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A 'conovenomic' analysis of the milked venom from the mollusk-hunting cone snail *Conus textile* – The pharmacological importance of post-translational modifications

Zachary L. Bergeron^{a,1}, Joycelyn B. Chun^a, Margaret R. Baker^a, David W. Sandall^c, Steve Peigneur^b, Peter Y.C. Yu^a, Parashar Thapa^a, Jeffrey W. Milisen^a, Jan Tytgat^b, Bruce G. Livett^c, and Jon-Paul Bingham^{a,1,*}

^aDepartment of Molecular Biosciences and Bioengineering, University of Hawai'i, Honolulu, HI, USA, 96822

^bLaboratory of Toxicology and Pharmacology, University of Leuven (KU Leuven), Campus Gasthuisberg O&N II, Leuven, Belgium, 3000

^cDepartment of Biochemistry and Molecular Biology, Bio21 Institute, University of Melbourne, Parkville, Victoria, Australia, 3010

Abstract

Cone snail venoms provide a largely untapped source of novel peptide drug leads. To enhance the discovery phase, a detailed comparative proteomic analysis was undertaken on milked venom from the mollusk-hunting cone snail, *Conus textile*, from three different geographic locations (Hawai'i, American Samoa and Australia's Great Barrier Reef). A novel milked venom conopeptide rich in post-translational modifications was discovered, characterized and named α -conotoxin TxIC. We assign this conopeptide to the 4/7 α -conotoxin family based on the peptide's sequence homology and cDNA pre-propeptide alignment. Pharmacologically, α -conotoxin TxIC demonstrates minimal activity on human acetylcholine receptor models (100 μ M, <5% inhibition), compared to its high paralytic potency in invertebrates, $PD_{50} = 34.2$ nMol Kg⁻¹. The non-post-translationally modified form, [Pro]^{2,8}[Glu]¹⁶ α -conotoxin TxIC, demonstrates differential selectivity for the $\alpha 3\beta 2$ isoform of the nicotinic acetylcholine receptor with maximal inhibition of 96% and an observed IC₅₀ of 5.4 ± 0.5 μ M. Interestingly its comparative PD_{50} (3.6 μ Mol Kg⁻¹) in invertebrates was ~100 fold more than that of the native peptide. Differentiating α -conotoxin TxIC from other α -conotoxins is the high degree of post-translational modification (44% of residues). This includes the incorporation of γ -carboxyglutamic acid, two moieties of 4-*trans* hydroxyproline, two disulfide

*Corresponding Author: Dr. Jon-Paul Bingham, jbingham@hawaii.edu, Fax: (808) 965-3542, Department of Molecular Biosciences and Bioengineering, College of Tropical Agriculture and Human Resources, University of Hawai'i, HI, 96822, USA.

¹Contributed equally to this work

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Supplementary Information: References to Table 3.

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bond linkages, and C-terminal amidation. These findings expand upon the known chemical diversity of α -conotoxins and illustrate a potential driver of toxin phyla-selectivity within *Conus*.

Keywords

α -conotoxin; conopeptides; *Conus textile*; mass spectrometry; milked venom; radula tooth; Post-translational modifications; nAChR

1. Introduction

The genus *Conus* (Family: *Conidae*, Subfamily: *Coninae*) has maintained a position of predatory superiority within tropical marine ecosystems for some 50 million years. The genus attributes its evolutionary success to the development and delivery of a venomous cocktail. The cone snail injects this venom through a hypodermic needle-like radula harpoon that can penetrate deep into the dermis of its prey [19]. These venoms comprise a potent pharmacopeia of individual bioactive peptide constituents, commonly referred to as conotoxins or conopeptides. New estimates indicate that half-a-million distinct biologically active molecules are expressed within this genus alone [17, 38, 49].

Stemming from their intended predatory use as potent neurotoxic agents for prey immobilization [12, 21], the chemical complexity and functional side-chain variability of conopeptides has been studied, whereby providing tools for probing ion channel function and structure-activity relationships. The majority of research has focused on the clinical significance and therapeutic potential of conopeptides. This is evident by the number of conopeptide derived molecules in pre-clinical development or clinical phase trials for the treatment of a broad spectrum of conditions ranging from neuropathic pain and Alzheimer's, to Parkinson's and epilepsy [5, 20, 22, 30, 47, 55].

Early investigations using milked venom from piscivorous species has fuelled rapid identification of a number of novel bioactive peptides including α A-conotoxin OIVA from *Conus obscurus* [50] and ω -conotoxin SIIIA from *Conus striatus* [48]. Most notably, in 2004, the United States Food and Drug Administration (FDA) approved Prialt™ (ω -Conotoxin MVIIA), a peptide naturally expressed in the milked venom of *Conus magus*, for the treatment of chronic neuropathic pain [7, 26]. Unfortunately, due to this heightened medical interest, in conjunction with intensive exploitation by the ornamental shell-trade industry, the over-harvesting of *Conus* has the potential to result in the depletion of population densities [9, 16]. Subsequently, the need for a rapid and biosustainable approach to identify novel, clinically significant conopeptides while simultaneously reducing pressure on native snail populations has emerged.

In order to increase our understanding of molluscivorous milked venoms, the current study was initiated to perform a comprehensive investigation into the venomous multiplicity of *Conus textile* using Reverse-Phase High Performance Liquid Chromatographic (RP-HPLC²) profiling and mass spectral analysis of both duct (DV) and milked venoms (MV) collected from non-captive specimens representing diverse locations throughout the Pacific. This widely distributed tropical species currently represents one of the most well-studied

molluscivore cone snail with countless molecular constituents, including 77 fully characterized conopeptides (see Table S1).

Our results from the combined ‘conovenomic’ approach revealed an unexpected level of diversity within the venom profiles of geographically unique populations of *C. textile*, and further demonstrated molecular consistency within individuals between the profiles of (i) MV, (ii) dissected whole DV, and (iii) Radula lumen Extract (RE). The comparison of geographically diverse venom profiles led to the identification of a previously uncharacterized peptide, α -conotoxin TxIC, which exhibits extensive post-translational modifications (PTMs). Here we demonstrate how PTM amino acids act as determining factors for selective targeting of the nicotinic acetylcholine receptor (nAChR) – a well-documented therapeutic target for the treatment of chronic neuropathic pain [8, 31, 44, 51, 52].

2. Materials and Methods

2.1 Snail milking

MV from non-captive *C. textile* was obtained within 24 hours of field collection. Envenomation was stimulated by the presence of live gastropods *Morula marginalba*, *Strombus luhuanus* or *Cypraea caputserpentis*. On extension of the cone snail's proboscis, a pipette fitted with a 5000 μ L tip was depressed and placed near the upturned aperture, close to the foot of the prey. On subsequent firing of the radula, envenomation was observed by the release of excess venom, typically as a visible ‘cloud’, which was carefully aspirated to avoid dispersion. The collected MV was acidified (1% v/v TFA) and either frozen (-20°C) or lyophilized for later analysis. Aliquots of seawater (blanks) were collected and processed in the identical manner.

2.2 Dissected duct venom extract preparation

Whole venom ducts were dissected from live specimens, individually dried by Speed-Vac, weighed and homogenized to obtain a fine powder. A mixture of (1000 μ L) 95% Solvent A (0.1% v/v TFA/aq.) and 5% Solvent B (90/10 v/v CH_3CN /0.08% v/v TFA/aq.) was used as the extracting solvent at a standardized concentration (1 mg mL^{-1}). Samples were vortexed (30 sec.), sonicated (10 min.) and then centrifuged (4,500g for 10 min.). The resulting supernatant was decanted, dried via Speed-Vac, weighed and then stored at -20°C until required. All materials were dissolved in 500 μ L of the above solvent, sonicated (5 min.), filtered (0.2 μ m membrane) and then centrifuged (12,000g for 10 min.) prior to chromatographic separation and analysis.

