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Hormone-like peptides in the venoms of marine cone snails

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ABSTRACT

The venoms of cone snails (genus *Conus*) are remarkably complex, consisting of hundreds of typically short, disulfide-rich peptides termed conotoxins. These peptides have diverse pharmacological targets, with injection of venom eliciting a range of physiological responses, including sedation, paralysis and sensory overload. Most conotoxins target the prey's nervous system but evidence of venom peptides targeting neuroendocrine processes is emerging. Examples include vasopressin, RFamide neuropeptides and recently also insulin. To investigate the diversity of hormone/neuropeptide-like molecules in the venoms of cone snails we systematically mined the venom gland transcriptomes of several cone snail species and examined secreted venom peptides in dissected and injected venom of the Australian cone snail *Conus victoriae*. Using this approach we identified several novel hormone/neuropeptide-like toxins, including peptides similar to the bee brain hormone prohormone-4, the mollusc ganglia neuropeptide elevenin, and thyrostimulin, a member of the glycoprotein hormone family, and confirmed the presence of insulin. We confirmed that at least two of these peptides are not only expressed in the venom gland but also form part of the injected venom cocktail, unambiguously demonstrating their role in envenomation. Our findings suggest that hormone/neuropeptide-like toxins are a diverse and integral part of the complex envenomation strategy of *Conus*. Exploration of this group of venom components offers an exciting new avenue for the discovery of novel pharmacological tools and drug candidates, complementary to conotoxins.
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1. Introduction

Marine cone snails (genus *Conus*) are venomous. They use their venoms for prey capture, self-defense and possibly intraspecific competition (Dutertre et al., 2014; Olivera, 1999). Cone snail venoms are remarkably complex cocktails containing hundreds of small cysteine-rich peptide toxins (conotoxins) (Olivera et al., 1990; Robinson and Norton, 2014). Many conotoxins have unmatched potency and selectivity profiles for their respective targets, including specific subtypes of voltage- and ligand-gated ion channels, G protein-coupled receptors and neurotransmitter transporters. As such, conotoxins represent a rich source of valuable pharmacological tools and drug candidates.

Conotoxins are produced in a specialized venom gland, where they are translated as precursor peptides. As a general rule, conotoxin precursor peptides are comprised of an N-terminal signal sequence for targeting to the cellular secretory pathway, an intermediate pro-region that plays a role in vesicular transport (Conticello et al., 2003), post-translational modification (Bandyopadhyay et al., 1998) and folding (Buczek et al., 2004) and is presumably cleaved following secretion, and a single copy of the mature toxin peptide at the C-terminus. In many ways this process mirrors the production of endogenous hormones/neuropeptides.

While conotoxins are the major, and by far the most studied, constituent of *Conus* venom, several cases of hormone/neuropeptide-like components have been reported (Table 1). Examples include the conopressins (vasopressin analogues) from

Abbreviations: MeCN, acetonitrile; MS, mass spectrometry; TFA, trifluoroacetic acid.

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Table 1
Hormones/neuropeptides identified in *Conus* venoms.[#]

Conopressins		
Conopressin-S	**C**IIRN**C**PRG[*]	Cruz et al., 1987
Conopressin-G	**C**FIRN**C**PKG[*]	Cruz et al., 1987
Conopressin-Vil	**C**LIQD**CP**γG[*]	Möller and Marí (2007)
Conopressin-T	**C**YIQN**C**LRV[*]	Dutertre et al. (2008)
Contulakin-G	ZSEEGGSNA<u>T</u>KKPYIL	Craig et al., 1999
Conorfamides		
CNF-Sr1	GPMGWVPVFYRF[*]	Maillo et al., 2002
CNF-Sr2	GPMγDPLγIIRI[*]	Aguilar et al. (2008)
CNF-Vc1	HSGFLLAWSGPRNRFVRF[*]	Robinson et al., 2015
Conomap-Vt	AfVKGSAQRVAHGY[*]	Dutertre et al., 2006
ConoCAP-a	PF**C**NSFG**C**YN[*]	Möller et al., 2010
Neuropeptide-F/Y	TVSDPPARPAVFHSREELMNYVRELNRYFAIVGRPRF[*]	Wu et al. (2010)
	TVSDPPARPAVFHSREELMNYVRELNLYFAIVGRPRY[*]	Wu et al. (2010)
Insulin		
Con-Ins G1 A-chain	GVVγH**CC**HRP**C**SNAEFKKY**C**[*]	Safavi-Hemami et al. (2015)
B-chain	TFDTOKHR**C**GSγITNSYMDL**C**YR	
Elevenin-Vc1	RRID**C**KVFVFAPI**C**RGVAA	This study
PH4-Vc1	IGFPGFSTPPR	This study

[#] Only those confirmed, in their entirety, at the peptide level are shown.
[*] C-terminal amidation, γ; gamma-carboxyglutamate, Z; pyroglutamic acid, <u>T</u>; O-glycosylated threonine, f; D-phenylalanine, O; hydroxyproline, cysteines are bolded.

