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REVIEW ARTICLE

Incorporation of post-translational modified amino acids as an approach to increase both chemical and biological diversity of conotoxins and conopeptides

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Abstract Bioactive peptides from *Conus* venom contain a natural abundance of post-translational modifications that affect their chemical diversity, structural stability, and neuroactive properties. These modifications have continually presented hurdles in their identification and characterization. Early endeavors in their analysis relied on classical biochemical techniques that have led to the progressive development and use of novel proteomic-based approaches. The critical importance of these post-translationally modified amino acids and their specific assignment cannot be understated, having impact on their folding, pharmacological selectivity, and potency. Such modifications at an amino acid level may also provide additional insight into the advancement of conopeptide drugs in the quest for precise pharmacological targeting. To achieve this end, a concerted effort between the classical and novel approaches is needed to completely elucidate the role of post-translational modifications in conopeptide structure and dynamics. This paper provides a reflection in the advancements observed in dealing with numerous and multiple post-translationally modified amino acids within conotoxins and conopeptides and provides a summary of the current techniques used in their identification.

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Abbreviations

Aph	4-Aminophenylalanine
Abu	α-Aminobutyric acid
Gla	γ-Carboxy glutamic acid
CRS	γ-Carboxylation recognition sequence
αα	Amino acid
CID	Collision-induced desorption
Da	Dalton
K_D	Dissociation constant
ESI–MS	Electrospray ionization mass spectrometry
ER	Endoplasmic reticulum
LSI–MS	Laser spray ionization
MS	Mass spectrometry
MALDI–MS	Matrix assisted laser desorption ionization mass spectrometry
MALDI–TOF–MS	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
nAChR	Nicotinic acetylcholine receptor
PPI	Peptidylprolyl isomerase
PFA	Performic acid
pK_a	Logarithmic acid dissociation constant
PSD	Post-source decay
PTM	Post-translational modification
PDI	Protein disulfide isomerase
RP–HPLC	Reverse phase high performance liquid chromatography
SPPS	Solid phase peptide synthesis

TCEP	Tris(2-carboxyethyl) phosphine
XRC	X-ray crystallography

Introduction

Post-translational modifications, or PTMs, of amino acids (αα) are covalent modifications typically pertaining to side chains or 'R' group functions of the 20 common ααs. These modifications are known to have considerable influence on the overall structure and bioactivity of a peptide. Peptide toxins isolated from the genus *Conus* demonstrate this phenomenon, with multiple documented peptides exhibiting diverse PTMs. Each PTM serves a specific purpose in the grand scheme of prey incapacitation, as seen with the glycosylated (bioactive) and non-glycosylated Threonine variants (reduced bioactivity) of Contulakin-G (Craig et al. 1999a). Numerous other examples exist in this carnivorous marine gastropod family, as will be discussed in this review. Known PTM classifications in *Conus* include the following: N-terminal modifications, C-terminal modifications, intramolecular and intermolecular disulfide bonding, hydroxylation, γ-carboxylation, sulfation, bromination *O*-glycosylation, and epimerization. PTMs found in toxins have either determinate or speculative effects on bioactivity in vivo and may be of great importance to the continued incorporation and advancement of proteomic screening. Many of these modifications are unobservable through some of the current genomic and proteomic discovery methodologies (e.g. αα epimerization) (Buczek et al. 2008). This review discusses various approaches that combine techniques that have been, or may be implemented to identify the array of PTMs present within the venoms of the *Conus* genus as well as other related peptides of interest.

History of identification

Investigation of conopeptides emerged in the late 1970s, prior to the emergence and standardization of modern proteomic techniques. The absence of these methodologies complicated venom characterization. Although it was determined that conopeptides were short sequences generally <30 ααs, complete delineation of peptide structure and activity proved to be a challenging endeavor due to the presence of various PTMs. Without the availability of contemporary genetic information, extensive cDNA or peptide libraries and innovative proteomic practices including reverse-phase high-pressure liquid chromatography (RP–HPLC) combined with mass spectrometry (MS), the problems in characterizing venom constituents were resolved using various classical biochemical techniques. Although many are now replaced by advanced MS methods, some of these paradigmatic methodologies are still used in conopeptide research. They serve as forerunners of the current multidisciplinary approach to conopeptide characterization and as the foundation of 'conovenomic' development.

Original methods of post-translational modification identification/detection: a biochemical prospective

Conotoxin GI (Table 1), discovered from the secretory duct venom of *Conus geographus*, was one of the first conotoxins to be isolated and completely biochemically characterized (Gray et al. 1981). Isolation of the peptide was commenced using a combination of phosphocellulose chromatography and electrophoresis; these techniques have since been replaced by RP–HPLC. The isolated peptide exhibited intramolecular disulfide bonding, which was one of many PTMs encountered in cone snail venom characterization. The inability to identify disulfide bonds through classical Edman degradation techniques was remedied using performic acid (PFA) oxidation, a method of breaking and inhibiting disulfide bond reformation via the oxidation of thiols to Cysteic acid. Here, four moieties of cystic acid were identified with α-conotoxin GI (31 % of the total sequence; Gray et al. 1981).

Further problems were encountered when it was discovered that the carboxyl terminus of the peptide could not be adequately DNS/dansyl labeled and that the use of carboxypeptidase Y had no degradative effect (Gray and Hartley 1963). This blockage, at the time, was hypothesized to be the result of C-terminal amidation. To demonstrate this, the use of two-dimensional electrophoresis and tryptic digestion was employed, comparing this to known standards matching the electrophoretic mobility of the hypothesized C-terminal fragment. Confirmation of C-terminal amidation established it as the second PTM to be discovered in *Conus*. This PTM was later found to be highly abundant within the genus (Kaas et al. 2012; "C-terminal: C-terminal amidation of conotoxins and conopeptides").

Although fundamental to the original elucidation of these peptide toxins, these methods have been succeeded by twenty-first century techniques in terms of genetic analysis as well as proteomics. Genetic determination of the "King-Kong" peptide (KK-1), a peptide from *Conus textile*, is the first reported isolation of a cone snail toxin being sequenced through the use of a cDNA library (Woodward et al. 1990). Since then, cDNA characterization has become a mainstream method of identification for many different conopeptides. However, this approach has its limitations; conopeptides contain an array of PTMs that are difficult to predict from cDNA alone. Even so, the resulting information can help provide tentative PTM assignment with identification of the parent αα. The

Table 1 N and C terminal modifications of select conotoxins/conopeptides

PTM	Toxin	Sequence	Receptor target of native toxin	Pharmacology PTM toxin	Pharmacology non-PTM toxin	Ref.
N-terminal modification						
Pyroglutamic acid, O-linked glycosylation	Contulakin-G	ZSEEGGSNATKKPYIL ↓ ZSEEGGSNA(gTr)KKPYIL	hNTR1 rNTR1 RNTR2 mNTR3	IC_{50}(nM) hNTR1: 23 rNTR1:79 RNTR2: 170 mNTR3: 171	IC_{50}(nM) hNTR1: 960 rNTR1: 524 RNTR2: 730 mNTR3: 250	Craig et al. (1999a, b)
C-terminal modification						
Amidation	α-GI	ECCNPACGRHYSC*	nAChR : α1β1γδ	IC_{50}(nM) 20	ND	Gray et al. (1981), Johnson et al. (1995)
Amidation	α-CnIA	GRCCHPACGKYYSC ↓ GRCCHPACGKYYSC*	nAChR: α1β1γδ	IC_{50}(nM) 190	ND	Favreau et al. (1999)
Amidation	ω-MVIIA	CKGKGAKCSRLMYDCCTGSCRSGKCG (ω-MVIIA-Gly) ↓ CKGKGAKCSRLMYDCCTGSCRSGKC* (ω-MVIIA)	Neuronal Ca^{2+} channels	K_d(M) 2.5×10^{-10} in SHTL buffer	K_d(M) 3×10^{-9} in SHTL buffer	Price-Carter et al. (1996)
Amidation	α-ImI	GCCSDPRCAWRC (α-ImI acid) ↓ GCCSDPRCAWRC* (α-ImI)	A-AChBP B-AChBP nAChR	K_d(M) Globular form: B-6.6, A-2.8 Ribbon form: B- >10 K, A-28 K Beaded form: B-220, A-220	K_d(M) Globular form: B-6.5, A-2.3 Ribbon form: B-95, A-48 Beaded form: B–N/D, A-140	Kang et al. (2007), Kang et al. (2005), McIntosh et al. (1994)
N- and C-terminal modification						
Pyroglutamic acid, amidation	α-Vi1359	ZCCITIPECCRI*	ND	ND	ND	Mandal et al. (2007)
Pyroglutamic acid, amidation	α-Vi1361	ZCCPTMPECCRI*	ND	ND	ND	Mandal et al. (2007)

Z Pyroglutamic acid, * C-terminal amidation, *gTr* glycosylated tryptophan, *NTR* neurotrophin receptor, *nAChR* nicotine acetylcholine receptor, *ND* not determined, *AChBP* acetylcholine binding protein

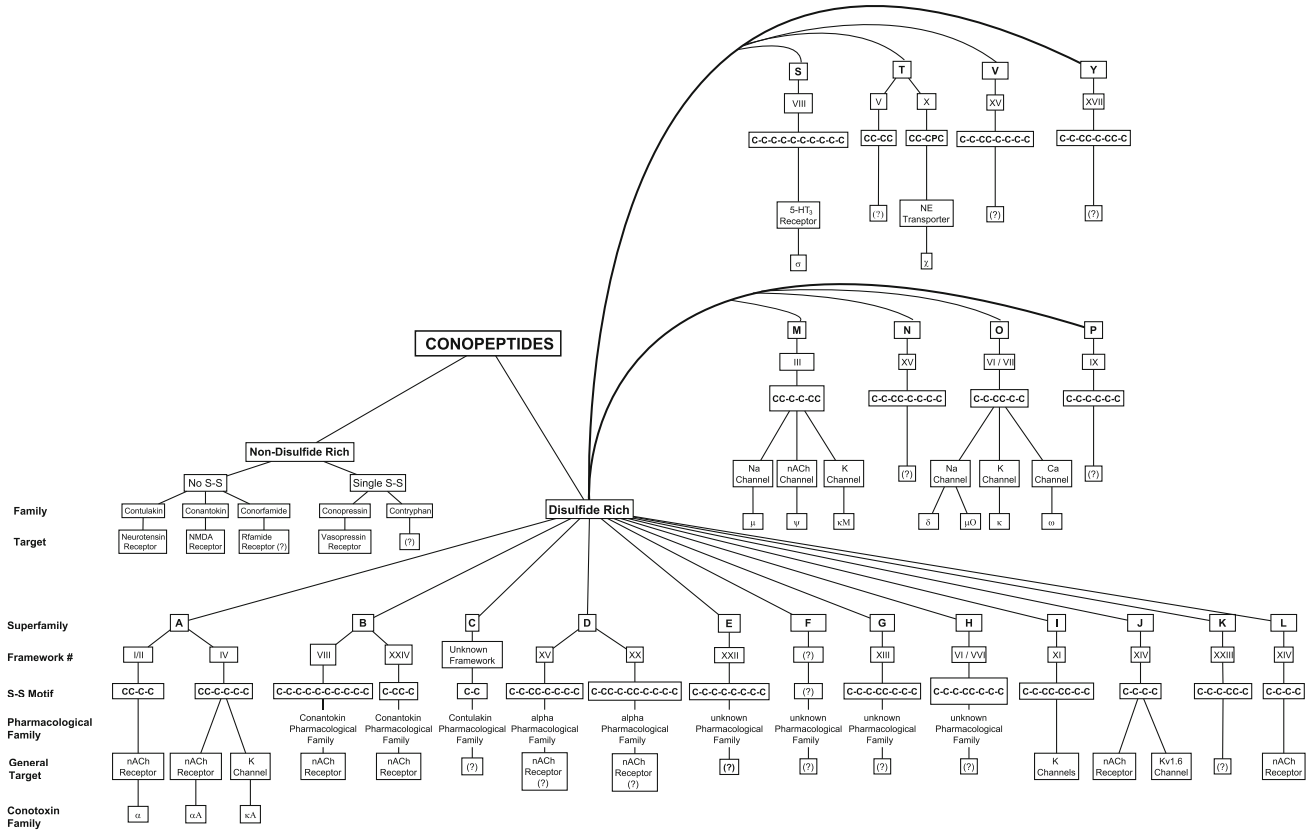


Fig. 1 The complexity of the conotoxin/conopeptide families. Classification of conotoxins and conopeptides has been an evolving process, with the main focus on peptide separation based on establishing peptide Superfamilies; these are classified on the basis of disulfide framework patterns. This classification has had strong correlation to pharmacological targeting—given their essential role in stabilizing 3-D structure. More interesting is the diversity of post-translational modification that extends to each superfamily, as illustrated in Tables 1, 3, 5, 6 and 8. Figure adapted and and modified from Terlau and Olivera (2004)

