Cell. Mol. Life Sci. 62 (2005) 3067–3079
1420-682X/05/243067-13
DOI 10.1007/s00018-005-5283-0

Cellular and Molecular Life Sciences

Review

Conotoxins and the posttranslational modification of secreted gene products

O. Buczek, G. Bulaj and B. M. Olivera*

Department of Biology, University of Utah, Salt Lake City, Utah 84112 (USA), Fax: +1 801 585 5010,
e-mail: olivera@biology.utah.edu

Received 24 June 2005; received after revision 13 August 2005; accepted 19 September 2005
Online First 28 November 2005

Abstract. The venoms of predatory cone snails (genus *Conus*) have yielded a complex library of about 50–100,000 bioactive peptides, each believed to have a specific physiological target (although peptides from different species may overlap in their target specificity). *Conus* has evolved the equivalent of a drug development strategy that combines the accelerated evolution of toxin sequences with an unprecedented degree of posttranslational modification. Some *Conus* venom peptide families are the most highly posttranslationally modified classes of gene products known. We review the variety and complexity of posttranslational modifications documented in *Conus* peptides so far, and explore the potential of *Conus* venom peptides as a model system for a more general understanding of which secreted gene products may have modified amino acids. Although the database of modified conotoxins is growing rapidly, there are far more questions raised than answers provided about possible mechanisms and functions of posttranslational modifications in *Conus*.

Key words. Posttranslational modification; conotoxin; D-amino acids; 6-bromotryptophan; O-glycosylation.

Introduction

The cone snails (*Conus*) are a large group (500–700 species) of predators that use venom as their major weapon for prey capture [1–3]. At least some cone snail species also use their venoms for defensive and competitive purposes [4]. The pharmacologically active components of these venoms are primarily small, conformationally constrained peptides ('conotoxins' or 'conopeptides') translated by ribosomes from the toxin-encoding genes expressed in the venom duct of that the snail [5, 6]. A majority of conotoxins characterized from venoms so far are 12–30 amino acids (AA) in length; however, these are initially translated as prepropeptide precursors, approximately 80–100 AA. The precursor organization is constant: a highly conserved signal sequence at the N-terminal end, an intervening propeptide region, with the mature toxin region, always in single copy, at the C-terminal end. The biologically active toxin is produced by proteolytic cleavage from the precursor, an essential posttranslational step in conopeptide maturation.

Conus venom peptides are ligands that affect the function of signaling molecules on the plasma membranes of cells; the majority of known targets are ion channels, primarily in neurons [5, 7]. *Conus* peptides are widely used tools in neuroscience, and have promising therapeutic applications. A number of reviews on various aspects of conotoxins have been published in the past year [7–10].

An ongoing theme in *Conus* peptide research has been the continuing discovery of new posttranslational modifications in these peptides [11]. As a group, *Conus* venom

* Corresponding author.

peptides are proving to be the most highly posttranslationally modified gene products known. Although the density of posttranslational modifications in *Conus* venom peptides is much higher than in typical gene products, most of these diverse conotoxin modifications are likely to have a widespread distribution in biological systems. Thus, *Conus* peptides are a potential model system for more general understanding of the posttranslational modification of secreted polypeptides. The posttranslational modifications defined in conotoxins are almost certainly going to be of broad biological significance, and it is far more facile to identify and define the more esoteric of these modifications in *Conus* peptides than it would be in most other families of modified gene products.

The high frequency of posttranslational modification of *Conus* peptides probably stems from the selective pressure on this group of predatory snails to evolve highly effective, fast-acting pharmacological agents [12, 13]; it is likely that sudden ecological changes are a major factor driving the selection for new conotoxins. The evolutionary history that gave rise to the high degree of posttranslational modification observed in conotoxins will be developed in the section that follows. The consequence is that both the frequency and diversity of these modifications is unprecedented.

Some posttranslational modifications in *Conus* peptides are found in a broad variety of secreted peptides (such as proteolytic processing, C-terminal amidation, disulfide bond formation). Others are unusual or rare in peptides, though well known in specialized secreted proteins – examples are hydroxylation of proline and O-glycosylation [11]. A number of unusual posttranslational modifications have been characterized in a more diverse set of *Conus* peptide gene products than in any other system. In one case (i.e., carboxylation of glutamate), a posttranslational modification was thought to be a highly specialized biochemical adaptation in the mammalian blood-clotting cascade (for a review see [14]). However, its discovery and characterization in *Conus* [15] led to the demonstration that it had arisen very early in the evolution of multicellular organisms (and therefore is expected to be much more widely distributed than has been shown so far). Another posttranslational modification, the bromination of tryptophan, was first discovered in *Conus* peptides [16–18] but was subsequently shown to occur in the mammalian central nervous system (CNS) [19].

As genomic and proteomic technologies advance, the need increases to predict when polypeptide products of genes are posttranslationally modified [20]. In many cases, unless the presence of a particular posttranslational modification is specifically assayed, it would easily be missed in a gene product that might require this modification for full biological activity. Although modern molecular biology has not reached a state where this has become a widespread concern (except for some commercial products of the biotechnology industry), it will be increasingly important to be able to predict when and where specific posttranslational modifications are likely to occur.

Some posttranslational modifications in *Conus* are exceedingly difficult to detect even by the most advanced analytical methodologies [21]. A dramatic case in point is the posttranslational epimerization of an L- to a D-amino acid; if a biologically active gene product required the D-amino acid for full function, present-day proteomic technology could not detect this, and it might be concluded that the gene product, for unknown reasons, cannot be expressed in an active form. The great diversity of posttranslationally modified *Conus* venom peptides provides the prospect for elucidating some general 'rules' for a number of posttranslational modifications, leading to more accurate prediction about when esoteric posttranslational modifications such as the bromination of tryptophan or the epimerization of an L- to D-amino acid may occur.

Conceptual biological framework: cone snails as specialists in neuropharmacology

The cone snails (*Conus*) are a large and successful group of marine predators that capture prey, defend against predators and interact with competitors primarily through their unusually complex and potent venoms. Three species (out of 500–700 species total) are shown in figure 1. In the venom ducts of cone snails, a large number of small peptide toxins can be expressed; the total repertoire of one species is probably ~100–200 different toxins. Because of the rapid divergence in toxin genes between species, virtually no molecular overlap between the toxins from the venoms of different species is observed.

Cone snails have little in the way of other offensive or defensive weaponry apart from their shells, venoms and a sophisticated venom-delivery system. The latter includes a harpoon-like hollow tooth used both as harpoon and hypodermic needle to inject venom [22, 23]. A framework for rationalizing the complexity of *Conus* venoms has been proposed [4]. The pharmacological strategy that cone snails evolved uses toxin combinations to achieve the speed and potency required for interactions with prey, predators and competitors [24]. In effect, cone snails use the equivalent of a combination drug therapy strategy.

