Peptidomic Analysis and Characterization of the Venom from *Conus purpurascens*

by

Alena Rodriguez

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This thesis was prepared under the direction of the candidate’s thesis advisor, Dr. Frank Mari, Department of Chemistry and Biochemistry, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Medicine and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

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The venom of cone snails is a potent cocktail of peptides, proteins, and other small molecules. Several of the peptides (conopeptides and conotoxins) target ion channels and receptors and have proven useful as biochemical probes or pharmaceutical leads. In this study, the venom of a fish-hunting cone snail, Conus purpurascens was analyzed for intraspecific variability; α-conotoxins from the venom were isolated by high performance liquid chromatography, identified by mass spectrometry and nuclear magnetic resonance, and tested in a electrophysiological assay in Drosophila melanogaster; the effects of diet change on venom composition was investigated. It has been determined that each specimen of C. purpurascens expresses a distinct venom, resulting in the expression of more than 5,000 unique conopeptides across the species. α-conotoxin PIA was shown to inhibit the Da7 nicotinic acetylcholine receptor.
Furthermore, when the diet is changed from fish to worms, the expression of conopeptides in the venom is modified in some cases.
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Chapter 1: Intraspecific Variation of Venom Components in *Conus purpurascens*

**Introduction**

Animal venoms are remarkable sources for biochemical probes and pharmaceutical drug leads because they target key biomolecular systems of medical interest. They are also extraordinarily complex and remain a largely untapped source of naturally active compounds. The greatest advantage of using natural products in drug discovery, particularly those found in venoms, is that they are the result of millions of years of evolutionary modification to allow them to specifically target a wide range of targets in a variety of systems, including the CNS and the vascular system. Venom components often reach clinical trials and some of them have received FDA approval for their commercial use. More recently, venom components have been incorporated into nutraceuticals preparations for cosmetic treatments and holistic medicine applications (Peter Thomas Roth Un-Wrinkle® Serum, patent pending and Escozine®, US Patent #US8097284 B2). Over the past four decades, intense studies have been performed on the composition and pharmacological properties of venom peptidomes and proteomes (venomics) of cone snails, a genus (*Conus*) of predatory marine mollusks. *Conus* venom is magnificent repository of highly modified peptides (conopeptides) whose biomedical value is exemplified by six conopeptides reaching clinical trials and the FDA-approved conopeptide, Prialt®, a chronic pain medication originally isolated from the venom *Conus magus*. 
Classic literature suggests that each of the 750+ Conus species worldwide contains an entirely unique venom made up of 50–200 distinct conopeptides. However, recent studies using more sensitive and better resolving techniques during conopeptide isolation and detection indicate that this number has been drastically underestimated. In most cases, conopeptides have been isolated from venom obtained from the dissected venom ducts of several specimens pooled together. The dissected venom contains cellular debris, unprocessed peptides, and other endogenous components in addition to conopeptides. Contrastingly, injected venom that is collected through the radular tooth during feeding is only composed of the toxic components that are delivered to prey; henceforth, injected venom is typically less complex than its dissected venom counterpart. Injected venom contains only the biologically relevant and mature conopeptides that are required to paralyze and capture prey and its components are unique for a given Conus species. The peptide profiles of dissected and injected venoms from the same species typically contain components in common, however comparisons are seldom drawn between the two type of venom.

The difference in venom composition among individuals of the same Conus species has been an evolving topic in cone snail research. Intraspecies variation has been previously described for the following cone snail species’ dissected venom: C. geographus, C. striatus, C. catus, C. ventricosus, C. textile, C. imperialis, C. marmoreus, C. vexillum, and C. ermineus. These variations have also been reported in injected venoms (C. striatus, C. catus, C. consors, C. ermineus, and C. purpurascens). Some studies accounted for variations seen in an individual specimen’s injected venom over time (C. consors, C. textile, and C. purpurascens).
HPLC combined with mass spectrometry (MS) techniques are the methods of choice to assess the complexity and variability of animal venoms. Invariably, venom fractionation by RP-HPLC is used in all cone snail venom analytical protocols, regardless of its subsequent use, which typically involves testing in a suitable biological assay. Venom fractions are best characterized either by matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) MS or electrospray ionization (ESI) MS. Complete venom profiling has been difficult by preparative methods alone because several conopeptides are lowly expressed and difficult to separate from the few abundant conopeptides present in the venom. Additionally, some venom components may be subjected to degradation, adsorption, or ion suppression effects during separation and mass spectrometric identification processes. These two factors can lead to an incomplete analysis of the venom variability and an overall undervalue of the sheer amount of conopeptides available. A better evaluation of the venom complexity can be achieved using hyphenated techniques with improved bioanalytical resolution. Using such deep venomics approaches more conopeptides can be detected, which may widen the range of compounds to be considered for therapeutic development and it will provide a more realistic scenario for the complexity of cone snail venom.

This study details the comprehensive analysis of the peptidic composition of one the most studied fish-hunting cone snail species, *C. purpurascens*. A comparative approach was used to analyze the venom components from: 1) RP-HPLC fractionation combined with offline MALDI-TOF MS aimed at sample recovery and 2) nanoflow LC-ESI-TripleTOF-MS used strictly as an investigative tool to detect the largest number of compounds possible. This resulted in a comprehensive repertoire of conopeptides
identified in the venom of *C. purpurascens*. The LC/MS and LC-ESI-MS profiles reveal significant intraspecific variations in both the injected venom and the dissected venom of *C. purpurascens* individuals. These variations dramatically increase the sheer number of conopeptides available in nature from the previously estimated 50-200 distinct conopeptides per species to more than 5,000 components. This complements the idea that cone snails are sources of incredibly large and ever-changing natural combinatorial peptide libraries that are useful for pharmaceutical research. Larger conopeptide libraries can be generated through the use of combined bioanalytical techniques that enhance the ability to separate and analyze complex *Conus* venoms.

**Materials and Methods**

*Specimen Collection.* Eleven specimens of *C. purpurascens* (20-50 mm in length) were collected from the Pacific shores of Costa Rica and were transferred to aquaria, where they were kept for venom extraction. All specimens were housed in 56.8-liter aquaria under similar temperature, salinity, and feeding time conditions.

*Injected Venom Extraction and Feeding.* The injected venom from the *C. purpurascens* individuals was extracted using the ‘milking’ procedure of Hopkins et al. Feeder gold fish (*Carassius auratus*) were acquired locally. The fish were placed in front of the cone snails until proboscis extension. Then, the fish was replaced with a microcentrifuge tube trap consisting of the hollowed out tube cap covered by latex glove membrane primed with pieces of a fish tail. Sensing the fish tail tissue, the cone snail injects its venom into
the collection tube. The tube was then separated from the snail’s harpoon and the snail was fed a gold fish. The cone snails were “milked” once a week and, on average, 5 – 10 μl of venom was collected per milking. The collected venom was centrifuged and stored at -80°C until further use.

_Dissected Venom Extraction._ The dissected venom from two _C. purpurascens_ individuals was isolated by removing the venom apparatus from live snails and then squeezing the venom out of the venom duct. These individuals previously provided injected venom, which was used for side-by-side comparisons. The dissected venom was dissolved in 0.1% TFA (Fisher Scientific, PA, USA). Extracts were stored at -80°C until further use.