advancement of 'conovenomics' is reliant on the expeditious turnover of conopeptide discovery and characterization. As a result, emerging proteomic techniques that promise rapid results are fast supplanting these old-fashioned and more laborious biochemical methods.

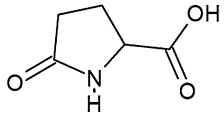
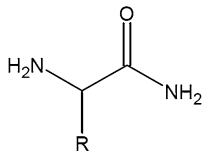
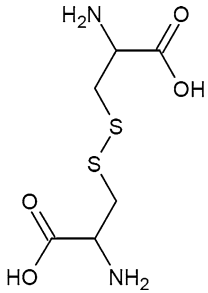
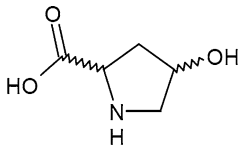
Advanced methods of post-translational modification identification/detection

The current proteomic approach of conotoxin/conopeptide research begins with toxin characterization through mass spectroscopy (MS) and αα analysis. Subesquently, the target peptide is purified and isolated by RP–HPLC. Confirmation is achieved through chemical synthesis and bioassays, while structural analysis is performed by Nuclear Magnetic Resonance spectroscopy (NMR) or X-ray crystallography (XRC). α-Conotoxin CnIA (Table 1), originating from the duct venom of *Conus consors*, was first identified through the utilization of each of these advanced techniques (Favreau et al. 1999). This peptide contains a similar 3–5 cysteine loop framework to that of α-conotoxin GI, a characteristic commonly used in conotoxin/conopeptide classification (Fig. 1). Peptide sequencing was carried out through a combination of αα analysis, automated Edman sequencing, and electrospray ionization mass spectrometry (ESI–MS). Disulfide connectivity, as well as overall three-dimensional structure of the peptide, was established through combining computational software and NMR. In this peptide, PTMs were again confined to disulfide bridges and the presence of the C-terminal amide; this represents one of the simpler examples in *Conus* venoms. The complexity of PTM ααs and the challenges they have presented in conotoxin/conopeptide discovery require individual examination to understand their role in biological function.

N-terminal: pyroglutamic acid of conotoxins and conopeptides

Pyroglutamic acid (Z) (Table 2), a PTM product of either Glutamic acid or Glutamine residues, is thought to be produced one of two ways: (a) by a nonenzymatic dehydration reaction (Chelius et al. 2006) or (b) by a glutamyl

Table 2 Documented modifications and methods of detection of terminal and side chain derivatized amino acids in conotoxins/conopeptides

Modification	Structure	Mass shift	Edman degradation	Enzymes	Mass spectrometry	Comments
Pyroglutamic acid		Glu(Gln) → pyroglu −17(−18 Da)	Blockage incompatible with sequencing	Pyro-glutamate amino-peptidase (EC 3.4.19.3)	ESI, LSI, MALDI	Aminopeptidase unblocks N-terminus for Edman degradation Will not be identified by amino acid analysis N-terminal blockage has little effect on proteomic approaches
C-terminal amidation		–COOH → –$CONH_2$ (−1 Da)	Unable to differentiate between the two forms	None	Oxazolone derivatization/ MALDI	Hydrazinolysis replaced by Oxazolone derivatization and mass spectrometry Will not be differentiated by amino acid analysis
Disulfide Bonding		–SH HS– → –S–S– (−2 Da)	Reduce and alkylate before Edman degradation	None	ESI, MALDI	Determine disulfide linkage by partial reduction and alkylation followed by mass spectrometry or Edman degradation If no reduction/alkylation undertaken, blank cycles will be observed in thiol containing positions
Hydroxyproline (4-*cis* and *trans*)		Pro → Hydroxypro (+16 Da)	Unhindered; use of standards allows for identification	None	ESI, MALDI	Hydroxylation determined by a combination of Edman degradation, amino acid analysis, and mass spectrometry

Refer to conotoxins/conopeptides containing modifications in Tables 1, 3 and 5 for references; If ionization mode is not specified, positive mode was used in mass spectrometry trials; Edman degradation: blockage, stopped Edman degradation; blank cycle, not enough residue collected at cycle to confirm amino acid

cyclase enzyme (Fischer and Spies 1987). It was originally thought that the non-enzymatic production of pyroglutamate used only Glutamine as a precursor. It has since been shown that pyroglutamic acid can form spontaneously in vitro, under appropriate conditions, with a Glutamate precursor (Chelius et al. 2006).

Pyroglutamic acid is known for its inherent ability to cause a blockage at the N-terminus of peptides thus complicating the use of Edman degradation. This characteristic side-chain cyclization to the α-N protects peptides from enzymatic digestion of exopeptidases (Chelius et al. 2006). It follows logically that the presence of this PTM in conopeptides may augment toxin bioavailability in prey. In terms of biochemical sequence analysis this blockage may be circumvented by treatment with pyroglutamate aminopeptidase (EC 3.4.19.3.) or by using a mild/partial acidic hydrolysis to cleave the offending αα at either the side-chain or neighboring peptide bond. Once treated, peptides may be submitted to normal sequential degradation procedures with the generation of a new α-N amino terminus (Chelius et al. 2006).

The presence of pyroglutamic acid eliminates the contribution of the αN-terminal pK_a, thus affecting the isoelectric focus point (PI) of the PTM peptide. In conopeptides, the difference in the overall charge state may influence three-dimensional structure that may contribute to a greater pharmacological selectivity within conotoxins (Nelson et al. 2008; Kapono et al. 2013). However, a complete assessment of effects in bioengineered conotoxins has yet to be investigated.

Pyroglutamic acid residues found within Contulakin-G (Table 1), a peptide isolated from the venom of *C. geographus,* have been identified by Electrospray Ionization (ESI–MS), Matrix Assisted Laser Desorption and Ionization (MALDI–MS), and Laser Spray Ionization (LSI–MS) Mass Spectroscopy in combination with conventional Edman degradation (Craig et al. 1999b).

Pyroglutamic acid residues have also been found within peptides in the venom of *Conus virgo* (Mandal et al. 2007). In this study, pyroglutamic acid residues were determined using a combination of ESI–MS and MALDI–MS on reduced peptides. These techniques illustrate the sequence abilities of MS in producing ladder fragmentation, which in turn can be interpreted into full sequences independent of most PTMs bypassing the sequencing issues associated with Edman chemistry (Mandal et al. 2007).

Other known pyroglutamic acid containing species include *Conus imperialis* (V; Craig et al. 1997), *Conus californicus* (O; Elliger et al. 2011), *Conus consors* (P; Zhang et al. 2006), *Conus magus* (P; Zhang et al. 2006), *Conus purpurascens* (P; Nielsen et al. 2002), *Conus striatus* (P; Craig et al. 1998), *Conus stercusmuscarum* (P; Santos et al. 2004), and *Conus stiolatus* (?; Walewska, and *Conus textile*) (M; Bhatia et al. 2012). [V = vermivorous, P = piscvorous, M = molluscivoroes, O = omnivorous, ? = unknown].

C-terminal: C-terminal amidation of conotoxins and conopeptides

C-terminal amidation (*) (Table 2) is a PTM present at the C-terminus of peptides in which an amide group replaces the hydroxyl group of the carboxylic acid terminal. As seen with N-terminal pyroglutamic acid modification, this C-terminal modification affects the overall isoelectric point and net charge state, neutralizing the potential for deprotonation. C-terminal modification is typically achieved by enzymatically cleaving a flanking C-terminal Glycine at the N–C bond, thus creating a new C-terminal that retains the truncated remains of the original peptide bond from the neighboring C-terminal amino acid (Price-Carter et al. 1996).

The presence of C-terminal signal sequences such a pre-PTM Glycine is commonly observed in the genetic analysis of conotoxins (Kaas et al. 2012). It is estimated that over 127 naturally occurring conopeptides contain a C-terminal amide. This encompasses a large number of gene superfamilies including: A, B, I_1, I_2, J, M, O_1, O_2, P, Q, S, T, and V superfamilies (Kaas et al. 2012; Fig. 1). C-terminal amidation has been shown to occur in vitro on short C-terminal Glycine containing peptides incubated with peptidylglycine α-amidating enzyme (EC 1.14.17.3), in an oxygen-rich environment and in the presence of reduced cofactors such as ascorbic acid (Tajima et al. 1990). This reaction is thought to form a hydroxyl glycine intermediate before the reaction goes to completion.

C-terminal amidation was first hypothesized as a PTM in α-conotoxin GI (Table 1), one of the earliest discovered venom peptides (Gray et al. 1981). Suspicion regarding the C-terminal assignment was not clearly demonstrated until α-conotoxin GI was chemically synthesized a few years later (Nishiuchi and Sakakibara 1982). As originally observed in α-conotoxin GI, the presence of a C-terminal amide may act to protect peptide toxins against carboxypeptidase activity, increasing the circulation half-life in prey (Gray et al. 1981).

The importance of C-terminal amidation within conotoxins was further recognized in the early- to mid-1990s when studies were first being conducted to understand their contribution to disulfide arrangement and folding, particularly that of the voltage-gated calcium channel blockers, or ω-conotoxins—(Price-Carter et al. 1996). Analysis of ω-Conotoxin MVIIA (Table 1) revealed an increased folding efficiency with the presence of the pre-PTM Glycine at the C-terminus. This challenged the original concept that the

larger N-terminal pre-pro sequence was the main source of thermodynamic favorability in enzyme-mediated folding and oxidation (Price-Carter et al. 1996).

C-terminal amidation is also thought to contribute to the formation of native globular structures within the α-conotoxin family. This is specifically illustrated with α-conotoxin ImI (Kang et al. 2005; Table 1). It has been shown that this conotoxin can be converted from a well-established nicotinic acetylcholine receptor (nAChR) antagonist to a newly defined noradrenaline receptor ligand through excision of the C-terminal amide. It is believed that the C-terminal amide strongly influences disulfide bond connectivity arrangement, causing major structural changes that in turn define biological function and selectivity.