The origin of these toxin combinations is best understood by analogy with the present-day pharmaceutical industry: cone snails use the equivalent of a powerful drug-discovery strategy that operates over evolutionary time scales. Pharmaceutical companies develop 'lead' candidates for further drug development by using a combinatorial library strategy [12, 13, 25]. Once the lead compound has been identified, it is further refined in its pharmacological

Figure 1. The shells of three species of cone snails are shown. These have been particularly significant in the discovery and characterization of posttranslational modifications of conotoxins. From left to right: the cloth-of-gold cone, *Conus textile*; the radial cone, *Conus radiatus*; and the geography cone, *Conus geographus*. The only modification that has been well characterized was an enzyme system from *Conus textile*; one of the most highly modified *Conus* peptides, tx5a, was also characterized from this species. Toxins from the radial cone were important in the discovery of brominated tryptophan residues, as well as D-amino acids. The geography cone, *Conus geographus*, was one of the first *Conus* species intensively studied, and modifications such as hydroxyproline and O-glycosylation were characterized from the first conotoxins purified from *Conus geographus* venom.

properties through sophisticated medicinal chemistry. In effect, cone snails carry out the equivalent of a combinatorial library strategy over evolutionary time by gene hypermutation focused on the mature toxin region of the conotoxin precursor (in contrast, the signal sequence and propeptide regions are relatively conserved). An apparent high rate of mutation occurs as species diverge from each other. By the time a new *Conus* species emerges, a very large number of different peptide sequences have presumably been tried out [26–31]. Thus, the accelerated evolution of mature conotoxins is equivalent to developing a lead compound through a combinatorial library strategy in a commercial drug company.

The equivalent of medicinal chemistry, the other aspect of drug development, has been achieved in the course of cone snail evolution by posttranslational modification. For the pharmacological ends of the cone snails, the chemistry that can be accessed is not restricted to the functional groups of the 20 standard amino acids: the increasingly sophisticated neuropharmacology required provided a biological impetus for recruiting more and more posttranslational modification systems to act on the toxins in *Conus* venoms [11]. Indeed, recent work to improve the permeability of neuroactive peptides has curious parallels with the *Conus* peptide system; similar types of posttranslational modifications (halogenation, glycosylation) have been shown to be effective [32, 33]. Thus, the diverse posttranslational modifications found in conotoxins are a consequence of selection for ever-more-potent and effective toxins, i. e., the same pharmaceutical imperative that leads a modern drug company to recruit a top-notch medicinal chemistry division.

Diversity of posttranslational modifications found in conotoxins

The posttranslational modifications that have been found in *Conus* peptides are shown in table 1, and the structures of modified amino acids are illustrated in figure 2. The distribution of the posttranslational modifications found in different groups of *Conus* peptides is highly nonuniform. While some groups of *Conus* peptides tend to have no posttranslational modifications at all (except for proteolytic processing and disulfide bond formation), others are heavily modified; examples of the latter are shown in table 2. Some of the smallest *Conus* peptides are the most highly modified. For example, Bromocontryphan-R (8 AA) has two modified tryptophan residues, one epimerized to a D-Trp, and the other brominated to 6-bromo-Trp; additionally, one out of two proline residues is hydroxylated [17]. In the tx5a peptide (13 AA), γ-carboxylation, bromination of tryptophan, O-glycosylation and hydroxylation of proline all occur [34, 35]. These peptides are posttranslationally modified to an unprecedented extent compared with other translation products of ribosomes. In many of the major well-known conotoxin families such as the α-, ω-, δ- and μ-conotoxins, the frequency of posttranslationally modified amino acids is far lower.

Although many intensively posttranslationally modified conotoxins are small, some larger conotoxins >20 amino acids in length also have multiple posttranslational modifications. The 34-amino acid 'light sleeper peptide' has multiple 6-bromotryptophan residues, as well as all four glutamate residues converted to γ-carboxyglutamate [36]. Another larger conotoxin, the 30-amino acid κA-conotoxin SIVA, undergoes five different modifications, including cyclization of N-terminal glutamine, O-glycosylation and hydroxylation of proline residues [37] (see table 2).

The unusually high density of posttranslational modification in *Conus* peptides has resulted in modifications that occur sequentially at a single locus. In one class of small peptides, D-hydroxyvaline was identified (for example, see table 1). The venom contained the isoforms of the same peptides with an unhydroxylated D-Val residue [38], suggesting that first an epimerase converted L- to D-Val, which was then the substrate for a hydroxylase with D-amino acid specificity. Even more widespread is the presence of an N-terminal pyroglutamate residue; this

Table 1. Posttranslational modifications of the *Conus* peptides.

Modification		Enzyme	Peptide example	Sequence	Ref.
Proteolytic processing of propeptide to mature peptide	cleavage of N-terminal prosequence	Tex31	δ-TxVIA	DDSKNGLENHFWKARDEMKNREASKLDKKEA CYAPGTFCGIKPGLCCSEFCLPGVCFGG	[66]
	cleavage of C-terminal 'tail'	carboxypeptidase	BtX	CRAEGTYCENDSQCCLNECCWGGCGHPCRHPG KRSKLQEFFRQR	[73]
Disulfide bridge formation/ oxidation of cysteines to cystine		*Conus* protein disulfide isomerase[48]	pro-GI	FPSERASDGRDDTAKDEGSDMEKLVEKKECCN PACGRHYSC*	[74]
Hydroxylation of:	proline to 4-*trans*-hydroxyproline	proline hydroxylase	μ-GIIIA	RDCCTOOKKCKDRQCKOQRCCA*	[75]
	D-valine to D-γ-hydroxyvaline	hydroxylase with D-amino acid specificity[a]	gld-V*	AOANS(D-Hyv)WS	[38]
	lysine to 5-hydroxylysine	lysyl hydroxylase	de13a	DCOTSCOTTCANGWECC(Hyk)GYOCVN(Hyk)* ACSGCTH	b
Amidation of C-terminus/ sequences: –XG, –XGR, –XGK, –XGRR etc. processed to –X-NH_2		protein amidating monooxygenase	α-MI	GRCCHPACGKNYSC*	[76]
Carboxylation of glutamic acid to γ-carboxyglutamate		γ-glutamyl carboxylase	Conatokin-G	GEγγLQVNQγLIRγKSN*	[15]
			Conantokin-L	GEγγVAKMAAγLARgDAVN*	[77]
Bromination of tryptophan to 6-bromotryptophan		bromo peroxidase	Bromocontryphan-R	GCD(D-W)EPWC*	[17]
			r7a/Light sleeper	WFGHγγCTYWLGPCγVDDTCCSASCγSKFCGLW	[36]
Epimerization of:	tryptophan to D-tryptophan	epimerase	Contryphan-R	GCO(D-W)EPWC*	[78]
	leucine to D-leucine		Leu-Contryphan-P	GCV(D-L)LPWC	[79]
			r11c	GOSFCKADEKOCKYHADCCNCCLGGICKOSTS WIGCSTNVFG(D-L)TT	c
	phenylalanine to D-phenylalanine		r11a	GOSFCKADEKOCEYHADCCNCCLSGICAOSTN WILPGCSTSSF(D-F)KI	[21]
	valine to D-valine		mus-V	SOANS(D-V)WS	[38]
Cyclization of N-terminal glutamine to pyroglutamate		glutaminyl cyclase	Bromoheptapeptide	ZCGQAWC*	[16]
Sulfation of tyrosine to sulfotyrosine		tyrosyl sulfotransferase	α-EpI	GCCSDPRCNMNNP(Y-SO_3H)C*	[80]
			α-AnIB	GGCCSHPACAANNQD(Y-SO_3H)C*	[81]
O-glycosylation		Polypeptide HexNAc transferase	κA-SIVA	ZKSLVPS$^+$VITTCCGYD(Hyp)GTMCOOCRCTNSC*	[37]

[a]Suggested by Pisarewicz et al. [38].
[b]Aguilar et al., in press.
[c]Buczek et al., in press.
The highlighted portions of the sequences are proteolytically cleaved during maturation of the toxin.
*, C-terminal amidation; O, 4-*trans*-hydroxyproline; D-Hyv, D-γ-hydroxyvaline; W, 6-bromotryptophan; Hyk, 5-hydroxylysine; γ, γ-carboxyglutamate; D-X, D-amino acid; Z, pyroglutamate; Y-SO_3H, sulfotyrosine; $^+$, O-glycosylation.