_RP-HPLC Analysis of Injected Venom._ Approximately 50 μL of injected venom from each specimen were pooled and dissolved in 0.1% TFA for fractionation. RP-HPLC was carried out using a SpectroMonitor 5000 Photodiode Array Detector (LDC Inc., Carlsbad, CA, USA) and a Series 200 LC pump (Perkin Elmer, Waltham, MA, USA). The chromatography was monitored using the PeakSimple system, software version 4.35 (SRI Instruments, Torrance, CA, USA). All fractionations were performed using a Kinetex C8 column (4.6 mm x 50 mm; 2.6 μm particle diameter; 100 Å pore size, Phenomenex, Torrance, CA, USA). Venom components were eluted over 100 min with an incremental linear gradient of 100% solution A (0.1% trifluoroacetic acid) to 100% solution B (60% acetonitrile in 0.1% trifluoroacetic acid). A flow rate of 1.0 mL/min was used. All fractions were collected manually under UV detection at λ = 205, 220 and 280 nm, respectively. Fractions were freeze-dried and kept at -20°C until further use.
MALDI MS Analysis of Injected Venom Fractions. MALDI-TOF MS spectra were acquired using an Applied Biosystems Voyager-DE PRO mass spectrometer (Framingham, MA, USA). Samples were run in reflector mode ($M/\Delta M = 10000$). Calibrations were carried out using Applied Biosystems/MDS Mass Standards Kit (Calmix 1) as an external calibration standard until mass tolerance of better than 10 ppm was achieved. Dried RP-HPLC fractions were dissolved in 25 $\mu$L of 60% acetonitrile/0.1% trifluoroacetic acid. 0.3 $\mu$L of $\alpha$-cyano-4-hydroxycinnamic acid (CHCA, 25 mg/mL in acetonitrile/methanol 54:31, ACROS Organics, NJ, USA) matrix was plated on an Applied Biosystems 384-well magnetic plate. 0.3 $\mu$L of each fraction was plated on top of the dried matrix in separate wells and allowed to dry before MALDI-TOF MS analysis. Fractions were analyzed in an 800 – 5,000 Da mass range.

LC-ESI-MS Analysis of Injected Venom and Dissected Venom. LC-ESI-TripleTOF-MS analysis of venom samples was performed using an AB SCIEX TripleTOF 5600 System (Framingham, MA, USA), equipped with a duospray ionization source coupled to a Shimadzu 30 series HPLC system. The same amount of injected venom (2 $\mu$L) was subjected to LC-ESI-TripleTOF-MS to obtain Total Ion Current (TIC) traces and complete mass lists of peptide components for the venom of each of the nine specimens. The LC venom separation was performed using a Thermo C18 4.6 x 150 mm column at a linear 1.3% B (90% acetonitrile/0.1% formic acid) gradient with a flow rate of 0.3 mL min$^{-1}$ over 20-25 minutes.
Bioinformatics. Mass spectrometry raw data was pre-processed before further analysis by using the “Remove duplicate masses” tool and the “Compare mass lists” tool found in the ConoServer website (http://www.conoserver.org). Duplicate masses within 0.5 Da were removed and the common masses between two mass lists were identified. Graph of the cumulative number of conopeptides present in *C. purpurascens* venom was constructed in GraphPad Prism 4 as described previously.

Results and Discussion

**RP-HPLC Chromatographic Profiles of Injected Venom and MALDI MS of Fractions.**

The injected venom of nine *C. purpurascens* specimens was separated by RP-HPLC (Fig. 1). All chromatograms display complex compositions and excellent resolution that compares to previously reported RP-HPLC profiles of injected venom from *C. purpurascens* and other fish-hunting *Conus* species (*C. geographus*, *C. consors*, *C. striatus*, and *C. ermineus*). There were between 97 and 150 fractions manually collected from the injected venom sample of each specimen. None of the chromatographic profiles are identical, however, most major components of the injected venom are polar compounds (Retention time, Rt = 10 – 45 min). Specimens 1, 3 and 4 show injected venom profiles with large, late eluting peaks containing hydrophobic components, around Rt = 60 – 80 min (Fig. 1). Whereas, specimens 2, 5, 6, 7, 8, and 9 show little to no major components in their injected venom past Rt = 60 min (Fig. 1). Each specimen exhibits a unique level of peptide expression, implying that each *C. purpurascens* individual may use unique molecular hunting strategies. There are known
molecular ‘cabals’, which are groups of conopeptides that work synergistically to produce a specific debilitating effect to the prey, such as neuromuscular block or excitotoxic shock \(^\text{21}\). It appears that each specimen brews its own “cabal” to effectively secure its prey.

Analysis of injected venom fractions analyzed using MALDI-TOF MS was optimized at various levels; most samples were recorded in reflector mode \((m/z \ 800 – 5000 \ \text{Da})\), since it allowed molecular mass measurement with higher resolution and accuracy than in the linear mode, albeit is lower sensitivity. The dried droplet spotting method was used to deposit CHCA matrix and then injected venom sample was utilized, because it resulted in a homogenous distribution of matrix crystals and sample solution for proper mass analysis.

A total of 513 components were identified with unique masses among the nine specimen samples. The masses obtained were compared with 22 published conopeptide masses of \(C. \ purpurascens\) and 16 of these were detected in the injected venom of at least one of the nine specimens. It is surprising that none of the known conopeptides were detected in all nine specimens since the known conopeptides are usually the most highly expressed components in the venom, hence their discovery (see Table 1). All specimens, except specimen 6, share three common molecular masses, 2616.48, 2632.52, and 2648.53 Da. These three components are likely variants of the same conopeptide that has been subjected to different levels of Pro hydroxylation because they differ by 16 Da each \(^\text{22}\). Hydroxylation, most typically of Pro, but occasionally of Lys \(^\text{23}\) and Val \(^\text{24}\), are among the most common post-translational modifications (PTMs) found in conopeptides and are significant contributors to the expanded diversity of venom components \(^\text{24-25}\). Using RP-
HPLC profiling combined with MALDI-TOF MS, it is clear that the injected venom of *C. purpurascens* has an extremely high degree of intraspecific expression variability.

**Figure 1.** Comparison of RP-HPLC chromatographic profiles of the injected venom of nine *C. purpurascens* specimens (Figs. A – I correspond to specimens 1–9 respectively). Injected venom samples were fractionated over a 100-minute gradient. The number of detected masses detected by MALDI-TOF MS is in parenthesis in the top right corner of each chromatogram.
Table 1. Expression of previously identified *C. purpurascens* conopeptides detected by MALDI-TOF MS in the injected venom of specimens 1 through 9.

<table>
<thead>
<tr>
<th>Conopeptide Name</th>
<th>MW (monoisotopic mass)</th>
<th>Reference</th>
<th>Specimen Number</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td>1</td>
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<td>Contraryphan-P [D5&gt;L]</td>
<td>887.40</td>
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<td>Contraryphan-P</td>
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<td></td>
<td></td>
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<tr>
<td>p1.9</td>
<td>1270.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVA</td>
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<tr>
<td>α-PIB</td>
<td>1758.68</td>
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<td>α-PIA</td>
<td>1979.82</td>
<td>28</td>
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<td>p1.10</td>
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<td>p3.9</td>
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<td></td>
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<tr>
<td>κA-PIVF</td>
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<td>□</td>
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<tr>
<td>μ-PIIIA</td>
<td>2603.11</td>
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<tr>
<td>αA-PIVA</td>
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</tr>
<tr>
<td>p21a</td>
<td>9313.98</td>
<td>35</td>
<td></td>
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</tbody>
</table>

Conopeptides that were identified in at least one specimen are bolded; □ indicates that the mass was detected.

*LC-ESI-MS Chromatographic Profiles of Injected Venom.* The injected venom of all specimens was analyzed using LC-ESI-TripleTOF-MS to produce a high-resolution snapshot of injected venom composition using a more rapid and direct approach (Fig. 2). Two injected venom samples from each specimen were blindly analyzed by LC-ESI-
TripleTOF-MS to determine if venom composition changes within the individual each time it injects its venom. The TIC traces of 18 injected venom samples (two per specimen) were perfectly paired as their ion chromatograms traces revealed unique profiles characteristic of each specimen (Fig. 2). The two injected venom samples of specimens 1 - 8 have impeccably matching venom profiles, whereas one of the injected venom samples of specimen 9 shows a lower level of conopeptide expression, which may be explained by a “blank” venom injection that has been reported in *C. purpurascens* venom

For most specimens, the highly expressed components in the injected venom are mostly hydrophilic (Rt = ~3 – 10 min), just as it was seen by standard RP-HPLC. Likewise, the injected venom LC-ESI-TripleTOF-MS profiles can be differentiated by the presence or absence of a large, late eluting peaks in the hydrophobic region (Rt = 17 min). LC-ESI-TripleTOF-MS analysis of the injected venom samples is highly advantageous because its high sensitivity and remarkable dynamic range of detection; these qualities allow the rapid detection of hundreds of conopeptides from minute venom samples. This is in notable contrast with the lengthier separation protocols used to fractionate sufficient quantities of conopeptides for use in bioassays.
Figure 2. Comparison of LC-MS Total Ion Current traces of the injected venom of *C. purpurascens* specimens 1 – 9 (A – I respectively) run on the TripleTOF 5600 system. Injected venom samples were fractionated over a 20-minute gradient.
Table 2. Expression of previously identified *C. purpurascens* conopeptides detected by ESI-TripleTOF-MS in the venom of specimens 3 and 7.