Structural studies using software modeling and NMR have revealed that C-terminal amidation in α-conotoxin ImI disrupts the folding of the ribbon conformation, particularly when proline is present within the first intercysteine loop (Kang et al. 2007). These characteristics seem to increase the probability of forming a globular structure in α-conotoxin ImI and also appear to be conserved in other toxins i.e. I-conotoxin MrvIA. The preponderance of this Proline/C-terminal amidation interaction among conotoxins suggests its efficacy in defining structural and biological properties.

C-terminal amides may be detected through the utilization of oxazolone derivatization (Kuyama et al. 2009). This selective chemical derivatization process in combination with tandem MS increases the C-terminal PTM fragment by 14 Daltons (Da), making detection much more evident. Typically, simple molecular mass deduction is employed. Accurate molecular mass analysis provides a high level of certainty in establishing the predicted sequence mass difference of 1 Da between the amide and carboxylic acid form. Numerous conotoxins with C-terminal modifications are known; over 170 are reported in Conoserver (Kaas et al. 2012).

Disulfide bonds within conotoxins and conopeptides

The pharmacological success of conotoxins as drug leads can be attributed to their receptor specificity and isoform selectivity (Adams et al. 1999). Their primary structures reveal pattern-specific cysteine frameworks that generate αα loops responsible for their receptor specificity (Armishaw and Alewood 2005; Dutton et al. 2002; Bingham et al. 2005). The peptide's unique receptor–ligand interaction is contingent on well-defined tertiary structures stabilized by disulfide bonds. Generally, conotoxins contain 1–3 disulfide bonds (Table 1); however, as many as ten have been observed (Bingham et al. 2005). Few conopeptides have demonstrated an absence in disulfide bond content. The smallest 'classical 3/5' molecular mass conotoxin, α-conotoxin MIC, contains only 12 αα, of which four (33 %) are cysteines involved in the formation of two disulfide bridges (Kapono et al. 2013).

The maximum possible combination of disulfide bridges within a peptide may be calculated using the formula $p = \frac{n!}{(\frac{n}{2})!2^{n/2}}$, where p is the number of possible disulfide bridges and n is the number of cysteines present within the peptide. These connections can have several permutations that generate new peptide isomers with distinct pharmacological and kinetic properties (Bingham et al. 2012). As such, disulfide bridges directly contribute to the efficacy and biological variability of conotoxins. Rarely do we see the random and spontaneous generation of all permutations, with dominance typically given to the lowest energy conformation. However, these yields can be greatly influenced by adding alcohols (Alewood et al. 2003).

Although the contribution of disulfide bonds to protein stability has been established, deletion of these cysteine bridges can have varying effects on biological activity. Through substitution of Alanine for Cysteine, Khoo et al. (2009) effectively induced a deletion of a single cystine bridge from 16 αα μ-conotoxin KIIIA to generate the synthetic analog μ-KIIIA [C1A, C9A] (Table 3). Despite having one less cysteine bridge, μ-KIIIA [C1A, C9A] exhibited comparable activity with native μ-KIIIA (3 disulfide bonds) toward rat $Na_v1.2$ with K_D values of 0.008 ± 0.002 μM and 0.005 ± 0.005 μM, respectively. NMR data coupled with molecular dynamic modeling indicated that both μ-KIIIA [C1A, C9A] and its native counterpart exhibited the retention of a key α-helix essential in binding to $Na_v1.2$ and $Na_v1.4$ receptors. Parallel studies done with scorpion toxins exhibited comparable results (Sabatier et al. 1996; Song et al. 1997). This finding suggests that biological activity of toxins is not absolutely dependent on disulfide bond stabilization, implying that toxin activity can be induced with conservation of partial or native-like motifs stabilized by non-covalent interactions.

In contrast, Flinn et al. (1999) demonstrated that deletion of a disulfide bond through synthetic substitution of Cys^{15} and Cys^{26} of 27-residue ω-conotoxin GVIA (3 native disulfide bonds) (Table 3), with serine residues resulted in a 8,000-fold loss of potency, with no measurable activity at 10 μM in rat vas deferens assay. NMR analysis of peptide $[Ser]^{15,26}$ ω-conotoxin GVIA revealed a loss of native structure, demonstrated by the existence of multiple isomers in solution and attributed to *cis–trans* isomerism of an amino acid stretch normally constrained by the deleted cystine bond (Flinn et al. 1999).

In addition to the selective disulfide formation of orthogonally protected $[Ser]^{15,26}$ω-conotoxin GVIA, Flinn et al. (1999) randomly oxidized other two-disulfide

Table 3 Disulfide bonding of select conotoxins/conopeptides

PTM		Toxin	Sequence	Receptor target of native toxin	Pharmacology PTM toxin	Pharmacology non-PTM toxin	Ref.
Disulfide bonds	Intramolecular	μ-KIIIA	CCNCSSKWCRDHSRCC* ↓ ACNCSSKWARDHSRCC* μ-KIIIA [C1A, C9A]	Na_V	Rat $Na_v1.2$ 0.008 ± 0.002 μM	Rat $Na_v1.2$ 0.005 ± 0.005 μM	Khoo et al. 2009
	Intramolecular, Amidation	ω-GVIA	CKSOGSSCSOTSYNCCRSCNOYTKRCY* ↓ CKSOGSSCSOTSYNSCRSCNOYTKRSY* [Ser 15,26]GVIA (P) CKSOGSSCSOTSYNSCRSCNOYTKRSY* [Ser 15,26]GVIA	Ca_V 2.2	Mouse activity assay in vas deferens (1μM) 50% inhibition	Mouse activity assay in vas deferens (1μM) No activity.	Flinn et al. 1999
Disulfide bonds, C-terminal modification	Intramolecular, Amidation	α-AuIB	GCCSYPPCFATNPDC* ↓ GCCSYPPCFATNPDC* Globular Isomer GCCSYPPCFATNPDC* Ribbon Isomer GCCSYPPCFATNPDC* Beaded Isomer	nAchR	IC_{50}(nM) Globular: 1.2	IC_{50}(nM) Ribbon: 0.1 Beaded: ND	Dutton et al. 2002
	Intermolecular, Amidation	Vt3.1	GPYRRYGNCYCPI* ↓ GPYRRYGNCYCPI* GPYRRYGNCYCPI* Crossed disulfide bonds or GPYRRYGNCYCPI* GPYRRYGNCYCPI* Parallel disulfide bonds	ND	Mouse activity assay Intraventricular injection (10-20μg/mouse) Cross-linked: activity Parallel: No activity	Linear sequence: no activity	Wu et al. 2010

* C-terminal amidation, *ND* not determined, *nAchR* nicotinic acetylcholine receptor, *Na_V* voltage gated sodium channel, *Ca_V 2.2* N-type calcium channel

analogs of ω-conotoxin GVIA and found isomers of the peptide with non-native disulfide connectivities. Given four cysteines, a total of three disulfide permutations are possible, yielding three forms: globular, ribbon, and beaded (Flinn et al. 1999; Jin et al. 2007; Dutton et al. 2002). Depending on the toxin, each isomer can yield variable degrees of biological activity. In the case of α-conotoxin AuIB (Table 3), the non-native ribbon isomer exhibited tenfold greater activity than its native counterpart (Armishaw and Alewood 2005; Dutton et al. 2002; Bingham et al. 2012). Recent investigation by Khoo et al. (2012) on μ-conotoxin KIIIA, containing six cysteines, reveal that glutathione-directed folding yielded two out of fifteen possible disulfide permutations. It was found that the minor isomer had a disulfide organization of 1–4/2–5/3–6 commensurate with μ-conotoxins. However, the major product that was believed to be the native conformer exhibited a 1–5/2–4/3–6 cysteine connectivity that has not been previously described for μ-conotoxins. Interestingly, both peptides were found to be bioactive against $Na_v1.2$ with K_D of 230 and 5 nM for the minor and major products, respectively.

In a natural setting, conotoxins usually adapt a globular formation after traveling through the venom duct (Dutton et al. 2002). Cystine formation is catalyzed in the endoplasmic reticulum (ER) lumen by protein disulfide isomerase (PDI), which can exist as the β subunit of prolyl hydroxylase or as a separate homodimer (Noiva and Lennarz 1992). Notably, PDI is able to donate disulfide bonds to peptides or catalyze reduction and isomerization of disulfide bridges in its reduced or oxidized state, respectively (Wang et al. 2007). As such, identification of the proper disulfide bond connectivity of the native toxin becomes imperative, especially for conopeptide bioengineering in which synthetic peptides are folded by selective disulfide bond formation.

Assignment of disulfide interlinkage can be done through selected disulfide reduction employing Tris(2-carboxyethyl) phosphine (TCEP). This allows for the production of a partially reduced 'captured' peptide (Gray 1993). TCEP reduction can be monitored in real-time through the gradual appearance of the partially reduced peptide mass target, as observed by ESI–MS (+2 Da/disulfide) (Bingham et al. 2005). To differentiate this single

reduced disulfide from others (i.e. fully reduced), an S-alkylation of the cysteine's free thiols is undertaken using a substituted or non-substituted maleimides, 4-vinylpriyindine or iodoacetamide, etc. The target alkylated/differentially alkylated material is then subjected to Edman degradation or MS sequencing techniques, such as collision-induced dissociation or Post-source decay MS (CID/PSD). Analysis of the consolidated data leads to the identification of cysteine moieties with matched individual modified thiols from which interconnectivity is deduced (Loughnan et al. 2009; Wu et al. 2010; Bingham et al. 2005). This strategy was utilized by Wu et al. (2010) in the characterization of Vt3.1, a conopeptide dimer with intermolecular disulfide crosslinks (Table 3). Furthermore, χ-conotoxin TxXIIIA from *C. textile* was initially identified as a homodimer by observing a mass shift between the oxidized and reduced peptide, resulting in the loss of $m/z = 2{,}785.7$ and the emergence of $m/z = 1{,}398.4$ with the reduction of the peptide (Quinton et al. 2009). Dimeric conotoxins are a recent discovery; as more are discovered these strategies will enable rapid inter- and intra-assignment of disulfide connectivity, a process that has in the past presented major hurdles. The abundance of disulfide bonds are represented equally throughout the genus.

Hydroxylation of conotoxins and conopeptides

The hydroxylation of ααs is a commonly observed PTM within conotoxins and is known to generally affect Proline or Valine residues as well as one currently documented instance of a Lysine modification. There are approximately 86 known naturally occurring conotoxins containing both *cis*- and *trans*-hydroxyprolines (Hyp/O) (Table 2) and 2 containing γ-hydroxy-D-valine (V*) (Pisarewicz et al. 2005; Table 4). Despite their abundance within *c*onopeptides and conotoxin families, the precise contributions of these hydroxylated ααs to biological activity and structure are not completely understood.

Hydroxylation of proline in conotoxins and conopeptides

The hydroxylation of Proline in conopeptides has been shown to have various effects among different gene families. *Cis*- and *trans*-hydroxylated Prolines in μ-conotoxin GIIIA (Table 5) were shown to increase the rate of inhibition of $Na_V1.4$ sodium channels anywhere from 2.5 to 4.5 times, while having little effect on oxidative folding (Lopez-Vera et al. 2008). In contrast, hydroxylation of α-conotoxin ImI and conotoxin GI displayed a lower activity when Proline was substituted with Hyp, despite increased stability in their overall structures (Lopez-Vera et al. 2008). The Hyp substitution in α-conotoxin ImI makes the conotoxin completely inactive against α7 nAChRs while substitution in α-conotoxin GI appears to have a greatly reduced affinity for its respective nAChRs (Lopez-Vera et al. 2008). A similar structural improvement was found in ω-conotoxin MVIIC, which experienced an approximate twofold yield increase in oxidative folding and retained biological activity (Lopez-Vera et al. 2008).