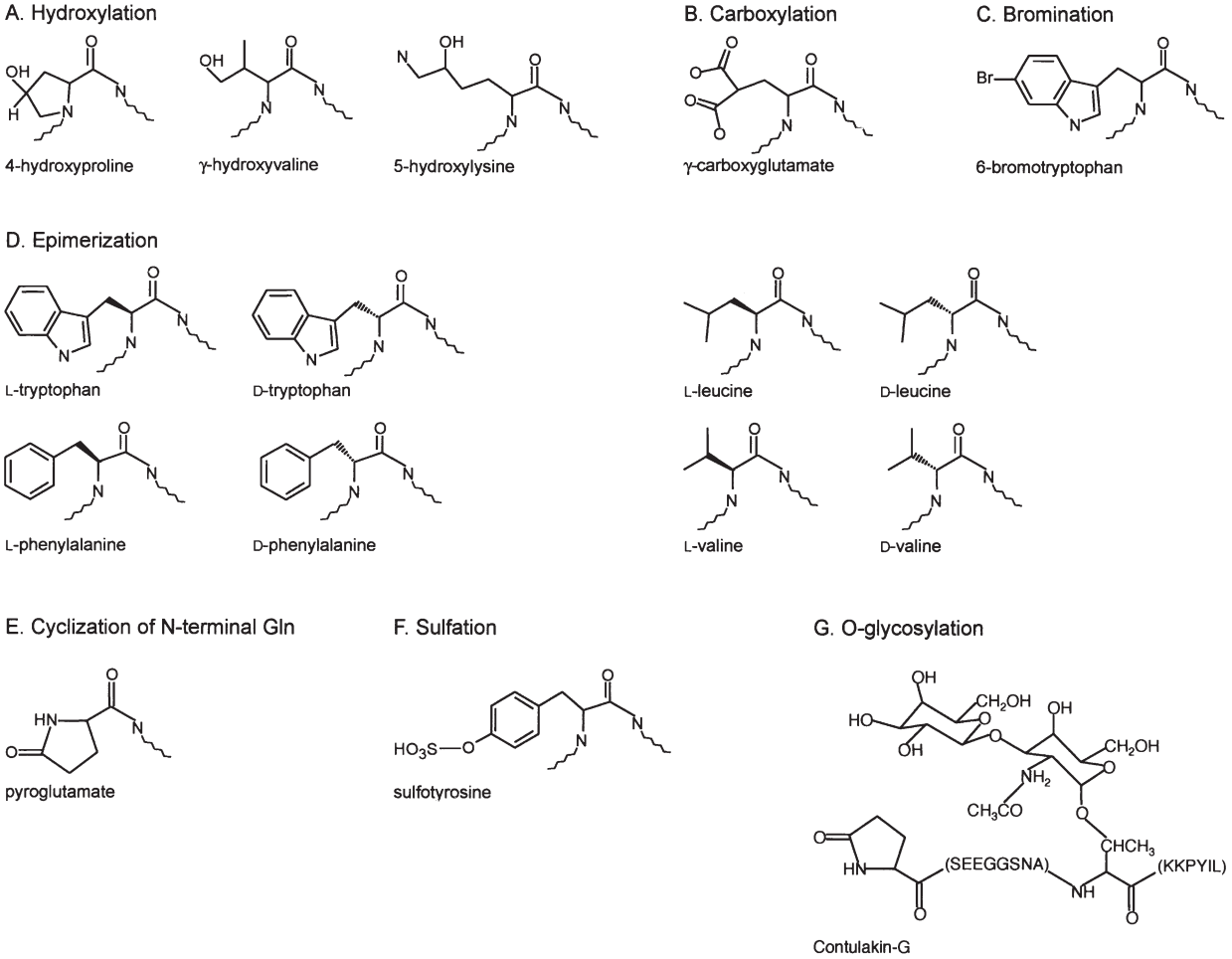

Figure 2. Review of the structures of posttranslationally modified amino acids documented in *Conus* peptides. For O-glycosylation, the sugars are shown in the context of the *Conus* peptide, Contulakin-G.

requires a proteolytic cleavage to generate an N-terminal Gln, which is the substrate for the glutaminyl cyclase.

Conus peptide posttranslational modifications: mechanisms and origins

A general mechanism of posttranslational modification of *Conus* peptides, as well as the potential evolutionary origins of modification enzyme systems has been suggested by the characterization of one specific *Conus* posttranslational modification enzyme, γ-glutamyl carboxylase. This enzyme catalyzes the carboxylation of specific glutamate residues to γ-carboxyglutamate (γ or Gla), which requires carbon dioxide, molecular oxygen and reduced vitamin K (see fig. 3A) [39–44]. This posttranslational modification was originally discovered and characterized in the mammalian blood clotting cascade. Certain blood clotting factors, such as prothrombin and Factor IX, must have γ-carboxylated glutamate residues for proper regulation of blood clotting [45, 46]. This posttranslational modification has enormous biomedical significance, since the predominant strategy for inhibiting blood coagulation is based on decreasing the proportion of γ-carboxylated glutamate residues in blood clotting factors.

The discovery of γ-carboxyglutamate in *Conus* peptides was completely unexpected – at the time, it was thought to be a specialized adaptation restricted to the mammalian blood clotting system [15]. This raised the question whether the two modification systems were related; perhaps these had evolved independently. However, the characterization of the γ-glutamyl carboxylase from *Conus*, and the analysis of the gene that encodes the enzyme provided an unusually definitive answer: the enzymes are not only homologs, but the *Conus* γ-glutamyl carboxylase gene has all introns found in the human gene at exactly the same loci [43].

Corresponding *Conus* and mammalian introns have diverged completely and exhibit no apparent homol-

Table 2. Variety of multiple posttranslational modifications within individual conotoxin.