<table>
<thead>
<tr>
<th>Conopeptide Name</th>
<th>MW (monoisotopic mass)</th>
<th>MW (specimen 3)</th>
<th>MW (specimen 7)</th>
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<td>887.40</td>
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<td>α-PIA</td>
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<td>2603.11</td>
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<tr>
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<td>P2a</td>
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<td>Conantokin-P</td>
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<tr>
<td>κ-PVIIA</td>
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<tr>
<td>P21a</td>
<td>9313.98</td>
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</tbody>
</table>

Conopeptides that were identified in at least one specimen are bolded; ☐ indicates that the mass was detected.

**Comparison of the Two Most Dissimilar Injected Venom Profiles.** Of all nine individuals, specimens 3 and 7 show the most dissimilar injected venom profiles. As seen in the overlaid preparative RP-HPLC chromatograms of the injected venom of specimens 3 and 7 (Fig. 3, black and red data lines respectively), there is little overlap of chromatographic peaks. The separated fractions from the injected venom of each individual contain unique...
peptide masses that display varying retention times. Using MALDI-TOF MS, 124 and 70 masses were detected in the venoms of specimen 3 and 7 respectively, with 26 masses in common between these two venoms. Interesting, specimen 7 has prominent hydrophobic components (Fig. 3, large peak at Rt = 66 min) that are not highly expressed in specimen 3 (Fig. 3). These results were confirmed by LC-ESI-TripleTOF-MS analysis (Fig. 4), which showed that specimen 3 has a late eluting cluster of peaks at Rt = 17 min, absent in specimen 7. This cluster contained the hydrophobic δ-conotoxin, PVIA, known as the ‘lock-jaw peptide’ 32. δ-PVIA is the first vertebrate-targeted excitotoxin described from cone snail venom and it is not always detected in all C. purpurascens individuals 14, 32, which correlated well with our findings (see Table 1).

The rapid separation of injected venom components using LC-ESI-TripleTOF-MS does not provide the same level of chromatographic resolution as standard RP-HPLC (Fig. 3). However, 1,916 and 1,849 masses were detected in the injected venom of specimen 3 and 7 respectively, using ESI-TripleTOF-MS, vastly exceeding the number of masses detected with MALDI-TOF MS. The peptide mass distribution of both specimens (Fig. 5) reveals that the most detected masses are in the 1-2 kDa range. While the total number of masses detected in both specimens was similar, there is little to no overlap of major components in each specimen’s injected venom. Of the 22 known conopeptides, 14 and 10 masses from specimens 3 and 7 respectively were found to correspond to known conopeptides. As part of their envenomation strategy, specimen 3 seems to rely mainly on κA-PIVF and p2a, paralytic components that inhibit nAChRs, as these are the major components of the venom. Contrastingly, specimen 7 relies mostly on ψ-PIIIE, also an inhibitor of nAChRs, and a series of variants of p21a. The molecular masses of venom
components of these two specimens were compared with the masses of known conopeptides from *C. purpurascens* (Table 1), revealing that both individuals contain αA-PIVA (nAChR inhibitor) and μ-PIIIA (skeletal muscle Na⁺ channel inhibitor). These conopeptides appear to be constant within the envenomation mechanism used by *C. purpurascens* to cause neuromuscular block. Likewise, the injected venom of both specimens contains κ-PVIIA (K⁺ channels inhibitor), a conopeptide involved in excitotoxic shock of the prey. While these few components have recognizable molecular targets, the rest of the venom contains hundreds of detected masses, which are novel *C. purpurascens* conopeptides. These results suggest that each individual cone snail acts as a unique ‘peptide engineer,’ producing a distinct blend of bioactive venom components fitted for a specific molecular hunting strategy.

**Figure 3.** Comparison of the two most dissimilar analytical RP-HPLC chromatographic injected venom profiles fractionated over a 100-min gradient. Chromatograms were overlaid with specimen 3 shown in black and specimen 7 shown in red. The Venn diagram shows the number of detected masses that were in common between the specimens.
Figure 4. Comparison of the most dissimilar LC-MS Total Ion Current traces run on the TripleTOF 5600 system. (A) specimen 3 and (B) specimen 7.

Figure 5. Molecular mass distribution (1 kDa bins) of injected venom fractions from specimens 3 and 7 obtained by LC-ESI-TripleTOF-MS.
Comparison of the Injected Venom and the Dissected Venom by LC-ESI-MS. Most studies on cone snail venom use dissected venom, therefore we decided to investigate the dissected venom of two *C. purpurascens* specimens, 10 and 11, and compared them with their injected venom counterparts (Fig. 6). LC-ESI-TripleTOF-MS yielded 2,566 masses in the dissected venom of specimen 10, compared to 941 masses found in the injected venom of the same specimen. There were 742 common masses between these two types of venom. For specimen 11, there were 1990 and 1,959 masses detected in the dissected and injected venom respectively, with 1,004 masses in common. The TIC traces of the injected and dissected venom of specimen 10 (Fig. 6A and 6B) show two major differences: there are more components are in the dissected venom overall and there is higher expression of hydrophobic components in the dissected venom compared with the injected venom (more peaks between Rt = 12-25 min in the dissected venom). Unexpectedly, the TIC trace of the dissected venom of specimen 11 (Fig. 6D and 6D) appears to be less complex that is injected venom counterpart. This could be due to partial degradation of venom components before separation.

The dissected venom of cone snails is usually considered more complex than its injected venom counterpart \(^8\text{b,37}\). Part of the added complexity of dissected venom is likely due to the presence of proteins (conoproteins) and enzymes that function in toxin maturation and processing, partly processed conopeptide precursors, endogenous peptides, and cellular debris that are mostly absent in the refined product that is injected into the prey. While in our current analysis we have emphasized the differential expression of peptides in the injected venom, we previously shown that conoproteins are important components in the injected venom of *C. purpurascens* and *C. ermineus* \(^38\).
Evaluating the differential expression of proteins in the venom will require dissimilar methodologies from our current approach.

Figure 6. Comparison of LC-MS Total Ion Current traces of the injected venom and the dissected venom of two *C. purpurascens* specimens run on the TripleTOF 5600 system. All venom samples were fractionated over a 25-minute gradient. (A) Injected venom and (B) dissected venom of specimen 10, (C) total masses detected in injected venom (941) and dissected venom (2,566) of specimen 10, (D) injected venom and (E) dissected venom of specimen 11, (F) total masses detected in injected venom (1,959) and dissected venom (1,004) of specimen 11.

**Total Number of Distinct Conopeptides found in the Injected Venom.** Regardless of the methods of extraction, most reports estimated that there are 50 – 200 conopeptides in the venom of each *Conus* species. However, it has been determined that the dissected venom of three *Conus* species (*C. textile, C. marmoreus, and C. imperialis*) contains between 1100 – 1900 conopeptides. It has been recently reported that the injected venom of *C. geographus* expresses between 1800 – 2500 conopeptide masses. Here we show that the overall number of detectable masses in *Conus* venoms is still underestimated, even when we used injected venom to investigate its variability in expression. A cumulative curve of total masses detected in *C. purpurascens* was generated from ranking specimens from the most diverse to least diverse and fitted using
an exponential equation. The conservative estimate of venom conopeptide diversity for this species levels off around 5,000 unique conopeptides (Fig. 7). The cumulative number of detectable conopeptide masses in the venom of *C. purpurascens* increases with increasing numbers of specimens. The number of detectable masses also increases with the use of multiple chromatography techniques and methods of mass spectrometry ionization. Thus, coverage of the molecular diversity of these venoms is improved with the use of different analytical methods.

The complexity of *C. purpurascens* venom is likely due to external factors such as selective pressures of prey type and availability, climate changes, and gender. Additionally, internal factors can give rise to conopeptide diversity, including focal hypermutation, recombination, or duplication of genes, post-translational modifications, and hypervariable processing (proteolytic and post-translational) at different loci within the same precursor. Combining bioanalytical methods of bioassay-guided fractionation and deep venomics approaches has resulted in a more comprehensive repertoire of conopeptides identified in the venom of *C. purpurascens*. 
Figure 7. Graph of the cumulative number of masses contained in the injected venom of all *C. purpurascens* specimens that were studied. Specimens were ranked from most diverse to least diverse and their respective number of detected masses was fitted using an exponential equation.

