JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2004; **39**: 548–557
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.624

JMS

Determining sequences and post-translational modifications of novel conotoxins in *Conus victoriae* using cDNA sequencing and mass spectrometry

Jennifer A. Jakubowski,[1] David A. Keays,[2] Wayne P. Kelley,[1] David W. Sandall,[3] Jon-Paul Bingham,[4] Bruce G. Livett,[3] Ken R. Gayler[3] and Jonathan V. Sweedler[1]*

[1] Department of Chemistry and the Beckman Institute, University of Illinois, Urbana-Champaign, Illinois 61801, USA
[2] Department of Human Anatomy and Genetics, University of Oxford, Oxford OX1 3QX, UK
[3] Department of Biochemistry and Molecular Biology, University of Melbourne, Victoria 3010, Australia
[4] Department of Pharmacology, Yale School of Medicine, New Haven, Connecticut 06520, USA

Received 26 December 2003; Accepted 11 February 2004

A combination of cDNA cloning and detailed mass spectrometric analyses was employed to identify novel conotoxins from *Conus victoriae*. Eleven conotoxin sequences were determined using molecular methods: one belonging to the A superfamily (Vc1.1), six belonging to the O superfamily (Vc6.1–Vc6.6) and four members of the T superfamily (Vc5.1–Vc5.4). In order to verify the sequences and identify the post-translational modifications (excluding the disulfide connectivity) of three *Conus victoriae* conotoxins, vc1a, vc5a and vc6a, deduced from sequences Vc1.1, Vc5.1, and Vc6.1, respectively, liquid chromatography/electrospray ionization ion trap mass spectrometry, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and nanospray ionization ion trap mass spectrometry with collisionally induced dissociation were performed on reduced and alkylated venom fractions. We report that vc1a, the native form of α-conotoxin Vc1.1 (an unmodified 16 amino acid residue peptide that has notable pain-relieving capabilities), includes a hydroxyproline and a γ-carboxyglutamate residue. Conotoxin vc5a is a 10-residue peptide with two disulfide bonds and a hydroxyproline and vc6a is a 25 amino acid peptide with three disulfide bonds. **Copyright © 2004 John Wiley & Sons, Ltd.**

KEYWORDS: electrospray ionization mass spectrometry; matrix-assisted laser desorption/ionization mass spectrometry; conotoxin; cDNA sequencing; post-translational modification

INTRODUCTION

Cone shells are predatory marine snails that can subdue prey such as fish, worms and molluscs by injecting a peptide-rich venom.[1] These conopeptides, which include conotoxins, conantokins, contulakins and contryphans,[2] demonstrate excellent specificity and affinity for a wide range of voltage- and ligand-gated receptors and closely related subtypes,[1,3] resulting in immobilization of the prey.[1,4] Conotoxins, the subject of this paper, are small, disulfide-rich peptides that are classified into superfamilies (A, O, M, T,[5] I,[6] S,[7,8] P[7]) based on their highly conserved protein precursor sequences and cysteine frameworks. The peptides are further categorized into families according to the pharmacological target.[9] The α-conotoxins, for instance, antagonize nicotinic acetylcholine receptors, the ω-conotoxins inhibit voltage-sensitive calcium channels and the δ-conotoxins target voltage-sensitive sodium channels.[9] This notable specificity makes conotoxins attractive as both basic neuroscience research tools and potential pharmacological agents,[3,4,9–11] providing the impetus to characterize the full complement of peptides in each *Conus* species. However, only a small fraction of the putative toxins have been fully characterized, perhaps as few as 150 peptides. This number is low considering that there are over 600 *Conus* species,[3] each containing an estimated 50–200 bioactive peptide components in the venom.[12] The dearth of characterized toxins is partly due to the complex nature of conotoxin sequence determination, which is further complicated by the high degree of post-translational modification (PTM) that exists in the majority of these peptides.[4,13,14] In addition to extensive disulfide bonding, common PTMs include C-terminal amidation, γ-carboxylation of glutamate, sulfation of tyrosine, pyroglutamylation, formation of hydroxyproline residues, bromination of tryptophan and glycosylation of serine and threonine residues.[14–16]

Techniques employed to sequence conopeptides and define their modifications include mass spectrometric analyses,[13,14,16–22] molecular approaches based on cDNA sequencing[4–6,16,23–25] and conventional methods[4] such as Edman degradation and amino acid analysis. Conventional

*Correspondence to: Jonathan V. Sweedler, Department of Chemistry, University of Illinois, 600 S. Mathews Avenue, Urbana-Champaign, Illinois 61801, USA.
E-mail: sweedler@scs.uiuc.edu
Contract/grant sponsor: National Institutes of Health; Contract/grant number: PHS 5 R01 NS31609-11.
Contract/grant sponsor: Australian Research Council.

Copyright © 2004 John Wiley & Sons, Ltd.

methods using Edman degradation are time consuming, labor intensive, require large amounts of duct venom and also do not always provide a complete sequence.[19,24,26] Molecular methods utilizing cDNA libraries created from venom duct messenger RNA are a more efficient means of identifying novel conotoxins;[24] however, PTMs, and therefore the native conotoxin peptides, generally cannot be ascertained.[4,16] In contrast, the isolation of peptides directly from the venom duct followed by detailed mass spectrometric experiments, typically with molecular or biochemical techniques, can allow the characterization of the mature toxins including PTMs.[4,14,17,18] Since modifications have been shown to affect conotoxin function,[2,27–29] it is important that sequencing studies include the determination of the PTMs in order to realize fully the biological significance of each peptide.

We utilized molecular methods to determine novel conotoxin sequences and mass spectrometry to verify the sequences and characterize the PTMs. Using this approach, we identified 11 conotoxin sequences from *Conus victoriae* belonging to the A, O and T superfamilies based on polymerase chain reaction (PCR) amplification of a cDNA library. We then confirmed the sequence and established the type and site of post-translational modifications in three of these conotoxins, vc1a, vc5a and vc6a, using a combination of liquid chromatography and electrospray ionization ion trap mass spectrometry (LC ESI-MS), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) and nanospray ionization ion trap mass spectrometry (NSI-MS) with collisionally induced dissociation (CID). Importantly, this study defines the modified peptide sequence deduced from sequence Vc1.1, whose non-modified form is reported to be a neuronal nicotinic acetylcholine receptor antagonist that acts as a powerful analgesic.[25]

EXPERIMENTAL

Chemicals

Trifluoroacetic acid (TFA), glacial acetic acid, formic acid, dithiothreitol (DTT), 4-vinylpyridine (4-VP), guanidine HSCN and ethylenediaminetetraacetic acid (EDTA, disodium salt) were obtained from Sigma. Ammonium bicarbonate, HPLC-grade acetonitrile and methanol were obtained from Fisher. Milli-Q water was used for HPLC solvents and Burdick and Jackson (Muskegon, MI, USA) water was used for duct venom sample preparation and peptide trap rinsing. Bovine insulin and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Aldrich. Acidic peptide and α-bag cell peptide (*Aplysia californica* peptides) were obtained from American Peptide (Sunnyvale, CA, USA). Immobilized tris-(2-carboxyethyl)phosphine disulfide reducing gel (TCEP) was purchased from Pierce (Rockford, IL, USA).