Peptide	Sequence	Posttranslational modification	Ref.
tx5a	γCCγDGWCCT⁺AAO	carboxylation of glutamic acid bromination of tryptophan O-glycosylation hydroxylation of proline disulfide bridge formation	[34] [35]
Bromocontryphan-R	GCO(D-W)EPWC*	hydroxylation of proline epimerization of tryptophan bromination of tryptophan amidation of C-terminus disulfide bridge formation	[17]
r7a/Light sleeper	WFGHAγγCTYWLGPCγVDDTCCSASCγSKFCGLW	bromination of tryptophan carboxylation of glutamic acid disulfide bridge formation	[36]
κA-SIVA	ZKSLVPS⁺VITTCCGYDOGTMCOOCRCTNSC*	cyclization of N-terminal glutamine O-glycosylation hydroxylation of proline amidation of C-terminus disulfide bridge formation	[37]
r11e	ECKTNKMSCSLHγγCCRFRCCFHGKCQTSVFGCWVDP*	carboxylation of glutamic acid bromination of tryptophan amidation of C-terminus disulfide bridge formation	[82]

γ, γ-carboxyglutamate; W, 6-bromotryptophan; ⁺, O-glycosylation; O, 4-*trans*-hydroxyproline; D-X, D-amino acid; *, C-terminal amidation; Z, pyroglutamate.

ogy, yet their locations are rigidly conserved: an intron that is between codons in the mammalian gene is found between the homologous codons in *Conus*, while an intron found between the first and second nucleotides of a codon in the mammalian gene has a corresponding location in the homologous codon of the *Conus* gene. The remarkable conservation of intron locations leaves no doubt that these enzymes must have had a common origin. The *Drosophila* enzyme has also been cloned; although the *Drosophila* γ-glutamyl carboxylase gene has lost most introns, the few that remain, albeit smaller, are found at precisely the same loci as in the human and *Conus* genes [42]. Thus, this posttranslational modification, which is found in a wide variety of gene products within the *Conus* peptide system, is ancient, and must have its origins more than 550 million years ago, before the Cambrian Explosion when molluscs, arthropods and chordates diverged.

A second notable feature is that a recognition signal sequence is present in the propeptide region of those conotoxin precursors which are substrates for γ-carboxylation [40]. Recently, however, another location for the recognition signal for carboxylation was found in novel conotoxins from *C. textile* – namely, within C-terminal 'postpeptide' [47]. The general overview of *Conus* peptide modification that emerges from the specific work on the γ-carboxylation of glutamate is illustrated in figure 3B.

A potentially similar situation may exist for disulfide bridge formation, promoted by protein disulfide isomerase (PDI) [48]. It was shown that PDI-assisted disulfide bridge formation in a conotoxin was more efficient during early folding in the presence of a propeptide sequence, compared with the mature toxin alone [49]. Thus, posttranslational modification enzymes may be recruited to specific *Conus* peptide precursors through recognition signals. Once bound to a recognition signal sequence, the enzyme then modifies a specific amino acid in the mature toxin region.

Although most other *Conus* posttranslational modification enzymes have not been characterized at all, the elucidation of the mechanism of γ-carboxylation provides a paradigm for posttranslational modification events that occur on conotoxin precursors. Preexisting enzymes, some or all of which may be distributed over a wide variety of biological systems, have been recruited in *Conus* venom ducts to modify conopeptides through recognition signals. Thus, conopeptide precursors may gain or lose a posttranslational modification either because of mutations in the putative recognition signal region (likely in the propeptide region), or mutations in the putative amino acids to be modified (in the mature toxin region).

A variety of data suggest that high specificity may evolve for targeting an amino acid for posttranslational modification. In one *Conus* peptide family, the Conantokins,

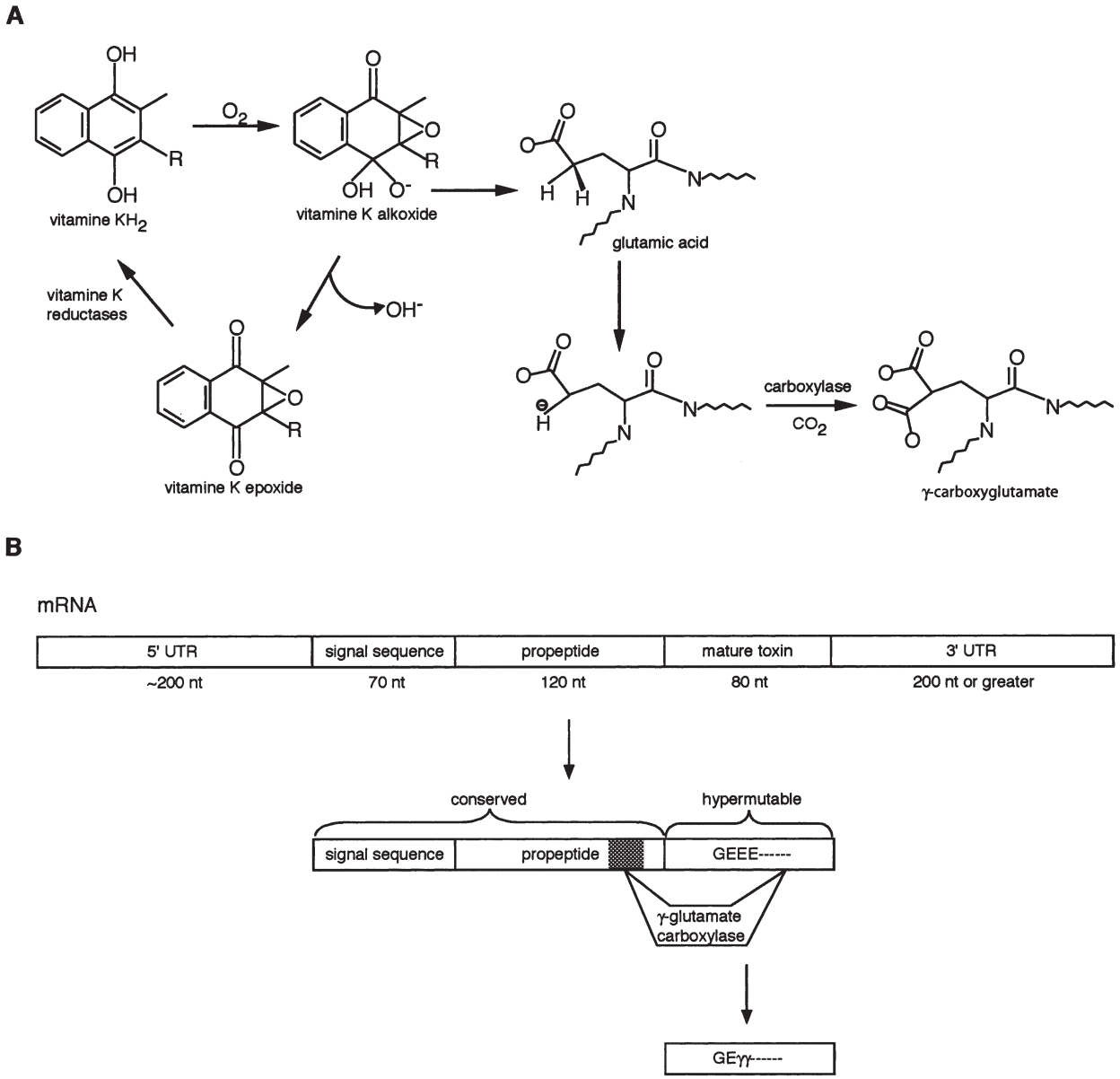

Figure 3. (*A*) Biosynthetic pathway for vitamin K-dependent synthesis of γ-carboxyglutamic acid. Vitamin K alkoxide subtracts the hydrogen from the γ-carbon of glutamic acid. Subsequently, carboxylase catalyses the formation of γ-carboxyglutamic acid by the addition of CO_2 to the γ-carbon of Glu. The activated vitamin K collapses into vitamin K epoxide, and is recycled to reduced vitamin K (KH_2). (*B*) A general overview of γ-carboxylation of glutamate in *Conus* peptides. The messenger RNA (mRNA) scheme shows the typical structure of a conotoxin precursor. The N-terminal signal sequence and propeptide are conserved parts of the molecule, while the mature toxin region is hypervariable. The *Conus* γ-carboxylase recognizes a sequence within the propeptide region of conotoxin precursors, in which the glutamic acid(s) is (are) modified to γ-carboxyglutamate(s).

there are typically four conserved amino acids at the N-terminus, which have the sequence GEEE--- before modification [40]. The first glutamate residue appears never to be modified, while the two more C-terminal Glu residues are always modified (to GEγγ--). As described above, in Bromocontryphan-R, one tryptophan residue is epimerized to a D-Trp, while the second tryptophan residue in the peptide is instead a substrate for post-translational bromination [17]. Recognition signals for two different modification enzymes may specify which Trp residue to modify; more complicated models are possible, and the intracellular location of the posttranslational modification enzymes may play a role. Thus, if one modification enzyme is localized such that it would act first, then in principle, the second enzyme acting on Trp could be less selective if it did not recognize the modified Trp residue produced by the prior-acting enzyme.