Conus geographus and *Conus striatus* (Cruz et al., 1987), contulakin-G (a neurotensin analogue) from *C. geographus* (Craig et al., 1999), RFamide neuropeptides from *Conus spurius* and *Conus victoriae* (Maillo et al., 2002; Robinson et al., 2015), conomap (a myoactive tetradecapeptide) from *Conus vitulinus* (Dutertre et al., 2006), conoCAPs (analogues of crustacean cardioactive peptide) from *Conus villepinii* (Möller et al., 2010) and neuropeptide-F/Y from *Conus betulinus* (Wu et al., 2010). Furthermore, we recently demonstrated that specialized insulins are an abundant and active component of some *Conus* venoms (Safavi-Hemami et al., 2015). When injected into fish, the venom insulin elicits hypoglycemic shock, thus facilitating capture of the physiologically impaired prey. It is thus becoming clear that *Conus* venoms are not limited to conotoxins targeting neuronal ion channels, but that other fascinating strategies are also being employed.

To systematically explore the full extent of hormone/neuropeptide-like venom components expressed, secreted and ultimately injected into the prey by cone snails, we used a combined transcriptomics/proteomics-based strategy (mass spectrometry (MS)-matching) on the extruded as well as the injected venom (venom collected from live snails during an envenomation event) of *C. victoriae*, a mollusc-hunting species endemic to the coast of north-western Australia. This combined approach led to the identification of several novel peptides that share high similarity with endogenous hormones/neuropeptides from other organisms. Furthermore, interrogation of venom gland transcriptomic data from additional cone snail species revealed that these peptides are widely distributed in the genus *Conus*.

2. Materials and methods

2.1. Venom gland transcriptome

Specimens of *C. victoriae* were collected from Broome, Western Australia. Specimens were collected specifically for research use, under a commercial fishing license of the Western Australian Specimen Shell Managed Fishery (license number 2577). Ethics approval is not required, in Australia, for taking samples from *Conus*.

Preparation of the venom gland transcriptome of *C. victoriae* has been described previously (Robinson et al., 2014). Briefly, whole venom glands of live specimens were removed, snap-frozen in liquid nitrogen and stored at −80 °C. Frozen venom glands were pulverized and homogenized prior to extraction of total RNA with Trizol (Invitrogen, Life Technologies). cDNA library preparation, normalization and 454 sequencing were performed by Eurofins, MWG Operon (Budendorf, GER). *De novo* transcriptome assembly was performed using MIRA3 (Chevreux et al., 2004) and annotated using BLAST+ (version 2.2.27+) (Altschul et al., 1990; Camacho et al., 2009).

The venom gland transcriptomes of *Conus marmoreus*, *C. geographus*, *C. bullatus*, *Conus tessulatus*, *Conus varius* and *Conus virgo* were sequenced on an Illumina HiSEq. 2000 platform at Cofactor Genomics as described previously (Safavi-Hemami et al., 2015). Reads were *de novo* assembled using Trinity (Grabherr et al., 2011) and annotated using BLASTx.

2.2. Venom extraction & preparation

Extruded venom samples were obtained by manually squeezing freshly dissected *C. victoriae* venom glands, then snap-frozen in liquid nitrogen and stored at −80 °C. Extruded venom (from several specimens) was reconstituted in 0.1% trifluoroacetic acid (TFA), pooled and homogenized using a glass Dounce tissue grinder. Insoluble material was pelleted by centrifugation, supernatant collected and lyophilized. Pellets were resuspended in 0.1% TFA/20% acetonitrile (MeCN), centrifuged, supernatant collected and lyophilized. This process was repeated with 40% and 60% MeCN. Lyophilized venom was resuspended in 2% MeCN, 0.1% TFA and pooled. Protein concentration was determined using a Bradford assay with ovalbumin as the standard. An aliquot of the venom was reduced in 20 mM tris(2-carboxyethyl)phosphine (pH 8) for 30 min at 60 °C, then alkylated by incubating in 40 mM iodoacetamide for 30 min. An aliquot of reduced and alkylated venom was further processed by tryptic digestion, essentially according to the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO, USA), with an incubation time of 4 h at 37 °C.

Lyophilized injected venom of *C. victoriae* was purchased from BioConus (www.bioconus.com). These specimens were also sourced from Broome, Western Australia and maintained in captivity where injected venom was collected using a procedure adapted from Hopkins et al. (1995). The injected venom samples were

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pooled from several individuals and prepared essentially as described above. Three samples of each of the extruded venom and injected venom of *C. victoriae* were prepared for MS: reduced and alkylated venom, reduced and alkylated venom with trypsin digestion, and unprocessed venom.