*Comparison of RP-HPLC/MALDI MS and LC-ESI-MS Techniques.* There was a degree of reproducibility between the techniques. For instance, both separation protocols revealed that specimens 1, 3, and 4 had the highest expression of hydrophobic components (see Rt = 66 min in Fig. 1A, 1C-D and Rt = 17 min in Fig. 2A, 2C-D). Also, at least half of the previously identified *C. purpurascens* peptide masses that were found in specimens 3 and 7 were detected by both techniques (Tables 1 and 2).

However, there are differences between the two approaches, each having its advantages and disadvantages. For example, TFA was used as an ion-pairing reagent in LC/MALDI-TOF-MS while FA was used in LC-ESI-TripleTOF-MS. TFA improves chromatographic resolution without any noticeable effects on MALDI-TOF MS sensitivity of peptides. On the other hand, TFA quenches the ionization on the ESI-MS
source and is therefore deleterious for ESI under LC-MS conditions. For this reason FA is used for the acidification of HPLC running buffers in LC-ESI-TripleTOF-MS experiments, but it is not as good ion-pairing reagent as TFA. RP-HPLC over a 100-minute gradient using TFA is more useful for isolation of conopeptides for the purpose of bioassays, as there is high resolution of separation (Fig. 1), while LC-ESI-TripleTOF-MS over a 20- or 25-minute gradient is better for overall rapid mass detection (Fig. 2).

The analytical approaches that we used rely on two different MS ionization sources: MALDI-TOF MS, which involves the use of a matrix that absorbs laser irradiation subsequently ionizing the sample and ESI-TripleTOF-MS, which is a flow-based protocol where the sample is aerosolized and then ionized. Some ions are detected in one ionization technique that are not detected in the other, giving rise to different sets of masses identified by MALDI-TOF MS versus ESI-TripleTOF-MS. The differences in the detected masses might be due to spectra that only show major components, whereas other peptides in the mixture are not detected due to poor ionization, or their abundance fall below the dynamic range capabilities of the instrument. This may explain why less masses were detected using MALDI-TOF MS in comparison to LC-ESI-TripleTOF-MS, and shows that the overlapping capabilities of different ion sources must be used to detect the most compounds possible in complex samples.

The diversity and complexity of Conus venoms warrants the use of several analytical methods to identify and characterize conopeptides, such as RP-HPLC followed by MALDI-TOF MS analysis and LC-ESI-TripleTOF-MS, which together provide better coverage of conopeptide masses. Here we compared a classical bioassay-guided protocol and a deep venomics approach to separate and identify as many conopeptide masses as
possible with the aim to assess the complexity of the venom of *C. purpurascens*. The results indicate that the number of unique conopeptides contained within the injected venom of a *Conus* species has been vastly underestimated. The classical claim that estimates 50 – 200 toxin components in the venom of each *Conus* species can be now expanded beyond 5,000 peptidic natural products.

Although intraspecific variations increase the opportunity for discovering novel conopeptides, they create challenges for characterization and assaying of these bioactive compounds. The conopeptide composition of *C. purpurascens* and other cone snail species tends to differ greatly from individual to individual, making it difficult to obtain a sufficient amount of a purified conopeptide to determine its pharmacological properties. Thus, the analysis of injected venoms from single specimens as opposed to pooling dissected venom from several individuals may allow the discovery of components that specifically target pharmaceutically important receptors and ion channels. Also, harvesting injected venoms does not require sacrificing the animals, which increases the amount of venom available for research.

Here we have compared current analytical methodologies to explore the venom of *Conus purpurascens*. This analysis demonstrates the enormous complexity of the cone snail venom and their molecular adaptability, which translates in efficient prey capture. Their evolutionary success has provided a massive library of active peptides, which can be utilized for discovery of molecular modulators with direct relevance to human therapeutics.
Chapter 2: Isolation and Characterization of α-conotoxins from *C. purpurascens*

Introduction

Conopeptides are classified in three schemes: gene superfamilies, cysteine frameworks, and pharmacological families. Gene superfamilies are grouped by the signal sequence of the peptide precursor that is cleaved from the mature peptide in the endoplasmic reticulum. The superfamilies A, O, and M are most commonly studied. The cysteine framework, the linkage of the disulfide bridges, can also be used to classify conopeptides. Conopeptides with two or more disulfide bridges are known as conotoxins. Moreover, conopeptides can be characterized pharmacologically by the ion channel or receptor that they target.\(^{47}\)

α-conotoxins are a group of conopeptides that range from 12 – 19 amino acids in length and belong to the A-superfamily and cysteine framework I. Their sequence contains four cysteines with the disulfide bond arrangement CC-C-C, where the dash indicates a certain number of amino acid residues that are not cysteine. These conotoxins are one of seven families of conotoxins that target nicotinic acetylcholine receptors (nAChRs) \(^{48}\). α-conotoxins are further classified by the number of amino acids within the two loops between cysteine residues and this can help predict the nAChR subtype specificity. For instance, α3/5-conotoxins have three residues in the first loop and five residues in the second loop and are selective inhibitors of muscle-type nAChRs \(^{49}\).
nAChRs are ligand-gated ion channels that can be divided into muscle receptors and neuronal receptors. Both types of nAChRs are made up of five subunits organized around a central pore. Adult muscle-type nAChRs are composed of two α1 subunits and one subunit each of β1, ε, and δ. Neuronal-type nAChRs contain variable compositions of α2-α10 and β2-β4 subunits and can be either homomeric or heteromeric.

Four α-conotoxins have been identified in the venom of *C. purpurascens*: α-PIA, α-PIB, α-PIC, and variant α-PIC[O7]. The bioactivity of α-PIA has been investigated in *in vitro* assays as well as α-PIB, which have also been reported to cause delayed, flaccid paralysis when injected into goldfish. α-PIA selectively inhibits nAChRs that contain the α6 subunit in *Xenopus* oocytes while α-PIB selectively inhibits skeletal muscle-type nAChRs. α-PIC and its variant α-PIC[O7] were previously discovered in Dr. Mari’s lab but have not been pharmacologically characterized. The average MWs, sequences, and selectivity for nAChR subtypes of known α-conotoxins from *C. purpurascens* are given in Table 3.

### Table 3. MWs, sequence alignment, and selectivity for nAChR subtypes of known α-conotoxins from *C. purpurascens*

<table>
<thead>
<tr>
<th>Conotoxin</th>
<th>Average MW (Da)</th>
<th>Sequence</th>
<th>Activity on nAChR Subtypes</th>
<th>IC50</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-PIA</td>
<td>1981.26</td>
<td>RDPCCSNPVCTVHNPQIC</td>
<td>α6/α3β2β3 (H. sapiens) α6/α3β4 (H. sapiens)</td>
<td>1.7 nM</td>
<td>Dowell, 2003</td>
</tr>
<tr>
<td>α-PIB</td>
<td>1760.00</td>
<td>ZSOGCCWNPACVKNR---C</td>
<td>α1β1δε (M. musculus) α1β1γδ (M. musculus)</td>
<td>36 nM 45 nM</td>
<td>Lopez-Vera, 2007</td>
</tr>
<tr>
<td>α-PIC</td>
<td>1459.61</td>
<td>SGCCKHPACGKNR----C</td>
<td>unknown</td>
<td>---</td>
<td>H. Cano, Thesis</td>
</tr>
<tr>
<td>α-PIC[O7]</td>
<td>1475.60</td>
<td>SGCCKHOACGKNR----C</td>
<td>unknown</td>
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</tbody>
</table>
It is important to continue to define the molecular targets of these conotoxins because they can be used as pharmaceutical probes to study diseases implemented by the dysfunction of nAChRs, such as Alzheimer’s disease, Parkinson’s disease, schizophrenia, epilepsy, and nicotine addiction. Traditionally, the characterization of conotoxins is done using patch clamp electrophysiology on neurons and oocytes or in vivo injections into small vertebrates to observe behavioral changes. The approach used in this study to characterize conotoxins is paired nanoinjection with in vivo electrophysiological recording of the Giant Fiber System (GFS) of Drosophila melanogaster, shown in Figure 8a. This method is advantageous because it requires picomole amounts of conotoxins and takes advantage of a well-characterized neuronal circuit that contains several molecular targets, including nAChRs, electrical GAP junctions, glutamatergic neuromuscular junctions, and other ion channels.