Sample collection

Conus victoriae animals were collected from Broome, Western Australia. Venom ducts were dissected from the live snails and the venom was removed by squeezing the duct of its contents. The venom extract was air-dried and left as a solid until it was reconstituted in water prior to LC/MS analysis. The venom ducts and intact venom bulbs were snap frozen in liquid nitrogen in preparation for mRNA extraction.

cDNA library construction and gene sequencing

mRNA was extracted from the venom ducts using a Dynabeads mRNA direct kit (Dynal, Norway). Double-stranded cDNA was then created utilizing a Marathon cDNA amplification kit (Clontech), which relies on a moloney murine leukemia virus reverse transcriptase. To allow rapid amplification of cDNA ends (RACE), a partially double-stranded adaptor was ligated on to the cDNA. To amplify conotoxin sequences belonging to the A, O and T superfamilies, primers were utilized that specifically target the conserved 5′ regions of the conotoxin precursors and the 3′ untranslated regions. A superfamily members were amplified as described previously.[25] O superfamily members were amplified using O_1 (ATG AAA CTG ACG TG(C/T) (A/G)TG (A/G)TG ATC GT)[23,24] and the adaptor primer (CCA TCC TAA TAC GAC TCA CTA TAG GGC), using the following PCR conditions: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 62.5 °C for 30 s and 72 °C for 45 s, concluding with a final step of 72 °C for 5 min. T superfamily members were amplified using T_1 (TTC (A/G)TC ATT CTT CTG CTG G) with the adaptor primer, using the following PCR conditions: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 45 s, concluding with a final step of 72 °C for 5 min. PCR products were visualized on 1.5% agarose gels and then cloned into the pCR2.1 vector (Invitrogen), each ligation reaction containing an insert to vector ratio of 2:1. pCR2.1 plasmids were transformed into competent INVαF′ *E. coli* cells (Original TA Cloning® Kit) and recombinants selected by blue/white screening on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Plasmids containing inserts of the expected size (250 bp for the A superfamily, 500 bp for the O superfamily and 750 bp for the T superfamily) were sequenced by the dideoxy termination method using an ABI PRISM dye terminator kit on a Perkin-Elmer 377 sequencer.

Duct venom purification

Owing to the large quantity of duct venom, a number of aqueous extractions of the solid were performed, rather than using the whole sample in one injection. For each aqueous extraction of the duct venom solid, ∼400–600 µl of water were added to the solid. After vigorous sonication and vortex mixing, the sample was centrifuged at 9000 rpm for 5 min and the supernatant used for injection. The supernatant was loaded onto a peptide trap (in-line with the injection loop) and rinsed with water (∼150 µl) prior to the LC separation. Purification of the duct venom extract was performed using a microbore HPLC system (Magic 2002, Michrom BioResources, Auburn, CA, USA) equipped with a reversed-phase polymer column (150 × 2.1 mm i.d.) with 5 µm particles and 30 nm pore size (Vydac, Hesperia, CA, USA). The hydrophobicity of the polymeric medium is approximately ranked between a C_8 and C_{18} silica-based reverse-phase adsorbent. A gradient separation was

Copyright © 2004 John Wiley & Sons, Ltd.

performed at 100 μl min^{-1} using solvents A and B (A, 98% H_2O, 2% acetonitrile, 0.1% acetic acid and 0.02% TFA (v/v); B, 95% acetonitrile, 5% H_2O, 0.08% acetic acid and 0.014% TFA (v/v)). The solvent gradient began by increasing the B content from 5 to 20% over 5 min. A gradient up to 65% B was performed over the next 60 min, followed by a 5 min gradient from 65 to 85% B. To complete the 80 min gradient, the system was ramped to 5% B over 5 min and maintained at 5% B for 5 min. Detection was performed via a dual UV/visible detector set at 220 and 280 nm. Fractions were collected on an FC 203B fraction collector (Gilson, Middletown, WI, USA) and subjected to MALDI-MS.

Fractions containing vc1a and vc5a were subjected to a second-stage separation after concentration of the fractions using a Speed Vac (ThermoSavant, Holbrook, NY, USA). The samples were loaded onto a peptide trap, rinsed with water and injected onto a Vydac Pepmap column (C_{18}, 150 × 1 mm i.d., 3 μm particle diameter, 30 nm pore size) at a uniform flow-rate of 20 μl min^{-1} using the microbore HPLC system with solvents A, 98% H_2O, 1.2% methanol, 0.8% acetonitrile, 0.1% formic acid and 0.02% TFA (v/v) and B, 57% methanol, 38% acetonitrile, 5% H_2O, 0.08% acetic acid and 0.014% TFA (v/v). A gradient was developed from 15 to 30% B over 30 min, from 30 to 40% B over the next 10 min, followed by a ramp up to 65% over 15 min and completed with a 5 min gradient back to 5% B. Again, fractions were collected and subjected to MALDI-MS.

MALDI-MS

MALDI-MS was used in order to obtain mass information used for putative peptide matches based on predicted cDNA sequences. A 0.3 μl aliquot of each fraction was spotted onto a gold-plated target along with an equal volume of a matrix (15 mg CHCA, 600 μl acetonitrile, 400 μl water and 3 μl TFA). Positive-ion mass spectra were acquired using the linear mode on a Voyager DE STR time-of-flight mass spectrometer (PE Biosystems, Framingham, MA, USA) equipped with delayed ion extraction. A pulsed nitrogen laser (337 nm) was used as the desorption/ionization source. External mass calibration was performed using a peptide standard mixture consisting of 60 pmol μl^{-1} of α-bag cell peptide (1–9), 120 pmol μl^{-1} of acidic peptide and 120 pmol μl^{-1} of bovine insulin.

LC/ESI-MS

Of 600 μl, a 30 μl aliquot of duct venom extract was injected onto the microbore-LC system for LC/ESI-MS. The same method as described above was used for the LC separation, except that the LC was connected to the ESI probe on the LCQ Deca Ion Trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). A spray voltage of 4.3 kV, a capillary temperature of 220 °C, a capillary voltage of 21 V and a tube lens offset of 10 V were employed. A triple play (full scan, ZoomScan, CID with 35% collisional energy) data-dependent acquisition method with dynamic exclusion was performed using the Xcalibur software. Spectra were processed using the base peak ion chromatogram and a seven-point Gaussian smoothing function. The multiply charged peaks detected by the ESI-MS experiments were manually deconvoluted based on the high-resolution mass scans (ZoomScan, which provided charge state information) and were consistent with masses obtained by MALDI-MS.

Reduction–alkylation procedure

A reduction–alkylation procedure was optimized for each individual peptide. For vc5a, a purified duct venom fraction was concentrated using the Speed Vac. A solution containing 6 M guanidine HSCN, 0.1 M ammonium bicarbonate and 10 μM EDTA (500 μl) was added to the sample. In a separate vial, 1000 μl of immobilized TCEP gel were mixed with 1000 μl of 20 mM EDTA. The TCEP vial was vortexed and centrifuged at 4800 rpm for 2 min. The supernatant was removed and discarded. Another 1000 μl of 20 mM EDTA were added to the gel and the rinse was repeated. The sample was then added to the gel and the vial vortexed on a low setting for 15 min. After this time, the sample vial was centrifuged at 4800 rpm for 2 min. The supernatant was removed and placed in a separate vial. A 500 μl aliquot of 0.05 M ammonium bicarbonate was added to the TCEP gel and the vial was vortexed and centrifuged for 2 min at 4800 rpm. This supernatant was added to the same vial. Room-temperature 4-VP (20 μl) was added to the sample vial, nitrogen was blown over the vial and then the sample was covered in foil and placed in a 45 °C air bath (using the Speed Vac in preheat mode without running the vacuum) for 2 h. The reduced and alkylated sample was then purified using the Vydac reversed-phase polymer column and the second-stage separation solvent system. The sample was loaded onto the peptide trap and rinsed with 250 μl of water and then injected onto the column at a uniform flow-rate of 100 μl min^{-1}. After running at 1% B for 10 min, a gradient was developed up to 5% B over 10 min, up to 10% B over the next 5 min, from 10 to 15% B for 10 min, from 15 to 25% B over 30 min and finally ramped up to 80% B for 10 min. Fractions were screened by MALDI-MS as described above after concentrating each to ∼20 μl in the Speed Vac.