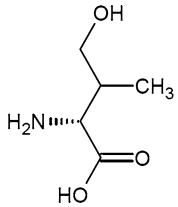
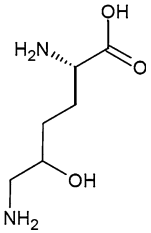
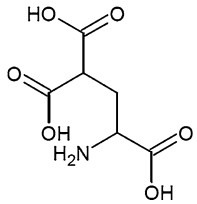
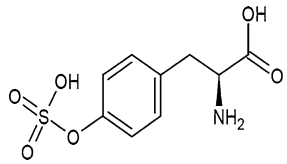
The appearance of Hyp in most forms of proteins and peptides is generally due to the presence of prolyl-4-hydrolase (EC 1.14.11.2; Gorres and Raines 2010). Presently, prolyl-4-hydrolase within the venom glands of the *Conus* genus has not been reported. It may also be possible that the enzyme responsible for the hydroxylation of Proline has a broader specificity that includes an ability to hydroxylate Lysine, based on previous research involving *Conus delessertii* (Aguilar et al. 2005). The production of partially or mixed PTM variants are a common occurrence within *Conus*, (Jones et al. 1996; Chun et al. 2012). This feature may represent a mechanism to increase chemical diversity and influence biological spectrum of conotoxins.

It has been shown that the folding of conopeptides containing more than one Hyp is assisted by a molecular chaperone enzyme known as peptidylprolyl *cis–trans* isomerase (EC 5.2.1.8) or PPI, which happens to be a subunit of prolyl-4-hydrolase, indicating that folding and hydroxylation may be a simultaneous or coupled process (Safavi-Hemami et al. 2010). Several isoforms of PPI have been isolated from *Conus novaehollandiae* including PPI B, an enzyme present in the snail's ER, and two cytosolic forms. PPI B is known for its ability to isomerize *trans*-hydroxyprolines to the *cis* conformation, which in turn may increase the stability of the peptide structure (Safavi-Hemami et al. 2010). Structural stability via isomerization was observed by NMR in vitro by comparing μ-conotoxin GIIIA (Table 5) that contained two *trans* Hyp, ω-conotoxin MVIIC that contains one Pro/Hyp residue and μ-conotoxin SIIIA that contained no Hyp residues (Safavi-Hemami et al. 2010). In this study, increased *Conus* PPIase caused a larger production of the native μ-conotoxin GIIIA while having little effect on the production of the non-hydroxyproline containing peptides. This provides evidence to the concept that the presence of Pro/Hyp gives rise to structural changes among conopeptides which can be facilitated by PPIases (Safavi-Hemami et al. 2010).

Hydroxylation of valine in conotoxins and conopeptides

The γ-hydroxylation and also epimerization of Valine are two key features of a novel structural motif (Ser-D-Hyv-Trp) found in two known vermivorous species of cone snails: *Conus gladiator* and *Conus mus* (Pisarewicz et al.

Table 4 Documented modifications and methods of detection of side chain derivatized amino acids in conotoxins/conopeptides

Modification	Structure	Mass shift	Edman degradation	Enzymes	Mass spectrometry	Comments
γ-Hydroxy-D-valine		Val → Hydroxyval (+16 Da)	Unhindered; use of standards allows for identification	None	ESI, MALDI	Can undertaken MS/MS or PSD analysis and assign sequence based on mass difference
5-Hydroxy-L-lysine		Lys → Hydroxylys (+16 Da)				Can detect via amino acid analysis if known to be present
γ-Carboxyglutamic acid		Glu → carboxyglu (+44 Da)	Blank cycle—may observe low levels of Glut	None	ESI, MALDI	γ-Carboxyglutamic acid-containing fragment also investigated by charge of −1 in paper electrophoresis Methylation of carboxylic acids Increase orifice potential during ESI
L-Sulfotyrosine		Tyr → Sulfotyr (+80 Da)	Unable to differentiate between 2 forms	Carboxy-peptidase Y (EC 3.4.16.1)	MALDI negative mode	Many mass spectrometry techniques used, but negative mode with lower collisional energies were optimal for observation. Amino acid analysis also used

Refer to conotoxins/conopeptides containing modifications in Tables 5 and 6 for references; If ionization mode is not specified, positive mode was used in mass spectrometry trials; Edman degradation: blank cycle, not enough residue collected at cycle to confirm amino acid

Table 5 Hydroxylation and carboxylation of select conotoxins/conopeptides

PTM		Toxin	Sequence	Receptor target of native toxin	Pharmacology PTM toxin	Pharmacology non-PTM toxin	Ref.
Hydroxylation, C-terminal modification	Hydroxyproline, Amidation	μ-GIIIA	RDCCTPPKKCKDRQCKPQRCCA* ↓ RDCCTOOKKCKDRQCKOQRCCA*	$Na_V1.4$	IC_{50}(nM) 870.3 (674–1,123)	IC_{50}(nM) 21.8 (18.4–25.7)	Lopez-Vera et al. (2008)
Hydroxylation, epimerization	Hydroxyvaline, epimerization	Conophan-gld-V	AOANSVWS	ND	ND	ND	Pisarewicz et al. (2005)
Hydroxylation, bromination	Hydroxylysine, hydroxyproline, 6-bromotyrptophan	De13a	DCPTSCPTTCANGWECCKGYPCVNKACSGCTH* ↓ DCOTSCOTTCANGWECCKGYOCVNKACSGCTH*	ND	ND	ND	Aguilar et al. (2005)
Carboxylation, C-terminal modification (non disulfide bonded)	γ-Carboxyglutamic acid, amidation	Conantokin-G	GEEELQENQELIREKSN* ↓ GEγγLQγNQγLIRγKSN*	NMDAR	IC_{50}(nM) 480	ND	Donevan and McCabe, (2000), Jimenez (2009), McIntosh et al. (1984)
Carboxylation, C-terminal modification	γ-Carboxyglutamic acid, amidation	Gla(1)-TxVI	GMWGECKDGLTTCLAPSECCSEDCEGSCTMW ↓ GMWGγCKDGLTTCLAOSγCCSγDCγGSCTMW	ND			Czerwiec et al. (2006)

* C-terminal amidation, *γ* gamma-carboxyglutamic acid, *O* hydroxyproline, *V* hydroxyvaline, *W* bromotryptophan, *K* hydroxylysine, *gThr* glycosylated threonine, Na_V voltage gated sodium channel, *NMDAR* N-methyl-D-aspartate receptor, *ND* not determined

2005). Conopeptides containing this motif are deemed hydroxyconophans and are known to contain γ-hydroxylated Valine (V*) (Conophan-gld-V; Table 5). Interestingly, this unique αα moiety necessitates epimerization for stability because it spontaneously cyclizes and forms a lactone as an L-αα. The present evidence indicates that the inhibition of lactone formation is dependent upon neighboring αα residues, which includes Tryptophan. It is believed that Tryptophan may influence the availability of the hydroxyl group on the γ-hydroxyvaline residue by sterically hindering lactone formation with its aromatic ring, thus structurally perturbing hydroxyvaline's conversion (Pisarewicz et al. 2005).

The D-form of hydroxyvaline found within *C. gladiator* appears to contribute to a linearization of the Conophan gld-V (Pisarewicz et al. 2005; Table 5) in which hyperhydroxylation of the residues is thought to increase hydrogen bonding and thus influence stability. A second unique characteristic of this hydroxyconotryphan is its short peptide length, consisting of only 8 residues (Pisarewicz et al. 2005). The placement of the D-γ-hydroxyvaline near the C-terminus of the peptide is atypical for epimerized conotoxins, which commonly exhibit epimers near the N-terminus. This suggests the presence of a unique enzyme for the modification of this class of peptides. This remains to be investigated.

Hydroxylation of lysine in conotoxins and conopeptides

A third hyrdoxylated αα, hydroxylysine (K) (Table 4), has also been found within conopeptides, specifically in De13a isolated from *C. delessertii* (Aguilar et al. 2005; Table 5). Despite the pervasiveness of hydroxyproline in other organisms such as mammals, it is considerably rarer in *Conus* venom. Consequently, its function in conopeptides remains to be elucidated. However, in other animal proteins, it is known to provide sites for glycosylation and sites for hydrogen bonding when considering receptor affinity and specificity (Aguilar et al. 2005). These functions could pertain to the hydroxylation of Lysine in the conopeptide De13a; however, supporting evidence has yet to be found (Aguilar et al. 2005).

Hydroxylation is observed in the following super families: O_1, O_2, O_3, I_2, Y, A, T, M, B_1, D, G, S, H, and also in the divergent families (Kaas et al. 2012; Fig. 1).

Isotopic identification and differentiation of hydroxyproline, leucine and isoleucine in conotoxins and conopeptides

The identification of isobaric αα Hydroxyproline, Leucine and Isoleucine by tandem MS has proven difficult due to their similar monoisotopic mass distributions (Grant 2002). Characterization of these residues may be improved through the use of w- or d-ion analysis (Soltwisch and Dreisewerd 2010) or by hydrolysis and αα analysis.

High-energy matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF–MS) can be utilized to generate side chain degradation of ααs, providing characteristic low abundant w- or d- ions. W- and d-ion analysis has been used on renin, fibrinopeptide A, and their Ileu/Leu substituted forms to accurately determine sequences containing isobaric αα (Soltwisch and Dreisewerd 2010). In contrast, researchers without access to a MALDI–TOF–MS may also perform primary identification through αα analysis. Langrock et al. (2006) were able to demonstrate the accurate determination of Leucine, Isoleucine, and Hydroxyproline isomers through the use of amino acid analysis and a N2-(5-fluoro-2,4-dinitrophenyl)-L-valine amide on collagen and the accuracy of their method was further confirmed through online ESI–MS.

Carboxylation of glutamic acid in conotoxins and conopeptides

The physiological importance of the γ-carboxyglutamic acid (Gla; γ) (Table 4) was underscored in 1974 when it was first identified as an essential component of the coagulation cascade. This PTM αα facilitates in vivo activation of prothrombin to thrombin by binding Ca^{2+} (Stenflo et al. 1974). Since its discovery, Gla's role has been described in other coagulation factors such as Factor IX (Aktimur et al. 2003) and in osseous tissue (Price et al. 1976).

γ-Carboxyglutamic acid was first discovered by studying the effects of dicoumarol (vitamin K antagonist) on prothrombin synthesis (Stenflo 1974). The resulting product was unable to bind Ca^{2+} and displayed no biological activity. N-terminal tetrapeptide fragments of normal prothrombin and dicoumarol-synthesized prothrombin (pH 6.5) showed that normal prothrombin had greater anodal migration (1.09) compared with its counterpart during electrophoresis. The difference in migration distances was originally attributed to an extra negative charge due to a prosthetic group. However, NMR and MS data indicated a methyl-ester substitution at the γ-carbon of each glutamatic acid residue, which corresponded to an addition of 58 Da per Glutamic acid. Further NMR analysis revealed a carboxyl group as the γ-carbon substituent (Stenflo et al. 1974).

