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Conotoxin truncation as a post-translational modification to increase the pharmacological diversity within the milked venom of *Conus magus*

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ABSTRACT

Milked venoms of *Conus* demonstrate direct lineage to US Food and Drug Administration approved and present in-trial drug leads. Yet the complexity of the milked venom has not been adequately investigated or characterized, in a sustainable manner. In this study we determine the extent of molecular mass differentiation in milked venom from captive *Conus magus* and confirm the expression of known conotoxin constituents. We demonstrate the presence of post-translational N-terminal peptide truncation, which differentiates the milked venom constituent α-conotoxin MI from the novel α-conotoxin MIC. This truncation has a direct effect on peptide bioactivity – K_i of 89.1 ± 9.1 and 248.7 ± 10.9 nM (α-conotoxin MI and MIC respectively) toward the muscle-type nAChR (*Torpedo*). These milked venom conotoxins demonstrated acute lethality in fish, with a LD_{50} of 12.24 and 23.29 μg kg^{-1} for α-conotoxin MI and MIC respectively. By synthesizing and investigating the synthetic intermediate variant *des*[Gly]^{1}α-conotoxin MI, it was demonstrated that retention of the N-terminal arginine residue increased affinity at the muscle-type nAChR site (binding K_i of 73.3 ± 5.8 nM and lethal toxicity level LD_{50} of 8.19 μg kg^{-1}). This post-translational modification event within the milked venom of *C. magus* represents a unique mechanism by which cone snails are able to increase the chemical and pharmacological diversity of their venoms.

Abbreviations: API, Atmospheric pressure ionization; Arg(Pbf), N-alpha-9-fluorenylmethoxycarbonyl-N-g-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl-L-arginine; Asn(Trt), N-alpha-9-fluorenylmethoxycarbonyl-N-beta-trityl-L-asparagine; Calc. MH^+, Calculated monoisotopic molecular mass; CnBr, Cyanogen bromide; Cys(Trt), N-alpha-9-fluorenylmethoxycarbonyl-S-trityl-L-cysteine; Da, Daltons; DCM, Dichloromethane; DIEA, *N,N*-Diisopropylethylamine; DMF, Dimethylformamide; DV, Duct venom; ESI-MS, Electrospray ionization mass spectrometry; Fmoc, 9-Fluorenylmethyloxycarbonyl; HCTU/DMF, 1H-benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate-(1-),3-oxide in dimethylformamide; HSS, HEPES-salt solution; Hyp, 4-*trans*-Hydroxyproline; LC/MS, Liquid chromatography interfaced mass spectrometry; LD_{50}, Lethal dose, 50%; Lys(Boc), N-alpha-9-fluorenylmethoxycarbonyl-N-ε-boc-L-lysine; MBHA, 4-(Methyl)benzhydrylamine; MH^+, Monoisotopic molecular mass; MV, Milked venom; nAChR, Nicotinic acetylcholine receptor; Obs. Alk. MH^+, Observed alkylated monoisotopic molecular mass; Obs. Red. MH^+, Observed reduced monoisotopic molecular mass; PTH, Phenylthiohydantoin; PTMs, Post-translational modification; RP-HPLC/UV, Reverse phase-high performance liquid chromatography interfaced ultra-violet detection; R_t, Retention time; TCEP, Tris(2-carboxyethyl)phosphine; TFA/aq., Trifluoroacetic acid/aqueous; TIPS, Triisopropylsilane; Tyr(tBu), N-alpha-9-fluorenylmethoxycarbonyl-O-t-butyl-L-tyrosine.

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1. Introduction

Diversity of peptides found in the milked venom (MV) of the carnivorous marine *Conus* spp. fundamentally increases their predatory success. Within the MV of *Conus geographus,* we have recently demonstrated the expression of conopeptide families and pharmacological classes previously identified in duct venom (DV) extracts (Bingham et al., 2012). The presence of such families in MV supports the proposal of Terlau and Olivera (2004) for pharmacological targeting strategies involving 'cabals'. The simplicity of MV profiles (Bingham, 1999; Dutertre et al., 2010; Bingham et al., 2012; Violette et al., 2012), together with their pharmacological refinement (Hopkins et al., 1995), and relative abundance (Chun et al., 2012), make them a natural and bio-sustainable resource that researchers can utilize to facilitate drug discoveries. However, the pharmacopoeia cannot be expanded without increasing our current understanding of the adaptive and/or expressional abilities of the MV itself and analyzing trends (within and between snails) that may emerge.