2.3. Mass spectrometry

Mass spectrometry was performed as described previously (Robinson et al., 2015). Briefly, 0.5 μg of each venom sample was centrifuged at 13,000 × g for 10 min and loaded onto a microfluidic trap column packed with ChromXP C18-CL 3 μm particles (300 Å nominal pore size; equilibrated in 0.1% formic acid/5% MeCN) at 5 μL/min using an Eksigent NanoUltra cHiPLC system. An analytical (15 cm × 75 μm ChromXP C18-CL 3) microfluidic column was then switched in-line and peptides separated using linear gradient elution of 0–80% MeCN/0.1% formic acid over 90 min (300 nL/min). Separated peptides were analyzed using an AB SCIEX 5600 TripleTOF mass spectrometer equipped with a Nanospray III ion source and accumulating 30 tandem MS (MS/MS) spectra per second.

2.4. MS-matching

Data were processed in ProteinPilot software (version Beta 4.1.46) using the Paragon algorithm. The search databases comprised the *C. victoriae* venom gland transcriptome database (the exact nature of which has been described previously (Robinson et al., 2014)). Some of the peptides described herein were not immediately identified by ProteinPilot and required further manual searching of the MS spectra. All hits, identified by ProteinPilot or manually, were then validated by comparison of experimentally-derived peaks against a theoretical peak list (Protein Prospector MS-Product, University of California, San Francisco).

2.5. Data availability

The nucleotide sequences of the *C. victoriae* elevenin-Vc1, PH4-Vc1, ILP-1 and thyrostimulin α2 and β5 subunit precursors have been deposited at DDBJ/EMBL/GenBank [accessions: GAIH01000102.1- GAIH01000106.1].

3. Results & Discussion

Matching of the *C. victoriae* venom gland transcriptome to MS/MS data of extruded venom revealed several novel peptides whose precursors did not belong to any conotoxin superfamilies (Robinson and Norton, 2014), but which belonged to hormone/neuropeptide families from other organisms. We were able to confirm the presence of two of these peptides in the injected venom of *C. victoriae*, unequivocally demonstrating their role as exogenous venom components. One of these peptides was related to prohormone-4, a neuropeptide first described in the bee brain. The other peptide was related to elevenin, a neuropeptide sequenced from several other invertebrates, although this is the first report of its identification at the peptide level and its presence in animal venom.

3.1. Elevenin

C. Victoriae elevenin (designated elevenin-Vc1) was identified as a 19 amino acid mature peptide with one pair of cysteines (RRIDCKVFVFAPICRGVAA) (Fig. 1A and Table S1) in the reduced and alkylated extruded venom (and as a tryptic fragment in the reduced, alkylated and trypsinized extruded and injected venom samples). The full-length disulfide-bonded elevenin-Vc1 was confirmed in both the unprocessed extruded venom as well as the unprocessed injected venom as a peak of m/z 530.54 corresponding to the $[M + 4H]^{+4}$ (theoretical m/z = 530.54) (Table S1). Consistent with a disulfide-constrained peptide, limited fragmentation was observed under CID, but several ions derived from the N- and C-termini that were diagnostic of the elevenin-Vc1 sequence were evident, including 90.05, 161.09, 271.16, 601.67, 644.36, 653.69 668.03 and 677.37 m/z, corresponding to the y1, y2, $b4^{+2}$, $b15^{+3}$, $a17^{+3}$, $b17^{+3}$, $a18^{+3}$ and $b18^{+3}$ ions. These data confirm the primary structure of elevenin-Vc1 in the venom of *C. victoriae*, as a single-chain, single disulfide-containing 19-residue peptide.

The precursor organization of elevenin-Vc1 differed from that typically reported for conotoxins: The mature peptide region was preceded immediately by a signal peptide and followed by a dibasic cleavage site and finally a longer propeptide region (Figs. 1B and 2). Elevenin precursors were also identified as venom gland transcripts in several other species of *Conus* (Fig. 1B); *C. marmoreus*, *C. tessulatus*, *C. varius* and *C. virgo*, suggesting that the use of this neuropeptide as a venom component may not be restricted to *C. victoriae*.

Elevenin-Vc1 showed sequence similarity (E = 0.003) to an abdominal ganglion L11 neuropeptide (elevenin) precursor of the gastropod mollusc *Aplysia californica* (Taussig et al., 1984). The *Conus* and *Aplysia* precursors share a similar overall structure but strong sequence similarity was only apparent for the mature peptide-encoding region (Fig. 1B). Other elevenin transcripts have been identified in the gastropod molluscs *Lottia gigantea* (Veenstra, 2010) and *Theba pisana* (Adamson et al., 2015), and the marine annelid *Platynereis dumerilii* (Conzelmann et al., 2011). The predicted *Platynereis* elevenin peptide has been synthesized, and affected larval locomotion by increasing ciliary beat frequency (in a similar fashion to cAMP) (Conzelmann et al., 2011), although the molecular target of the peptide remains to be determined. Significantly, our data not only demonstrate that this neuropeptide is used as a venom component in *C. victoriae* but also confirm for the first time, in any species, a mature peptide product of an elevenin precursor.