**Figure 8.** (a) Electrophysiological arrangement of the GFS in D. melanogaster. Tungsten electrodes are placed through the eyes into the brain of the fly to stimulate the Giant Fibers (GF) and into the abdomen to serve as a ground (not shown in figure). Glass electrodes are placed in the TTM and DLM to record the muscle responses to GF stimulation. The conotoxins are injected through the ocelli while simultaneously stimulating the GF circuit. (b) Wild-type response latency of the TTM and DLM.
The GFS of *D. melanogaster* is composed of four neurons and two muscles responsible for the escape response of the fly (Figure 9)\textsuperscript{54}. When a drug or conotoxin is injected into the open circulatory system of the fly, hints of its molecular target can be deduced from changes in the wild-type electrophysiological response latency (Figure 8b) of the two muscles, the Dorsal Longitudinal Muscle (DLM) and the Tergo Trochanteral Muscle (TTM)\textsuperscript{53a}. The synapse between the Peripheral Synapsing Interneurons (PSI) and the DLM motor neurons (DLMn) is dependent on the homomeric *Drosophila* α7 (Da7) nAChR\textsuperscript{55}. Another cholinergic synapse is located between the Giant Fibers (GF) and the TTM motor neurons (TTMn), however, this synapse also contains an electrical GAP junction component\textsuperscript{54-55}. It is predicted that when an α-conotoxin that can target Da7 nAChR is injected into the fly, the GF-TTM pathway would remain intact while the GF-DLM would be disrupted by the inhibition of the cholinergic synapse between the PSI and DLMn. Since the Da7 receptor is most homologous to that of the human α7 (Ha7) nAChR (Figure 10), sharing 82% amino acid homology in the ligand-binding domain\textsuperscript{53c}, this bioassay is a promising characterization tool to discover chemical probes or pharmaceutical leads.
Figure 9. Diagram of the GFS. The GFs (shown in red) synapse electrically (Gap Junctions) as well as chemically (cholinergic) onto a Peripheral Synapsing Interneuron (PSI, shown in green) and the TTM neuron (TTMn, shown in yellow). The PSI to DLM neuron (DLMn, shown in blue) connection is dependent on Da7 nAChR subtype. The neuromuscular junction (NMJ) of the TTMn and the DLMn onto the jump (TTM, shown in purple) and flight muscles (DLM, shown in purple) is glutamatergic\textsuperscript{53c}.
In this study, α-PIA, α-PIC, and α-PIC[O7] were isolated from the venom of *C. purpurascens* and tested in the *D. melanogaster* GFS bioassay. Nanoinjection of α-PIA into the fly causes disruption of the GF-DLM pathway but not the GF-TTM pathway at various concentrations (ranging from 21-88 pmol/fly), indicating that cholinergic synapses are most likely being inhibited. α-PIC shows very low inhibition activity at high concentrations while α-PIC[O7] does not show any significant effects in the GFS.
Materials and Methods

*Specimen Collection, Injected Venom Extraction and Feeding, RP-HPLC, and MALDI MS.* Refer to Chapter 1. Fractions from several RP-HPLC separation runs containing α-PIA, α-PIC, or α-PIC[O7] were pooled together by rinsing each fraction two times each with solution A, 50:50 solution A: solution B, and solution B. All solutions containing the same conotoxin were combined in a 5 mL centrifuge tube and freeze-dried for subsequent purification RP-HPLC runs. Purification HPLC runs were under the same conditions described in Chapter 1.

*Determination of α-conotoxin Concentration and Total Yield.* Concentrations were calculated using the molar extinction coefficient at absorbance of λ = 205nm (ε205) with a previously described method 56. The absorbance at 205nm of 1 μL of conotoxin was measured in a DS-11 spectrophotometer (DeNovix Inc.) The calculated ε205 and concentrations of α-PIA, α-PIC, and α-PIC[O7] are shown in Table 4.

*Fly Stocks and Husbandry.* Wild type flies (w118) were kept at room temperature in vials containing standard media. The stocks were transferred to new vials with fresh media weekly. Male flies of 1-6 days old were used in the assays.

*Paired Electrophysiology/Nanoinjection Bioassay in Drosophila melanogaster.* Bioassays in flies were performed as previously described 53a-c. Briefly, flies were immobilized in dental wax and five electrodes were placed into the fly to acquire standard electrophysiological recordings. Two tungsten electrodes were placed into each
eye to stimulate the GF through the brain. Another tungsten electrode was placed into the fly’s abdomen as a ground. Recording glass electrodes were filled with a saline solution that mimics the hemolymph of the fly (101 mM NaCl, 1 mM CaCl2, 4 mM MgCl2, 3 mM KCl, 5 mM glucose, 1.25 mM NaH2PO4, 20.7 mM NaHCO3, pH 7.2) and placed in the TTM and DLM muscles.

To establish which flies had baseline wild-type recordings, the GF was tested electrophysiologically. Before, during, and after conotoxin injection, the GFs were stimulated with single pulses at 1 Hz for 100 sweeps to examine any immediate effects on the GF-pathways. Within 1 minute after conotoxin injection the integrity of the GF-TTM and GF-DLM pathway responses were tested with 10 sweeps of 10 stimulus trains at 100 Hz with 1 second between each sweep (total of 100 stimuli). This was repeated every 5 minutes for a total of 20 minutes. A muscle response percentage was calculated by the ability to follow the trains of stimuli and compared to baseline responses before conotoxin injection and to control flies injected with 0.7% saline.

Injection glass micropipettes were filled with α-conotoxins and inserted into the ocelli of the fly’s head for injection. For α-PIA injections, flies (n = 10) were injected with 46, 35, 23, and 11 nl of pure isolated α-PIA (88, 67, 44, and 21 pmol/fly respectively) to generate a dose response curve. For α-PIC injections, flies (n = 10) were injected with 46 nl of pure isolated α-PIC (112 pmol/fly). For α-PIC[O7] injections, flies (n = 10) were injected with 46 nl of pure isolated α-PIC[O7] (84 pmol/fly). The same amounts of 0.7% saline was injected into control flies (n = 10).
Homology Modeling of α-PIA Bound to the Da7 Receptor. Homology models of α-PIA bound to the Da7 nAChR were created from the crystal structure of the acetylcholine binding protein (AChBP) in complex with α-conotoxin ImI as previously described using Modeller v9.11 and analyzed using Chimera v1.6.1.

Results and Discussion

Isolation of α-conotoxins. The IV of several C. purpurascens specimens was separated by analytical RP-HPLC to collect enough conotoxin; a representative chromatogram can be seen in Figure 11. It should be noted that not every specimen of C. purpurascens expresses all α-conotoxins, correlating with the results from Chapter 1, were individuals exhibit intraspecies variation of venom components. A total of 37, 49, and 29 fractions containing α-PIA, α-PIC, and α-PIC[O7] respectively were combined from several RP-HPLC separation runs for further purification. α-PIB was rarely detected in the venom and therefore was not collected. Figure 12A, B, and C show the purification chromatographic profiles of the combined fractions of α-PIA, α-PIC, and α-PIC[O7] respectively. The single large peak of Figure 12A and 12B corresponds to α-PIA and α-PIC. While the largest peak of Figure 12C corresponds to α-PIC[O7], a second peak is present that contains α-PIC. This occurred because α-PIC and α-PIC[O7] only differ by one amino acid residue so they are very close in hydrophobicity and elute from the column consecutively. The retention times of these peaks are given in Table 3.
Figure 11. Representative RP-HPLC chromatographic profile of the injected venom from a *C. purpurascens* specimen that expresses α-PIA, α-PIC, and α-PIC[O7] at 220nm and range = 1. α-PIC[O7] eluted from 11.76 – 12.41 min. α-PIC eluted from 13.78 – 14.76 min. α-PIA eluted from 34.08 – 34.55 min.