The selectivity of posttranslational modification has been shown to extend beyond *Conus* in one example. The enzymatic glycosylation of 16-amino-acid Contulakin-

G using mammalian ppGalNAc-transferase yielded a product with O-glycosylated Thr^{10}, but not Ser^{2} or Ser^{7} [50]. Interestingly, the presence of a propeptide sequence in pro-Contulakin-G slightly improved specificity for the Thr^{10} residue. Thus, the mammalian enzyme exhibits the selectivity expected for the endogenous *Conus* enzyme.

Functional aspects of posttranslational modification

Although the occurrence of diverse posttranslational modifications in *Conus* peptides is now well established, the functions of most of these remain undefined; insufficient structure/function work is available to provide mechanistic insight. However, a clear demonstration for the functional importance of one posttranslationally modified amino acid has been obtained [21]. The excitatory peptide, r11a, which belongs to the I-conotoxin superfamily, has a single D-amino acid, a Phe residue in position 44, three amino acids from the C-terminus. The functional assay used was the induction of repetitive action potentials in amphibian axons. The natural peptide (with D-Phe^{44}) is highly potent in eliciting the repetitive activity; in contrast, the L-Phe analog was not biologically active using this assay, even at 10-fold higher concentrations. Interestingly, a homologous peptide with a leucine residue at the position of D-Phe in r11a was recently characterized [O. Buczek et al., in press]. This peptide, r11c, also elicited repetitive action potentials when D-Leu^{42} was present. Surprisingly, when the r11c[L-Leu^{42}] analog was synthesized, it proved to be almost as active as the D-amino-acid-containing natural form. Thus, it appears that the D-amino-acid-containing natural peptides are highly potent, and for at least some amino acids, substituting the corresponding L-isomer results in a loss of biological activity. This system holds promise for understanding in molecular detail how the epimerization of an L- to a D-amino acid affects biological activity.

It has generally been assumed that posttranslational modifications improve the functional efficacy and/or specificity of the conopeptides in which they are found. An alternative role to be considered is that posttranslational modification may facilitate the correct folding of a *Conus* peptide, particularly if modification occurs early in the secretory process. Results suggesting this possibility were obtained in a study of venom peptides from two mollusc-hunting cone snails, *Conus textile* and *Conus gloriamaris*. It was found that the tx9a *(C. textile)* and gm9a (*C. gloriamaris*) peptides with three disulfide bonds were identical in sequence except at three loci: in the *C. textile* peptide, two of these loci had Gla residues, but in the *C. gloriamaris* peptide, these were substituted by standard amino acids [51]. This provided an opportunity to evaluate functional differences that might arise because of Gla residues. Surprisingly, no differences in function could be discerned [48]. Somewhat unexpectedly, however, clear differences were observed in the kinetics of peptide folding into the correct conformation. Under standard conditions (in the absence of calcium), the peptide without Gla was more efficiently folded. With increasing concentrations of Ca^{++}, the *C. textile* peptide, tx9a, which contained two Gla residues, was progressively folded more efficiently relative to the *C. gloriamaris* peptide. In this case, the presence of Gla residues in a *Conus* peptide did not affect the pharmacological activity of the peptide directly once it was folded correctly. Rather, Gla residues appear to function to facilitate proper folding within the endoplasmic reticulum (which has an elevated [Ca^{++}]). It was further hypothesized that γ-glutamyl carboxylase might be a relict from the precellular world when polypeptide chains were relatively short, serving as a mechanism to orient polypeptide chains for proper formation of disulfide bonds; Gla residues could chelate Ca^{++}, forming a transient crosslink. If this speculation was valid, it would suggest that in the *Conus* peptide system, the primordial function of γ-glutamyl carboxylase could have been recapitulated as these snails evolved ever smaller disulfide-rich peptides as pharmacological agents in their venoms.

Glycosylation of proteins and peptides increases their biological stability by protecting polypeptides from proteolytic degradation, as well as by stabilizing their tertiary structures [52]. Although glycosylation has been documented for a number of conotoxins, its biological function was only extensively studied in the case of Contulakin-G [53]. This 16-amino-acid peptide contains the Gal-GalNAc moiety attached to Thr^{10} (fig. 2, table 3). Nuclear magnetic resonance (NMR) studies indicated that the disaccharide moiety of Contulakin-G did not stabilize its three-dimensional conformation [54, 55]. However, the presence of glycosylation was shown not only to dramatically improve the peptide's stability against proteolytic degradation, but was also to be directly important for its pharmacological properties ([56]; A. G. Craig, unpublished results).

All known functions of posttranslational modifications in *Conus* peptides are summarized in table 4.

Predicting posttranslational modification: bioinformatics aspects

The ability to anticipate purely from gene sequences when a posttranslational modification may occur is one facet of bioinformatics addressed in this section. Many posttranslational modifications discussed in this review are too insufficiently characterized for their occurrence to be accurately predicted. However, we discuss the progress made so far, and problems that need to be addressed.

Table 3. Structural diversity and complexity of posttranslational modifications in *Conus* peptides.

A. Epimerization: diversity of the chemical nature of modified residue and its position in primary sequences

Peptide	Sequence	*Conus* species	Ref.
gld-V / no disulfides	AOANS(D-V)WS	*C. gladiator*	[38]
mus-V* / no disulfides	SOANS(D-Hyv)WS	*C. mus*	[38]
Contryphan-R / one disulfide	GCO(D-W)EPWC*	*C. radiatus*	[78]
Leu-Contryphan-P / one disulfide	GCV(D-L)LPWC	*C. purpurascens*	[79]
Contryphan-Vn / one disulfide	GNCP(D-W)KPWC*	*C. ventricosus*	[86]
r11a / four disulfides	GOSFCKADEKOCEYHADCCNCCLSGICAOSTNWILPGCSTSSF(D-F)KI	*C. radiatus*	[21]
r11c / four disulfides	GOSFCKADEKOCKYHADCCNCCLGGICKOSTSWIGCSTNVF(D-L)T	*C. radiatus*	a

B. O-glycosylation: diversity of conotoxin sequences containing glycosylated residue(s)

Peptide	Sequence	*Conus* species	Ref.
Contulakin-G / no disulfides	ZSEEGGSNAT^{+}KKPYIL	*C. geographus*	[53]
tx5a / two disulfides	γCCγDGWCCT^{+}AAO	*C. textile*	[34, 35]
κA-SIVA / three disulfides	ZKSLVPS^{+}VITTCCGYDOGTMCOOCRCTNSC*	*C. striatus*	[37]
κA-MIVA / three disulfides	AOγLVVT^{+}AT^{+}TNCCGYNOMTICOOCMCTYSCOOKRKO*	*C. magus*	[30]

[a] Buczek et al., in press.
*, C-terminal amidation; O, 4-*trans*-hydroxyproline; D-Hyv, D-γ-hydroxyvaline; W, 6-bromotryptophane; γ, γ-carboxyglutamate; D-X, D-amino acid; Z, pyroglutamate; $^{+}$, O-glycosylation.

