C RSGR- C G
C. catus	AF174238	C QGRGAS C RRTSYD CC TGS C RSGR- C G
C. catus	AF174238	C QGRGAS C RKTMYN CC SGS C RSGR- C G
C. magus	MVIIA	C KGKGAK C SRLMYD CC TGS C RSGK- C*
C. magus	MVIIB	C KGKGAS C HRTSYD CC TGS C NRGK- C*
C. achatinus	**Ac 6.4**	C KGKGAS C SRTMYN CC TGS C NRGK- C G
C. magus	MVIIC	C KGKGAP C RKTMYD CC SGS C RRGK- C*
C. magus	MVIID	C QGRGAS C RKTMYN CC SGS C NRGR- C*

calcium channels leading to a block in nerve impulse propagation and a resulting long acting paralysis whose effects can only partially be reversed by *in vitro* buffer washes.[44] The paralysis brought about by the ω-conotoxins gradually builds up over minutes with their effects occupying a nonoverlapping time frame relative to the δ-conotoxins.[45] The need for these slower acting ω-conotoxins by the cone snail during feeding forms a point of speculation since, in principle, the snail would have incapacitated the prey by the action of other more rapidly acting conotoxins much before the ω-conotoxin-derived paralysis takes hold. Table 3 shows a comparative alignment of Ac 6.4 with other closely related

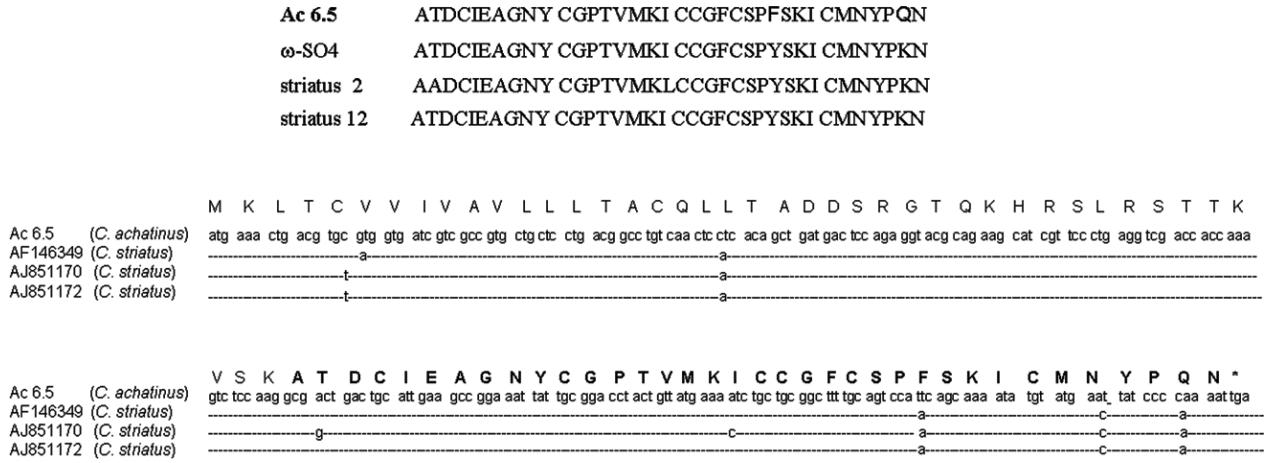

Figure 10. ω-like conotoxin Ac 6.5 conservation. Alignments of the *C.achatinus* mature ω-like conotoxin Ac 6.5 with very similar sequences isolated from piscivorous *C.striatus*, showing how well the conotoxin has remain conserved. The lower figure shows the nucleotide sequence of Ac 6.5 along with the observed nucleotide differences found in the other sequences from *C.striatus* (AF146349 are from *Lu et al* 1999, AJ851170 and AJ851172 are from *Kauferstein et al* 2005).

ω-conotoxin sequences from three piscivorous *Conus* species (*C. striatus*, *C. catus*, and *C. magus*). Sequence Ac 6.4 from *C. achatinus* shows the greatest similarity to the ω-conotoxin MVIIB (80%). This latter conotoxin belongs to a group of closely related conotoxins isolated from the piscivorous *C. magus* (ω-MVII A, B, C, and D) known to target different subtypes of voltage-activated calcium channels. Tyr13 in ω-MVIIA, and other ω-conotoxins, is a conserved residue and has been shown to be important for CaV receptor binding.[46,47] This conserved residue is also present in ω-Ac 6.4. A characteristic feature of ω-Conotoxins is their high content of basic amino acid residues. Sequence Ac 6.4, with five positively charged groups, conforms to this observation. Glycine at the C-terminal residue for Ac 6.4 suggests that in the mature peptide this residue is postranslationaly converted to a C-terminal amide. This latter modification has been found in the ω-MVII (A–D) series of conotoxins and has been suggested to help in increasing the overall charge of the peptide for binding to their target CaV receptors.[39,48] The similarity of Ac 6.4 to ω-MVII (A–D) series of conotoxins leads one to expect that it would at least block CaV channels, though the subtype specificity is unknown at present.