γ-Carboxyglutamic acid containing non-disulfide bonded conotoxins and conopeptides

Initially thought to be unique to mammalian systems, the discovery of Gla residues in the sleeper peptide (Conantokin G; Table 5) of *C. geographus* provided evidence that

this PTM permeated other animal phyla (McIntosh et al. 1984). Carboxylation of Glutamic acid residues are catalyzed by γ-glutamyl carboxylase (E.C.4.1.1.90) in a vitamin K-dependent reaction with a requirement for carbon dioxide and molecular oxygen, localized in the lumen of the ER (Buczek et al. 2005a, b, c; Esmon et al. 1975; Wu et al. 1991). Substrates for carboxylation contain a propeptide region, known as the γ-carboxylation recognition sequence (CRS), that facilitates enzyme binding and increases substrate-enzyme affinity (Stanley et al. 1997; Bandyopadhyay et al. 1998).

Identification of γ-Carboxyglutamic acid in *C. geographus* Conantokin G proceeded in a similar fashion as prothrombin. Digestion of the peptide and paper electrophoresis revealed that the fragment believed to be Gln–Lys contained a net charge of −1. This observation ruled out Gln–Lys as a possibility, with a predicted net charge of +1. Furthermore, Pyr–Lys and Glu–Lys were also eliminated as prospects since these fragments would exhibit a neutral charge. It was concluded by McIntosh et al. (1984) that the extra negative net charge gained was due to an additional modification. Base hydrolysis of the peptide revealed a reciprocal relationship between the amount of Glu produced and Gla degraded. Determination of Conantokin G's primary structure relied on sequential enzymatic digestion and Edman degradation. In all, this discovery of Gla in *C. geographus* venom challenged traditional beliefs about γ-carboxylation, while reaffirming the efficacy of conventional biochemical methods of αα analysis.

γ-Carboxyglutamic acid containing disulfide bonded conotoxins and conopeptides

As a wider array of conotoxins were discovered, the previously reigning classical techniques were coupled with MS for ascertainment of uncommon/unusual amino acids such as Gla, *4-trans*-hydroxyproline and 6-L-bromotryptophan, based on their characteristic monoisotopic distributions as demonstrated in the discovery of Gla(1)-TxVI, Gla(2)-TxVI/A, Gla(2)-TxVI/B and Gla(3)-TxVI from *C. textile* (Czerwiec et al. 2006; Table 5). Data collected may be supplemented with the use of ESI–MS high orifice potential analysis, which induces the decarboxylation of the weaker γ-carbon in Gla, resulting in a characteristic loss of 44 Da (Nakamura et al. 1996). The advent of advanced MS techniques has aided in the identification of many other *Conus* peptides, most notably the contryphans, which contain numerous modified ααs in its primary sequence (Thakur and Balaram 2007).

The discovery of Gla residues in two non-related peptides hinted at a primitive purpose to K-dependent γ-carboxylation in the biological schema (Bandyopadhyay et al. 2002). Clarifying the role of vitamin K in the carboxylation process triggered the proliferation of enzymatic studies on γ-glutamyl carboxylase (Czerwiec et al. 2002, 2006; Bandyopadhyay et al. 2002). Subsequent investigations have identified this enzyme in other animal species including toadfish (*Opsanus tau*) and beluga whale (*Delphinapterus leucas*; Begley et al. 2000).

The role of Gla residues on peptide folding and stability has also been investigated. *Conus* peptides are ideal for this study because they contain multiple Gla residues within their short primary αα sequence. Modification of Gla residues can have a direct impact on folding and three-dimensional structure, which then influences the conopeptide's pharmacological properties. McIntosh et al. (1984) demonstrated that decarboxylation of Gla residues of Conantokin-G (known originally as Conotoxin-GV; Table 5) terminated its biological function (McIntosh et al. 1984; Jimenez 2009). Gla residues are proposed to bind Ca^{2+} which aids in the stabilization of the Conantokin-G α-helix (Jimenez 2009). Its ability to bind divalent ions may help direct peptide folding into the necessary three-dimensional conformation to retain bioactivity. Bulaj et al. (2003) demonstrated that chelation of Ca^{2+} by Gla residues aided native-like folding of the *spasmodic* peptide from *C. textile*, whereas it had no effect on a homologous, *Conus gloriamaris* (M) peptide lacking Gla. Furthermore, it was found that chelation of Ca^{2+} in solution yielded low quantities of native-like folded spasmodic peptide, again leading to the conclusion that Gla residues assist in peptide folding efficiency (Bulaj et al. 2003).

Conus species have widely incorporated Gla into their peptide venoms; the PTM is present in most of the pharmacological classes known. These peptides may be broken into two classifications: (a) Non-disulfide bond and (b) Mixed. The non-disulfide groups pertain to peptides containing Gla only and lack cystine bonds. These include Conantokins and Conorfamide Sr2 from the species: *Conus parius* (P; Teichert et al. 2007), *Conus tulipa* (P; Malmberg et al. 2003), and *Conus spurius* (V; Aguilar et al. 2008). Peptides in the mixed group contain both Gla and disulfide bonds; these include conopeptides from the species *Conus radiatus* (P; Klein et al. 2001) and Contryphans from *Conus marmoreus* (M; Hansson et al. 2004).

Sulfation of conotoxins and conopeptides

Tyrosine sulfation (Y) (Table 4) is a widespread PTM, occurring in common biomolecules such as gastrin (Huebner et al. 1991) and cholecystokinin (Durieux et al. 1983). Sulfation involves the addition of sulfate to ααs, introducing a side chain functional group that can undergo

selective deprotonation; this in turn impacts the pK_a of the parent peptide. Its presence has been documented in numerous species, as seen in humans (Shworak et al. 1999), zebra fish (Mohammed et al. 2012), and plants—*Arabidopsis thaliana* (Hashiguchi et al. 2013). Previous research points to Tyrosine sulfotransferases (E.C. 2.8.2.20) for production of numerous natural compounds with the reported purposes of bioactivation (Weinshilboum et al. 1997) and modulation of protein–protein interactions (Kehoe and Bertozzi 2000).

The interconnectivity of conotoxins and sulfation supports the theory of bioactivation due to sulfation, suggesting that sulfated conotoxins may also mimic physiological interactions for increased bioactivity. Unsulfated α-conotoxin EpI (Table 6) was observed to inhibit nAChRs in a similar fashion to sulfated α-conotoxin EpI. Yet sulfated α-conotoxin EpI was more effective at inhibiting the release of catecholamines (Loughnan et al. 1998). Similarly, α-conotoxin AnIB (Table 6) was found to have greater bioactivity than its unsulfated synthetic analog, with a ten-fold loss of activity in unsulfated α-conotoxin AnIB in targeting α7 nAChRs, but no change in activity when targeting α3β2 nAChRs (Loughnan et al. 2004). These pharmacological differences may become important when examining phylogenetic differences between ion channels and conotoxins reported selectivity amongst different phyla (Bingham et al. 2010).

A number of studies in the literature have focused on sulfation identification and analysis. An accepted method arose from these studies: observing negatively charged ions in soft ionization at lower collision energies during ESI–MS and MALDI–MS. Positive ionization mass spectrometry, a general practice in peptide analysis, causes sulfate groups to dissociate from tyrosine residues. Switching to negative ionization preserves a fraction of the native peptide mass including the PTM, while observation in negative ion mode at lower collision energies yield even higher proportions of native peptide mass (Önnerfjord et al. 2004; Wolfender et al. 1999). Use of negative-ion mode is thus essential in observing native peptide masses for these reasons.

Characterization of α-conotoxin PnIA and PnIB (Table 6) from *Conus pennaceus* represents some of the earlier analyses undertaken in sulfated compounds. Numerous MS techniques were compared for the observation of intact sulfated peptides. Wolfender et al. (1999) posited the possible presence of phosphorylation versus sulfation (both having a mass of 80 Da). Phosphorylation, which also occurs on Tyrosine residues, although not presently documented in *Conus*, has a tendency to be cleaved in positive-ion MALDI–MS analysis similar to sulfation; although M + H is still the dominant over M + H-80 mass peak.

The identities of the moieties in-question were differentiated by observing phosphorylated caerulin standard in reflectron-positive and -negative MALDI–MS modes. Caerulin was observed in both modes as a dominantly intact phosphorylated peptide, disparate from α-conotoxin PnIB and α-conotoxin PnIA spectras that exclusively yielded a peak with loss of 80 Da in positive mode (Wolfender et al. 1999). Wolfender et al. (1999) states that this observation was due to phosphorylated peptides having an increased stability in comparison to the labile sulfated peptides. Additionally, when RP–HPLC elution of unsulfated α-conotoxin PnIB, natural α-conotoxin PnIB, and *C. pennaceus* venom were compared, it was found that unsulfated analogs did not have a noticeable difference in retention time (Wolfender et al. 1999). This observation highlights the importance of alternative methods in suspected cases of peptide sulfation.

It is possible that assigned non-sulfated conotoxins may, in actuality, be sulfated. A large fraction of sequence assignment is accomplished through MS, RP–HPLC, and Edman degradation. More often than not, MS is done in positive mode, where no trace of sulfation would be found, even if sulfation were present. Confirmation by comparative RP–HPLC of native and synthetic peptides leaves further room for error as sulfation minimally changes RP–HPLC elution time, as in the case of α-conotoxin EpI where an elution time change of ~1 min was observed (Loughnan et al. 1998). Edman degradation is unhindered by sulfation, which may also be problematic as hindrance in degradation usually incurs further investigation into possible modifications at the given residue (Loughnan et al. 1998). Without Edman degradation hindrance, a noticeable change in elution time, or an observable mass shift, investigators would not suspect a PTM until the bioassay of synthetic and native toxin. Accordingly, a simple sequencing oversight may compound other errata. In this case, identifying sequence homology (even if consensus sequences are not concrete) would be the first option, followed by confirmation through methods fitting for the proposed modification (Fig. 2).