2.3 Chromatographic separation and analysis, RP-HPLC

Representative MV peptides and DV extracts were individually separated as follows: (i) Capillary Scale (Phenomenex; C_{18} , 5 μ m, 300 $\text{\AA}$, 1.0×250 mm, flow 100 μ L min^{-1}) – used for comparative RP-HPLC/UV profiling, quality control of peptide purity, peptide quantification and peptide co-elution experiments. (ii) Analytical Scale (Vydac; C_{18} , 5 μ m, 300 $\text{\AA}$, 4.2×250 mm, flow 1 mL min^{-1}) – used for the isolation and purification of native peptides for MALDI-TOF-(TOF)-MS and Edman Degradation (Sections 2.4 & 2.5). (iii)

Preparative Scale (Vydac; C₁₈, 10 µm, 300 Å, 22 × 250 mm, flow 5 mL min.⁻¹) – used for the desalting and preparative separation of native MV peptides for sequencing and pharmacological assay (Sections 2.9 & 2.12). Systems (i) and (ii) used a Waters 2695 Alliance RP-HPLC System interfaced with a 996 Waters Photo Diode Array Detector for automated sample analysis and detection. Data was acquired and analyzed using Waters Millennium³² (v3.2) software. Samples were eluted using a linear 1% min.⁻¹ gradient of organic (90/10% v/v CH₃CN/0.08% v/v aq. TFA) Solvent B against aqueous (0.1% v/v TFA aq.) Solvent A for 65 min., excluding a terminating high organic wash (80% Solvent B for 5 min.), and pre-equilibration step (5% Solvent B) for 10 min. prior to sample injection. Eluent was monitored from 200–300 nm and extracted at 214 nm. Preparative RP-HPLC/UV, system (iii), used a 625 Waters HPLC pump and controller interfaced with a 996 Waters Photo Diode Array Detector. Both gradient control and data acquisition were facilitated by the use of the Waters Millennium³² software. Filtered (Nylon 0.22 µm) crude MV and DV peptide extracts were manually loaded and eluted from the preparative scale column using the same 1% gradient at 5 mL min.⁻¹ and monitored at 214 and 280 nm. Fractions were collected manually and stored at – 20°C or freeze-dried until required.

2.4 Direct ESI-MS infusion

AB/MDS-Sciex API 3000 triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) was used in this investigation as previously described by Chun et al. [10]. The ESI-MS system was calibrated manually in positive mode with PPG 3000 (AB/MDS-Sciex) to achieve <5-ppm mass accuracy, as per manufacturer's protocol.

2.5 MALDI-TOF MS venom analysis

ZipTipTM or RP-HPLC/UV purified venom fractions in Solvent A (0.1% v/v aq. TFA) were mixed 1:1 with matrix solution (40 g L⁻¹ 2,5-dihydroxybenzoic acid (DHB) in 1:1 0.1% v/v aq. TFA: CH₃CN) and 1 µL was spotted on a MTP 384 polished steel target plate (Bruker Daltonics). The spots were dried under a stream of N₂ gas. Mass spectra were acquired on the Ultraflex III (Bruker Daltonics), controlled by the Compass 1.2 SR1 software package (Bruker Daltonics), in positive reflector mode from *m/z* 500 to 5000. Mass spectra were summed (400 to 1200 laser shots) until no further improvement to the signal to noise ratio of peaks was achieved. Peptide II Calibration Mix (Bruker Daltonics) was used for external calibration, with a mass accuracy of approximately 50-ppm. Analysis of the spectra was completed using FlexAnalysis v3.0 (Bruker Daltonics).

2.6 MALDI-TOF/TOF MS peptide sequencing and PTM characterization

The reduced venom peptide, α-conotoxin TxIC, was desalted via ZipTipTM (C₁₈ reversed-phase media, Millipore) or RP-HPLC/UV isolated and spotted onto the target plate with DHB matrix, as described above (Section 2.5). Tandem mass spectra (MS/MS) were acquired in reflector positive LIFT mode on the UltraflexIII (Bruker Daltonics), externally calibrated with Peptide Calibration Mix II (Bruker Daltonics) with a MS/MS accuracy of 0.04 Da. FlexAnalysis v3.0 (Bruker Daltonics) was used for manual inspection and annotation of the LIFT-spectra. The RapiDeNovo module in BioTools (Bruker Daltonics) was used to make additional assignments to the amino acid sequence.

2.7 MALDI-TOF MS radula analysis

Conus textile radulae were collected from dissected radula sacs. Harpoons were prepared for MALDI-TOF MS analysis as described previously by Chun et al. [10].

2.8 Peptide reduction and thiol alkylation

For complete peptide reduction, *Conus* extracts and peptide(s) (typically 0.02–3 mg) were exposed to 100 μ L of 200 mM Tris(2-carboxyethyl)phosphine (TCEP; Pierce Chemicals, USA) in 25 mM NH_4OAc (pH 4.5) and heated for 5–30 min. at 50°C. Alkylation of the RP-HPLC purified, reduced peptide(s) was achieved by dissolving the peptide in 90–150 μ L of 25 mM NH_4OAc (pH 4.5) and adding 100 mM *N*-phenylmaleimide or Maleimide (Fluka, Switzerland) in isopropanol. Typically 20–40 fold excess (w/w) of the alkylating agent was used. Alkylation was allowed to proceed at 50°C for 15 min., prior to RP-HPLC/UV purification.

2.9 Sequencing – Edman degradation

Non-alkylated and Maleimide alkylated derivatives were applied to Polybrene-treated glass fiber support filters for automated Edman degradation on a gas-phase sequencer (Model 470A; Applied Biosystems, Foster City, CA, USA). Assignment of the amino acid sequence was essentially as described by Atherton *et al.* [2] and Matsudaira [34].

2.10 Peptide synthesis

α -Conotoxin TxIC [$\text{ROQC}^4\text{CSHOAC}^{10}\text{NVDHP}\gamma\text{IC-NH}_2$; γ = γ -carboxyglutamic acid (Gla), O = 4-*trans* hydroxyproline (Hyp)] was manually assembled using a 0.5 mmole scale and Fmoc SPPS via *in-situ* neutralization with 2 mmole Fmoc-amino acid per 10–30 min. single coupling, as adapted from Schnölzer *et al.* [46] and described in detail in Kapono *et al.* [26]. A Fmoc-Cys(Trt)-Rink-Amide MBHA Resin (0.55 meq. g^{-1} ; Peptides International, Louisville, KY, USA) was used for peptide assembly to give the desired C-terminal amide function. Side chain protecting groups were: Cys(Trt), Asp(tBu), Arg(Pbf) and Gln(Trt). Gla(otBu)₂, His(Trt), Asn(otBu) and Hyp(tBu) (as supplied by Peptides International, Louisville, KY, USA). An additional 0.5 mmole scale synthesis of α -conotoxin TxIC was undertaken using orthogonally protected Cys(Acm) being placed in positions 4 and 10 in the above sequence. This synthesis used the same synthetic strategy as previously mentioned, but deviated using an alternative directive/sequential oxidation process (see below). Finally, a non-PTM amino acid variant of α -conotoxin TxIC, (i.e. [Pro]^{2,8}[Glu]¹⁶ α -conotoxin TxIC), was produced and folded using the same orthogonally Cys(Acm) protected scheme as above. Incorporated were the Fmoc amino acids Pro and Glu(OtBu) used as PTM substituents.