3.2. Prohormone-4

Three peptides derived from a precursor with sequence similarity ($E = 8 \times 10^{-11}$) to the prohormone-4 precursor of the honey bee *Apis mellifera* (Hummon et al., 2006) were identified in the unprocessed extruded venom of *C. victoriae*, with two of the three peptides identified in the injected venom (Fig. 2A and B and Table S1). Prohormone-4 precursors were also identified in the venom gland transcriptomes of several other species of *Conus*; *C. geographus*, *C. tessulatus*, *C. varius* and *C. virgo* (Fig. 2C).

In the honeybee, prohormone-4 was identified in the brain and has been implicated in the regulation of behavior (Brockmann et al., 2009), although the molecular target of this peptide remains to be determined. Prohormone-4 has only been described recently in molluscs, where a sequence (Uniprot: K1QBC8) was predicted from the genome of the Pacific oyster *Crassostrea gigas* (Zhang et al., 2012) (Fig. 2C). To investigate whether this neuropeptide might be more widespread in molluscs, we performed a search of the *A. californica* EST database using blastn with the *C. victoriae* prohormone-4 sequence as the query. Indeed, this search revealed that a prohormone-4 sequence (PEG001-C-001879) is expressed in the neuronal transcriptome of this species (Moroz et al., 2006), specifically that of the pedal-pleural ganglia (Fig. 2C).

The honeybee prohormone-4 precursor consists of a signal peptide followed immediately by the short mature peptide region, a cysteine-rich low-density lipoprotein (LDL) receptor class A

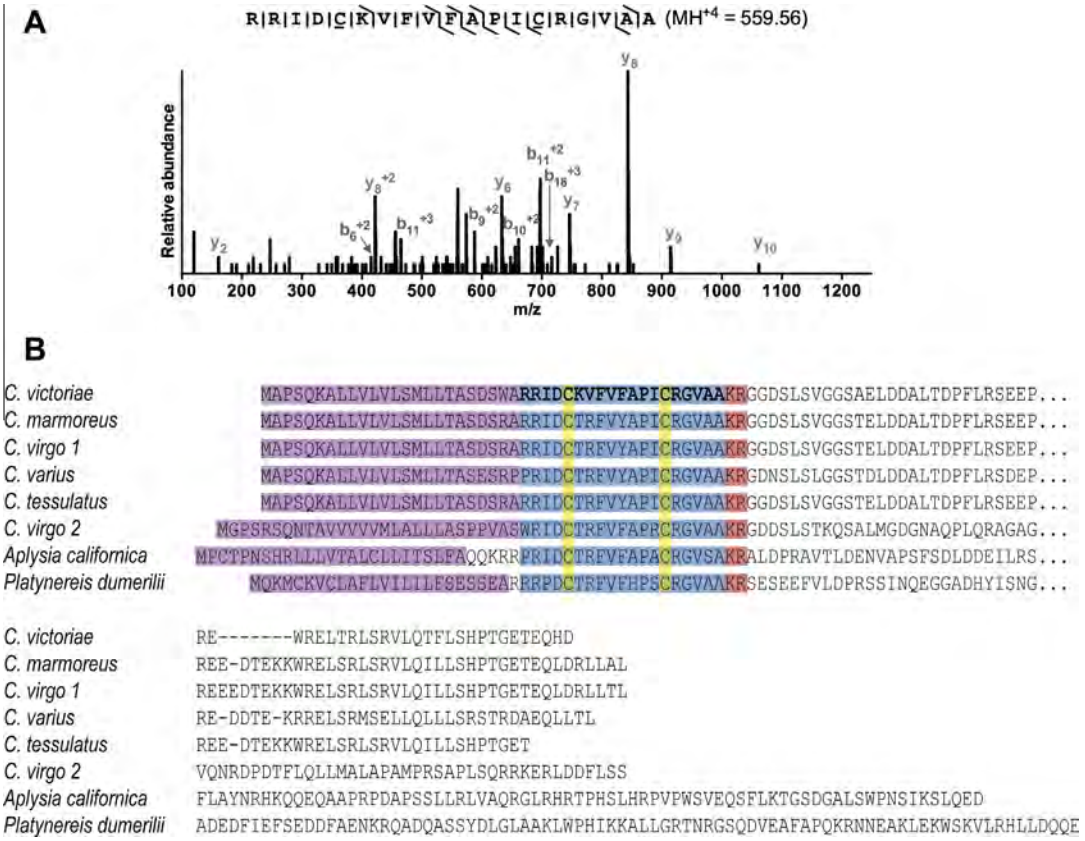

Fig. 1. *C. victoriae* venom elevenin. (A) MS/MS spectrum of reduced and alkylated elevenin-Vc1. The precursor ion selected for MS/MS had a monoisotopic m/z of 559.55 ($z = 4$) with a predicted monoisotopic m/z of 559.55 ($z = 4$). For clarity, only b and y ions are labeled. Inset b/y ladder diagrams summarize observed b- and y-ions. C; carbamidomethylated cysteine. (B) The aligned precursor sequences of elevenin-Vc1, and those of *C. marmoreus*, *C. virgo*, *C. varius*, *C. tessulatus*, *A. californica* and *P. dumerilii*. Precursor signal peptides are highlighted in purple, dibasic cleavage sites in red and the putative/confirmed encoded mature peptides are in blue and the confirmed elevenin-Vc1 is in boldface.