One fundamental issue is whether the posttranslational modification of interest is carried out by a single enzyme system. Recognition elements that dictate whether or not a posttranslational modification will occur may diverge between enzyme systems, if several are involved. Among the posttranslational modifications for which multiple enzymes may be involved are proteolytic processing (discussed below), hydroxylation of amino acids, conversion of L- to D-amino acids and O-glycosylation; the complexity of sequence contexts in *Conus* peptides for the last two modifications is illustrated by the examples in table 3.

Some posttranslational modification enzymes may be invariant in their recognition mechanisms across phyla; however, other modification enzymes may evolve new specificity rapidly, with recognition signals potentially changing even between related species of *Conus*. An additional issue is the penetrance of recognition signals: for a particular locus where a posttranslational modification occurs, will the amino acid always be modified, or is modification conditional, depending on physiological factors? These issues are considered below.

Among the more predictable posttranslational modifications are disulfide bond formation and the processing events that generate the N- and C-termini of mature conotoxins. Disulfide bonding is the most predictable of posttranslational processing events; cysteine residues in conopeptides are even in number, and no case has yet been discovered in which Cys residues did not form disulfide crosslinks in any natural conotoxin. Which specific disulfide bonds are actually formed is part of the more general issue of how conotoxins are folded, a topic beyond the scope of this review [57, 58]. The formation of disulfide bonds is presumably catalyzed by protein disulfide isomerases; these are the major large polypeptidic constituents found in venom-producing cells [48, 59].

In general, the arrangement of cysteine residues in the primary sequence can be predicted by the gene superfamily the conotoxin belongs to, which is in turn predictive of the actual disulfide bonding framework. Recently, however, it has been established that the same arrangement of cysteine residues in the primary sequence of peptides in the same conotoxin gene family can lead to different disulfide frameworks [30, 60–63]. The shift from one disulfide framework to another is correlated with the number of amino acids between Cys residues. Thus, the A- and M-superfamilies have two alternative arrangements of cystine crosslinks [30].

The processing that generates the N- and C-terminal ends of the mature toxin region, including proteolytic processing to generate the N-terminus of the mature toxin and C-terminal amidation, also has high predictability. The primary sequence is a reliable guide to whether C-terminal amidation will occur; thus, a C-terminal sequence which ends with ---XG$(X_1)_n$, where X_1 is either a Lys or Arg residue and n is a number between 0–4, is reliably processed to ---X-NH_2 in both *Conus* and other biological systems. To date, only two *Conus* peptides have been purified and characterized that violate this rule: a small excitatory peptide belonging to the M-superfamily [64] and the K-channel inhibitor from *Conus virgo*, ViTx [65],

Table 4. Functions of posttranslational modifications in *Conus* peptides.

Modification	Function	Example	Ref
Proteolytic processing of precursor	maturation of bioactive toxin	proteolytic cleavage of the precursor is obligatory in the maturation of all *Conus* peptides.	[7]
Disulfide bridge formation	structure formation of bioactive toxin	cysteines occur as disulfides in all conopeptides; proper disulfide connectivity is essential for bioactivity	[7]
		the exception: non-native disulfide bond connectivity in α-AuIB increases bioactivity	[83]
Carboxylation of glutamic acid	structure stabilization	induction of α-helix formation in conantokins	[84]
		Conantokin-G self-associates by multiple interchain calcium coordination	[85]
		GEγγLQVNQγLIRγKSN*	
	improving oxidative folding in the presence of calcium	calcium increases folding efficiency of tx9a, but has no effect on gm9a folding	[48]
		tx9a GCNNSCQγ HSDCγ SHCICTFRGCGAVN*	
		gm9a SCNNSCQSHSDCASHCICTFRGCGAVN*	
Epimerization of phenylalanine	biological activity	r11a (D-F^{44}) induces repetitive action potentials in motor nerve, whereas r11a (L-F^{44}) has no such activity	[21]
		GOSFCKADEKOCEYHADCCNCCLSGICAOSTNWILPGCSTSSF(**D-F**)KI	
		r11b (D-F^{44}) induces repetitive action potentials in motor nerve, whereas r11b (L-F^{44}) has no such activity.	a
		GOSFCKANGKOCSYHADCCNCCLSGICKOSTNVILPGCSTSSF(**D-F**)RI	
Glycosylation of threonine	biological stability and activity	Contulakin-G; glycosylation protects the peptide from proteolytic degradation and improves its pharmacological properties	
		ZSEEGGSNAT^{+}KKPYILb	

[a]Buczek et al., in press.
[b]([56]; A. G Craig, unpublished result).
γ, γ-carboxyglutamate; *, C-terminal amidation; O, 4-trans-hydroxyproline; D-X, D-amino acid; $^{+}$, O-glycosylation.

were predicted to be processed to an amidated C-terminal end; the native peptides purified from venoms had the unprocessed C-terminal sequences instead.

Proteolytic processing of the precursor to the mature toxin is also largely successfully predicted. The analysis of the frequency of amino acids at positions from P6 to P1, based on 35 known conotoxin precursor sequences of different families, shows the following preferences: (i) arginine is the most frequent residue at P1, corresponding to 77%; (ii) the other basic amino acid, lysine, is found at P2 in 40% of sequences; the alternative to lysine being a hydrophobic amino acid, such as Val, Leu or Ile; (iii) at P4, leucine is preferred with a frequency of 40%. Thus, for *Conus* peptides, the signals ---LXKRX, ---LXXRX, ---XXKRX or ---XXXRX are predictive of proteolytic cleavage after the arginine residue. If any of these sequences are found before the first cysteine residue following the signal sequence, this is strongly predictive of a proteolytic cleavage site to generate an N-terminal residue in the mature toxin. Since mature sequences of *Conus* peptides are hypervariable, there is no amino acid specificity observed on the P' site of the substrate-conotoxin precursor. However, these consensus sequences, when found after the first cysteine residue do not direct a proteolytic cleavage. As disulfide bond formation occurs first, one possible explanation for this is that the compact folding of amino acids between the disulfide crosslinks protects the polypeptide chain from proteolysis.

Only one enzyme so far has been postulated to process conotoxin precursors, a protease Tex31, belonging to the CRISP family [66]. This protease was found in the extracellular venom fluid. Much of the protein content of venom collected from a dissected venom duct is in granules believed to contain unprocessed toxin precursors; however, the venom duct also contains fully processed peptides [67]. A number of synthetic peptide substrates were used to determine the specificity of Tex31 [66]. Apart from dibasic specificity at P1 and P2 positions, the protease preferred Leu at P4. As mentioned earlier, Leu was a frequent amino acid (40%) at this position in conotoxin precursor sequences. It is believed that Tex31 is not the only protease, and that more enzymes with different specificity are involved in the posttranslational processing of conotoxin precursors.