2.11 TFA cleavage

Fmoc peptidyl-resins were subjected to a cleavage with 82.5% v/v TFA in the presence of thioanisole (5% v/v), Phenol (5% v/v) H_2O (5% v/v) and TIPS (2.5% v/v), acting as protecting group scavengers, for 2.5 h at 24°C. The resulting cleaved peptide material was recovered by filtration and cold *t*-butyl ether precipitation. Resulting crude peptide was stored at –20°C, as lyophilized powder, until required.

2.12 Random disulfide bond formation

Peptide oxidation was achieved with 15 mg of TCEP reduced C₁₈ RP-HPLC purified peptide, using 0.1 M NH₄HCO₃ pH 8.7 (5 days stirring; room temp.). The oxidized peptide was preparative RP-HPLC/UV isolated and re-purified, and the oxidized molecular mass was verified by ESI-MS.

2.13 Directed disulfide bond formation

Cleaved α -conotoxin TxIC and [Pro]^{2,8}[Glu]¹⁶ α -conotoxin TxIC, both containing Cys(Acm) in positions 4 and 10, were RP-HPLC/UV purified, and then air oxidized, as above. Partially oxidized materials, as confirmed by ESI-MS, were then subjected to spontaneous thiol deprotection and disulfide bond formation. Deprotection was achieved by dissolving the partially folded peptide in 50% v/v acetic acid (1 mg mL⁻¹) and by adding a solution of freshly saturated I₂ in 50% v/v acetic acid to the stirring peptide (25% reaction vol.). Reaction was quenched after 5 min. with the addition of 10 μ L aliquots of 1M Na₂S₂O₃ until the stirring solution became clear, which was then followed by the addition of 200 μ L TFA. Resulting acidified material was centrifuged (12,000g, 5 min.) and directly purified by preparative RP-HPLC/UV (as above) with mass confirmation provided by ESI-MS.

2.14 Genetic analysis of α -conotoxin TxIC

The venom duct and bulb of *C. textile* specimens (Great Barrier Reef, Australia) were removed by dissection and snap frozen in liquid N₂. The secretory venom duct and bulb were ground and mRNA was extracted using a Dynabeads mRNA direct kit (Dyna, Norway). A cDNA library was created from this mRNA using a Marathon cDNA amplification kit (Clontech) as previously described [44]. All PCRs utilized the primers Uni α -1 (universal α -conotoxin primer) 5' ATGGGCATGCGGATGATGTT 3'; and Uni α -2 (universal α -conotoxin primer) 5' CGGAAAGTGAAGCAGGTCAG 3', designed against conserved sequences found on the 5' and 3' conserved regions of known conotoxins. Reaction mixtures contained Taq polymerase (Roche) and deoxynucleotides in a buffer supplied by the manufacturer. Samples were incubated at 94°C for 2 min.; followed by 30 cycles of 94°C for 30 sec, an annealing step for 30 sec, 72°C for 45 sec; concluding with a final step of 72°C for 5 min. Amplification products were purified after separation on a 1.5% agarose gel using a Qiaquick gel extraction kit (Qiagen). The purified PCR products were transformed into competent INV α F' *Escherichia coli* cells in accordance with the manufacturer's specifications (Invitrogen, Netherlands). Recombinant plasmids containing inserts of approximately 250 bp were sequenced by the di-deoxy chain termination method using the ABI PRISM Big-Dye Terminator Cycle Sequence Ready Reaction Kit (Perkin Elmer, USA). Sequences were analyzed on a Perkin Elmer 377 sequencer at the Australian Genome Research Facility (AGRF).

2.15 Pharmacology

2.15.1 Expression of voltage-gated ion channels in *Xenopus laevis* oocytes—

For the expression of nAChR (α 1, α 3, α 4, α 5, β 2, β 4, γ ; δ ; ϵ) in *Xenopus* oocytes, the linearized plasmids were transcribed using the T7 or SP6 mMESSAGE-mACHINE

transcription kit (Ambion®, Carlsbad, CA, USA). The harvesting of stage V–VI oocytes from anaesthetized female *Xenopus laevis* frog was previously described by Liman *et al.* [29]. Oocytes were injected with 50 nL of cRNA at a concentration of 1 ng nL⁻¹ using a micro-injector (Drummond Scientific®, Broomall, PA, USA). The oocytes were incubated in a solution containing (in mM): NaCl, 96; KCl, 2; CaCl₂, 1.8; MgCl₂, 2 and HEPES, 5 (pH 7.4), supplemented with 50 mg L⁻¹ gentamycin sulfate.

2.15.2 Electrophysiological recordings—Two-electrode voltage-clamp recordings were performed at room temperature (18–22°C) using a Geneclamp 500 amplifier (Molecular Devices®, Downingtown, PA, USA) controlled by a pClamp data acquisition system (Axon Instruments®, Union City, CA, USA). Whole cell currents from oocytes were recorded 1–4 days after injection. Bath solution composition was (in mM): NaCl, 96; KCl, 2; CaCl₂, 1.8; MgCl₂, 2 and HEPES, 5 (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.5–1.5 MΩ. During recordings, the oocytes were voltage-clamped at a holding potential of –70 mV and superfused continuously with ND96 buffer via gravity-fed tubes at 0.1–0.2 mL min.⁻¹, with 5 min. incubation times for the bath-applied peptides. Acetylcholine (ACh) was applied via gravity-fed tubes until peak current amplitude was obtained (1–3 sec.), with 1–2 min. washout periods between applications. Data was sampled at 500 Hz and filtered at 200 Hz. Peak current amplitude was measured prior to and following incubation of the peptide.

To assess the concentration-response relationships, data points were fitted with the Hill equation: $y = 100/[1 + (EC_{50}/[\text{toxin}])^h]$, where y is the amplitude of the toxin-induced effect, EC_{50} is the toxin concentration at half maximal efficacy, $[\text{toxin}]$ is the toxin concentration and h is the Hill coefficient. Comparison of two sample means was made using a paired Student's t test ($p < 0.05$). All data is presented as mean $\pm$ standard error (S.E.M) of at least 4 independent experiments ($n = 4$). All data was analyzed using pClamp Clampfit 10.0 (Molecular Devices®, Downingtown, PA, USA) and Origin 7.5 software (Originlab®, Northampton, MA, USA).

2.16 Whole animal bioassay

Standardized concentrations of native α -conotoxin TxIC (2.5, 5, 10, 20, 40, 80, 160, 320 and 640 pMol g⁻¹) and synthetic [Pro]^{2,8}[Glu]¹⁶ α -conotoxin TxIC (0.16, 0.32, 0.64, 1.28, 2.56, 5.12 and 10.24 nMol g⁻¹) in 10 μ L volumes (PBS), were injected intramuscularly (IM - foot) into Hawaiian snakehead cowries (*Cypraea caputserpentis*) using a Hamilton 10 μ L syringe at a depth of 2 mm. Following injection, animals were placed in glass Petri dishes filled with fresh aerated seawater, where paralysis was determined by the inability of the animal to cling to the substrate. Dosage and paralysis were recorded and plotted according to Reed and Muench [40], and the PD₅₀ was extrapolated using GraphPad Prism Software (v5.02). All dose experiments were repeated in triplicate or greater ($n = 3$).