domain (flanked by two dibasic cleavage sites) and an extended cysteine-free propeptide region (Fig. 2B). The mature peptide region yields six neuropeptides (N- and C-terminal truncation variants) (Hummon et al., 2006). The *C. victoriae* sequence (designated PH4-Vc1), those of other *Conus*, and those of *C. gigas* and *A. californica*, show a very similar precursor organization (Fig. 2B). As in the *Apis* prohormone-4, each of the PH4-Vc1 peptides was derived from the same region of the precursor, with the two shorter peptides (GFPGFSTPPR and FPGFSTPPR) being N-terminal truncation variants of the longer (IGFPGFSTPPR) (Table S1). The sequences identified in other *Conus* each appear to encode similar short neuropeptides (IDFSRYSLPSR in *C. geographus* and IDFSRLSLPSR in *C. tessulatus*, *C. varius* and *C. virgo*), as do those of *C. gigas* (VDFSRLRK) and *A. californica* (IDLSRLAFQR). Interestingly, the prohormone-4 cysteine-rich LDL-receptor class A domain of the *Conus* and *Aplysia* sequences appear to contain an additional cysteine (seven in total) compared with those of *A. mellifera* and *C. gigas* (six). Together, these data suggest that prohormone-4 functions as a neuropeptide in molluscs, and that it appears to be used as a venom component by several species of cone snail.

It is noteworthy that in all species of *Conus* examined, including *C. victoriae,* a second class of "prohormone-4-like" sequences was also identified (Supplemental Fig. 1). These sequences shared a similar cysteine-rich LDL-receptor class A domain that contained six rather than seven cysteines. This domain was flanked at its C-terminus by a putative dibasic cleavage site, followed by a long (~100 residues) region as in prohormone-4. Cleavage at this site would leave a C-terminal glycine residue on the cysteine-rich domain that would presumably be removed, resulting in C-terminal amidation of this domain. These sequences differ from prohormone-4 in that, preceding the cysteine-rich domain, is a long (~400 residues) Pro/Met-rich domain, which is in turn preceded by another propeptide region (~50 residues) and the 22-residue signal peptide. While this class of sequences shares some similarity to prohormone-4, particularly in the cysteine-rich domain and C-terminal propeptide region, the annotation as prohormone-4 may be misleading as it is unclear whether in fact they encode any mature neuropeptides. A search of the non-redundant protein database suggested that this class of prohormone-4-like sequences appears, so far, to be unique to *Conus* and it will be of considerable interest to explore the role this class of sequences plays in the venom gland.

3.3. Insulin

The most well-known role of insulin is the hormonal control of glucose metabolism, although there is increasing evidence that it also functions as a neurotransmitter within the central nervous system (Schulingkamp et al., 2000). In molluscs insulin plays a role in glucose homeostasis and also as a neurotransmitter (Smit et al., 1998), and as we have recently demonstrated, insulin peptides are expressed abundantly in the venom glands of *Conus*, and are a major venom component in *C. geographus* and other *Conus* species (Safavi-Hemami et al., 2015). In fact, they represent one of the most abundant classes of transcript in the distal segment of the venom gland of *C. geographus*, where they are expressed almost exclusively, and it was recently demonstrated that the distal venom gland of several *Conus* species is responsible solely for the production of venom peptides involved in predation (rather than defense) (Dutertre et al., 2014). The fish-hunting species *C. geogra-*