Bromination of conotoxins and conopeptides

Brominated compounds are commonly found in marine organisms due to the abundance of bromine in the marine environment (Steen and Mann 2002). Unsurprisingly, a number of brominated conopeptides have been observed, each of which are believed to produce a 6-bromo-tryptophan (w) (Table 7). Craig et al. (1997) were the first to document the occurrence of bromination of Tryptophan residues in *Conus* venom constituents. This analysis was achieved through various combined approaches utilizing

Table 6 Sulfation and bromination of select conotoxins/conopeptides

PTM	Toxin	Sequence	Receptor target of native toxin	Pharmacology PTM toxin	Pharmacology non-PTM toxin	Ref.
Sulfation, C-terminal modification						
Sulfotyrosine, Amidation	α-EpI	GCCSDPRCNMNNPDYC* ↓ GCCSDPRCNMNNPDYC*	α3β2 nAChR, α3β4 nAChR	pIC_{50} (M) 7.1 ± 0.12 (adrenaline release), 6.7 ± 0.006 (noradrenaline release)	pIC_{50} (M) 6.1 ± 0.1 (adrenaline release), 6.0 ± 0.08 (noradrenaline release)	Loughnan et al. (1998)
Sulfotyrosine, Amidation	α-AnIB	GGCCSHPACANNQDYC* ↓ GGCCSHPACANNQDYC*	α3β2 nAChR, α7 nAChR	IC_{50} (nM) 0.28 (α3β2), 76 (α7)	IC_{50} (nM) 0.64 (α3β2), 836 (α7)	Loughnan et al. (2004)
Sulfotyrosine, amidation	α-PnIA	GCCSLPPCAANNPDYC* ↓ GCCSLPPCAANNPDYC*	α3β2 nAChR α7 nAChR	Paralytic $Dose_{50}$ (pmol/100 mg body mass) 14.6	IC_{50} (nM) 14	Fainzilber, et al. (1994), Hogg et al. (1999)
Bromination						
Bromotryptophan	*C. imperialis* bromoheptapeptide	ZCGQAWC*	ND	No observed effects	No observed effects	Craig et al. (1997)
Bromotryptophan	*C. radiatus* Bromosleeper peptide	WATIDγCγγTCNVTFKTCCGOOGDWQCVγACPV	ND	40–50 pmol/g body weight affects mice of mixed ages	ND	
Bromotryptophan	Bromocontryphan	GCO*W*EPWC*	ND	Observed effects at 4–7 nmol/g body weight	ND	Craig et al. (1997)

* C-terminal amidation, γ gamma-carboxyglutamic acid, *O* hydroxyproline, W bromotryptophan, Y sulfotyrosine, *Z* pyroglutamic acid, *W* epimerization of tryptophan, *ND* not determined, *nAchR* nicotinic acetylcholine receptor

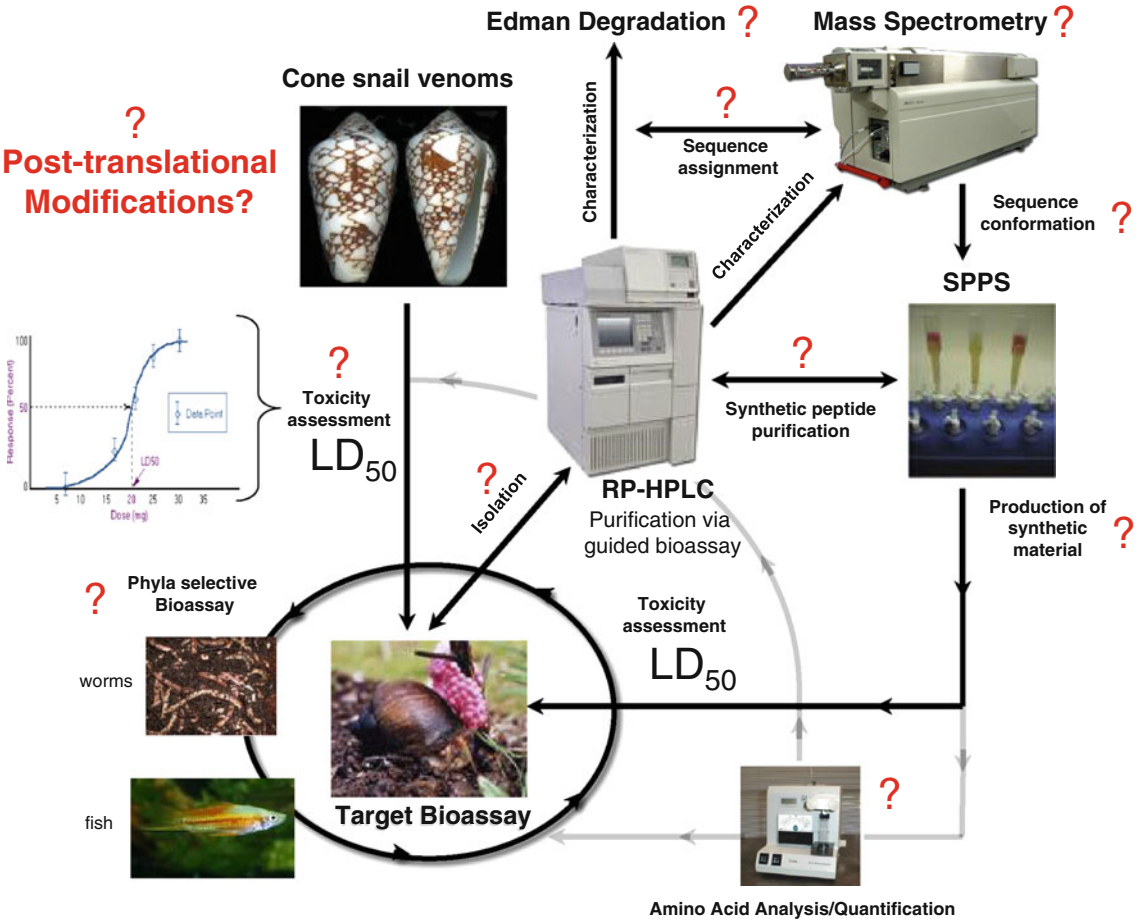


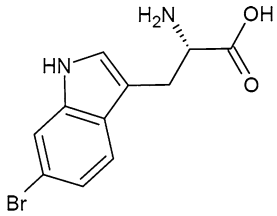
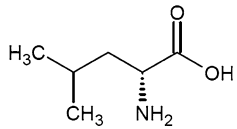
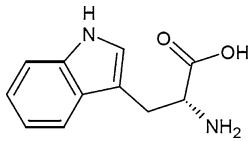
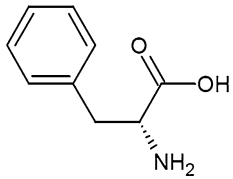
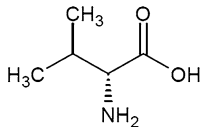
Fig. 2 The impact of post-translational modifications in the discovery, assignment and synthesis of conotoxins and conopeptides. The high relative abundance of PTMs within conotoxins/conopeptides has implications at each step of scientific investigation. Their present in de novo sequencing presents hurdles in their classification and assignment; their incorporation into synthetic approaches can be challenging, and; their interpretation regarding the impact on pharmacological targeting and phyla-selectivity is complex, and poorly understood. Careful and often combined biochemical, proteomic, and genomic approaches have been used in their identification, and to provide confirmation to their assignment

enzyme digestion, solid-phase peptide (SPPS), comparative RP–HPLC, and MS. These initial studies were focused on two peptides: bromoheptapeptide isolated from the vermivorous *Conus imperialis*, and bromosleeper peptide isolated from piscivorous *Conus radiatus* (Craig et al. 1997; Table 6).

Suspicion of a modification on the 5th residue of bromoheptapeptide arose after a 'blank' or non-identifiable cycle was observed during classical Edman degradation (Craig et al. 1997). Assignment of peptide's sequence through cDNA encoding resulted in the determination of a parent Tryptophan residue at this position, and thus commenced the search to define this novel PTM, as no modification for Tryptophan had been previously reported in *Conus* species.

It also became apparent that in the bromocontryphans (Table 6), the Tryptophan modification always produced two distinct mono-isotopic forms of the parent mass differing by 2 Da under standard MS conditions (Jimenez et al. 1997). Bromination of Tryptophan was postulated because of known prevalence of bromination in other marine compounds and the observed MS spectra behavior. This behavior is attributed to the relative abundance in which natural isotopic forms of bromine exist, as seen with the near equal distribution of Br^{79} (50.69 %) and Br^{81} (49.31 %) (Nair et al. 2006). The hypothesis was tested by the SPPS of bromoheptapeptide and a section of the bromosleeper peptide with a brominated Tryptophan incorporated at the 5th position (Craig et al. 1997). The constructed sections of bromoheptapeptide and the bromosleeper peptide were subjected to comparative RP–HPLC with the native bromoheptapetide and bromosleeper peptide fragments that were believed to have identical primary sequences. RP–HPLC co-elution was observed, along with the now characteristic staggered mono-isotopic distribution seen with bromine incorporation into both

Table 7 Documented modifications and methods of detection of L-6-bromotryptophan and D-amino acids in conotoxins/conopeptides

Modification	Structure	Mass shift	Edman degradation	Enzymes	Mass spectrometry	Comments
L-6-Bromotryptophan		Trp → Bromotrp +78/+ 80 Da (native isotopes)	Blank cycle	Digestion with α-chymotrypsin (EC 3.4.21.1), Carboxy-peptidase Y (EC 3.4.16.1) implied L conformation	ESI, MALDI	Addition of Br isotopes produces two isomers two Daltons apart. Contryphan has been observed without bromination prior to bromocontryhan
D-Leucine		L-Leu → D-Leu (0 Da)	Unable to differentiate between L- and D-forms	None	Non-indicative of L- or D-forms	Bioassay, co-elution of synthetic and native peptide to determine presence
D-Tryptophan		L-Trp → D-Trp (0 Da)	Unable to differentiate between L- and D-forms	None, possible to use α-chymotrypsin (EC 3.4.21.1) to differentiate	No observable mass change	Enzyme specificity used in some cases, in which digestion is inhibited due to D-forms
D-Phenylalanine		L-Phe → D-Phe (0 Da)	Unable to differentiate between L- and D-forms	Failure of α-chymotrypsin (EC 3.4.21.1) cleavage implied D-Phe		Unable to differentiate L- or D-forms by amino acid analysis
D-Valine		L-Val → D-Val (0 Da)	Unable to differentiate between L- and D-forms	Possible use of aminopeptidase to differentiate		

Refer to conotoxins/conopeptides containing modifications in Tables 6 and 8 for references; If ionization mode is not specified, positive mode was used in mass spectrometry trials; Edman degradation: blank cycle, not enough residue collected at cycle to confirm amino acid

native and synthetic peptide forms. The same comparative approach was then used to assign the bromination position within the Tryptophan side chain in the bromoheptapeptide, while enzymatic digestions were used to determine its chirality.

No mechanism for the bromination of Tryptophan in cone snails has been proposed. Yet selectivity has been examined with PTMs in the bromosleeper peptide; Trp^{1} has been found to undergo bromination, while Trp^{25} does not (Craig et al. 1997). The presence of a signaling site within the pre-pro-sequence has been suggested from the observed brominated conotoxins, but this remains to be identified (Craig et al. 1997; Jimenez et al. 1997).

The purpose and advantages of bromination are currently unknown. Craig et al. (1997) assessed the biological activities of brominated peptides in mice models. It was found that effects varied greatly; effects of the bromosleeper peptide were highly dependent on the age of injected mice. Nine-day-old mice slept for 10 h upon inter-cranial injection of 40–50 pmol/g body weight, while at the same dosage 17-day-old mice and 22-day-old mice became lethargic, but did not sleep. No activity in mice was observed from the polychaete-hunting *C. imperialis* bromoheptapeptide, possibly due to phyla selectivity (Craig et al. 1997). In studies of native contryphan as well as bromocontryphan, activity seems to be unaffected by the addition of bromine, leaving investigators with questions as to why this modification occurs in the natural world, given the energetic and enzymatic recruitment required (Craig et al. 1997).

Epimerization of conotoxins and conopeptides

Unlike other conopeptide PTMs, epimerization (Tables 7 and 8) of ααs from L- to D-configuration are particularly difficult to assign. The first difficulty arises in the use of classical Edman degradation, a methodology typically implemented early on in peptide characterization. 'Blank cycles' during Edman degradation are typically used as indications of PTMs, and an investigation of the modification ensues (see "Carboxylation of glutamic acid in conotoxins and conopeptides", "Bromination of conotoxins and conopeptides"). Investigating epimerization using this method is problematic as D-ααs do not hinder sequencing/chemical degradation process, nor do they produce a 'blank cycle'. Their degradation will typically produce an identifiable parent αα, in which chirality is not assigned but assumed to be L-configuration. In conopeptides this may represent an incorrect assignment, which may be carried through to SPPS.