3. Results

3.1 Conformational analysis of δ -conotoxin TxVIA

The MH^+ 3035.4 Da was identified by ESI-MS from RP-HPLC/UV fractions of DV, confirming the identity of δ -conotoxin TxVIA (Calc. MH^+ 3035.3 Da). TCEP reduction, RP-HPLC/UV separation and ESI-MS analyzed demonstrated a mass shift of 6 Da (Obs. Red. MH^+ 3041.5 Da), which reflects disulfide content. This same material (~ 300 nmole) was then subjected to 27 cycles of Edman degradation, and a single unambiguous sequence that corresponded to δ -conotoxin TxVIA W(-)KQSGEM(-)NLLDQN(-)(-)DGY(-)IVLV(-)T was obtained. 'Blanks' (-) were observed at degradative cycles 2, 9, 16, 17, 21 and 26. These corresponded to the 6 underivatized cysteine moieties as predicted within this specific sequence. This confirmed the expression of δ -conotoxin TxVIA – a species-specific duct venom 'biomarker' for all specimens examined in this study. The methionine sulfoxide derivative (+16 Da) of this peptide was also observed by MALDI-TOF MS (m/z 3051.3; Figs. 2A & B).

3.2 Geographic biodiversity - RP-HPLC/UV non-captive milked venom profiling

Typical representative MVs from non-captive specimens of *C. textile* demonstrate an abundance of chromatographic peaks ranging from 20–70 perMV profile (Fig. 1). Multiple MV samples from Hawai'i (n=30 specimens) and the Great Barrier Reef (n=50 specimens; GBR), Australia contained similar chromatographic content, with varying relative peptide concentrations as observed in the moderately hydrophilic region (25–40% CH_3CN , 25 to 40 min.; see Figs. 1A & C, respectively). No observable RP-HPLC/UV correlation to cone snail sex or collection/milking season could be established from the MV profiles from any of the three locations examined (not shown).

The moderate to hydrophobic RP-HPLC/UV profile region (40–80% CH_3CN , 40 to 80 min.) accounted for >30% of milked venom variability, with the highest proportion of peak variability seen in the representative MV of non-captive specimens from American Samoa (n=20 specimens) (Fig. 1B). The hydrophobic δ -conotoxin TxVIA (R_t 65.3 min.) was observed in both non-captive Hawai'ian and American Samoan representative samples (see arrow in Fig. 1A & B respectively), but was absent in non-captive GBR Australian MV (Fig. 1C). One previously undocumented peak (underlined), with a retention time (R_t) = 26.6 min. (α -conotoxin TxIC), was observed in all three locations (n=100, total specimens examined) (Fig. 1A–C). This observed geographic commonality lends credence to the importance of that particular conopeptide.

3.3 Geographic biodiversity - Molecular mass profiling of non-captive milked venom

MALDI-TOF MS profiles of MV from 5 random specimens of non-captive *C. textile* from each geographic location cover the entirety of the examined mass range (Figs. 2A–C). In the examples illustrated, a combined total of 178 non-overlapping peaks were observed in the three MVs (Table 1 & Table S2). Of these, 42 peaks ($\sim 24\%$) were common to two collection sites, with only 11 ($\sim 6\%$) being common to all three. Importantly, 27 of the 178 non-captive MV constituents ($\sim 15\%$) correlated to known *C. textile* peptides (Table 1; Tables S1 & S2), excluding potential PTM variants including C-terminal processing (± 1.0 Da for free-acid

vs. amide). Expression of 2 of these known conopeptides, TxIA and TxIIIC, were previously confirmed by Edman degradation sequencing [4].

3.4 Single specimen venom source consistency – Non-captive RP-HPLC/UV profiling

RP-HPLC/UV venom peptide profiles from a single non-captive specimen of *C. textile* (Oahu, Hawai'i) is shown in Figs. 3A–C. Results illustrate a high level of peptide R_t consistency between the whole duct venom (DV) extract, the milked venom (MV), and the Radula lumen Extract (RE). The relative abundance of peptides in the different venom extracts was highly variable as seen with the relative intensity of the hydrophobic δ -conotoxin TxVIA (R_t 65.3 min.; Figs. 3B & C).

3.4.1 Duct venom (DV) extracts—In the DV, δ -conotoxin TxVIA was one of the major components (Fig. 3C). The presence of α -conotoxin TxIC in DV (underscored, Fig. 3C) was observed at R_t = 26.6 min., corresponding to an identical elution profile from the MV of the same specimen (Fig. 3B) as well as in the representatives from geographic MV sampling (Fig. 1).

3.4.2 Milked venom (MV) extracts—In contrast to the DV, MV profiles naturally correspond to a more accurate representation of bioactive constituents used in prey immobilization. δ -Conotoxin TxVIA was one of only a few hydrophobic peptides observed in the MV (Fig. 3B), which exhibited direct correlation to the DV. These results are reiterated in the geographic specimens that exhibit few hydrophobic peptides (Fig. 1); most notably, Australian specimens – which may reflect venom processing. Highly abundant hydrophilic peptides within both DV and MV venom extract profiles were examined (Fig. 3B & C), which identified α -conotoxin TxIC (underscored, R_t = 26.6 min.).

3.4.3 Radula lumen (RE) extracts—Analysis of the RE demonstrated poorly resolved peaks with limited abundance (Fig. 3A), yet the two major peaks correlated with the two highly abundant peaks in the MV (Fig. 3B). Both peaks showed marked differences in the DV extract.

3.5 Single specimen venom source consistency – Non-captive molecular mass profiling

MALDI-TOF-MS was used to detect and compare identifiable peptide masses in extracts of the RE, MV and DV, from a single non-captive representative specimen of *C. textile* (Fig. 4). A total of 159 unique molecular masses were observed (Table S3). Common to all three extracts were 16 individual masses (~10%). Showing the greatest similarity were the MV and DV profiles that shared 38 masses (~24%). The RE mass spectrum, exhibiting masses within m/z 945 to 3850, contained fewer masses than the MV and DV. The MV mass spectrum was the most complex in peak number of the three.

Most notable are the high intensity masses observed in both the DV and MV extracts which correspond to important and previously characterized conopeptides (Table 2 & Fig. 4). The dominant peak in the MV and DV mass spectra (m/z 2488.2 $\pm$ 0.3) corresponds to the *C. textile* Convulsant peptide. This peak is also present in the RE mass spectrum. Other

identified peptides (i.e., TxIA and TxIIIC) were also observed as major contributors in the geographic analysis (Table 1, Fig. 2).

3.6 Mass spectrometric analysis of α -conotoxin TxIC disulfide bonds

A geographically consistent peptide ($R_t = 26.6$ min.; Fig. 3) was isolated from MV by RP-HPLC/UV. A single parent mass of MH^+ 2080.8 Da was assigned by ESI-MS (Obs. $[M+3H]^+ 694.6$ Da; data not shown). After TCEP reduction, the observed parent mass was MH^+ 2084.7 Da, which indicated the presence of 2 disulfide bridges. This was confirmed by alkylation of the reduced material with N-phenylmaleimide, after which an increase in 692 Da was observed, corresponding to 4 alkylation groups (173 Da for each free thiol [6]).

3.7 CID analysis of α -conotoxin TxIC

The parent ion of α -conotoxin TxIC was not observed in MALDI-TOF MS analyses of geographic and source extracts (Figs. 2 & 4). This indicated decay of the parent ion, which is common in MALDI-TOF MS of peptides containing labile PTMs. Direct observation of this suspected decay was achieved via MALDI-TOF MS of the purified material in its reduced form (Fig. 5A). Two well resolved peaks, with a difference of 44 Da, indicated the presence of a carboxyl group. Similar decay of the parent ion could be reproduced with ESI-MS, a softer ionization method, by increasing the orifice potential voltage outside of the normal operating range (data not shown).