Signals for proteolytic cleavage are generally conserved across biological systems. Most enzymes responsible

for proteolytic processing of the precursors of secreted polypeptides belong to a family of dibasic- and monobasic-specific serine proteases called subtilisin/kexin-like proprotein convertases (PCs) [68]. The PCs have a preference for basic amino acids (lysine or arginine) at positions P1 and P4; these are also most favorable residues at P2 position [69]. Thus, a combination of CRISP-type and more conventional subtilisin/kexin-type proteases may be involved in conotoxin maturation.

Further posttranslational processing may occur at a nascent N-terminus if an N-terminal glutamine residue is generated; this is invariably cyclized to pyroglutamate. The substrate specificity of a human glutaminyl cyclase (hQC) was investigated by Schilling and co-workers [70]; QC is physiologically active in the pyroglutamate formation of most, if not all, N-terminal-Gln-containing hormones. However, the K_M value was extremely high for a peptide containing Pro at the position next to Gln. Of the conotoxins (13 sequences) with the proven presence of N-terminal pyroglutamate, there was no sequence that contained an adjacent proline reside. Thus, the *Conus* and human enzymes may have similar specificity.

Another posttranslational modification for which a substantial database is available both in *Conus* and other biological systems is the γ-carboxylation of glutamate residues. Identifying a recognition signal is straightforward experimentally: the presence of such a signal increases the affinity of a potential substrate for the γ-glutamyl carboxylase [40]. Although recognition signals have been identified experimentally, it has still not been possible to predict from amino acid sequence alone whether or not a conotoxin precursor has a physiologically relevant recognition signal for the γ-carboxylase. Some structure/function work has been done on *Conus* recognition signals, as well as a very much more extensive set of experiments on blood clotting factors [40, 43, 47, 71, 72]. In the case of the latter, a consensus sequence that predicts which blood clotting factors will be modified has been postulated; however, these consensus sequences do not appear to work well for mammalian proteins outside the blood clotting cascade known to have Gla. For both blood clotting factors and conotoxin precursors, large hydrophobic residues appear to be a feature important for recognition. The general picture that emerges is that the recognition signals are not rigidly sequence-specified; the situation may be somewhat analogous to the loose consensus of signal sequences for transport across the endoplasmic reticulum and recognition by the signal peptidase.

Summary overview

A striking and novel feature of conotoxins is their high density of posttranslational modifications. Ten classes of such modifications have been discovered so far, almost certainly involving more than 15 different modification enzyme systems. As the 500–700 *Conus* species evolved, preexisting modification enzymes were likely recruited to be highly expressed in their venom ducts. Particular toxin genes gained recognition signals for particular modification enzymes, rendering the encoded toxin precursor a substrate for modification. Thus, in addition to the accelerated evolution of peptide sequences, chemical diversity was introduced through posttranslational modification of the 20 standard amino acids. This combination of rapidly diverging amino acid sequences and a high density of posttranslational modification is equivalent to a drug discovery strategy involving a combinatorial library strategy coupled with sophisticated medicinal chemistry.

1 Kohn A. J., Saunders P. R. and Wiener S. (1960) Preliminary studies on the venom of the marine snail *Conus*. Ann. N. Y. Acad. Sci. **90:** 706–725
2 Olivera B. M., Gray W. R., Zeikus R., McIntosh J. M., Varga J., Rivier J. et al. (1985) Peptide neurotoxins from fish-hunting cone snails. Science **230:** 1338–1343
3 Röckel D., Korn W. and Kohn A. J. (1995) Manual of the Living Conidae, Christa Hemmen, Wiesbaden
4 Olivera B. M. (2002) *Conus* venom peptides: reflections from the biology of clades and species. Annu. Rev. Ecol. Syst. **33:** 25–42
5 Olivera B. M., Rivier J., Clark C., Ramilo C. A., Corpuz G. P., Abogadie F. C. et al. (1990) Diversity of *Conus* neuropeptides. Science **249:** 257–263
6 Woodward S. R., Cruz L. J., Olivera B. M. and Hillyard D. R. (1990) Constant and hypervariable regions in conotoxin propeptides. EMBO J. **1:** 1015–1020
7 Terlau H. and Olivera B. M. (2004) *Conus* venoms: a rich source of novel ion channel-targeted peptides. Physiol. Rev. **84:** 41–68
8 Grant M. A., Morelli X. J. and Rigby A. C. (2004) Conotoxins and structural biology: a prospective paradigm for drug discovery. Curr. Protein Peptide Sci. **5:** 235–248
9 Lewis R. J. (2004) Conotoxins as selective inhibitors of neuronal ion channels, receptors and transporters. IUBMB Life **56:** 89–93
10 Livett B. G., Gayler K. R. and Khalil Z. (2004) Drugs from the sea: conopeptides as potential therapeutics. Curr. Med. Chem. **11:** 1715–1723
11 Craig A. G., Bandyopadhyay P. and Olivera B. M. (1999) Post-translationally modified peptides from *Conus* venoms. Eur. J. Biochem. **264:** 271–275
12 Olivera B. M. (1997) *Conus* venom peptides, receptor and ion channel targets and drug design: 50 million years of neuropharmacology (E.E. Just Lecture, 1996). Mol. Biol. Cell **8:** 2101–2109
13 Jones R. M. and Bulaj G. (2000) *Conus* peptides – combinatorial chemistry at a cone snail's pace. Curr. Opin. Drug Discov. Dev. **3:** 141–154
14 Stenflo J. (1999) Contributions of Gla and EGF-like domains to the function of vitamin K-dependent coagulation factors. Crit. Rev. Eukaryot. Gene Expr. **9:** 59–88
15 McIntosh J. M., Olivera B. M., Cruz L. J. and Gray W. R. (1984) g-Carboxyglutamate in a neuroactive toxin. J. Biol. Chem. **259:** 14343–14346
16 Craig A. G., Jimenez E. C., Dykert J., Nielsen D. B., Gulyas J., Abogadie F. C. et al. (1997) A novel post-translational modification involving bromination of tryptophan: identification