For vc1a and vc6a, pure, concentrated fractions were individually subjected to the same reduction–alkylation procedure, modified from a published method.[30] A 230 μl aliquot of the same guanidine mixture as described above was added to the sample, along with 20 μl of 20 mM EDTA and 10 μl of 0.4 M DTT. This mixture was vortex mixed on a low setting for 20 min. After the addition of 10 μl of 4-VP, the sample was wrapped in foil and placed in an air bath maintained at ∼45 °C for 20 min. The same solvent and column system as used for the first-stage duct venom separation was employed to purify reduced and alkylated vc1a and vc6a. The gradient was optimized in each case to purify the peptides appropriately. For vc1a, after a 15 min isocratic separation at 1% B, a gradient was developed up to 8% B over 15 min, followed by a gradient up to 18% B over 40 min and then a ramp up to 80% B over 10 min. For vc6a, after running at 1% B for 10 min, a gradient was developed up to 5% B over 10 min, up to 15% B over 10 min, from 15 to 25% B over 10 min, from 25 to 35% over 30 min and finally ramped up to 80% B for 10 min. In all cases, LC separations incorporating lengthy shallow-gradient rinse procedures were required in order to remove high concentrations of

Copyright © 2004 John Wiley & Sons, Ltd.

guanidine HSCN. Fractions were screened by MALDI-MS as described above after concentrating each to about 20 μl in the Speed Vac.

NSI-MS with CID

Once the fractions of interest had been determined, 3–4 μl aliquots of each fraction were individually loaded into static PicoTips (2 μm tip diameter, New Objective, Woburn, MA, USA). For vc1a and vc6a, a spray voltage of 1.6 kV and an auxiliary gas flow of 5 units were employed. For vc5a, a spray voltage of 2.0 kV and an auxiliary gas flow of 10 units were employed. CID was performed on several charge states and various modified forms of each of the toxins in order to complete the sequence verification. The sequence summaries are based on a combination of CID experiments; however, only one CID spectrum for each peptide is presented. Collisional energies (CE) of 35–180% were used in order to optimize fragmentation. Fragment masses were matched with predicted fragmentation patterns of the sequence (with a mass tolerance of ±0.3 u) determined using Protein Prospector.[31]

Nomenclature

For this study, we employ nomenclature based on ion channel naming, as reported previously.[5] Briefly, sequences obtained from clones are abbreviated as follows: two letters representing non-fish hunting species (Vc, for *Conus victoriae*), a number designating the disulfide framework (for C–C–CC–C–C, this number is 6), and another number indicating the order of discovery, separated by a decimal. Peptides isolated from the venom are labeled with lower-case letters and arabic numerals, such that sequence Vc6.1 corresponds to peptide vc6a. Once the target has been determined, the appropriate Greek letter is placed before the name, the first letter is capitalized, the disulfide designation is given as a roman numeral and the order of discovery is represented by a capital letter (when there are multiple peptides). Thus, if vc6a is determined to be an ω-conotoxin, it would be named ω-VcVI. However, synthetic peptide Vc1.1 (deduced from the gene sequence) is known to target neuronal nicotinic acetylcholine receptors and is therefore designated α-conotoxin Vc1.1, as reported previously.[25] The sequence of the venom-isolated peptide, vc1a, as determined by MS has the same amino acid sequence as synthetic α-conotoxin Vc1.1, but additionally includes PTMs.

RESULTS AND DISCUSSION

Molecular cloning

We obtained two specimens of *Conus victoriae*, removed the venom ducts and extracted mRNA. This sample was then used to construct a cDNA library to which adaptors were attached. Putative conotoxins belonging to the A, O and T superfamilies were identified by PCR amplification of this cDNA library using primers targeted to the conserved prepro regions of the conotoxin precursors. Cloning and sequencing of the PCR products resulted in the identification of 11 conotoxin sequences: one belonging to the A superfamily, six belonging to the O superfamily and four belonging to the T superfamily. Table 1 shows the translated precursor sequences for these peptides. In accordance with current nomenclature,[5] the sequences are named Vc1.1, Vc5.1, Vc5.2, Vc5.3, Vc5.4, Vc6.1, Vc6.2, Vc6.3, Vc6.4, Vc6.5 and Vc6.6. The cloning of Vc1.1 has been reported previously, resulting in a sequence of GCCSDPRCNYDHPEIC-amide,[25] while the T and O superfamily toxins are novel. Consistent with a number of other studies,[24,32,33] we observe significant homology in the prepro regions of the superfamilies, while the predicted toxin regions are hypervariable within a conserved cysteine framework. Vc6.1 to Vc6.6 (O superfamily) have six cysteine residues in a C–C–CC–C–C arrangement and the smaller T superfamily members, Vc5.1 to Vc5.4, have four cysteine residues in a CC–CC arrangement. Notably, Vc6.4, with the exception of a single residue, is identical with ω-PnVIB, a calcium channel antagonist isolated from the molluscivorous *Conus pennaceus*.[34]

Table 1. Conotoxin precursor sequences belonging to the A, O and T superfamilies determined by cDNA cloning[a]

A superfamily

Vc1.1 MGMRMMFTVFLLVVLATTVVSSTSGRREFRGRNAAAKASDLVSLTDKKRG**CC**SDPR**C**NYDHPEI**C**G

O Superfamily

Vc6.1 **MKLTC**VV**IVAVLFLTANTF**A**TA**DDPRNGLENLFLKAHHEMNPEASKLNER. . . .**C**LSGGEV**C**DFLFPK.**CC**NY.**C**ILLF**C**S

Vc6.2 **MKLTC**MM**IVAVLFLTANTF**V**TA**DDSGNGMENLFPKAGHEMENLEASNR . . .GKP**C**HEEGQL**C**DPFLQN.**CC**LGWN**C**VFV**C**I

Vc6.3 **MKLTC**VM**IVAVLFLTANTF**A**TA**DDPRNGLRDLFSIAHHEMKNPEASKLNEK . . .**C**YGFGEA**C**LVLYTD.**CC**GY.**C**VLAV**C**L

Vc6.4 **MKLTC**VM**IVAVLFLTANTF**V**TA**VPHSSNVLENLYLKARHEMENPEASKLNTRYD**C**EPPGNF**C**GMIKVGPP**CC**SGW.**C**FFA**C**A

Vc6.5 **MKLTC**MV**IVAVLFLTANTF**V**TA**DDSGNGLENLFSKAHHEIKNPEASNLNKR . . .**C**IPFLHP**C**TFFFPD.**CC**NSI.**C**AQFI**C**L

Vc6.6 **MKLTC**MV**IVAVLFLTANTF**V**TA**VPHSSNALENLYLKAHHEMNNPKDSELNKR.**C**YDGGTG**C**DSGNQ. . .**CC**SGW.**C**IFV**C**L

T superfamily

Vc5.1 **VILLL**ITSTPSVDARLKAKDNMPLASFHDNAKRTLQTRLINTR.**CC**PGKP.**CC**RIG

Vc5.2 **VILLL**LIASIPSDAVQLKTKDDMPLASFHGNARRTLQMLSNKRI**CC**YPNEW**CC**D

Vc5.3 **VILLL**LTASAPSVDARPKTEDVPLSSFRDNTKSTLQRLLKR.VN**CC**GIDES**CC**S

Vc5.4 **VILLL**LIASAPSVDAQPKTKDDVPLAPLHDNAKSALQHLNQR.**CC**QTFYW**CC**GQGK

[a] Conserved amino acids within each superfamily are in bold. Underlined residues represent the cleavage boundary of the prepro region and the mature peptide.