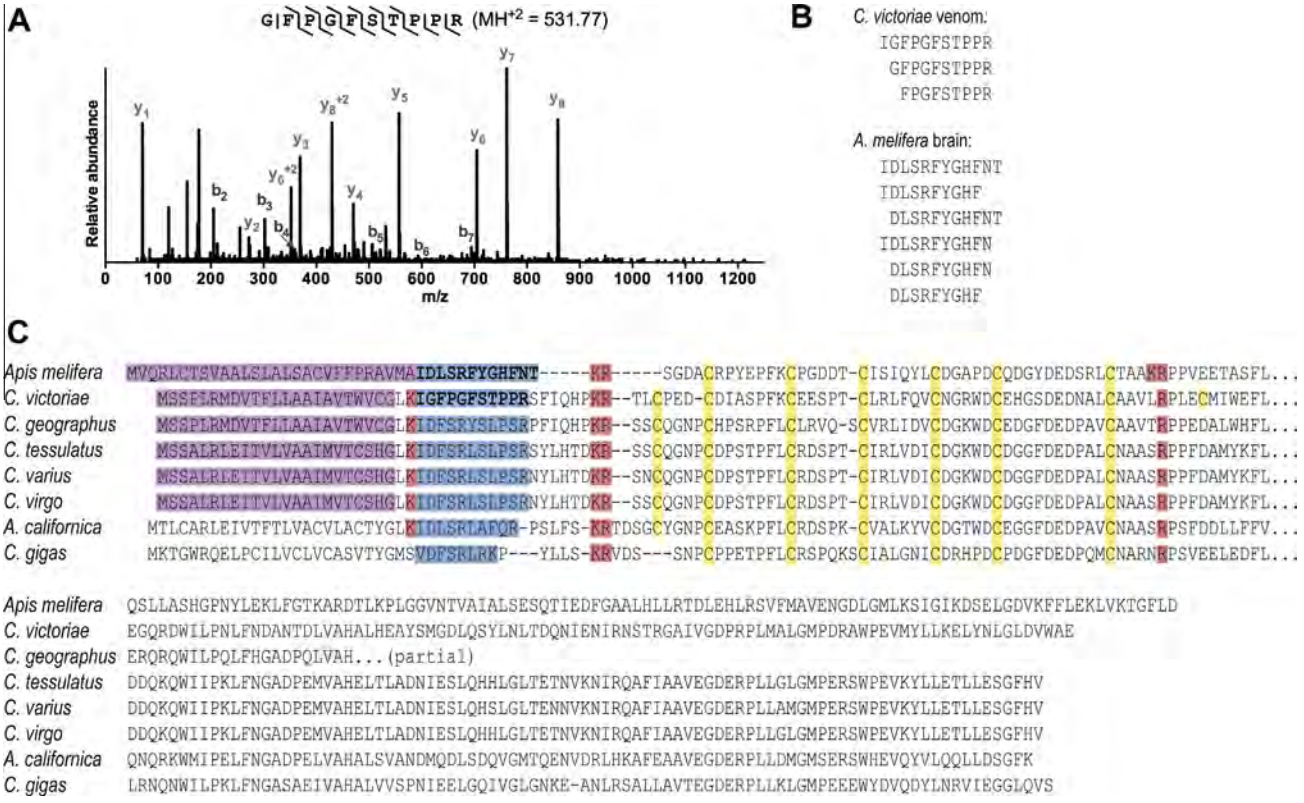

Fig. 2. *C. victoriae* venom prohormone-4. (A) MS/MS spectrum of PH4-Vc1 peptide. The precursor ion selected for MS/MS had a monoisotopic m/z of 531.77 ($z = 2$) with a predicted monoisotopic m/z of 531.77 ($z = 2$). For clarity, only b and y ions are labeled. Inset b/y ladder diagrams summarize observed b- and y-ions. (B) The mature peptide variants derived from prohormone-4 in *C. victoriae* venom and *A. mellifera* brain. (C) The aligned precursor sequences of *A. mellifera* prohormone-4, PH4-Vc1 and those of *C. geographus*, *C. tessulatus*, *C. varius*, *C. virgo*, *A. californica* and *C. gigas*. Precursor signal peptides are highlighted in purple, cleavage sites in red, cysteine residues in yellow, putative/confirmed encoded mature peptides are in blue and identified mature peptides of *A. mellifera* prohormone-4 and PH4-Vc1 are in boldface.

phus and *Conus tulipa* appear to be using insulin peptides alongside other venom peptides to elicit hypoglycemic shock and facilitate capture of their fish prey. Insulin sequences identified in several cone snail species appear to correlate with their feeding preferences, with fish-hunting cone snails expressing fish-like sequences and mollusc-hunting snails expressing molluscan-like insulins (Safavi-Hemami et al., 2015).

MS-matching on the reduced/alkylated and trypsinized sample of extruded *C. victoriae* venom revealed a single tryptic fragment (GIVCγCCK) derived from one of two insulin-like peptide (ILP) precursors present in the *C. victoriae* venom gland transcriptome (Fig. 4A). This peptide fragment corresponded to the N-terminus of the peptide A-chain. Interestingly, Glu5 of this fragment was modified to γ-carboxyglutamate (confirmed by peaks at 467.18 and 640.21 m/z corresponding to the y_3 and y_4 ions, respectively), consistent with our observation of this modification at the equivalent position in other *Conus* venom insulins (Safavi-Hemami et al., 2015). The precursor sequences of each *C. victoriae* ILP are typical of other insulins; an N-terminal signal peptide, the B-chain, followed by one or more propeptide regions flanked by dibasic cleavage sites (which may encode insulin C-peptides), and finally the A-chain. Unlike the *A. californica* insulin precursor, those of *C. victoriae* do not encode a C-terminal D-peptide (Jakubowski et al., 2006). When compared with vertebrate insulins and the *C. geographus* venom insulins, both *C. victoriae* ILPs have an additional cysteine residue on each chain, which presumably form an additional interchain disulfide bond. This additional pair of cysteines is seen in molluscan insulins, including that of *A. californica* (Floyd et al., 1999) (Fig. 3) as well as in the venom insulins of other mollusc-hunting *Conus* (Safavi-Hemami et al., 2015). Although other roles are possible, together these data suggest that insulin peptides may serve as exogenously targeted hormones/neuropeptides in *C. victoriae*.