Ac 6.5

Ac 6.5 is an unusual conotoxin of the O-superfamily in two aspects. Firstly, the precursor retains the characteristic O-superfamily cysteine distribution pattern in the mature toxin region and its pro-sequence is similar to other ω-conotoxin precursors, as shown in Fig. 10. This strongly suggests that sequence Ac 6.5 is an ω-conotoxin. However, while the mature ω-conotoxins are typically ~25–29 amino acid residues in length, Ac 6.5 and its homologs (ω-SO4, striatus 2, striatus 12) are longer (36 residues). Furthermore, while the ω-conotoxins have a large number of charged residues, Ac 6.5 has a significant number of hydrophobic residues reminiscent of the δ-conotoxins. The second unusual feature of Ac 6.5 is that it appears to be quite well conserved. As shown in Fig. 10, conotoxins having very similar sequences have earlier been isolated by cDNA cloning from *C. striatus*, another fish-eating snail in two independent studies. These include the ω-type conotoxin, ω-SO4 with 94% identity (*C. striatus*) collected from the Hanan islands in South China[23] and striatus 2 and striatus 12 isolated from the same species from the Hawaiin islands.[24] A comparative alignment of the mature regions of the 3 sequences shows 34 out of 36 are identical and the 2 changes, F26Y and Q35N are conservative. It is most probable that the gene in *C. achatinus* and *C. striatus* coding for these conotoxins have in some way been conserved in stark contrast to the variability that can be seen among the δ- and ω-conotoxins from the same two species (Table 3).

Deconvolution of complex natural peptide libraries is aided by combined application of the methods of cDNA sequencing and direct mass spectral fragmentation of peptide components in crude venom. The results in this article emphasize the utility of this approach in identifying posttranslational modifications in the mature peptides of *Conus* venom permitting the characterization of five new conotoxins from the piscivorous snail *C. achatinus*. Rapid peptide profiling may be useful in monitoring both intra-species and inter-species variation in venom components. The methods described in this article are readily applicable to the venom samples collected from a single snail, thus obviating the need for a large collection of snails.[49–51]

Acknowledgment

We acknowledge Y.K. Subrahmanya Prakash for mass spectrometry. K.K.D. acknowledges facilities at the Unichem R & D Research Centre and the encouragement for carrying out this work provided by Dr C.S. Ramadoss and Dr P.R. Krishnaswamy.

REFERENCES

1. Biemann K. Mass spectrometry of peptides and proteins. *Annual Review of Biochemistry* 1992; **61**: 977.
2. McLafferty FW, Fridriksson EK, Horn DM, Lewis MA, Zubarev RA. Biomolecule mass spectrometry. *Science* 1999; **284**: 1289.
3. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature* 2003; **422**: 198.
4. Domon B, Aebersold R. Mass spectrometry and protein analysis. *Science* 2006; **312**: 212.