The identities of the purified α-conotoxin fractions from Figure 12 were confirmed by their MW using MALDI-TOF MS in reflector mode. The MALDI-TOF MS spectra of purified α-PIA, α-PIC, and α-PIC[O7] can be seen in Figure 13. The observed MWs are given in Table 4.
Figure 12. RP-HPLC chromatographic profiles of purified α-conotoxins at 220nm and range = 1. (A) α-PIA (B) α-PIC (C) α-PIC[O7]
Figure 13. MALDI-TOF MS spectra of isolated α-conotoxins in reflector mode. (A) α-PIA (B) α-PIC (C) α-PIC[O7]
**Determination of α-conotoxin Concentration and Total Yield.** The concentration of each α-conotoxin was calculated using the molar extinction coefficient at absorbance of 205nm ($\varepsilon_{205}$), which are given in Table 4. Beer-Lambert’s Law in a rearranged form (Equation 1) was used to determine the concentration of α-PIA and serves as an example of how the concentrations of α-PIC and α-PIC[O7] were determined.

$$
c = \frac{A_{205}}{\varepsilon_{205} \times l}
$$

\[
c (M) = \frac{2.954}{59410 \text{ M}^{-1}\text{cm}^{-1} \times 1 \text{ cm}} = 4.972 \times 10^{-5} \text{ M}
\]

The total yield of the α-conotoxins was determined by conversions. To measure the $A_{205}$, purified α-conotoxins were diluted in variable amounts of HPLC-grade water. The total yield of α-PIA diluted in 500 μL was calculated as follows

$$
\left(\frac{4.972 \times 10^{-5} \text{ mol}}{\text{L}}\right) \left(\frac{1980.94}{1 \text{ mol}}\right) \left(\frac{1000 \text{ mg}}{1 \text{ g}}\right) \left(\frac{1 \text{ L}}{1000 \text{ mL}}\right) = 0.0985 \text{ mg/mL}
$$

$$
0.0985 \mu\text{g}/\mu\text{L} (500 \mu\text{L}) = 49.25 \mu\text{g}
$$

The α-conotoxins were freeze-dried and diluted in variable amounts of 0.7% saline to create desired picomolar concentrations to inject into *D. melanogaster*. Below is an example of how the injectable concentration of α-PIA was calculated using 13 μL of 0.7% saline.

$$
\left(\frac{49.25 \mu\text{g}}{13 \mu\text{L}}\right) \left(\frac{1 \text{ g}}{1 \times 10^6 \mu\text{g}}\right) \left(\frac{1 \times 10^6 \mu\text{L}}{1 \text{ L}}\right) \left(\frac{1 \text{ mol}}{1980.94 \text{ g}}\right) = 1.912 \times 10^{-3} \text{ M}
$$

The largest volume that can be reliably injected into *D. melanogaster* is 46 nl. Therefore, the highest possible concentration of α-PIA that can be injected in each fly was calculated as follows
\[
\left( \frac{1.912 \times 10^{-3} \text{ mol}}{\text{L}} \right) \left( \frac{46 \text{ nl}}{\text{fly}} \right) \left( \frac{1 \text{ L}}{1 \times 10^9} \right) \left( \frac{1 \times 10^{12} \text{ pmol}}{1 \text{ mol}} \right) = 87.95 \text{ pmol/fly}
\]

The volume injected into the fly was reduced to test lower concentrations of α-PIA.

Table 4. Observed MWs, retention times from purification runs, and calculated ε<sub>205</sub>, total yields, and concentrations of isolated α-conotoxins from *C. purpurascens*

<table>
<thead>
<tr>
<th>Conotoxin</th>
<th>Observed MW (Da)</th>
<th>Retention Time (min)</th>
<th>ε&lt;sub&gt;205&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Total Yield (μg)</th>
<th>Calculated Concentration (mM)</th>
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<tr>
<td>α-PIA</td>
<td>1980.94</td>
<td>35.35 - 37.14</td>
<td>59410</td>
<td>1.91</td>
<td>1.912</td>
</tr>
<tr>
<td>α-PIC</td>
<td>1460.13</td>
<td>13.68 - 15.36</td>
<td>47490</td>
<td>2.44</td>
<td>2.443</td>
</tr>
<tr>
<td>α-PIC[O7]</td>
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<td>12.64 - 14.52</td>
<td>47490</td>
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*In vivo Effects of α-conotoxins in the Drosophila melanogaster GFS.* To have enough volume to test in multiple flies, each α-conotoxin was diluted in 8 – 15 μL of 0.7% saline, producing various stock concentrations. First, 46 nl of each α-conotoxin was tested in the flies (n = 10) to produce starting concentrations of 88, 112, and 84 pmol/fly for α-PIA, α-PIC, and α-PIC[O7] respectively.

Figure 14 shows the effect of α-PIC and α-PIC[O7] on the DLM. At 112 pmol/fly, α-PIC inhibits the DLM response to approximately 60% by 20 minutes post-injection. α-PIC[O7] does not exhibit a significant inhibition of the DLM response at 84 pmol/fly. The TTM response was unaffected by α-PIC and α-PIC[O7] as seen in Figure 15. We would expect α-conotoxins to cause disruption in the DLM response without affecting the TTM response. As seen in Figure 9, the GF-DLM pathway contains a synapse between the PSI and the DLMn that is dependent on Da7 nAChR while the GF-TTM pathway contains a synapse between the GF and TTMn that is both cholinergic and electrical in nature. Therefore, if a α-conotoxin is present that is selective to the Da7 nAChR, the synapse of the GF-DLM that is strictly cholinergic will be inhibited, causing a loss in
muscle response of the DLM. Meanwhile, the α-conotoxin can also inhibit the cholinergic component of the synapse in the GF-TTM, but the electrical GAP junctions are still able to carry the TTM muscle response \textsuperscript{53a, 53c, 53d}.

α-PIB, α-PIC, and α-PIC\textsuperscript{[O7]} are all α4/4-conotoxins with four amino acid residues in between each cysteine loop. Therefore, it can be hypothesized that α-PIC and α-PIC\textsuperscript{[O7]} may have similar selectivity to nAChRs to α-PIB, an inhibitor of muscle-subtype nAChRs located in mammalian neuromuscular junctions (NMJs) \textsuperscript{27}. The Da7 nAChR is a neuronal-type nAChR and the NMJs of flies are glutaminergic rather than cholinergic like in mammalians \textsuperscript{54}. Although, it is necessary to perform more experiments to characterize the pharmacological target of these conotoxins, this may partially explain the poor selectivity of α-PIC and α-PIC\textsuperscript{[O7]} for the Da7 nAChR.

On the other hand, α-PIA displayed significant disruption of the DLM response at 88 pmol/fly, as seen in Figure 16, but did not affect the TTM response (Figure 17). At this concentration, α-PIA causes the DLM muscle response to reduce to an average of 10\% by 10 minutes post injection. By the end of the 20 minutes, the response is lowered to 4\% on average. To determine the effects of α-PIA at lower concentrations, 35, 23, and 11 nl of α-PIA were injected into the flies (n = 10), resulting in approximately 67, 44, and 21 pmol/fly injections respectively. As the concentration of α-PIA is decreased, there is less inhibition of the GF-DLM pathway. At the lowest concentration that was tested, 21 pmol/fly, the DLM response was reduced on average to 68\% by 10 minutes and 47\% by 20 minutes post-injection.

The difference in bioactivity of α-PIA in the GFS of \textit{D. melanogaster} in comparison to α-PIC and α-PIC\textsuperscript{[O7]} can be explained by the difference in amino acid
residues within the cysteine loops as α-PIA is a α4/7-conotoxin. Several α4/7-conotoxins are known to have selectivity towards either α3-containing nAChRs (i.e. α-MII, α-PnIA, α-EpI, α-GIC, α-GID) or α7 nAChRs (i.e. α-EpI, α-PnIB, α-GID) 49. Although not all α4/7-conotoxins inhibit these nAChR subtypes, αPIA has been reported to have low selectivity towards α3-containing nAChRs and has not yet been tested on α7 nAChRs. Synthesized α-PIA has been tested in vitro in Xenopus oocytes and was found to be most selective towards the chimeric α6/α3β2β3 nAChR. It was also shown to be non-active on α2β2 and α4β2 and the muscle subtype 28. It is important to note that synthetic conotoxins may not be properly folded and/or may have missing post-translational modifications that occur in the native conotoxin, which can affect its selectivity. Also, it is likely that conotoxins behave differently in whole organisms as opposed to single cells. This study accounts for the first time that native α-PIA has been isolated from C. purpurascens and tested in an in vivo assay.
Figure 14. DLM response curve for α-PIC and α-PIC[O7] at 112 pmol/fly and 84 pmol/fly respectively.