3.4. Thyrostimulin

Two tryptic fragments observed in the reduced/alkylated and trypsinized sample of extruded *C. victoriae* venom were derived from a transcript encoding a glycoprotein hormone α2-subunit (Fig. 4 and Table S1). The glycoprotein hormone family consists of several members characterized by a common α-subunit non-covalently bound to a β-subunit with the β-subunit defining hormone activity. The glycoprotein hormone α2-subunit combines with a β5-subunit (encoded on a separate transcript) to become thyrostimulin (Nakabayashi et al., 2002). In humans, thyrostimulin influences body metabolism, via stimulation of the thyrotropin G protein-coupled receptor. A thyrostimulin analogue was recently identified in the mollusc *A. californica* (Heyland et al., 2012).

A search of the *C. victoriae* venom gland transcriptome yielded a transcript encoding the accessory β5-subunit of thyrostimulin (Fig. 4), and thyrostimulin subunit transcripts were also present in the venom gland transcriptomes of other *Conus* species; *C tessulatus* and *C. virgo* (Fig. 4). It seems probable that, like the human analogue, *Conus* thyrostimulins form functional non-covalent heterodimers.

4. Conclusions

Our finding of hormone/neuropeptide-like components in the venom of *C. victoriae*, together with previous reports for this genus, clearly demonstrates that the *Conus* venom gland is not only highly specialized in the biosynthesis and secretion of conotoxins but also

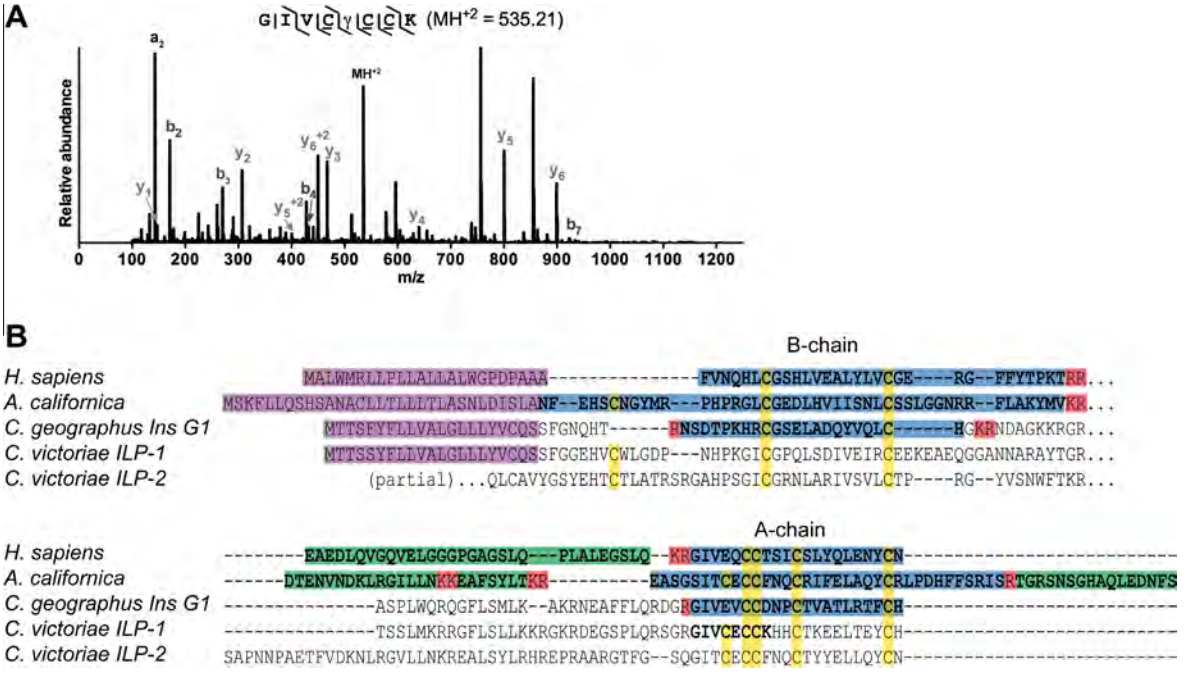

Fig. 3. *Conus* venom insulins. (A) MS/MS spectrum of *C. victoriae* ILP-1 A-chain tryptic fragment. The precursor ion selected for MS/MS had a monoisotopic m/z of 535.21 ($z = 2$) with a predicted monoisotopic m/z of 535.21 ($z = 2$). Inset b/y ladder diagrams summarize observed b- and y-ions. C; carbamidomethylated cysteine, γ; gamma-carboxyglutamate. (B) The aligned precursor sequences of human insulin, *A. californica* insulin, the *C. geographus* venom insulin Ins-G1 and the *C. victoriae* insulins. Precursor signal peptides are highlighted in purple, confirmed cleavage sites in red and the confirmed encoded mature peptides are in boldface with the insulin A- and B-chains in blue and the C/D peptides in green.