5. Quinton L, Demeure K, Dobson R, Gilles N, Gabelica V, Pauw ED. New method for characterizing highly disulfide-bridged peptides in complex mixtures: application to toxin identification from crude venoms. *Journal of Proteome Research* 2007; **6**: 3216.
6. Jakubowski JA, Sweedler JV. Sequencing and mass profiling highly modified conotoxins using global reduction/alkylation followed by mass spectrometry. *Analytical Chemistry* 2004; **76**: 6541.
7. Neitz S, Jurgens M, Kellmann M, Schulz-Knappe P, Schrader M. Screening for disulfide-rich peptides in biological sources by carboxyamidomethylation in combination with differential matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* 2001; **15**: 1586.
8. Legros C, Celerier ML, Henry M, Guette C. Nanospray analysis of the venom of the tarantula *Theraphosa leblondi*: a powerful method for direct venom mass fingerprinting and toxin sequencing. *Rapid Communications in Mass Spectrometry* 2004; **18**: 1024.
9. Jakubowski JA, Keays DA, Kelley WP, Sandall DW, Bingham JP, Livett BG, Gayler KR, Sweedler JV. Determining sequences and post-translational modifications of novel conotoxins in *Conus victoriae* using cDNA sequencing and mass spectrometry. *Journal of Mass Spectrometry* 2004; **39**: 548.
10. Jakubowski JA, Kelley WP, Sweedler JV. Screening for post-translational modifications in conotoxins using liquid chromatography/mass spectrometry: an important component of conotoxin discovery. *Toxicon* 2006; **47**: 688.
11. Terlau H, Olivera BM. *Conus* venoms: a rich source of novel ion channel- targeted peptides. *Physiological Reviews* 2004; **84**: 41.
12. Olivera BM, Hillyard DR, Marsh M, Yoshikami D. Combinatorial peptide libraries in drug design: lessons from venomous cone snails. *Trends in Biotechnology* 1995; **13**: 422.
13. Myers RA, Cruz LJ, Rivier JE, Olivera BM. *Conus* peptides as chemical probes for receptors and ion channels. *Chemical Reviews* 1993; **93**: 1923.
14. Olivera BM, Rivier J, Clark C, Ramilo CA, Corpuz GP, Abogadie FC, Mena EE, Woodward SR, Hillyard DR, Cruz LJ. Diversity of *Conus* neuropeptides. *Science* 1990; **249**: 257.
15. Olivera BM. *Conus* venom peptides, receptor and ion channel targets and drug design: 50 million years of neuropharmacology (EE Just Lecture, 1996). *Molecular Biology of the Cell* 1997; **8**: 2101.
16. Sudarslal S, Majumdar S, Ramasamy P, Dhawan R, Pal PP, Ramaswami M, Lala AK, Sikdar SK, Sarma SP, Krishnan KS, Balaram P. Sodium channel modulating activity in a δ-conotoxin from an Indian marine snail. *FEBS Letters* 2003; **553**: 209.
17. Sudarslal S, Singaravadivelan G, Ramasamy P, Ananda K, Sarma SP, Sikdar SK, Krishnan KS, Balaram P. A novel 13 residue acyclic peptide from the marine snail, *Conus monile*, targets potassium channels. *Biochemical and Biophysical Research Communications* 2004; **317**: 682.
18. Gowd KH, Sabareesh V, Sudarslal S, Iengar P, Franklin B, Fernando A, Dewan K, Ramaswami M, Sarma SP, Sikdar SK, Balaram P, Krishnan KS. Novel peptides of therapeutic promise from Indian conidae. *Annals of the New York Academy of Sciences* 2005; **1056**: 462.
19. Sabareesh V, Gowd KH, Ramasamy P, Sudarslal S, Krishnan KS, Sikdar SK, Balaram P. Characterization of contryphans from *Conus loroisii* and *Conus amadis* that target calcium channels. *Peptides* 2006; **27**: 2647.
20. Mandal AK, Ramasamy MR, Sabareesh V, Openshaw ME, Krishnan KS, Balaram P. Sequencing of T-superfamily conotoxins from *Conus virgo*: pyroglutamic acid identification and disulfide arrangement by MALDI mass spectrometry. *Journal of the American Society for Mass Spectrometry* 2007; **18**: 1396.
21. Turner RJ, Freeman SE. Factors affecting the muscle depolarization due to *Conus achatinus* toxin. *Toxicon* 1974; **12**: 49.
22. Liu L, Chew G, Hawrot E, Chi C, Wang C. Two potent α3/5 conotoxins from piscivorous *Conus achatinus*. *Acta biochimica et biophysica Sinica (Shanghai)* 2007; **39**: 438.
23. Lu BS, Yu F, Zhao D, Huang PT, Huang CF. Conopeptides from *Conus striatus* and *Conus textile* by cDNA cloning. *Peptides* 1999; **20**: 1139.
24. Kauferstein S, Melaun C, Mebs D. Direct cDNA cloning of novel conopeptides precursors of the O-superfamily. *Peptides* 2005; **26**: 361.
25. Jiang H, Xu CQ, Wang CZ, Fan CX, Zhao TY, Chen JS, Chi CW. Two novel O-superfamily conotoxins from *Conus vexillum*. *Toxicon* 2006; **15**: 425.
26. Luo S, Zhangsun D, Lin Q, Xie L, Wu Y, Zhu X. Sequence diversity of O-superfamily conopeptides from *Conus marmoreus* native to Hainan. *Peptides* 2006; **27**: 3058.
27. Luo S, Zhangsun D, Feng J, Wu Y, Zhu X, Hu Y. Diversity of the O- superfamily conotoxins from *Conus miles*. *Journal of Peptide Science* 2007; **13**: 44.
28. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* 1987; **163**: 156.
29. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *Journal of Molecular Biology* 1990; **215**: 403.
30. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 1997; **25**: 3389.
31. Thompson JD, Plewnial F, Thierry JC, Poch O. Rapid and reliable global multiple alignments of protein sequences detected by database searches. *Nucleic Acid Research* 2000; **28**: 2919.
32. Boeckmann B, Bairoch A, Apweiler R, Blatter MC, Estreicher A, Gasteiger E, Martin MJ, Michoud K, O'Donovan C, Phan I, Pilbout S, Schneider M. The swiss-prot protein knowledge base and its supplement TrEMBL. *Nucleic Acids Research* 2003; **31**: 365.
33. Cohen SL, Chait BT. Influence of matrix solution conditions on the MALDI-MS analysis of peptides and proteins. *Analytical Chemistry* 1996; **68**: 31.
34. Buczek O, Bulaj G, Olivera BM. Conotoxins and the posttranslational modification of secreted gene products. *Cellular and Molecular Life Sciences* 2005; **62**: 3067.
35. Prigge ST, Mains RE, Eipper BA, Amzel LM. New insights into copper monooxygenases and peptide amidation: structure, mechanism and function. *Cellular and Molecular Life Sciences* 2000; **57**: 1236.
36. Biemann K. Sequencing of peptides by tandem mass spectrometry and high-energy collision-induced dissociation. *Methods in Enzymology* 1990; **193**: 455.
37. Jakubowski JA, Kelley WP, Sweedler JV, Gilly WF, Schulz JR. Intraspecific variation of venom injected by fish-hunting *Conus* snails. *Journal of Experimental Biology* 2005; **208**: 2873.
38. Bulaj G, DeLaCruz R, Azimi-Zonooz A, West P, Watkins M, Yoshikami D, Olivera BM. δ-Conotoxin structure/function through a cladistic analysis. *Biochemistry* 2001; **40**: 13201.
39. McCleskey EW, Fox AP, Feldman DH, Cruz LJ, Olivera BM, Tsien RW, Yoshikami D. ω-conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proceedings of the National Academy of Sciences of the United States of America* 1987; **84**: 4327.
40. Hodgkin AL, Huxley AF. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *Journal of Physiology* 1952; **116**: 440.
41. Clay JR. Axonal excitability revisited. *Progress in Biophysics and Molecular Biology* 2005; **88**: 59.
42. Terlau H, Schon KJ, Grilley M, Stocker M, Stuhmer W, Olivera BM. Strategy for rapid immobilization of prey by a fish-hunting marine snail. *Nature* 1996; **381**: 148.
43. Leipold E, Hansel A, Olivera BM, Terlau H, Heinemann SH. Molecular interaction of δ-conotoxins with voltage-gated sodium channels. *FEBS Letters* 2005; **579**: 3881.