MALDI-TOF/TOF MS/MS fragmentation spectra of the suspected decarboxylated (m/z 2041.06; Fig. 5B) and 'intact' form (m/z 2805.07; data not shown) of TCEP reduced α -conotoxin TxIC were nearly indistinguishable. In both cases MS/MS fragmentation displayed a 44 Da deficient a-, b-, and y-ion series. Analysis of the overlapping ion series allowed for interpretation of the amino acid sequence as R-[I/L/O]²-Q-C-C-S-H-[I/L/O]⁸-A-C-N-V-D-H-P-E-[I/L/O]¹⁷-C. The isobaric nature of isoleucine and leucine (I and L) and their similarity in mass to 4-*trans*-hydroxyproline (O) (-0.036 Da) resulted in ambiguities at positions 2, 8 and 17, which were resolved with Edman degradation (Section 3.8).

The CID fragment ions also indicated heterogeneity of the C-terminus, the predominant form being amidated. The presence of low abundant C-terminal free acid form was verified using a narrow precursor ion selection window of ± 0.5 Da. The MS/MS fragmentation spectrum was interpreted to have the same amino acid sequence as the amidated form (data not shown). Additional verification was obtained by comparing predicted and observed isotopic distribution patterns for the respective parent ions.

3.8 Edman degradation of α -conotoxin TxIC

RP-HPLC/UV purified N-phenylmaleimide thiol alkylated α -conotoxin TxIC, ~300 nmole, was subjected to 20 cycles of automated Edman degradation. PTH-4-*trans*-hydroxyproline was added to the amino acid standard mixture to aid identification. Sequential degradation provided an unambiguous 18 amino acid sequence: RO²QCCSHO⁸ACNVDP(-)¹⁶IC. Cycle 16 indicated a low recovery level PTH-Glu (<1 nmole) and was designated as a 'blank cycle' based on the expected sequential cycle yields observed within the normal degradative process.

Edman degradation assigned 17 of the 18 amino acids conclusively, including positions 2 and 8 as 4-*trans*-hydroxyproline and 17 as isoleucine. The presence of low levels of glutamic acid in cycle 16, combined with the observed mass difference of 44 Da ($-\text{CO}_2$) under various mass spectrometric conditions, provided further corroborated the presence of a γ -carboxylated glutamic acid within α -conotoxin TxIC at position 16.

3.9 Genetic analysis of α -conotoxin TxIC

The peptide sequence was confirmed genetically by identifying the α -conotoxin transcript in *C. textile* venom duct cDNA (GBR, Australia) using oligonucleotides based on regions of conserved sequence in the pre-region and C-terminal flanking region of α -conotoxins [44]. The resulting 19 amino acid toxin- or mature-sequence region was deduced: RPQCCSH PACNV DHPEIC[R] (see Fig. 6). A C-terminal arginine was absent following Edman analysis; its inclusion would also be at variance with native molecular mass as observed by either ESI-MS, MALDI-TOF MS or by sequence analysis using MALDI-TOF/TOF MS LIFT.

The N-terminal pro-region of α -conotoxin TxIC also possesses an arginase site for mature conopeptide cleavage, an occurrence common to conotoxin processing [24]. In α -conotoxin TxIC the pro-peptide cleavage site is located between two neighboring arginine moieties – see Fig. 6. This is confirmed by the recovery/identification of an N-terminal arginine in α -conotoxin TxIC both by MALDI-TOF/TOF MS LIFT and Edman Degradation. Independent identification of [Glu]¹⁶, via genetic analysis, conclusively confirmed the original parent PTM amino acid assignment.

3.10 Peptide synthesis of α -conotoxin TxIC

Based on the assigned C-terminal amide sequence above, α -conotoxin TxIC was synthetically constructed and folded by random air oxidation. Upon completion, the correct target molecular mass was observed by ESI-MS: Obs. MH^+ 2080.9 Da (Calc. MH^+ 2080.8 Da). RP-HPLC/UV demonstrated the presence of 3 peaks, with the two dominant peaks separated by <0.3 min. (Fig. 7A). All three peaks were determined to be disulfide isomers of α -conotoxin TxIC, as confirmed by ESI-MS. Separation of the two main isomers proved difficult. Changes to gradient, ion pair agents and column failed to achieve their full separation (data not shown).

A second synthesis was performed using the established disulfide connectivity pattern reported for the 4/7 α -conotoxin family [31]. This was achieved by incorporation of differential orthogonally protected cysteine moieties at positions 4 and 10 (Fig. 7B). This allowed for the step-wise production of a single oxidized peptide peak that retained target characteristics of mass (Obs. MH^+ 2080.9 Da) and RP-HPLC/UV using a slower chromatographic gradient ($0.5\% \text{ min}^{-1}$), without production of the previously observed isomeric materials (Fig. 7A). Co-injection of the RP-HPLC/UV purified synthetic material with α -conotoxin TxIC isolated from the native milked venom, in a ratio of 1:2 respectively, demonstrated chromatographic homogeneity at 33.3 min., Fig. 7B. RP-HPLC/UV co-injection of the TCEP reduced material also demonstrated chromatographic homogeneity

(not shown). This confirmed the original peptide sequence assignment, including C-terminal assignment, and provided an independent synthetic standard for RP-HPLC/UV.

3.11 Pharmacological assessment

Native α -conotoxin TxIC (100 μ M) had minimal inhibitory action on human nicotinic acetylcholine receptor (nAChR) subunit combinations $\alpha 4\beta 2$ (brain), $\alpha 4\beta 4$ (neuronal), $\alpha 7$ (neuronal), $\alpha \beta \gamma \delta$ (muscle), $\alpha \beta \gamma \epsilon$ (muscle) or the human peripheral nAChR subtypes $\alpha 3\beta 2$, $\alpha 3\beta 2\alpha 5$ and $\alpha 3\beta 4$. Biological activity was observed with [Pro]^{2,8}[Glu]¹⁶ α -conotoxin TxIC, the synthetic non-PTM form (Fig. S1A & B). This analogue inhibited the human nAChR subtypes ($\alpha 3\beta 2$, $\alpha 3\alpha 5\beta 2$ and $\alpha 3\beta 4$), with the $\alpha 3\beta 4$ isoform demonstrating the highest sensitivity (IC₅₀ 2.1 $\pm$ 0.2 μ M) and exhibiting a 77% maximum inhibition. Isoform $\alpha 3\beta 2$ demonstrated the highest level of inhibition (96%) with an IC₅₀ of 5.4 $\pm$ 0.5 μ M (Table 4). No significant inhibition was observed at the remaining isoform targets ($\alpha 4\beta 2$, $\alpha 4\beta 4$, $\alpha 7$, $\alpha \beta \gamma \delta$, $\alpha \beta \gamma \epsilon$) at concentrations up to 100 μ M (Table 4).