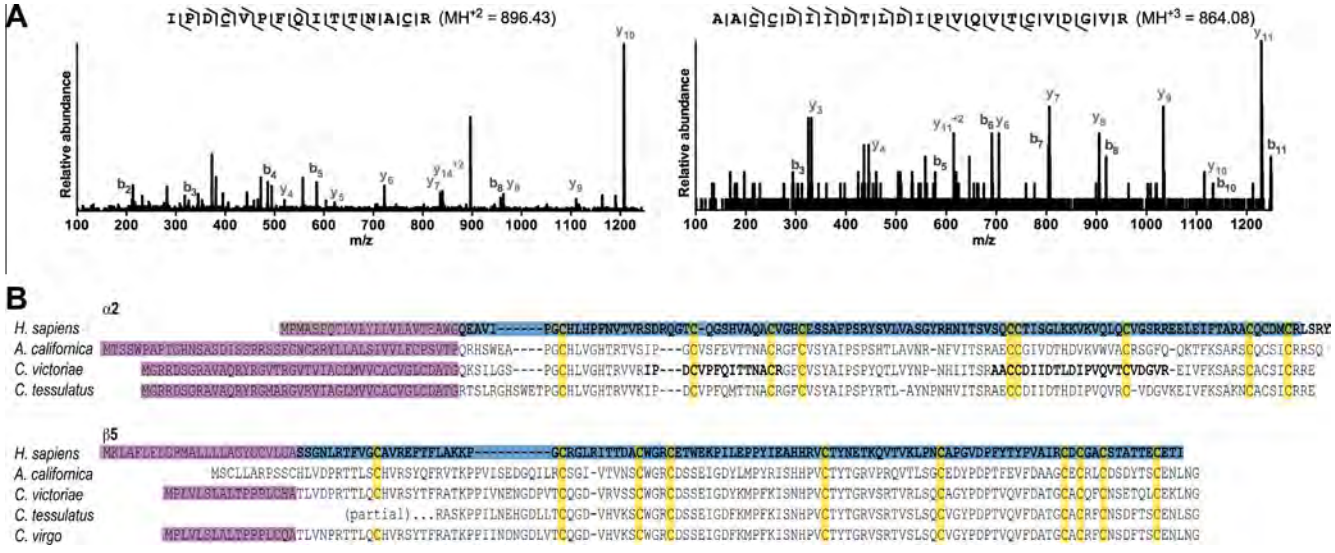

Fig. 4. *Conus* venom thyrostimulins. (A) MS/MS spectra of *C. victoriae* thyrostimulin α2-subunit tryptic fragments. The precursor ions selected for MS/MS had monoisotopic m/z of 896.43 ($z = 2$) and 864.08 ($z = 3$), with predicted monoisotopic m/z of 896.43 ($z = 2$) and 864.08 ($z = 3$), respectively. For clarity, only b and y ions are labeled. Inset b/y ladder diagrams summarize observed b- and y-ions. C; carbamidomethylated cysteine. (B) The aligned precursor sequences of the α2- and β5-subunits of human, *A. californica*, *C. victoriae*, *C. tessulatus* and *C. virgo* (β5 only) thyrostimulins. Precursor signal peptides are highlighted in purple, confirmed peptide sequences are in boldface and the confirmed encoded subunits of human thyrostimulin are highlighted in blue.

a rich source of hormone/neuropeptide-like peptides. Two of these peptides (elevenin and prohormone-4) were identified in the injected venom of *C. victoriae*, unambiguously demonstrating their role as exogenously targeted venom components. Notably, this is the first study to report the presence of these particular hormones in animal venom. These and several other previously-reported venom components appear to have been recruited into the venom from endogenous processes (insulin, elevenin, prohormone-4, thyrostimulin, conopressin, conoCAPs, conomap), a phenomenon that has been proposed for larger polypeptides in other animal venoms (Fry et al., 2009).

Together with previous observations on the presence of hormone/neuropeptide-like peptides in cone snail venoms, our findings strongly suggest that these are not sporadic incidences but that these molecules form a diverse and integral part of the complex molecular envenomation strategy of *Conus*. The rapid duplication and divergence of conotoxin-encoding genes and the incorporation of diverse post-translational modifications are two

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major factors considered to contribute to cone snail venom diversification (Olivera, 1997). It appears that venom diversification may involve an additional mechanism, the recruitment of endogenous hormones/neuropeptides.

Numerous *Conus* venom components have proven valuable as pharmacological tools and several have potential as drug leads or therapeutics. For example, ziconotide (Prialt®), the synthetic equivalent of ω-MVIIA from the venom of C*onus magus*, a potent and selective blocker of the $Ca_V2.2$ calcium channel, is being used to treat chronic pain (Miljanich, 2004). Some of the hormone/neuropeptide-like components of *Conus* venom also show similar therapeutic potential: contulakin-G, a neurotensin analogue from the venom of *C. geographus*, is a potent analgesic (Allen et al., 2007) and at one point had progressed as far as human clinical trials. Further exploration of this group of venom components may offer an exciting new avenue for the discovery of novel pharmacological tools and drug candidates, complementary to conotoxins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2015.07.012.

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