Alternatively, sequence analysis via proteomic approaches, i.e. collision-induced desorption mass spectrometry (CID–MS) or post-source decay (PSD) PSD–MALDI–MS, etc. would not indicate αα epimerization due to their identical molecular masses and backbone fragmentation patterns (Buczek et al. 2005a, b, c). On a genomic level, translation of cDNA will be unable to inform the investigator of epimerization as a consequence of the physical disjoint in peptide assembly and sequestration (Buczek et al. 2005a, b, c). Thus, the investigators may be unaware of their presence until the native and synthetic materials are biologically assessed. Only then may αα epimerization be considered as a potential candidate. Here HPLC/UV co-injection experiments of native and synthetic materials in uneven ratios (2:1) provide certainty in their combined homogeneity.

Researchers in the past have searched for homologous sequences within the conotoxins with documented epimerized ααs for possible clues in predicting their position(s) (Buczek et al. 2008). Until the collection of D-αα-containing conopeptides is increased, establishing specific trends is highly subjective (Buczek et al. 2008).

In studies on the I_1 conotoxin superfamily (defined as peptides with the precursor sequences MKLCVTFLLVLM ILPSVTG or EKSSERTLSGALLRGVKRR), Buczek et al. (2005a, b, c) found that the third αα position from the C-terminus in precursor sequences were epimerized in multiple peptides. Curiously, homologous I_1 sequences R11d and Ar11a (Table 8), which were hypothesized to contain epimerized residues, were found to be comprised solely of L-αα's (Buczek et al. 2008). Han et al. (2008) mentioned that in addition to the -3 and -5 positions (C-terminus side), the +2 (N-terminus side) position appeared favorable for epimerization. Observed epimerized residues in these three positions are typically Leucine, Phenylalanine, Tryptophan, and Valine (Buczek et al. 2008; Han et al. 2008).

Peptides under high suspicion of containing D-ααs may be synthesized with the proposed sequence and subjected to comparative RP–HPLC analysis with the native peptide, as discussed above. Bioassays comparing the native to synthetic material also run the chance of exhibiting similar bioactivity, as seen with R11c and Ar11a (Table 8) (Buczek et al. 2005a, b, c, 2008). Therefore, the possibility arises that unknown epimerization may exist in well-established peptides, as the scheme of synthesis, comparative RP–HPLC, MS, and bioassay may leave epimerized residues undocumented. While αα hydrolysis, in an endeavor to resolve both D- and L-αα via chiral chromatography, has demonstrated merit in detection and assessment (Frank et al. 1977). The use of this specialized technique is poorly recorded in conotoxins. Alternatively, utilization of protease L-αα specificities has been demonstrated to be highly informative, as cleavage is terminated once a D-αα is encountered (Buczek et al. 2005a, b, c).

Finally, NMR can be utilized to analyze peptides containing D-ααs, as epimerization will be apparent in the

Table 8 Epimerization and glycosylation of select conotoxins/conopeptides

PTM		Toxin	Sequence	Receptor target of native toxin	Pharmacology PTM toxin	Pharmacology non-PTM toxin	Ref.
Hydroxylation, Epimerizaiton	D-Leucine, Hydroxyproline	R11c	GOSFCKADEKOCKYHADCCNCCLGGICKOSTSWIGCSTNVFLT ↓ GOSFCKADEKOCKYHADCCNCCLGGICKOSTSWIGCSTNVFLT	ND	Tail raising, convulsions at 20 pmol	Tail raising, convulsions at 100 pmol	Buczek, et al. (2008)
Epimerization?	None	Ar11a	RTCSRRGHRCIRDSQCCGGMCCQGNRCFVAIRRCFHLPF ↓ RTCSRRGHRCIRDSQCCGGMCCQGNRCFVAIRRCFHL̲PF	ND	110 pmol/g body weight caused sensitivity to touch and body tremor; at 20 μM, no action potential in nerve-muscle preparation		Buczek, et al. (2008)
N-terminal modification, glycosylation	Pyroglutamic acid, O-linked glycosylation	Contulakin-G	ZSEEGGSNATKKPYIL ↓ ZSEEGGSNA(gTr)KKPYIL	hNTR1 rNTR1 RNTR2 mNTR3	IC_{50}(nM) hNTR1: 23 rNTR1:79 RNTR2: 170 mNTR3: 171	IC_{50}(nM) hNTR1: 960 rNTR1: 524 RNTR2:730 mNTR3: 250	Craig et al. (1999a, b)
N-terminal modification, C-terminal modification, glycosylation, hydroxylation	Pyroglutamic acid, $Hex_3HexNAc_2$, hydroxyproline, amidation	κA-SIVA	ZKSLVPSVTTTCCGYDOGTMCOOCRCTNSC* ↓ ZKSLVP(gSer)VTTTCCGYDOGTMCOOCRCTNSC*		IP injection in fish (>50 pmol/g) and IC (>400 pmol/g) in mice caused death, voltage-clamp attenuations at 3 μM	No data published, authors mention lower potency in comparison to glycosylated peptide	Craig et al. (1998)
Carboxylation, hydroxylation, bromination, glycosylation	γ-Carboxyglutamic acid, hydroxyproline, 6-bromotyrptophan, GalNAc-Gal	ε-TxIX	ECCEDGWCCTAAP ↓ γCCγDGW̲CC(gThr)AAO	ND	0.20 ± 0.029 nmol	ND	Rigby et al. (1999), Walker et al. (1999)

Z pyroglutamic acid, * C-terminal amidation, *gTr* glycosylated tryptophan, *gSer* gycosylated serine, *O* hydroxyproline, *V̲* hydroxyvaline, *W̲* bromotryptophan, *L̲* epimerization of leucine, *ND* not determined, *NTR* neurotrophin receptor

structure of the peptide. Arguably the most universal method to determine stereospecific modifications, NMR has been used in multiple instances for characterization of conopeptides carrying D-ααs (Han et al. 2008, 2009).

It has been suggested that epimerization in conotoxins may serve multiple purposes. Analysis has shown that epimerization increases structural stability (Pisarewicz et al. 2005) and conveys resistance to protease activity (Craig et al. 1997). It is suspected that this follows the similar protective abilities of N-terminal cyclization, as seen with pryoglutamic acid ("N-terminal: pyroglutamic acid of conotoxins and conopeptides") and C-terminal amidation ("C-terminal: C-terminal amidation of conotoxins and conopeptides"). Epimerization also increases the chemical and structural diversity of conotoxins from a single gene (Hansson et al. 2004) and could contribute to the high specificity of conotoxins toward their native receptor targets (Pisarewicz et al. 2005). Pisarewicz et al. (2005) also state that D-isomerization reduces the probability of lactonization by hydroxylated residues, which can cause undesirable cleavage of peptides.

The mechanism behind the genesis of epimerized residues has not yet been identified, but some insight may come from the historical analysis of spider ω-agatoxin IVB and IVC. Slow isomerization of a serine residue by peptide isomerase in agatoxin was observed, with the proposed mechanism including two bases in a deprotonation and reprotonation chain of events (Kreil 1997). Future work promises insights into these stealthy modification and leads to more reliable methods of prediction.

Glycosylation of conotoxins and conopeptides

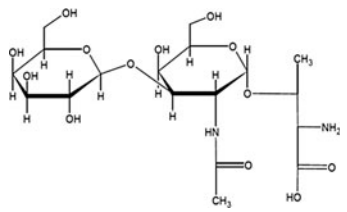
Among PTMs, none rival the diversity and complexity of glycosylation. More than ten different carbohydrate moieties have been observed in conopeptides, all of which have been conjugated to Threonine (T) (N-linked) or Serine (S) (O-linked) residues (Walker et al. 1999, Craig et al. 1998, respectively; Table 9). Despite the number of incidences in conotoxins, only a few have been completely characterized (Gerwig et al. 2013). In most cases the number of hexoses and associated groups has been determined through tandem MS, but the exact configuration and connectivity are still to be assigned; this presents the largest difficulty in analysis of glycosylated conopeptides.

Methods of enzymology have been implemented to elucidate details (including connectivity to the conopeptide being studied) of the glycan moiety, but NMR is almost always utilized for positive identification. One of the few completely characterized glycosylated moieties associated with a conopeptide not initially characterized by NMR was found linked to Contulakin-G (Table 8). Additional studies have been focused on this peptide documenting bioactivity and possible mechanisms of enzymatic conjugation. For these reasons, Contulakin-G will serve as the model for discussion here. A more comprehensive review has recently been published discussing glycosylation (Gerwig et al. 2013), with recent published work including an in-depth NMR analysis of the most complex conopeptide glycan moiety observed thus far, linked to the *Conus consors* conopeptide CcTx (Hocking et al. 2013).

Named for the sluggish behavior observed in mice after intercranial injection, Contulakin-G was the first conotoxin in which both the toxin and the glycosylated moiety were characterized (Craig et al. 1999a, b). Glycosylation was originally suspected after the observation of a blank cycle (with slight traces of threonine) in Edman degradation. The presence of a codon for Threonine at position 10 in cDNA encoding the peptide in combination with MS data indicated large hexose-sized masses bound to the αα. Multiple Contulakin-G species with differences between glycan moieties were also observed, including one with a hypothesized sulfated glycan moiety. The main species, which was believed to carry an *N*-acetylated disaccharide, was chosen for further characterization.

Utilizing β-galactosidase (EC 3.2.1.23), cleavage of native Contulakin-G disaccharide moiety was observed,

Table 9 Documented modifications and methods of detection of glycosylation in conotoxins/conopeptides

Modification	Structure	Mass shift	Edman degradation	Enzymes	Mass spectrometry	Comments
Contulakin-G's β-D-Gal*p*-(1 → 3)α-D-Gal*p*NAc-(1 → O)-L-Threonine		Thr → glycosylated Thr (+364 Da—varies)	Blank cycle	β-Galactosidase (EC 3.2.1.23) and further digestion by O-glycosidase (EC 3.2.1.97)	MALDI	Digestions combined with MALDI analysis of cleavages determined the disaccharide moiety

Refer to conotoxins/conopeptides containing modifications in Table 1 for references; If ionization mode is not specified, positive mode was used in mass spectrometry trials; Edman degradation: blank cycle, not enough residue collected at cycle to confirm amino acid

implying that the terminal hexose was β-galactose. O-glycosidase (EC 3.2.1) was applied and another cleavage from the same Threonine residue was observed, which supported the hypothesis of the glycan moiety being O-linked due to enzyme specificity. Post-digest Contulakin-G was analyzed on MALDI–MS, with masses observed consistent with an unmodified Threonine. Confirmation of structure was carried out by synthesis of Contulakin-G with the hypothesized structure. Native and synthetic Contulakin-G coeluted on RP–HPLC, with similar MALDI–MS spectra observed for each compound.

Glycan moieties found in Contulakin-G and other conopeptides have been hypothesized to increase peptide stability, in vivo transportation, and/or increase the selectivity of toxin (Craig et al. 1999a, b). These hypotheses were based on the following observations: (a) glycosylation inhibited proteolytic cleavage and (b) bioassays studying Contulakin-G activity (membranous and soluble) in relation to rat neurotensin type receptors 1 and 2 demonstrated a disparity between effects of glycosylated and non-glycosylated Contulakin-G in vitro and in vivo. In vitro, glycosylation did not show increases in binding affinity, while in vivo studies showed an increase in potency from non-glycosylated to glycosylated Contulakin-G (Craig et al. 1999a, b).