3.13 Whole animal bioassay

Whole animal bioassay using the native α -conotoxin TxIC demonstrated a dose-dependency in its ability to produce animal paralysis (see Fig. S2). α -Conotoxin TxIC produced total paralysis at 640 pMol g⁻¹ (n=14), this dose did not cause lethality in test animals. A PD₅₀ of 34.2 pMol g⁻¹ (34.2 nMol Kg⁻¹; whole snail weight) was determined for the native toxin (Table 4). The synthetic non-PTM analogue of α -conotoxin TxIC demonstrated an inability to cause paralysis at same PD₅₀ concentration (n=15). Total paralysis was achieved with 10.24 nMol g⁻¹ (n=7), this being $\sim 16\times$ that of the native. A similar dose-dependent trend was observed (see Fig. S2), with the maximal dose used not causing animal lethality. A PD₅₀ of 3.6 nMol g⁻¹ (3.6 μ Mol Kg⁻¹; whole snail weight) was calculated (Table 4), this PD₅₀ concentration being $\sim 100\times$ more than observed with the native PTM toxin.

4. Discussion

This study is the first reported analysis comparing the venom profile of a single specimen of *Conus* from milked venom (MV), to duct venom (DV), and finally to radula lumen extract (RE) by RP-HPLC/UV analysis and molecular mass profiling. This has provided greater insight into the complexity of molluscivorous venom from a single specimen through to the gross variation observed at a species level from wide-ranging geographical locations.

The importance of *Conus textile* to conopeptide research cannot be underestimated. Early studies on *C. textile* [21, 27, 36] made major contributions to the present understanding of conopeptide processing and expression [11, 23, 54], and led to the discovery of novel PTM amino acids [14, 41] and unique biological activities. The specie's relative abundance, population distribution and access has allowed for an intensive level of research, covering specimens collected from the Philippines [28], Red Sea [21], South China Sea [33], Japan [27, 36] and the GBR, Australia [15, 25]. In the present study, we have expanded this geographic range to include specimens collected from Hawai'i and American Samoa (Fig. 1 and 2), making this the largest geographic study of a single *Conus* species to date – this

provides an indication to their regional biodiversity, an area poorly addressed in *Conus* research.

A ‘conovenomic’ comparison of MV from distant geographic locations, illustrates the true extent of venom peptide diversity present within *C. textile*, as alluded to by Lu *et al.* [33]. Our evidence further demonstrates the intraspecific venom variability identified in DV of other members of the genus [1, 15, 42, 43] – a characteristic that now extends to MV. This may increase earlier estimates of bioactive venom constituents [18, 35, 36, 49], and expand the therapeutic potential of *Conus* [5, 7, 20, 30, 47].

This is only further demonstrated with the identification of a novel, highly PTM-modified conopeptide, α -conotoxin TxIC, despite the intensive level of research that *C. textile* has received historically. The newly observed α -conotoxin demonstrates homology to Vc1.1 (~66 %; Table 3), and an experimentally confirmed level of selectivity (in its non-PTM form) to human isoforms of neuronal type nAChR (Table 4), a well-documented target for the treatment of chronic neuropathic pain [8, 31, 44, 51].

Within *C. textile*, considerable diversity is found primarily within the hydrophobic region of the RP-HPLC/UV venom profiles (Fig. 1), and prominently in specimens from American Samoa. Mass spectral analysis revealed a level of molecular mass consistency across all locations, however the relative proportion of venom constituents varies markedly (Fig. 2; Table 1; Table S2). Peptide expressional variability supports an important biological role in the predator envenomation strategy, a factor that may aid the discovery of a novel conopeptides, as illustrated with α -conotoxin TxIC.

To further refine this approach, the analysis of a single *C. textile* specimen facilitated the tracking of integral venom constituents from DV to MV and then RE. This strengthens the proteomic correlation, end-point detection and characterization of the essential secretory venom constituents used during envenomation [51]. It is these identified end-point MV peptides that have shown the strongest correlation to both potential biological activity and proven drug potential in piscivores [7, 26]. Using RP-HPLC/UV we established that α -conotoxin TxIC is represented in DV extract (Fig. 3C), and that expressional continuity continues through the MV (Fig. 3B). Interestingly α -conotoxin TxIC was absent from the RE (Fig. 3A). For this species at least, the radula lumen extract is not representative of the venom content as a whole – however some level of conopeptide continuity, by m/z comparison, is clearly observable (Table 2; Fig. 4).

α -Conotoxin TxIC represents a novel toxin that highlights some of the issues that have plagued conopeptide discovery and sequencing, necessitating a combined ‘conovenomic’ approach to obtain a thorough and accurate sequence analysis. The sole use of mass spectrometry for *Conus* venom analysis can lead to misidentification of sequence and molecular mass arising from inconsistencies associated with sample preparation, matrix selection and the ionization technique used. However we are unsure of the extent of PTM ‘miss assignment’ that may exist in the 1000’s of conopeptides already documented in the publically available conopeptide database (<http://www.conoserver.org>). Yet, such errors are

highly possible given the relative abundance and differential incorporation of PTM amino acids in conotoxins/conopeptides [10].

The ability to detect the presence of α -conotoxin TxIC only by its decarboxylated and fragmented products, and not its parent molecular mass, illustrates the susceptibility of the parent peptide to in-source decay. This process was exacerbated for α -conotoxin TxIC by the presence of the PTM amino acid γ -carboxyglutamic acid (Gla). Similar observations have been described involving the loss of the PTM Tyr-SO₄ desulfation (-80 Da) in α -conotoxins PnIA (*Conus pennaceus*) and EpI (*Conus episcopatus*) [32, 53]. Although ESI-MS is less harsh, a similar degree of peptide degradation can occur if working outside normal operating parameters. Our observations highlight the necessary awareness that needs to be undertaken when dealing with complex and highly PTM processed DV extracts and native MVs.

Irrespective of MS technique, each method has advantages and disadvantages in the performance of mass detection and sequencing. If suspicion of PTMs arise, it is best to employ a combined approach for analysis and verification; this would include the use of molecular biology techniques along with more traditional biochemical methods such as Edman degradation. The latter technique becomes a necessity when considering the isobaric CID fragments generated from Hyp and Ile and Leu (± 0.04 Da). Three such unambiguous assignments were observed in the *de-novo* sequencing analysis of α -conotoxin TxIC (Fig. 5B). Residues were later confirmed and assigned by Edman degradation (Section 3.8) and via genetic analysis, as shown in Fig. 6 (Section 3.9).

Comprising of 18 amino acids with two disulfide bonds, 44% of residues display one or more forms of modification, α -conotoxin TxIC is one of the most highly PTM conopeptides documented to date. This novel 4/7 conopeptide (as represented by its cysteine framework) shares sequence homology with other α -conotoxins [18, 31] and demonstrates sequence commonality to other molluscivorous conopeptides, specifically Ai1.2 (*C. ammiralis*) and Vc1.1 (*C. victoriae*) (Table 3). This homology is seen explicitly in Vc1.1 in its native PTM form: [Hyp]⁶[Gla]¹⁴Vc1.1 [24], and more so within the last cysteine loop (89% homology; Table 3). Both *C. ammiralis* (Ai) and *C. victoriae* (Vc) are close relations of *C. textile* – with *C. victoriae* being endemic to Australia. Genetic analysis of α -conotoxin TxIC strengthens its relationship to the established 4/7 α -conotoxins, as seen by the homology of the pre/pro-peptide regions of α -conotoxins MII and Vc1.1 (Fig. 6) [17, 33, 39, 44].