Figure 15. TTM response curve for α-PIC and α-PIC[O7] at 112 pmol/fly and 84 pmol/fly respectively.
Figure 16. DLM dose-response curve for α-PIA at 88, 67, 44, and 21 pmol/fly.

Figure 17. TTM dose-response curve for α-PIA at 88, 67, 44, and 21 pmol/fly.
Homology Modeling of α-PIA Bound to the Da7 Receptor. The interactions between α-PIA and the Da7 nAChR was visualized by a molecular model that was built based upon the crystal structure of AChBP, a homolog of the extracellular domain of nAChRs, bound to α-conotoxin ImI. A pictorial representation of the pentameric Da7 receptor is shown in Figure 18A and a top down view of two of the subunits (light and dark blue) with the toxin (yellow) bound is shown in Figure 18B. Figure 18C shows a side view of a single α-PIA molecule bound to the interface of two of the five subunits of the receptor.

This model predicts that the ligand binds to both the principal (Figure 18C, light blue) and complementary (Figure 18C, dark blue) sides of the binding pocket. According to this model, the residues of α-PIA that determine inhibitory binding are predicted to be Asn7 and Val9 on the principal side and Ser6 and Val12 on the complementary side. These residues are predicted to bind to the receptor primarily by hydrogen bonding (red) and hydrophobic interactions (orange). The details of these interactions are displayed in Table 5. It appears that α-PIA ‘glues’ itself between the interface of two subunits in the receptor, disabling the receptor from making the conformational change that will open the channel pore to allow the flux of ions. When the channel pore is closed, no synaptic transmission can occur, thus reducing the DLM response that is seen in Figure 16.

Although homology modeling can help us visualize how α-PIA complexes with the Da7 nAChR, it is important to note that it is just a model and the interactions that are displayed may be entirely accurate. The modeled structure of the Da7 nAChR subunits is based off the most homologous crystal structure to a nAChR to date, the AChBP. Although the crystal structure of the AChBP offers three-dimensional information about the arrangement of the ligand binding site homologous to that of an α-subunit of a
nAChR$^{58a}$, ligand binding to the whole pentameric transmembrane nAChR may occur in a different way. Also, this model was based off the crystal structure of AChBP in complex with a different $\alpha$-conotoxin, ImI$^{58b}$, which is much shorter than $\alpha$-PIA at 12 amino acid residues in length; it is possible that the interactions between $\alpha$-PIA and D$a7$ are different than what is depicted because $\alpha$-PIA is a considerably larger peptide, with 18 amino acid residues in length.
**Figure 18.** Homology model of α-PIA bound to the wild-type Da7 nAChR. (A) Pictorial representation of two out of the five Da7 subunits that are shown in the homology models (B) Top-down view of homology model, where light blue is the principal subunit, dark blue is the complementary subunit, and yellow is α-PIA (C) Side view of homology model.
Table 5. Amino acid residues and bond distances of predicted hydrogen bonds and hydrophobic interactions of α-PIA bound to the wild-type Dα7 nAChR

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HYDROPHOBIC INTERACTIONS

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Chapter 3: Diet Change Effects in the venom of *C. purpurascens*

**Introduction**

Cone snail species can be classified as worm-hunters (vermivorous), fish-hunters (piscivorous), or mollusk-hunters (molluscivorous)\(^{39a}\). Some *Conus* species prey on more than one type; a prime example is *C. californicus*, a hunter of worms, fish, and mollusks\(^{39a, 61}\). The exact diet of cone snails has a tendency to be species-specific and it is believed that all species originated from a vermivorous diet\(^5\).

Shifts in prey-type from errant polychaetes to sedentary polychaetes, fishes, and gastropods may be linked to the evolution of the various venom compositions by different *Conus* species\(^62\). However, it has been suggested that the feeding modes of cone snail species today has been evolutionarily conserved and have not changed very much since the first great speciation event in the Miocene time period\(^63\). The results of a recent phylogenetic study suggests that variation of venom composition at the superfamily level is not associated with diet preference, meaning that most superfamilies of conopeptides have been found in all cone snails regardless of their prey type\(^64\). Therefore, it is believed that differences in venom composition of cone snails that target different types of prey occur at the species and intra-superfamily level\(^{2a, 65}\). In addition to feeding mode, venom variability of *Conus* species has also been attribute to several
genetic processes such as alternative splicing, unequal crossing-over, exon shuffling, lack of a mismatch repair system, and recombination mechanisms and also external factors such as environmental changes.

The effect of changing the prey of a Conus species in captivity has not yet been studied in detail. In this study, the venom of C. purpurascens, a fish-hunter of the Americas that is widely distributed along the Pacific coast of Mexico to northern Peru, was analyzed during a diet-shift to the common earthworm, Lumbricus terrestris, over the course of nine months. Out of the four specimens that were fed worms during this time, the venom composition of one specimen was drastically altered during the prey-shift. Immediately after changing the prey back to fish at the end of the nine-month period, the venom composition of this specimen returned to nearly the same state as before the prey change.

Materials and Methods

Specimen Collection, Injected Venom Extraction and Feeding, RP-HPLC, and MALDI MS. Refer to Chapter 1. IV ‘milking’ were combined by date to separate approximately 40 μL of venom at a time using RP-HPLC.

 Injected Venom Extraction while Feeding Worms. The injected venom from the C. purpurascens individuals was extracted using the ‘milking’ procedure of Hopkins et al as described in Chapter 1. However, the cone snails were fed locally acquired common
earthworms (*L. terrestris*) instead of feeder gold fish after injected venom was collected. The collected venom was centrifuged and stored at -80°C until further use.

*Nuclear Magnetic Resonance.* Two conotoxins corresponding to two fractions of the venom that was collected during the time when the cone snails were on a worm diet were prepared in HPLC-grade water (Fisher Scientific) containing 38 pmol trimethylsilyl propionic acid (TSP) as an internal reference and 10% deuterium oxide (D$_2$O) (Sigma Aldrich) for a total volume of 40 μL in 1.7 mm capillary tubes (Wilmad-Labglass). The pH was recorded for each sample. Both conotoxin samples were subjected to 1-D $^1$H NMR experiments at 25°C using a Varian Inova 500 MHz instrument. One of the conotoxin samples was subjected to 2-D $^1$H-$^1$H TOCSY NMR experiments at 0°, 10°, 25°, and 35°C. The other conotoxin sample was not concentrated enough to produce 2-D spectra.

*Reduction and Alkylation of Conotoxins.* Reduction and alkylation of cysteine residues was performed using a previously described method. Briefly, 10 μL of each sample was freeze-dried and then reconstituted in 0.1 M Tris-HCl (pH 7.4). The samples were reduced with 20 mM dithiothreitol (DTT) and incubated for 30 minutes at 60°C. The samples were alkylated with 5 mM iodoacetamide (IAM) and NH$_4$OH (pH 10.5) and incubated at room temperature in the dark for one hour. Reduced and alkylated samples were recovered using a pre-equilibrated Zip Tip (C-18, Millipore).
Results and Discussion

**RP-HPLC Chromatographic Profiles of IV and MALDI MS of Fractions.** The IV of four specimens, named Pur 2-L, Pur 2-2L, Pur 2-M, and Pur 2-2M, was analyzed during this study. The IV of each specimen before the prey change to worms, during the prey change, and after the pray was changed back to fish was separated by RP-HPLC and masses analyzed by MALDI-TOF MS for comparison of venom composition. Two of the four specimens displayed significant changes in venom composition when they were preying upon worms.