One of the driving mechanisms increasing the diversity of these venom peptides is the incorporation of post-translational modifications (PTMs) (Craig et al., 1999). The influence of PTMs on peptides are commonly met with questions such as; "Could the PTM events that transpire in the MV (Hopkins et al., 1995; Teichert et al., 2007; Violette et al., 2012) expand the existing range of peptide sequences by augmenting their pharmacological potential?", "Have past studies using DV reported prematurely processed PTM constituents?", and "How stable is the expression of the MV over time?". Investigating these questions may impact how we utilize the MVs as a novel source of bioactive compounds and potential new drug leads.

To address this, we have examined the relative simplicity of the MV obtained from piscivorous *Conus magus* using liquid chromatography interfaced Mass Spectrometry (LC/MS). Herein, we provide the first detailed study of MV molecular mass differentiation in *C. magus* during a 12-month period of captivity. We then demonstrate comparative peptide profiling, which examines the extent of changes within captive cone snail MV using Reverse Phase High Performance Liquid Chromatography/Ultraviolet detection (RP-HPLC/UV). Combined with biochemical analyses of the MV peptides, we have confirmed the expression of known α- and ω-conotoxins, including the US Food and Drug Administration approved analgesic Prialt® (ω-conotoxin MVIIA). Innovatively, we have identified two inter-related α-conotoxins within the captive cone snail MV: a novel 12 amino acid peptide α-conotoxin MIC (the smallest reported 3/5 α-conotoxin to date), and its N-terminal variant α-conotoxin MI (14 amino acids). Their total chemical synthesis including the production of *des*[Gly]^{1}α-conotoxin MI, a 13 amino acid intermediate is described. These three peptides are used to illustrate how cone snails are able to increase the chemical and pharmacological diversity of their venoms through post-translational modification, thereby providing a strategy for more effective prey immobilization.

2. Materials and methods

2.1. Conus *housing, milking and venom extraction*

Nine specimens of *C. magus* were collected from Night Island, Far North Queensland, Australia (13°11′3″S 143°34′25″E). Specimens were fed weekly, using juvenile *Carassius auratus* (goldfish; weight 1–2 g) and 'milked' of venom for a period of 12 months (Hopkins et al., 1995). Individual MV volumes were measured, acidified with 0.1% v/v aqueous Trifluoroacetic acid (TFA/aq.; 50 μL) and stored at −20 °C to inhibit any enzymatic activity and/or degradation. For longer-term storage samples were Speed-Vac dried.

2.2. RP-HPLC/UV – peptide purification

MV and synthetic peptides were profiled using C_{18} Narrow-bore RP-HPLC/UV (Vydac; 5 μm, 300 Å, 2.1 × 250 mm) and later quantified using C_{18} capillary-bore RP-HPLC/UV (Phenomenex; 5 μm, 300 Å, 1.0 × 250 mm), as previously described by Chun et al. (2012).

2.3. Conotoxin reduction

MV was Speed-Vac dried and re-suspended in 100 mM Tris(2-carboxyethyl)phosphine (TCEP; Pierce Chemicals) in 25 mM NH_4OAc, pH 4.5. To aid reduction, samples were heated at 60 °C for 15 min, and then RP-HPLC/UV purified.

2.4. Thiol alkylation and N-terminal Edman degradation

TCEP reduced RP-HPLC purified peptides were dissolved in 25 mM NH_4OAc (90–150 μL), pH 4.5, and alkylated by adding 100 mM *N*-phenylmaleimide (Fluka, Switzerland) in isopropanol. Typically 20–40 fold excess w/w of the alkylating agent were used. Alkylation was allowed to proceed at 50 °C for 15 min, prior to RP-HPLC/UV re-purification. Non-alkylated and *N*-phenylmaleimide alkylated peptides were applied to Polybrene-treated glass fiber support filters for automated N-terminal (Edman) degradation on a gas-phase sequencer (Model 470A; Applied Biosystems, Foster City, CA, USA). Phenylthiohydantoin (PTH) derivatives of 4-*trans*-Hydroxyproline (Hyp) and *N*-phenylmaleimide-alkylated cysteine were added into the amino acid standard mix to aid identification and sequence assignment.

2.5. Conotoxin synthesis, cleavage and oxidation

Conotoxins were manually assembled on 4-(Methyl) benzhydrylamine (MBHA) Rink-amide resin (0.44 meq g^{-1}) using 9-Fluorenylmethoxycarbonyl (Fmoc) chemistry in the following manner. MBHA Rink-amide resin (0.5 mM scale) was swelled overnight in 4 mL dimethylformamide (DMF). The resin was washed three times with DMF (5 mL), deprotected twice with 50% (v/v) piperidine in DMF (5 mL, 2 × 1 min), and then re-washed two times with 5 mL DMF. Fmoc amino acids, 4 fold excess (2 mM), were activated *in-situ* using 2 mL 0.4 M HCTU/DMF