44. Feng ZP, Hamid J, Doering C, Bosey GM, Snutch TP, Zamponi GW. Residue Gly1326 of the N-type calcium channel α_{1B} subunit controls reversibility of ω-conotoxin GVIA and MVIIA block. *Journal of Biological Chemistry* 2001; **276**: 15728.
45. Olivera BM, Rivier J, Scott JK, Hillyard DR, Cruz LJ. Conotoxins. *Journal of Biological Chemistry* 1991; **266**: 22067.
46. Kim J, Takahashi M, Ogura A, Kohno T, Kudo Y, Sato K. Hydroxyl group of Tyr13 is essential for the activity of ω-conotoxin GVIA, a peptide toxin for the N-type calcium channel. *Journal of Biological Chemistry* 1994; **269**: 23876.
47. Flinn JP, Pallaghy PK, Lew M, Murphy R, Angus JA, Norton RS. Role of key functional groups in ω-conotoxin GVIA: synthesis structure and functional assay of selected peptide analogues. *European Journal of Biochemistry* 1999; **262**: 447.
48. Price-Carter M, Gray WR, Goldenberg DP. Folding of ω-conotoxins. 2 influence of precursor sequences and protein disulfide isomerase. *Biochemistry* 1996; **35**: 15547.
49. Chivian E, Roberts CM, Bernstein AS. The threat to cone snails. *Science* 2003; **302**: 391.
50. Duda TF Jr, Bingham JP, Livett BG, Kohn AJ, Massilia GR, Schulz JR, Down J, Sandall D, Sweedler JV. How much at risk are cone snails? *Science* 2004; **303**: 955.
51. Nelson L. Venomous snails: one slip, and you're dead. *Nature* 2004; **429**: 798.

DOI: 10.1002/jms