Copyright © 2004 John Wiley & Sons, Ltd.

JMS

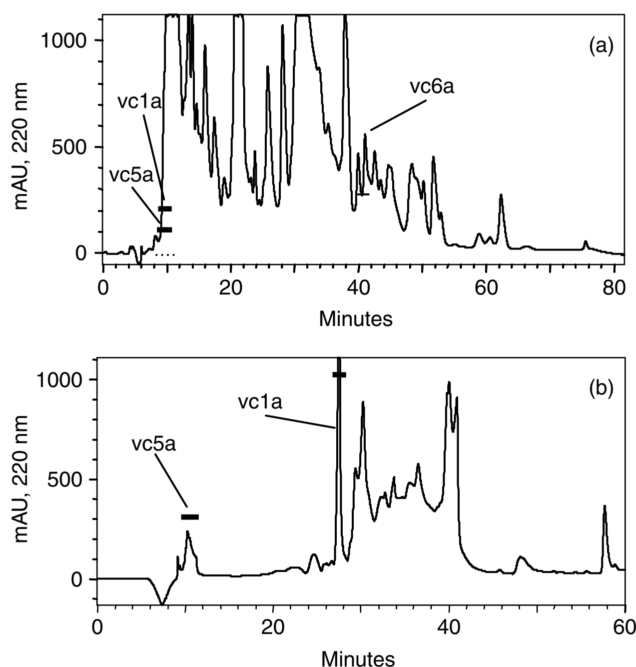

Figure 1. Venom duct purification. Liquid chromatograms showing (a) the first-stage separation of the duct venom and (b) the second-stage separation of fractions (indicated by a dotted line, ~8–12 min) collected from the first-stage separation. Conotoxins identified by MS in this study are labeled by name.

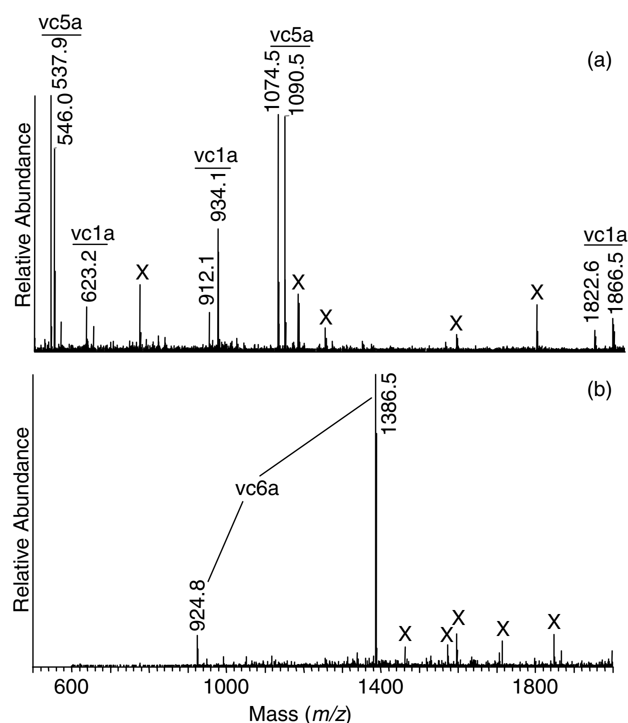

Figure 2. Using ESI-MS to assess post-translational modifications. Full mass spectra demonstrating post-translational modifications for (a) vc1a and vc5a and (b) vc6a. Predicted charge states of each form are given in Table 2. X = co-eluting, unrelated peptide.

Table 2. Predicted charge state masses of vc1a, vc5a and vc6a[a]

Pentide	M + H	+2 charge	+3 charge
vc1a (minus Gla)	1822.6	911.8	608.2
vc1a	1866.7	933.8	622.9
vc5a (minus Hyp)	1074.4	537.7	n/a
vc5a	1090.4	545.7	n/a
vc6a	2771.2	1186.1	924.4

[a] Shaded masses were observed in Fig. 2. Masses are monoisotopic.

Duct venom purification and peptide screening

In order to determine putative fractions containing the cDNA-predicted toxins, duct venom was subjected to an LC separation followed by MS to create a mass profile of the duct conotoxins. Figure 1(a) demonstrates the complexity of the duct venom, which contained at least 100 putative peptides as determined by ESI-MS and MALDI-MS. These two complementary MS techniques were employed in order to ascertain the peptide masses with confidence and to maximize the information on PTMs. Putative peptide masses were calculated based on the cDNA sequences and potential modifications, and these masses were used to screen for matches with the MS data. Due to the number of potentially modified sites (such as proline and glutamate residues) and the uncertainty of the exact N- and C-termini in some cases, there was an extensive list of predicted masses. Putative peptides vc1a, vc5a and vc6a, corresponding to the deduced peptide sequences Vc1.1, Vc5.1 and Vc6.1, respectively, were observed as noted on the chromatograms in Fig. 1.

Post-translational modification determination

Analysis of the MS data enabled putative PTMs in the mature form of the peptides to be assessed, as illustrated by the ESI-MS data in Fig. 2. Table 2 presents the predicted masses of the three modified peptides where the shaded entries represent the charge states that were observed in Fig. 2. A distribution of the different modified forms of each toxin was observed as a result of the labile modifications being partially removed by the MS process and also because native venom duct material probably contains a collection of partially modified products. The mature form of each peptide is taken to be the most modified form observed. These preliminary MS data were coupled with a mass analysis based on the cDNA sequences, knowing the amino acids (such as proline and glutamate) that are commonly modified in conotoxins, to achieve the final PTM assignments. Thus, vc1a is modified by amidation (as reported previously[25]), a hydroxyproline residue and a γ-carboxyglutamate residue (Fig. 2(a)). Conotoxin vc5a is amidated with one hydroxyproline residue (Fig. 2(a)). Amidation was also predicted based on the presence of a C-terminal glycine in the cDNA sequences of vc1a and vc5a,[25,35] supporting the MS observations. Peptide vc6a, despite having several potential modification sites, is unmodified except for disulfide bond formation (Fig. 2(b)). The observed heterogeneity of the PTMs on these peptides demonstrates a combinatorial approach to peptide design whereby PTMs rapidly diversify the conotoxin repertoire without re-inventing toxin genes.[10,12]

Copyright © 2004 John Wiley & Sons, Ltd.