As the majority of these illustrated α -conotoxin sequences are deduced from genomic sequences, the current inclusion of the observed PTMs at positions [Pro→Hyp]², [Pro→Hyp]⁸ and [Glu→Gla]¹⁶ in Table 3 increases the known chemical diversity of α -conotoxin TxIC. Modifications may be affecting *in vivo* characteristics including kinetic properties and/or pharmacological selectivity of conotoxins. The absence of these PTMs in piscivorous 4/7 α -conotoxins, such as α -conotoxin MII (*Conus magus*), potentially indicates a phyla-selective targeting differentiation. By examining both the native α -conotoxin TxIC, as well as a synthetic unmodified isoform ([Pro]^{2,8}[Glu]¹⁶ α -conotoxin TxIC), we have here substantiated these claims, highlighting the role of PTMs *in vivo*. While remaining biologically active in whole animal bioassay, native α -conotoxin TxIC is inactive when

tested human nAChR channel isoforms (Table 4). The synthetic unmodified α -conotoxin TxIC (i.e. [Pro]^{2,8}[Glu]¹⁶ α -conotoxin TxIC) however remains active at the nAChR, demonstrating isoform selectivity (Table 4). These features highlight the importance of PTMs in the selective targeting of α -conotoxins. This is further illustrated at a phylogenetic level with the observed switching in potency in invertebrate models that represents the native prey target (Fig. S2).

The phyla-selectivity and reduced potency of the native α -conotoxin TxIC towards human nAChR isoforms demonstrates an underlying differentiation between receptor selectivity and pharmacodynamic properties in ion channels from different phyla. This is an area reiterated by the PTM of the nAChR in the Egyptian mongoose (*Herpestes ichneumon*) that provides a level of resistance to α -bungarotoxin [3]. Here, genomic and proteomic investigation into invertebrate nAChR isoforms, as well as other receptors (e.g. N-type (Ca_v2.2) calcium channels and GABA_B [13]), may provide insight into the determinants for pharmacological potency and phyla selective characteristics observed commonly with *Conus*. Such information will provide insights into toxin target specificity enhancement through peptide bioengineering and the potential manipulation/incorporation of PTMs.

5. Conclusions

The complexity of MV profiles from specimens of *C. textile* revealed in this study serve to remind us of the extent we have underestimated the biodiversity and value of these unique venomous marine snails. The analysis of venoms necessitates a specifically tailored approach, requiring special attention to the analytical methods employed, and further accentuating the need to employ a combined ‘conovenomic’ approach. The lessons learned and approaches outlined will facilitate peptide prospecting, toxinological correlations and the future discovery of new, clinically relevant conopeptides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

$\alpha\alpha$	amino acid
CH₃CN	acetonitrile
Acm	Acetamidomethyl
API	Atmospheric pressure ionization
DHB	Dihydroxybenzoic acid
cDNA	complimentary DNA
CHCA	α -Cyano-4-hydroxycinnamic acid
CID	Collision induced dissociation
DCM	Dichloromethane

DIEA	<i>N,N</i> -diisopropylethylamine
DMF	Dimethylformamide
DV	Duct venom
ESMS	Electro spray mass spectrometry
FDA	Food and Drug Administration
Fmoc	9-Fluorenylmethoxycarbonyl
Fmoc-Asn(Trt)-OH	N-alpha-9-Fluorenylmethoxycarbonyl-N-beta-Trityl-L-Asparagine
Fmoc-Asp(OtBu)-OH	9-Fluorenylmethoxycarbonyl-L-Aspartic Acid-beta-t-Butyl Ester
Fmoc-Arg(Pbf)-OH	N-alpha-9-Fluorenylmethoxycarbonyl-N-g-2,2,4,6,7-Pentamethyldihydrobenzofuran-5-Sulfonyl-L-Arginine
Fmoc-Cys(Acm)-OH	9-Fluorenylmethoxycarbonyl-S-Acetamidomethyl-L-Cysteine
Fmoc-Cys(Trt)-OH	9-Fluorenylmethoxycarbonyl-S-Trityl-L-Cysteine
Fmoc-Gla(otBu)₂-OH	N-Fmoc-L- γ -Carboxyglutamic Acid γ,γ -Di-t-Butyl Ester
Fmoc-Gln(Trt)-OH	N-alpha-9-Fluorenylmethoxycarbonyl-N-gamma-Trityl-L-Glutamine
Fmoc-His(Trt)-OH	N-alpha-9-Fluorenylmethoxycarbonyl-Nim-Trityl-L-Histidine
Fmoc-Hyp(tBu)-OH	9-Fluorenylmethoxycarbonyl-O-t-Butyl-L-Hydroxyproline
GBR	Great Barrier Reef
(Gla)	γ -gammacarboxyglutamic acid
HCTU	1H-Benzotriazolium-1-[bis(Dimethylamino)Methylene]-5-Chloro Hexafluorophosphate-(1-),3-Oxide
IND	Investigational new drug
MALDI-MS	Matrix assisted desorption/ionization Mass spectrometry
MALDI-TOF-(TOF)-MS	Matrix assisted desorption/ionization-Time of light-(Time of flight)-Mass spectrometry
M_r	average molecular mass
mRNA	Messenger RNA
MS	Mass spectrometry

MV	Milked venom
<i>m/z</i>	Mass to charge ratio
nAChR	Nicotinic acetylcholine receptor
PCR	Polymerase chain reaction
PSD	Post-source decay
PTM	Post-translational modification
RE	Radula Extract
RV	Radula Venom
RP-HPLC	Reverse phase - High performance liquid chromatography
R_t	Retention time
SEM	Scanning electron micrograph
SPPS	Solid phase peptide synthesis
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
UV	Ultra-violet
TIPS	Triisopropylsilane

Highlights

Single Cone snail analysis shows m/z continuity in duct, milk and radula extracts.

Geographic specimen analysis shows peptide diversity in the milked venoms.

α -Conotoxin TxIC represents a highly post-translationally modified conotoxin.

Post-translational modifications influence biological and phylogenetic selectivity.

Fig. 1. RP-HPLC/UV comparison of peptides from the milked venom of representative non-captive *C. textile* specimens sampled from different geographic locations in the South Pacific: (A) Hawai'i; (B) American Samoa; and (C) the Great Barrier Reef, Australia. The expression of δ -conotoxin TxVIA (arrow), was detected in MV samples from Hawai'i (n=30, from 5 sites) and American Samoa (n=20, from 5 sites), but was absent from those from GBR, Australia (n=50, from 32 sites). α -Conotoxin TxIC (underscore) was detected in MV from all three locations, indicating its importance in the envenomation strategy of *C. textile*. MALDI-TOF MS analysis of these MV samples is shown in Fig. 2. In each map, indicated are cone snail collection sites (filled circle) and individual representative samples (arrow).

Fig. 2. MALDI-TOF MS mass spectra of of representative non-captive milked venoms from *C. textile* specimens obtained from: (A) Hawai'i; (B) American Samoa; and (C) the Great Barrier Reef, Australia

Peaks corresponding to known conopeptides (*) and peaks common to all 3 spectra () are indicated Table S2 provides an extensive list comparing all peaks observed in these venom profiles.

Fig. 3. RP-HPLC/UV comparison of venom obtained from (A) Radula Lumen Extract; (B) Milked Venom; and (C) Duct Venom from a representative specimen of *C. textile*
 (A) The RE revealed limited similarity of peptides within the hydrophilic region to those in the hydrophilic regions of MV and DV. Note that α -conotoxin TxIC (underscore) and δ -conotoxin TxVIA (arrow) were not detected in the RE. Both (B) MV and (C) DV demonstrated a high level of peptide abundance and continuity. These samples were profiled via MALDI-TOF MS (Fig. 4).