Mechanisms for in vivo production of glycosylated conopeptides have been primarily hypothetical. Two possible modes of glycosylation (enzyme and epithelial cells of the venom duct) are theorized, but neither has been fully identified. General motifs for predicting O-linked glycosylation in conopeptides have been proposed including specific positions for acidic and basic residues, but consensus sequences have been as elusive as the mode of production (Craig et al. 2001). In an effort to elucidate possible mechanisms involved, Craig et al. (2001) implemented mammalian UDP-*N*-acetyl-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase (EC 2.4.1.41). Glycosylation of unglycosylated Contulakin-G and extended Contulakin-G precursor was successful, leaving the possibility open for a yet unknown *Conus* enzyme to utilize a similar mechanism. In addition, scintillation counting of [^{3}H]-GalNAc-contulakin-G and [^{3}H]-GalNAc-pre-Contulakin-G implied pre-Contulakin-G was glycosylated at a higher percent than mature Contulakin-G (25–20 %, respectively) (Craig et al. 2001).

In addition to Contulakin-G, κ-conotoxin SIVA from *C. striatus* (a voltage-gated K^+ channel inhibitor; Table 6) has been characterized without the specific structure of the glycan (Craig et al. 1998). ε-Conotoxin TxIX (Table 8), a *C. textile* isolate peptide hypothesized to target presynaptic Ca^{2+} channels (Rigby et al. 1999), was also found to contain an O-linked glycan moiety. This was later determined to be α-D-Gal-(1 → 3)-α-D-GalNAc, which is similar to the Contulakin-G glycan moiety previously discussed, but dissimilar in the α-configuration of a galactose (Kang et al. 2004). The targets of these conopeptides highlight the great range of bioactivities encountered when studying glycosylation in *Conus* venoms. In addition, κ-conotoxin SIVA studies again showed higher in vivo effects of the native glycosylated peptide versus non-glycosylated peptide, demonstrating that the greater in vivo activity of native Contulakin-G was not an isolated case (Craig et al. 1998).

Non-native amino acids: bioengineering of conotoxins and conopeptides

The diversity of conopeptides and their characteristic receptor specificity make them prime targets for biological engineering (Table 10). The gradual transformation from natural product to drug candidate entails the chemical manipulation of the various levels of peptide structure with the aim of enhancing the desired properties of these peptides (Craik and Adams 2007). As outlined by Bingham et al. (2012), conopeptide bioengineering can be divided into six generations based on the extent of chemical manipulation. The simplest of these strategies involves αα substitutions, where αα moieties are exchanged for other ααs based on side-chain charge, size, and hydrophobicity. Luo et al. (1999) established that a single αα substitution of Alanine for Leucine on position 10 of α-conotoxin PnIA can alter its receptor selectivity from α3β2 to α7 nAChRs (Luo et al. 1999; Table 10). Later studies demonstrated that [A10L] α-conotoxin PnIA exhibited the greatest potency and selectivity for α7 nAChRs, showing the impact a single αα substitution can have on receptor-ligand interactions (Hogg et al. 1999). This finding was later utilized to design probes with affinity for α7 receptors and acetylcholine-binding proteins of *Aphysia californica* and *Lymnea stagnalis* (Kasheverov et al. 2011).

In some instances, PTM ααs are strategically incorporated in SPPS so that the synthetic variants can be compared with the PTM native counterparts. This technique was utilized in the elucidation of α-conotoxin Vc1.1's analgesic properties versus native α-conotoxin Vc1a with regard to their action on α9α10 nAChRs (Nevin et al. 2007; Table 10). Furthermore, in the delineation of specific receptor–ligand interactions and construction of probes for target receptors, substitutions in a peptide's primary structure are not limited to native ααs. Armishaw et al. (2010) appropriated this concept in the creation of synthetic combinatorial libraries of α-conotoxin ImI, in which non-native ααs such as 4-aminophenylalanine (Aph) and α-aminobutyric (Abu) acid supplant various ααs in the peptide's primary sequence (Table 10). It was found that [Leu^9–Aph^{10}–Abu^{11}]-α-conotoxin ImI (Peptide 56;

Table 10 Select bioengineered conotoxins/conopeptides

		Toxin	Sequence	Receptor target of native toxin	Pharmacology modified toxin	Pharmacology native toxin	Ref.
Bioengineered	Amino Acid Substitution	α-PnIA	GCCSLPPCAANNPDYC* ↓ GCCSLPPCALNNPDYC* [A10L]PnIA	α3β2 nAChRs	IC_{50} (nM): 99.3 ± 0.57 for α3β2 nAChR; 12.6 ± 1.2 for α7 nAChR	9.56 ± 1.0 for α3β2 nAChR; 252 ± 1.2 for α7 nAChR	Luo et al. 1999
	Substitution of PTM Amino Acids	α-Vc1.1 (Synthetic)	GCCSDPRCNYDHPEIC* ↓ GCCSDORCNYDHPγIC* Synthetic Vc1a	α9α10 nAChRs	Vc1a IC_{50} 62.9 ± 5.2 nM	Vc1.1 IC_{50} 64.2 ± 15.0 nM	Nevin et al. 2007
	Non-Native Amino Acid Substitution	α-ImI	GCCSDPRCAWRC* ↓ GCCSDPRCLBJC* B = Aph J = Abu	α7 nAChR at α7-GH3 cell line	IC_{50} 0.19 μM	IC_{50} 2.92 μM	Armishaw et al. 2010
	Selenocysteine incorporation	α-AuIB	GCCSYPPCFATNPDC* ↓ GUCSYPPUFATNPDC* Sec[1,3]-AuIB	α3β4 nAChR	IC_{50} 260 ± 20 nM	IC_{50} 3100 ± 1002 nM	Muttenthaler et al. 2010
	Cystathione Bridges	α-ImI	GCCSDPRCAWRC* ↓ GXCSDPRXAWRC* X = L-allylglycine (Agl)	nAChR of Bovine Adrenal Chromaffin Cells	IC_{50} of Noradrenaline Release (μM): Cis Isomer 17 ± 12; Trans Isomer 20 ± 15	0.87 ± 0.04	MacRaild et al. 2009
	Peptide Prosthesis	μ-KIIIA	CCNCSSKWCRDHSRCC* ↓ CNC(Aopn)KWCRDHSRCC* [desC1]KIIIA[S3/4Aopn,C9A]	$Na_v1.2$	K_d 46 nM	K_d 0.004 ± 0.004 μM	Han et al. 2009

* C-terminal amidation, γ gamma-carboxyglutamic acid, *O* hydroxyproline, W bromotryptophan, Y sulfotyrosine, *Aph* 4-aminophenylalanine, *Abu* α-aminobutyric acid, *U* selenocysteine, *Agl* L-allylglycine, *Aopn* 5-amino-3-oxapentanoic acid

Table 10) exhibited 14-fold potency than native α-conotoxin ImI with IC_{50} of 0.19 and 2.9 μM, respectively, toward α7-GH3 cell lines (Armishaw et al. 2010). The amalgamation of native and non-native ααs coupled with a combinatorial library design can give rise to peptides not ordinarily found in nature, allowing for diversity of potential pharmaceutical leads.

Ideally, drug candidates must be stable within the physiological environment of the body. However, the peptidic nature of conotoxins makes them prone to degradation (Craik and Adams 2007). Through peptide truncation experiments, it was established that the disruption of secondary structures, notably the α-helix of α-conotoxin PnIA resulted in the loss of bioactivity and increased susceptibility to disulfide bond scrambling (Jin et al. 2008). Given the contingency of pharmacological properties with peptide structure, efforts have been made to replace Cystine bridges with more stable bonds. Walter and Du Vigneaud (1966) successfully incorporated Selenocysteine in the synthesis of a potent oxytocin isolog. This strategy was adopted in the SPPS of various conotoxins because Selenocysteine bonds have a higher redox potential than disulfide bridges allowing for greater bond stability in reductive conditions as evidenced by the resistance of Selenoconotoxins Sec[1,3]-α-conotoxin AuIB and Sec[2,4]-α-conotoxin AuIB towards disulfide scrambling by glutathione and rat plasma (Muttenthaler et al. 2010; Table 10). Selenocysteine is an isosteric substitute for Cysteine with greater nucleophilicity, translating to a preferential formation of diselenide over disulfide bonds in acidic conditions, a beneficial attribute in directed bond-formation strategies (de Araujo et al. 2011). Studies on [Sec^3,Sec^{11},Nle^7]-endothelin indicate that it shared the same folding propensity, comparable structural conformation, and bioactivity as endothelin-1 (Pegoraro et al. 1998). Similar results were observed for the Selenocysteine-analogs of α-conotoxin ImI (Armishaw et al. 2006; Table 10).

Alternatively, allylglycine may also be used as a Cysteine substitute, creating an unsaturated, non-reducible dicarba linkage after joining of two allyl groups (MacRaild et al. 2009). Like diselenide bridges, this strategy has been incorporated in the synthesis of bioactive peptides such as oxytocin (Oka et al. 1975) and has since been applied to conotoxins. McRaild et al. (2009) synthesized a dicarba-analog of α-conotoxin ImI and found 2 isomers representing a *cis* and *trans* dicarba configuration (Table 10). This difference in geometry accounted for the divergent activities of the two isomers against rat α7 nAChRs and

their loss of potency at inhibiting catecholamine release from bovine chromaffin cells as compared with the native peptide (MacRaild et al. 2009). Other disulfide replacements that hold promise include thioether and lactam bridges, which were demonstrated using α-conotoxin GI and SI, respectively. The thioether analog of α-GI was found to be less potent than its native counterpart. Interestingly, the lactam-bridged α-SI analogs exhibited various degrees of bioactivity ranging from complete loss, moderate loss, and augmentation of bioactivity depending on the Cys–Cys combination that the lactam bridge replaced (Bondebjerg et al. 2003; Hargittai et al. 2000).

As key αα residues to receptor-ligand interactions are identified, peptide backbone substitutions deviate from ααs to chemical moieties that act as spacers that maintain the spatial positions of key amino residues in their interaction with target receptors. This innovative peptide prosthesis approach has seen success with μ-conotoxin SIIIA and KIIIA using amino-3-oxapentanoic acid as a flexible peptide backbone substitute for various amino acids (Green et al. 2007; Han et al. 2009; Table 10). The creation of these peptide–polymer amalgamations marks a shift to minimalistic peptide-analog design while maintaining maximal pharmacological efficacy, the impetus for the creation of peptidomimetics, or more specifically 'conomimetics'.

Conclusions/future perspectives

Characterizing and identifying the array of PTMs comes part and parcel with conovenomic analysis. The inherent challenges in PTM identification necessitate a concerted approach between classical biochemical techniques and novel proteomic and genomic methodologies. It is only through these combined, complementary, deductive, and, and analytical procedures that researchers have been able to gain the necessary supporting evidence for confirmation of the occurrence and positioning of these PTMs. The complexity of peptides within these venoms makes it likely that other PTMs remain to be discovered. A vigilant and methodical approach is essential to PTM analysis.

The relatively small conopeptides provide a unique opportunity to develop novel techniques, confirm efficacy of methods, and help to understand PTM importance in biological function. Research expanding upon the fundamental 20 building blocks of biological form and function in *Conus* has not only yielded great insight but also leads investigators to acknowledge the fragmented understanding of this field.

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Conflict of interest The authors state that there is no conflict of interest.

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