JMS

Sequence verification using CID

As mass matches alone do not confirm the conotoxin sequences, CID data from the ESI-MS triple play were analyzed with the aim of verifying the sequences and elucidating the residues that undergo PTM. The initial CID spectra demonstrated that the highly constrained, untreated peptides were not amenable to collisional fragmentation.[16,19,36] In the presence of intact disulfide linkages, fragmentation is minimized since the production of fragment ions requires two cleavages, one at the disulfide bond and another at the peptide bond.[36] Figure 3 shows a CID spectrum from the experiment, corresponding to vc1a with a hydroxyproline and a γ-carboxyglutamate residue. The carboxylate group was labile under the low-energy collisions performed in the ion trap, while little fragmentation of the peptide backbone occurred. This simplified spectrum certainly aided in the assignment of PTMs; however, the fragmentation was insufficient for sequence verification. Similar spectra were observed for other *Conus victoriae* peptides (data not shown), demonstrating that an essential part of the sequence elucidation was to develop a method to reduce and alkylate the disulfide bonds.[13,16]

The reduction–alkylation process was found to be peptide-specific such that several methods were tested to establish an effective method for alkylating each individual peptide. The success of the procedure was determined based on the mass shifts observed in the MALDI-MS of the fractions (data not shown). Mass shifts of +424 and +637 Da were observed for two disulfide-bond-containing (vc1a and vc5a) and three disulfide-bond-containing peptides (vc6a), respectively (based on reduction of each disulfide and addition of ethylpyridine, 105.1 Da, to each free thiol). Therefore, reduction–alkylation not only is necessary for CID analysis but also provides information on the number of cysteine residues.

NSI-MS with CID was then performed on the purified, reduced and alkylated peptides in order to verify the amino acid sequences and establish the sites of the modifications.

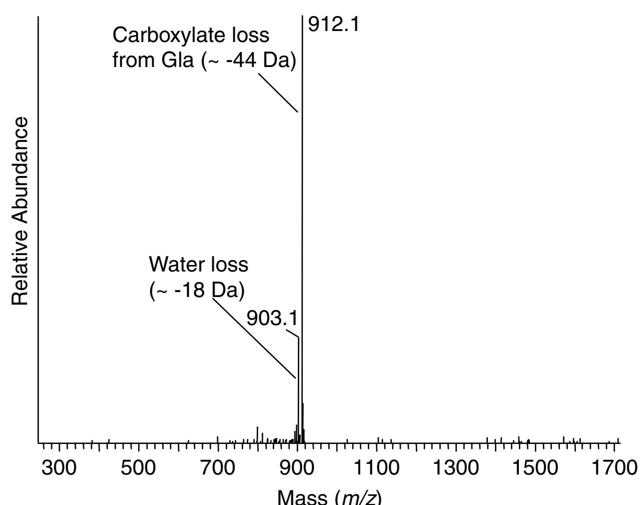

Figure 3. ESI-MS with CID of vc1a before reduction-alkylation. CID data of the *m/z* 934.2 ion (vc1a, +2) from an LC/ESI-MS experiment demonstrating minimal fragmentation (except for decarboxylation and water loss) with intact disulfide bonds.

Table 1 shows that sequence Vc1.1 has a single glutamate residue, and Fig. 2 demonstrates a loss of 44 Da from vc1a that is indicative of γ-carboxyglutamate. Thus, the presence of γ-carboxyglutamate at residue 14 of peptide vc1a is confirmed. Additionally, sequences Vc1.1 and Vc5.1 each have two proline residues; however, the MS results given in Fig. 2 demonstrate that only one proline is modified in peptides vc1a and vc5a. Therefore, using the ability of the ion trap to isolate specific masses, CID was performed on a number of different modified forms of the peptides in order to deduce the site of modification.[37] Figure 4(a), (b) and (c) show the results of NSI-MS with CID of vc1a with one hydroxyproline, vc5a with one hydroxyproline and vc6a without modifications except for disulfide bonds, respectively. Figure 4, in comparison with Fig. 3, illustrates the dramatic increase in fragmentation after reduction and alkylation of the disulfide bonds. Observed fragment ions are labeled based on mass matches to predicted ions (b- and y-ions) by Protein Prospector.[31] For vc1a and vc5a, the data were further analyzed for matches to predicted fragments of both potential hydroxyproline forms. The boxed ions in Fig. 4(a) and (b) were used to determine the modification site (i.e. the boxed ions would not have been identified if the modification were on the alternate proline residue.) Thus, the abundance and number of boxed ions provide strong support for the site of the hydroxyprolines. For both vc1a and vc5a, the hydroxyproline is the sixth residue of the sequence. The insets in Fig. 4(a)–(c) summarize the results of the sequence verification, where detected b- and y-ions are indicated by the numbers above the brackets.

Although several b- and y-ions could be identified, Fig. 4 demonstrates the added complexity due to ethylpyridine losses, as noted by several other researchers.[13,17,19,38] In all cases, these ions were the most intense, unfortunately complicating the detection of important peptide backbone fragments. In order to minimize this detrimental effect, the collisional energy was optimized for each experiment and is noted in Fig. 4. Increased collisional energies generally resulted in greater backbone fragmentation; however, when high collisional energy was used, there was a decline in the mass accuracy such that ions could not be identified within the 0.3 u tolerance. Furthermore, when using greater collisional energies, the data had to be averaged for several more minutes in order to obtain high-quality CID spectra. Hence there is a tradeoff of fragmentation efficiency with time and mass accuracy.

Additionally, the abundance of certain b- and y-ions is notable and conforms to known cleavage rules.[39–41] In particular, ions corresponding to cleavage N-terminal to proline (and also hydroxyproline) were consistently more abundant than other b- and y-ions, supporting statistical reports that demonstrate enhanced fragmentation N-terminal to proline.[40,41] For instance, in Fig. 4(a), the highly abundant b_5, b_{12}, y_4 and y_{11} ions result from cleavage N-terminal to proline or hydroxyproline. Furthermore, these ions represent D–P and H–P cleavages, following the statistical observation that D–P and H–P cleavages are among the most likely N-terminal proline fragmentations.[40,41] Similarly,

Copyright © 2004 John Wiley & Sons, Ltd.