The IV of one specimen, Pur 2-2L, showed drastic changes in venom composition when their prey was changed to worms, as seen in Figure 19. Before changing the prey to worms the RP-HPLC chromatographic profile of the IV shows a complex mixture of several major components, as seen in the top trace of Figure 19, comparable to that seen in Chapter 1. Further, this specimen’s venom shows significant expression of large, late eluting peaks (Rt = 60 – 80 min) that correspond to hydrophobic components. Within 0 – 2 months of changing the prey to worms, the cone snail’s venom appears to downregulate the expression of several of its major components that elute between 20 – 30 min (for the exception of two major peaks labeled 1 and 2 in Fig. 19) while maintaining the expression the hydrophobic components eluting between 60 – 80 min, as seen in the trace below the top trace of Figure 19. This trend in venom composition continues from 5 – 7 months of preying upon worms, as seen in the second to bottom trace in Figure 19. However, within 0 – 2 months of changing the prey back to fish the venom composition changed once again (bottom trace of Figure 19); several major components eluting
between 20 – 30 min were once again being expressed as they were before the prey change.

All fractions that were collected during RP-HPLC separation were analyzed by MALDI-TOF MS to identify venom components. The masses that were detected from the IV before, during, and after the prey change of specimen 2-L were compared (Figure 20) to see which venom components remain during the prey change. The Venn diagram reveals that only 4 detected masses were in common between the IV collected before, during, and after the prey change; two of these masses was identified as αA-PIVA, a muscle nAChR inhibitor, and δ-PVIA, a NaV channel inhibitor. Considering that the chromatographic IV profiles from before and after the venom change appear to be similar, to our surprise only 7 masses were in common between the IV collected from the specimen while it was preying on fish (before and after the prey change). We were especially interested in the two major peaks that remained in the IV during the prey change (labeled 1 and 2 in Fig. 19). The MALDI-TOF spectra of the two peaks displayed in Figures 23 and 24 show novel masses that do not match that of any published C. purpurascens conotoxins. Further investigation of the venom from this specimen after the prey was changed back to fish is necessary to see if the IV composition fully reverts back to its original state.

A less drastic change in venom composition occurred in specimen Pur 2-2L. The RP-HPLC chromatographic profiles of the IV of Pur 2-2L before, during, and after the prey change (Figure 21) reveals and change in expression of major components eluting between 15 – 35 minutes. Also, the specimen’s venom shows an upregulation of late eluting, hydrophobic components when it was asked to prey upon worms instead of fish.
The full extent of this change was not seen until 6 – 7 months after the prey change to worms, as seen in the second to the bottom trace in Figure 21. Remarkably, the venom composition appears to revert back to the state it was in before the prey change just like Pur 2-L, as seen in the bottom trace of Figure 21.

To determine the extent of variations in the venom composition in specimen 2-2L, the masses detected before, during, and after the prey change were compared using a Venn diagram (Figure 22). A small subset of 5 masses was found in common between the IV collected before, during, and after the prey change; two of these masses were identified as excitotoxic conotoxin κA-PIVE (exact pharmacological target is unknown) and α-PIB, a muscle nAChR inhibitor. It will be interesting to see if this specimen reverts its venom back to its original state as time passes. The venom composition of specimens Pur 2-M and Pur 2-2M changed very little during the prey change to worms (data not shown) and were not examined further.
Figure 19. RP-HPLC chromatographic profiles of the injected venom of *C. purpurascens* specimen Pur 2-L from before prey change to worms (*top*), during prey change (*middle*), and after changing the prey back to fish (*bottom*). Injected venom samples were fractionated over a 100-minute gradient and fractions were subjected to MALDI-TOF MS. Previously discovered conopeptides that were detected are labeled.

Figure 20. Venn diagram of total masses detected in the IV of specimen Pur 2-L before the prey change, during the prey change (5 – 7 months of preying on worms), and after the prey change.
Figure 21. RP-HPLC chromatographic profiles of the injected venom of *C. purpurascens* specimen Pur 2-2L from before prey change to worms (*top*), during prey change (*middle*), and after changing the prey back to fish (*bottom*). Injected venom samples were fractionated over a 100-minute gradient and fractions were subjected to MALDI-TOF MS. Previously discovered conopeptides that were detected are labeled.

Figure 22. Venn diagram of total masses detected in the IV of specimen Pur 2-2L before the prey change, during the prey change (6 – 7 months of preying on worms), and after the prey change.
Figure 23. MALDI-TOF MS spectra of isolated conopeptides from peak 1 of the injected venom of specimen 2-L during prey change in linear mode. There are two peaks at 3271.56 and 1636.70 Da.

Figure 24. MALDI-TOF MS spectra of isolated conopeptides from peak 2 of the injected venom of specimen 2-L during prey change in reflector mode. (A) 2657.46 Da peptide (B) 2469.27 Da peptide. The corresponding zoomed-in spectra showing the isotopic distribution of each peptide peak is displayed on the right.
Nuclear Magnetic Resonance. Specimen 2-L exhibited the most diverse changes in venom composition in response to a change in prey. Therefore, we decided to study the changes in this individual’s venom in more detail. The two major components, corresponding to the peaks that remained (between Rt = 20 – 40 minutes) when the prey was changed to worms (labeled 1 and 2 in Fig. 21) have masses of 3271.56 and 2657.46 Da, matching that of κ-PVIIA and κA-PIVE respectively.

The NMR spectrum of peak 1 is shown in Figure 25. This sample was not very concentrated but still shows signals indicative of NH and aromatic groups of amino acid residues between ~6.5 – 9.0 ppm. When compared to the literature $^1$H NMR spectra for κ-PVIIA $^{68}$, several signals are in common. For instance, in our spectrum there is unusual signal at 10.4 ppm that matches the HN assignment for Cys16 of the literature spectrum for κ-PVIIA (10.41 ppm). Based on our NMR analysis so far, it is hypothesized that the major component of peak 1 is κ-PVIIA.

Figure 25. $^1$H NMR spectrum of peak 1 containing the 3271.56 Da component at 25°C.
The NMR spectrum of peak 2 containing the 2657.46 Da component is shown in Figure 26 and shows characteristics of several amino acid residues. For instance, the spectrum shows several signals between ~6.5 – 9.0 ppm, indication of NH-groups and aromatic groups within the peptide structure. Further, as seen by the zoomed-in panel of Fig. 26 between 7.0 – 9.0 ppm, there are two-proton aromatic doublets indicative of a tyrosine residue at ~7.0 and 7.3 ppm \(^{69}\); this agrees with the published sequence of κA-PIVE, which contains one tyrosine residue.

**Figure 26.** \(^1\)H NMR spectrum of peak 2 containing the 2657.46 Da component at 25°C. The zoomed-in insert between 7.0 – 9.0 ppm (top) is from the \(^1\)H NMR spectrum of the same sample at 0°C, which allowed the visualization of a pair of doublet signals at ~7.0 and 7.3 ppm that are indicative of a tyrosine residue.

*Reduction and Alkylation of Conotoxins.* Reduction and alkylation of peaks 1 and 2 (labeled in Fig. 21) was preformed to determine how many cysteine residues are present in each component. Denaturation was achieved by treating the samples with DTT and
heat and “alkylation” of free thiol groups of cysteines was achieved by the addition of IAM. Each cysteine residue that is “alkylated” causes an addition of 57.07 Da to the MW. The MALDI-TOF MS spectra of both peaks after the reduction and alkylation reactions are shown in Figures 27 and 28.

![MALDI-TOF MS spectra](image)

**Figure 27.** MALDI-TOF MS spectra of isolated conopeptides from peak 1 of specimen 2-L during prey change after reduction and alkylation in linear mode. There are two peaks at 3615.91 and 1808.91 Da.

The MW of the major component in peak 1 increased from 3271.56 to 3615.91 Da (difference of 344.35 Da), indicative of six cysteine residues. This agrees with the published sequence of κ-PVIIA. The MW of the minor component of peak 1 increased from 1636.70 to 1808.91 Da (difference of 172.21 Da), indicative of three cysteine residues.
Figure 27. MALDI-TOF MS spectra of isolated conopeptides from peak 2 of specimen 2-L during prey change after reduction and alkylation in linear mode. There are two peaks at 3005.09 and 2816.09 Da.

The MW of the major component in peak 2 increased from 2657.46 to 3005.09 Da (difference of 347.63 Da), indicative of six cysteine residues. This agrees with the published sequence of κA-PIVE. Likewise, the MW of the minor component of peak 2 increased from 2469.27 to 2816.09 Da (difference of 346.82 Da), also indicating that this component has six cysteines. Further biochemical characterization of peaks 1 and 2 is necessary to determine complete sequences.
References