Fig. 4. MALDI-TOF MS profiles of peptide extracts from a single representative *Conus textile* specimen obtained from: (A) Radula Lumen Extract; (B) Milked Venom and (C) Whole Duct Venom

Peptides exhibiting common peaks are listed in Table 2 and indicated () in each profile. These data illustrate expressional consistency, although some unique peaks are observed from each venom source. Absence of α -conotoxin TxIC is apparent, but is suspected to be present as an in-source decay fragment. δ -Conotoxin TxVIA was observed at m/z 3035.6 ± 0.2 . Peaks corresponding to other known *C. textile* peptides are indicated (*). Table S1 provides an extensive list of observed peaks corresponding to known *C. textile* venom peptides.

Fig. 5.

(A) The decarboxylation of TCEP Reduced α -conotoxin TxIC as seen by MALDI-TOF MS. Observed molecular masses differ by 44 Da. The ratio could be influenced by choice of MALDI-MS matrix (not shown). **(B) CID analysis of α -conotoxin TxIC.** Using overlapping a-, b-, and y-ion series, the sequence was interpreted to be R-[I/L/O]-Q-C-C-S-H-[I/L/O]-A-C-N-V-D-H-P-E-[I/L/O]-C. Fragment ions also indicated decarboxylation and heterogeneity of the C-terminal amino acid.

Fig. 6. Genetic analysis of α-conotoxin TxIC and its comparison to α-conotoxins MII and Vc1.1
The Pre/Pro region of α-conotoxin TxIC demonstrates a high level of amino acid identity to established members of the 4/7 α-conotoxin family. *N*-terminal processing of α-conotoxin TxIC shares similarities with the processing of α-conotoxin Vc1.1 required to produce the mature toxin. Similar mechanisms operate to process α-conotoxin MII. C-terminal brackets [] represent stop codons within the formal cDNA sequence.

Fig. 7.

(A) Random folding of synthetic α-conotoxin TxIC. Production of isomeric materials was achieved upon random peptide folding, which when resolved by RP-HPLC/UV demonstrated difficulties in purification. **(B) Selective folding of α-conotoxin TxIC and its co-elution with native material.** Undertaking a separate synthetic, together with a step-wise directed folding strategy, allowed for the production of a single peak/isomer upon full oxidation. RP-HPLC/UV co-elution in oxidized and TCEP reduced from (not shown), demonstrated identical chromatographic behaviors.

Table 1
MALDI-TOF MS analysis of *C. textile* MV data from different geographical locations,
showing masses that correlate to know conopeptides

Obs. <i>m/z</i>	HI	AS	GBR	Mass correlation
879.309	✓			Leu-contryphan-Tx
1091.051		✓		TeAr193
1238.410		✓		TeAr151
1239.434			✓	
1305.405			✓	TxIIIC (Tx3.4)
305.443	✓			
1357.499			✓	Tx10b or Tx10c
1357.537	✓			
1392.411	✓			TxXIIIA
1447.593	✓			Tx5b
1448.557			✓	
1474.604			✓	Tx5c
474.620	✓			
1592.509			✓	Tx3d
1656.531	✓			Tx1c
1657.754		✓		TxIA
1678.968		✓		Tx-D0111
1679.718	✓			
1712.485			✓	TxIIIA
1712.570	✓			
1723.566			✓	TxMLKM-021
1723.636	✓			
Obs. <i>m/z</i>	HI	AS	GBR	Mass correlation
1757.642	✓			TxMMSK-03
1756.444			✓	
1761.518			✓	TxMRCL-D012
1779.609	✓			TxMMSK-04
1844.578			✓	Tx3e
1889.753			✓	Tx3f (Tx3.5)

Obs. <i>m/z</i>	HI	AS	GBR	Mass correlation
1889.808	✓			
2151.783	✓			Tx3h (TxMLKM-011)
2487.917			✓	Convulsant Peptide
2488.002		✓		
2488.044	✓			
2642.073	✓			Tx6.3
2942.164	✓			TxMKLT1-015
2961.308	✓			Tx05
2995.234	✓			
2995.311		✓		Tx6.2
3035.342	✓			TxVIA (King-Kong 0)
3066.942			✓	
3067.313		✓		TxMLKLT1-0111
3067.339	✓			

Obs. *m/z* are within ± 1.0 Da of the identified peptide mass to account for possible amide and free acid variants.

Abbreviations are: Hawai'i (HI); American Samoa (AS); and Great Barrier Reef, Australia (GBR).

Table 2
Comparative analysis of MALDI-TOF MS *C. textile* data from different venom sources (RE; MV; and DV)

Obs. <i>m/z</i>	RE	MV	DV	Mass correlation
1305.460			✓	
1305.528		✓		TxIIIC
1305.689	✓			
1357.487			✓	
1357.660		✓		Tx10b/Tx10c
1357.801	✓			
1392.422			✓	
1392.533		✓		TxXIIIA (TxMRCL-02)
1447.628			✓	
1447.747		✓		Tx5b
1447.879	✓			
1474.753		✓		
1474.906	✓			Tx5c
1521.536			✓	TxIIIB
1592.538			✓	
1592.716		✓		Tx3d
1592.896	✓			
1642.756			✓	
1642.927		✓		Tx5d
1643.013	✓			
1657.766			✓	
1657.914		✓		TxIA
1658.034	✓			
1679.701			✓	
1679.850		✓		Tx-D0111
1710.766			✓	TxIIIA
1723.635			✓	
1723.787		✓		TxMLKM-021
1757.609			✓	
1757.801		✓		TxMMSK-03
1757.981	✓			

Obs. <i>m/z</i>	RE	MV	DV	Mass correlation
1844.824		✓		Tx3e
1889.819			✓	
1889.980		✓		Tx3f
1890.178	✓			
1931.063	✓			TxVA
2151.757			✓	
2151.973		✓		Tx3h
2152.196	✓			
2488.586	✓			
2488.269		✓		Convulsant Peptide
2487.992			✓	
2557.971			✓	
2558.240		✓		Tx02
2558.667	✓			
2642.027			✓	
2642.319		✓		Tx6.3
2944.225			✓	
2944.594		✓		TxMKLT1-015
3011.221			✓	King-Kong 2
3035.290			✓	
3035.647		✓		TxVIA (KK-0)
3035.892	✓			
3067.747		✓		TxMKLT1-0111
3107.684		✓		Tx04

Obs. *m/z* are within ± 1.0 Da of the identified peptide mass to account for possible amide and free acid variants.

Table 4
Pharmacological analysis of α -conotoxin TxIC and [Pro]^{2,8}[Glu]¹⁶ α -conotoxin TxIC on nAChR isoforms

nAChR subtype ^a	IC ₅₀ ^b [μ M]	Maximum inhibition %	PD ₅₀ ^c [nMol Kg ⁻¹]
α -Conotoxin TxIC			34.2
$\alpha 3\beta 2$, $\alpha 3\alpha 5\beta 2$, $\alpha 3\beta 4$	100	<5	
$\alpha 4\beta 2$, $\alpha 4\beta 4$, $\alpha 7$, $\alpha \beta \gamma \epsilon$, $\alpha \beta \gamma \epsilon$	50	0	
[Pro] ^{2,8} , [Glu] ¹⁶ α -Conotoxin TxIC			3600
$\alpha 3\beta 2$	5.4 $\pm$ 0.5	96	
$\alpha 3\alpha 5\beta 2$	4.9 $\pm$ 0.8	91	
$\alpha 3\beta 4$	2.1 $\pm$ 0.2	77	
$\alpha 4\beta 2$, $\alpha 4\beta 4$, $\alpha 7$, $\alpha \beta \gamma \epsilon$, $\alpha \beta \gamma \epsilon$	100	<10	

^aHuman variants;

^bhalf-maximal inhibitory concentration (human);

^cparalytic dose (mollusk)