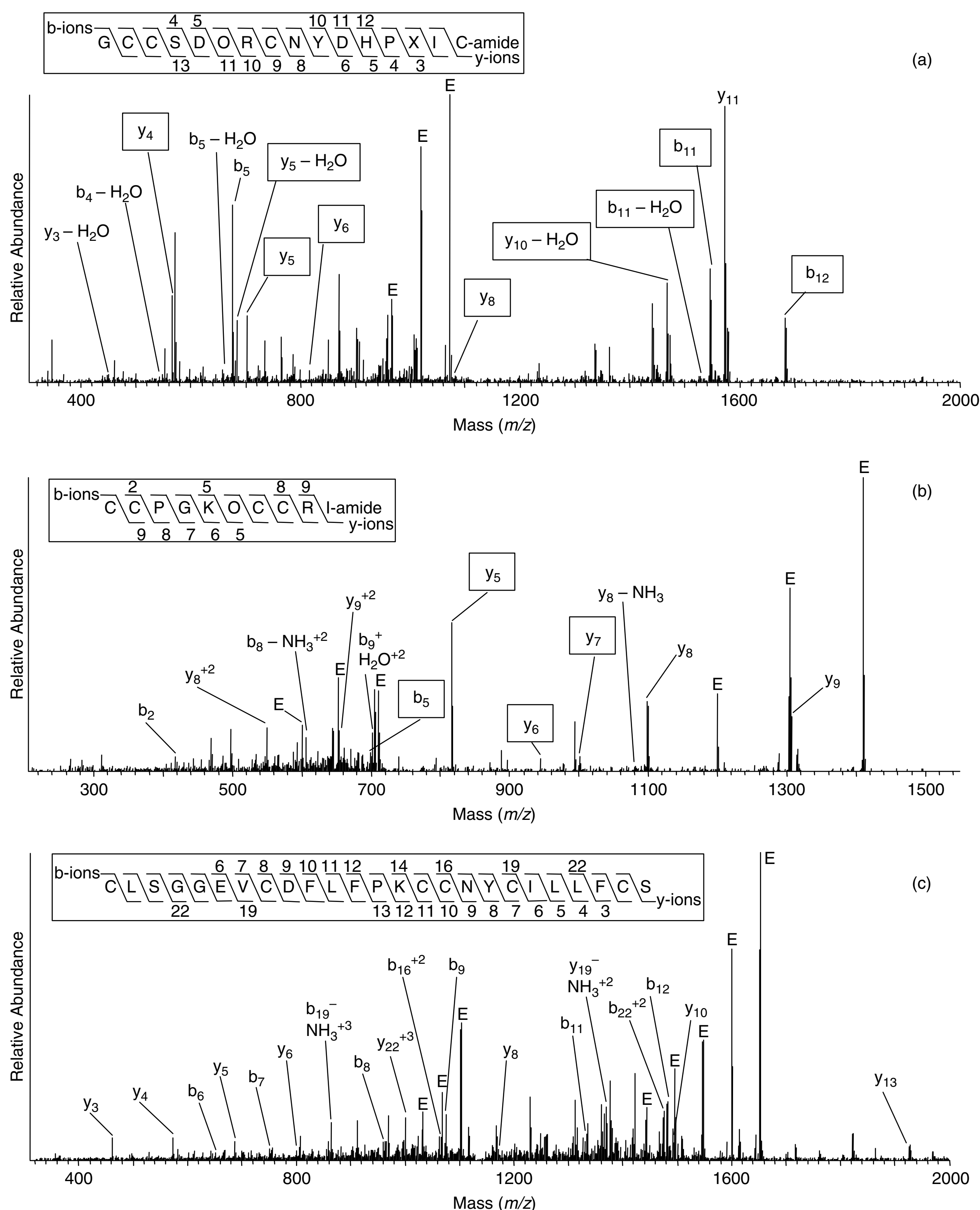

Figure 4. Sequence verification and determination of PTM sites. NSI-MS with CID on pure fractions of reduced and alkylated (a) vc1a without Gla ($m/z = 1125.0$, +2, CE = 60%), (b) vc5a without Gla ($m/z = 757.9$, +2, CE = 120%) and (c) vc6a ($m/z = 1137.7$, +3, CE = 60%). Peaks are labeled with b/y-ions based on mass matches to predicted fragments. For vc1a and vc5a, boxed ions are those that were used to determine the site of the hydroxyproline. E = ethylpyridine losses. The inset in each part shows a ladder diagram with the b/y-ion numbers labeled that were determined in all CID experiments (including additional data not shown). X = γ-carboxyglutamate; O = hydroxyproline.

the abundant y_5 and y_8 ions and the moderately abundant b_{12} and y_{13} ions given in Fig. 4(b) (vc5a) and 4(c) (vc6a), respectively, demonstrate the favored cleavage N-terminal to proline (or hydroxyproline) residues. Importantly, observing preferential fragmentation adds another dimension to the sequence verification of the peptides; rather than relying solely on mass matches to predicted fragments, the abundances of the ions can also be used to support the site of proline residues in the sequence.

The three sequenced peptides vc1a, vc5a and vc6a from *Conus victoriae* demonstrate significant homology to known conotoxins from other *Conus* species, as shown in Table 3.

Copyright © 2004 John Wiley & Sons, Ltd.

Table 3. Conotoxin homology: conotoxins exhibiting the most similarity to vc1a, vc5a and vc6a[a]

Conotoxin	Species	Prey	Sequence	Reference
vc1a	***C. victoriae***	Molluscs	– G – C C S D O R C N Y D H P X I C*	This work, 5, 49
α-EpI	*C. episcopatus*	Molluscs	– G – C C S D P R C N M N N P D Y^ C*	44
α-PnIA	*C. pennaceus*	Molluscs	– G – C C S L P P C A A N N P D Y^ C*	18
α-PnIB	*C. pennaceus*	Molluscs	– G – C C S L P P C A L S N P D Y^ C*	18
α-MII	*C. magus*	Fish	– G R C C S N P V C H L E H S N L C*	42
α-PIA	*C. purpurascens*	Fish	R D P C C S N P V C T V H N P Q I C*	45
α-ImI	*C. imperialis*	Worms	– G – C C S D P R C A W R – – – – C*	43
vc5a	***C. victoriae***	Molluscs	– C C P G K O – C C R I*	This work
p5a	*C. purpurascens*	Fish	G C C P K Q M R C C T L*	5
vc6a	***C. victoriae***	Molluscs	C L S G G E V C D F L F P K C C N – Y C I L L F – – C S	This work
TxKK2	*C. textile*	Molluscs	C A P F L H P C T F F F P N C C N S Y C V Q – F I – C L	33
ω-TVIA	*C. tulipa*	Fish	C L S O G S S C S O T S Y N C C R S – C N O Y S R K C	47
ω-GVIIA	*C. geographus*	Fish	C K S O G T O C S R G M R D C C T S – C L L Y S N K C R R Y	48
ω-GVIIB	*C. geographus*	Fish	C K S O G T O C S R G M R D C C T S – C L S Y S N K C R R Y	48
ω-TxVII	*C. textile*	Molluscs	C K Q A D E P C D V F S L D C C T G I C L G V – – – C M V	26

[a] Conserved amino acids are shaded. X = γ-carboxyglutamate, O = hydroxyproline,
* = amidated C-terminus and Y^ = sulfated tyrosine.

Copyright © 2004 John Wiley & Sons, Ltd.

Peptide vc1a is similar to α-EpI, α-PnIA, α-PnIB, α-MII, α-PIA and α-ImI, all of which are known to antagonize the neuronal nicotinic acetylcholine receptors.[18,42–45] Peptide vc5a, which has the cysteine framework of the recently discovered T superfamily, is most similar to the uncharacterized p5a from *Conus purpurascens*.[5] Peptide vc6a is most homologous to the King Kong 2 peptide from *Conus textile*,[33] whose target remains undetermined, and the calcium channel antagonist ω-TVIA.[46,47] Omega-conotoxins GVIIA, GVIIB[48] and TxVII[26] are also similar to vc6a.

Using a combination of molecular approaches and MS, we have defined putative amino acid sequences for 10 novel *Conus victoriae* conotoxins and also verified the sequence and identified the site and type of PTMs of three peptides, vc1a, vc5a and vc6a, as shown in Fig. 4. We have determined that vc1a, in addition to being amidated at the C-terminus,[25] has a hydroxyproline at residue 6 and a γ-carboxyglutamate at residue 14. Defining the PTMs of venom-isolated peptide vc1a is important because the unmodified synthetic peptide Vc1.1, whose sequence was deduced from the cloned cDNA sequence, has been shown to antagonize mammalian neuronal nicotinic acetylcholine receptors and was found to be a potent analgesic in a rat model of neuropathic pain.[25,49] Interestingly, there are studies demonstrating that post-translationally modified conotoxins are more potent than the respective unmodified forms.[2,27,29] Craig and co-workers have reported that the glycosylated forms of conotoxin κA-SIVA[27] and contulakin-G[2] are more potent. Similarly, McIntosh *et al*. have shown that γ-decarboxylation inactivates conantokin G.[29]

In order to assess the effect of PTMs on bioactivity, a peptide with the sequence of vc1a, which incorporates the modified amino acids, has been synthesized.[49] Unlike the reported synthetic α-conotoxin Vc1.1 peptide, synthetic vc1a does not inhibit the neuronal-type nicotinic receptor response in primary monolayer cultures of bovine chromaffin cells, nor does it act as an analgesic *in vivo* in two rat models of neuropathic pain.[49] However, we are unable to state if this observed difference in biological activity of the two synthetic peptides is due to altered disulfide bonding, altered folding during oxidation or the presence of PTMs alone. Disulfide connectivity can have an unpredictable impact on the biological function of α-conotoxins, as demonstrated by a report showing that a non-native disulfide bond isomer of α-AuIB was 10 times more potent than the native form.[28] Furthermore, the ion channel properties can vary depending on the host cell system in which the channel subunits are expressed, adding another level of complexity to understanding the specific function of conotoxins.[50–52] Given that *Conus victoriae* is a molluscivorous species,[53] it would be informative to explore the affinity of the native vc1a peptide and its non-modified forms, including different disulfide isomers, for invertebrate nicotinic acetylcholine receptors.

Acknowledgements

This research was supported by the National Institutes of Health (grant PHS 5 R01 NS31609-11) and the Australian Research Council (K.R.G., B.G.L.). This work was also supported in part by a fellowship from Merck Research Laboratories. We thank Michael Ewing for creating an Excel macro that aided in CID data analysis.

REFERENCES

1. 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.
2. Craig AG, Norberg T, Griffin D, Hoeger C, Akhtar M, Schmidt K, Low W, Dykert J, Richelson E, Navarro V, Mazella J, Watkins M, Hillyard D, Imperial J, Cruz LJ, Olivera BM. Contulakin-G, an *O*-glycosylated invertebrate neurotensin. *J. Biol. Chem.* 1999; **274**: 13 752.
3. Le Gall F, Favreau P, Richard G, Benoit E, Letourneux Y, Molgo J. Biodiversity of the genus *Conus* (Fleming, 1822): a rich source of bioactive peptides. *Belg. J. Zool.* 1999; **129**: 17.
4. Adams DJ, Alewood PF, Craik DJ, Drinkwater RD, Lewis RJ. Conotoxins and their potential pharmaceutical applications. *Drug Dev. Res.* 1999; **46**: 219.
5. Walker CS, Steel D, Jacobsen RB, Lirazan MB, Cruz LJ, Hooper D, Shetty R, DelaCruz RC, Nielsen JS, Zhou LM, Bandyopadhyay P, Craig AG, Olivera BM. The T-superfamily of conotoxins. *J. Biol. Chem.* 1999; **274**: 30 664.
6. Jimenez EC, Shetty RP, Lirazan M, Rivier J, Walker C, Abogadie FC, Yoshikami D, Cruz LJ, Olivera BM. Novel excitatory *Conus* peptides define a new conotoxin superfamily. *J. Neurochem.* 2003; **85**: 610.
7. Lirazan MB, Hooper D, Corpuz GP, Ramilo CA, Bandyopadhyay P, Cruz LJ, Olivera BM. The spasmodic peptide defines a new conotoxin superfamily. *Biochemistry* 2000; **39**: 1583.
8. England LJ, Imperial J, Jacobsen R, Craig AG, Gulyas J, Akhtar M, Rivier J, Julius D, Olivera BM. Inactivation of a serotonin-gated ion channel by a polypeptide toxin from marine snails. *Science* 1998; **281**: 575.
9. Jones RM, Bulaj G. Conotoxins—new vistas for peptide therapeutics. *Curr Pharm. Des.* 2000; **6**: 1249.
10. Myers RA, Cruz LJ, Rivier JE, Olivera BM. *Conus* peptides as chemical probes for receptors and ion channels. *Chem. Rev.* 1993; **93**: 1923.
11. Shen GS, Layer RT, McCabe RT. Conopeptides: from deadly venoms to novel therapeutics. *Drug Discov. Today* 2000; **5**: 98.
12. Olivera BM. E.E. Just Lecture, 1996—*Conus* venom peptides, receptor and ion channel targets, and drug design—50 million years of neuropharmacology. *Mol. Biol. Cell* 1997; **8**: 2101.
13. Krishnamurthy T, Prabhakaran M, Long SR. Mass spectrometric investigations on *Conus* peptides. *Toxicon* 1996; **34**: 1345.
14. Nakamura T, Yu ZG, Fainzilber M, Burlingame AL. Mass spectrometric-based revision of the structure of a cysteine-rich peptide toxin with gamma-carboxyglutamic acid, txviia, from the sea snail, *Conus textile. Protein Sci.* 1996; **5**: 524.
15. Craig AG, Bandyopadhyay P, Olivera BM. Post-translationally modified neuropeptides from *Conus* venoms. *Eur. J. Biochem.* 1999; **264**: 271.
16. Craig AG. The characterization of conotoxins. *J. Toxicol. Toxin Rev.* 2000; **19**: 53.
17. Kalume DE, Stenflo J, Czerwiec E, Hambe B, Furie BC, Furie B, Roepstorff P. Structure determination of two conotoxins from *Conus textile* by a combination of matrix-assisted laser desorption/ionization time-of-flight and electrospray ionization mass spectrometry and biochemical methods. *J. Mass Spectrom.* 2000; **35**: 145.
18. Wolfender JL, Chu FX, Ball H, Wolfender F, Fainzilber M, Baldwin MA, Burlingame AL. Identification of tyrosine sulfation in *Conus pennaceus* conotoxins alpha-PnIA and alpha-PnIB: further investigation of labile sulfo- and phosphopeptides by electrospray, matrix-assisted laser desorption/ionization (MALDI) and atmospheric pressure MALDI mass spectrometry. *J. Mass Spectrom.* 1999; **34**: 447.
19. Fainzilber M, Nakamura T, Gaathon A, Lodder JC, Kits KS, Burlingame AL, Zlotkin E. A new cysteine framework in sodium channel blocking conotoxins. *Biochemistry* 1995; **34**: 8649.
20. Wolfender JL, Chu F, Ball H, Wolfender F, Fainzilber M, Baldwin MA, Burlingame AL. Combination of mass spectrometric methods for the identification of novel conotoxins

Copyright © 2004 John Wiley & Sons, Ltd.

in the venom of the molluscivorous gastropod *Conus pennaceus*. *FASEB J.* 1999; **13**: A1408.
21. Rigby AC, Lucas-Meunier E, Kalume DE, Czerwiec E, Hambe B, Dahlqvist I, Fossier P, Baux G, Roepstorff P, Baleja JD, Furie BC, Furie B, Stenflo J. A conotoxin from *Conus textile* with unusual posttranslational modifications reduces presynaptic Ca2+ influx. *Proc. Natl. Acad. Sci. USA* 1999; **96**: 5758.
22. Craig AG, Fischer WH, Rivier JE, McIntosh JM, Gray WR. In *Techniques in Protein Chemistry VI*, Crabb JW (ed). Academic Press: San Diego, 1995; 31.
23. Lu BS, Yu F, Wang JH, Zhao SQ, Zhao D, Dai QY, Huang PT, Huang CF. New O-superfamily conotoxins from *Conus striatus* inhabited near Chinese Hainan Island. *Chin. Sci. Bull.* 2000; **45**: 432.
24. Lu BS, Yu F, Zhao D, Huang PT, Huang CF. Conopeptides from *Conus striatus* and *Conus textile* by cDNA cloning. *Peptides* 1999; **20**: 1139.
25. Sandall DW, Satkunanathan N, Keays DA, Polidano MA, Liping X, Pham V, Down JG, Khalil Z, Livett BG, Gayler KR. A novel alpha-conotoxin identified by gene sequencing is active in suppressing the vascular response to selective stimulation of sensory nerves in vivo. *Biochemistry* 2003; **42**: 6904.
26. Fainzilber M, Lodder JC, Vanderschors RC, Li KW, Yu ZH, Burlingame AL, Geraerts WPM, Kits KS. A novel hydrophobic omega-conotoxin blocks molluscan dihydropyridine-sensitive calcium channel. *Biochemistry* 1996; **35**: 8748.
27. Craig AG, Zafaralla G, Cruz LJ, Santos AD, Hillyard DR, Dykert J, Rivier JE, Gray WR, Imperial J, Delacruz RG, Sporning A, Terlau H, West PJ, Yoshikami D, Olivera BM. An O-glycosylated neuroexcitatory *Conus* peptide. *Biochemistry* 1998; **37**: 16 019.
28. Dutton JL, Bansal PS, Hogg RC, Adams DJ, Alewood PF, Craik DJ. A new level of conotoxin diversity, a non-native disulfide bond connectivity in alpha-conotoxin AuIB reduces structural definition but increases biological activity. *J. Biol. Chem.* 2002; **277**: 48 849.
29. McIntosh JM, Olivera BM, Cruz LJ, Gray WR. Gamma-carboxyglutamate in a neuroactive toxin. *J. Biol. Chem.* 1984; **259**: 14 343.
30. Lundell N, Schreitmuller T. Sample preparation for peptide mapping—a pharmaceutical quality-control perspective. *Anal. Biochem.* 1999; **266**: 31.
31. Clauser KR, Baker P, Burlingame AL. Role of accurate mass measurement (± 10 ppm) in protein identification strategies employing MS or MS MS and database searching. *Anal. Chem.* 1999; **71**: 2871.
32. McIntosh JM, Corpuz GO, Layer RT, Garrett JE, Wagstaff JD, Bulaj G, Vyazovkina A, Yoshikami D, Cruz LJ, Olivera BM. Isolation and characterization of a novel *Conus* peptide with apparent antinociceptive activity. *J. Biol. Chem.* 2000; **275**: 32 391.
33. Woodward SR, Cruz LJ, Olivera BM, Hillyard DR. Constant and hypervariable regions in conotoxin propeptides. *EMBO J.* 1990; **9**: 1015.
34. Kits KS, Lodder JC, Vanderschors RC, Li KW, Geraerts WPM, Fainzilber M. Novel omega-conotoxins block dihydropyridine-insensitive high voltage-activated calcium channels in molluscan neurons. *J. Neurochem.* 1996; **67**: 2155.
35. Bradbury AF, Smyth DG. Peptide amidation. *Trends Biochem. Sci.* 1991; **16**: 112.
36. Stephenson JL, Cargile BJ, McLuckey SA. Ion trap collisional activation of disulfide linkage intact and reduced multiply protonated polypeptides. *Rapid Commun. Mass Spectrom.* 1999; **13**: 2040.
37. Afonso C, Modeste F, Breton P, Fournier F, Tabet JC. Peptide toxin identification by liquid chromatography/mass spectrometry using an external electrospray ionization source combined with ion trap mass spectrometry. *Euro. J. Mass Spectrom.* 2000; **6**: 429.
38. Wolf SM, Biemann K. Previously uncharacterized ions observed in the high energy collision-induced dissociation mass spectra of peptides containing *S*-alkyl cysteine. *Int. J. Mass Spectrom. Ion Processes* 1997; **160**: 317.
39. Huang YY, Wysocki VH, Tabb DL, Yates JR. The influence of histidine on cleavage C-terminal to acidic residues in doubly protonated tryptic peptides. *Int. J. Mass Spectrom.* 2002; **219**: 233.
40. Tabb DL, Smith LL, Breci LA, Wysocki VH, Lin D, Yates JR. Statistical characterization of ion trap tandem mass spectra from doubly charged tryptic peptides. *Anal. Chem.* 2003; **75**: 1155.
41. Breci LA, Tabb DL, Yates JR, Wysocki VH. Cleavage N-terminal to proline: analysis of a database of peptide tandem mass spectra. *Anal. Chem.* 2003; **75**: 1963.
42. Cartier GE, Yoshikami DJ, Gray WR, Luo SQ, Olivera BM, McIntosh JM. A new alpha-conotoxin which targets alpha-3-beta-2 nicotinic acetylcholine receptors. *J. Biol. Chem.* 1996; **271**: 7522.
43. McIntosh JM, Yoshikami D, Mahe E, Nielsen DB, Rivier JE, Gray WR, Olivera BM. A nicotinic acetylcholine receptor ligand of unique specificity, alpha-conotoxin ImI. *J. Biol. Chem.* 1994; **269**: 16 733.
44. Loughnan M, Bond T, Atkins A, Cuevas J, Adams DJ, Broxton NM, Livett BG, Down JG, Jones A, Alewood PF, Lewis RJ. Alpha-conotoxin EpI, a novel sulfated peptide from *Conus episcopatus* that selectively targets neuronal nicotinic acetylcholine receptors. *J. Biol. Chem.* 1998; **273**: 15 667.
45. Dowell C, Olivera BM, Garrett JE, Staheli ST, Watkins M, Kuryatov A, Yoshikami D, Lindstrom JM, McIntosh JM. Alpha-conotoxin PIA is selective for alpha 6 subunit-containing nicotinic acetylcholine receptors. *J. Neurosci.* 2003; **23**: 8445.
46. Fox JA. Novel omega-conopeptides reduce field potential amplitudes in the rat hippocampal slice. *Neurosci. Lett.* 1994; **165**: 157.
47. Miljanich GP, Ramachandran J. Antagonists of neuronal calcium channels—structure, function, and therapeutic implications. *Annu. Rev. Pharmacol. Toxicol.* 1995; **35**: 707.
48. Olivera BM, Gray WR, Zeikus R, McIntosh JM, Varga J, Rivier J, de Santos V, Cruz LJ. Peptide neurotoxins from fish-hunting cone snails. *Science* 1985; **230**: 1338.
49. Livett BG, Khalil Z, Gayler KR, Down JG, Sandall DW, Keays DA. Alpha conotoxin peptides with analgesic properties. WO 02/079 236 A1, 2002.
50. Nicke A, Samochocki M, Loughnan ML, Bansal PS, Maelicke A, Lewis RJ. Alpha-conotoxins EpI and AuIB switch subtype selectivity and activity in native versus recombinant nicotinic acetylcholine receptors. *FEBS Lett.* 2003; **554**: 219.
51. Lewis T, Harkness P, Sivilotti L, Colquhoun D, Millar N. The ion channel properties of a rat recombinant neuronal nicotinic receptor are dependent on the host cell type. *J. Physiol. (London)* 1997; **505**: 299.
52. Sivilotti L, McNeil D, Lewis T, Nassar M, Schoepfer R, Colquhoun D. Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behavior. *J. Physiol. (London)* 1997; **500**: 123.
53. Nishi M, Kohn AJ. Radular teeth of Indo-Pacific molluscivorous species of *Conus*: a comparative analysis. *J. Mollusc. Stud.* 1999; **65**: 483.

Copyright © 2004 John Wiley & Sons, Ltd.