of the residue, L-6-bromotryptophan, in peptides from *Conus imperialis* and *Conus radiatus* venom. J. Biol. Chem. **272:** 4689–4698
17 Jimenez E. C., Craig A. G., Watkins M., Hillyard D. R., Gray W. R., Gulyas J. et al. (1997) Bromocontryphan: post-translational bromination of tryptophan. Biochemistry **36:** 989–994
18 Rigby A. C., Lucas-Meunier E., Kalume D. E., Czerwiec E., Hambe B., Dahlqvist I. et al. (1999) A conotoxin from *Conus textile* with unusual posttranslational modifications reduces presynaptic Ca^{2+} influx. Proc. Natl. Acad. Sci. USA **96:** 5758–5763
19 Fujii R., Yoshida H., Fukusumi S., Habata Y., Hosoya M., Kawamata Y. et al. (2002) Identification of a neuropeptide modified with bromine as an endogenous ligand for GPR7. J. Biol. Chem. **277:** 34010–34016
20 Nakai K. (2001) Review: prediction of in vivo fates of proteins in the era of genomics and proteomics. J. Struct. Biol. **134:** 103–116
21 Buczek O., Yoshikami D., Bulaj G., Jimenez E. C. and Olivera B. M. (2005) Posttranslational amino acid isomerization: a functionally important D-amino acid in an excitatory peptide. J. Biol. Chem. **280:** 4247–4253
22 Olivera B. M., Hillyard D. R., Rivier J., Woodward S., Gray W. R., Corpuz G. et al. (1990) Conotoxins: targeted peptide ligands from snail venoms in Marine Toxins: Origin, Structure and Molecular Pharmacology, pp. 256–278, Hall S. and Strichartz G. (eds.), American Chemical Society, Washington, DC
23 Marshall J., Kelley W. P., Rubakhin S. S., Bingham J. P., Sweedler J. V. and Gilly W. F. (2002) Anatomical correlates of venom production in *Conus californicus*. Biol. Bull. **203:** 27–41
24 Terlau H., Shon K., Grilley M., Stocker M., Stühmer W. and Olivera B. M. (1996) Strategy for rapid immobilization of prey by a fish-hunting cone snail. Nature **381:** 148–151
25 Jones R. M. and Bulaj G. (2000) Conotoxins – new vistas for peptide therapeutics. Curr. Pharm. Des. **6:** 1249–1255
26 Duda T. F. Jr. and Palumbi S. R. (1999) Molecular genetics of ecological diversification: duplication and rapid evolution of toxin genes of the venomous gastropod *Conus*. Proc. Natl. Acad. Sci. USA **96:** 6820–6823
27 Duda T. F. Jr. and Palumbi S. R. (2000) Evolutionary diversification of multigene families: allelic selection of toxins in predatory cone snails. Mol. Biol. Evol. **17:** 1286–1293
28 Conticello S. G., Gilad Y., Avidan N., Ben-Asher E., Levy Z. and Fainzilber M. (2001) Mechanisms for evolving hypervariability: the case of conopeptides. Mol. Biol. Evol. **18:** 120–131
29 Espiritu D. J. D., Watkins M., Dia-Monje V., Cartier G. E., Cruz L. J. and Olivera B. M. (2001) Venomous cone snails: molecular phylogeny and the generation of toxin diversity. Toxicon **39:** 1899–1916
30 Santos A. D., McIntosh J. M., Hillyard D. R., Cruz L. J. and Olivera B. M. (2004) The A-superfamily of conotoxins: structural and functional divergence. J. Biol. Chem. **279:** 17596–17606
31 Olivera B. M., Walker C., Cartier G. E., Hooper D., Santos A. D., Schoenfeld R. et al. (1999) Speciation of cone snails and interspecific hyperdivergence of their venom peptides. Potential evolutionary significance of introns. Ann. N.Y. Acad. Sci. **870:** 223–237
32 Witt K. A., Gillespie T. J., Huber J. D., Egleton R. D. and Davis T. P. (2001) Peptide drug modifications to enhance bioavailability and blood-brain barrier permeability. Peptides **22:** 2329–2343
33 Elmagbari N. O., Egleton R. D., Palian M. M., Lowery J. J., Schmid W. R., Davis P. et al. (2004) Antinociceptive structure-activity studies with enkephalin-based opioid glycopeptides. J. Pharmacol. Exp. Ther. **311:** 290–297
34 Walker C., Steel D., Jacobsen R. B., Lirazan M. B., Cruz L. J., Hooper D. et al. (1999) The T-superfamily of conotoxins. J. Biol. Chem. **274:** 30664–30671
35 Kang J., Low W., Norberg T., Meisenhelder J., Hasson K., Stenflo J. et al. (2004) Total chemical synthesis and NMR characterization of the glycopeptide tx5a, a heavily post-translationally modified conotoxin, reveals that the glycan structure is a-D-Gal-(1→3)-a-D-GalNAc. Eur. J. Biochem. **271:** 4939–4949
36 Jimenez E. C., Watkins M. and Olivera B. M. (2004) Multiple 6-bromotryptophan residues in a sleep-inducing peptide. Biochemistry **43:** 12343–12348
37 Craig A. G., Zafaralla G., Cruz L. J., Santos A. D., Hillyard D. R., Dykert J. et al. (1998) An O-glycosylated neuroexcitatory *Conus* peptide. Biochemistry **37:** 16019–16025
38 Pisarewicz K., Mora D., Pflueger F. C., Fields G. B. and Mari F. (2005) Polypeptide chains containing D-gamma-hydroxyvaline. J. Am. Chem. Soc. **127:** 6207–6215
39 Stanley T. B., Stafford D. W., Olivera B. M. and Bandyopadhyay P. K. (1997) Identification of a vitamin K-dependent carboxylase in the venom duct of a *Conus* snail. FEBS Lett. **407:** 85–88
40 Bandyopadhyay P. K., Colledge C. J., Walker C. S., Zhou L.-M., Hillyard D. R. and Olivera B. M. (1998) Conantokin-G precursor and its role in g-carboxylation by a vitamin K-dependent carboxylase from a *Conus* snail. J. Biol. Chem. **273:** 5447–5450
41 Begley G. S., Furie B. C., Czerwiec E., Taylor K. L., Furie G. L., Bronstein L. et al. (2000) A conserved motif within the vitamin K-dependent carboxylase gene is widely distributed across animal phyla. J. Biol. Chem. **275:** 36245–36249
42 Walker C. S., Shetty R. P., Clark K., Kazuko S. G., Letsou A., Olivera B. M. et al. (2001) On a potential global role for vitamin K-dependent g-carboxylation in animal system. J. Biol. Chem. **276:** 7769–7774
43 Bandyopadhyay P. K., Garrett J. E., Shetty R. P., Keate T., Walker C. S. and Olivera B. M. (2002) g-Glutamyl carboxylation: an extracellular post-translational modification that antedates the divergence of molluscs, arthropods and chordates. Proc. Natl. Acad. Sci. USA **99:** 1264–1269
44 Czerwiec E., Begley G. S., Bronstein M., Stenflo J., Taylor K. L., Furie B. C. et al. (2002) Expression and characterization of recombinant vitamin K-dependent g-glutamyl carboxylase from an invertebrate, *Conus textile*. Eur. J. Biochem. **269:** 6162–6172
45 Aktimur A., Gabriel M. A., Gailani D. and Toomey J. R. (2003) The factor IX gamma-carboxyglutamic acid (Gla) domain is involved in interactions between factor IX and factor XIa. J. Biol. Chem. **278:** 7981–7987
46 Schmidt A. E. and Bajaj S. P. (2003) Structure-function relationships in factor IX and factor IXa. Trends Cardiovasc. Med. **13:** 39–45
47 Brown M. A., Begley G. S., Czerwiec E., Stenberg L. M., Jacobs M., Kalume D. E. et al. (2005) Precursors of novel Gla-containing conotoxins contain a carboxy-terminal recognition site that directs gamma-carboxylation. Biochemistry **44:** 9150–9159
48 Bulaj G., Buczek O., Goodsell I., Jimenez E. C., Kranski J., Nielsen J. S. et al. (2003) Efficient oxidative folding of conotoxins and the radiation of venomous cone snails. Proc. Natl. Acad. Sci. USA **100 Suppl. 2:** 14562–14568
49 Buczek O., Olivera B. M. and Bulaj G. (2004) Propeptide does not act as an intramolecular chaperone but facilitates protein disulfide isomerase-assisted folding of a conotoxin precursor. Biochemistry **43:** 1093–1101
50 Craig A. G., Park M., Fischer W. H., Kang J., Compain P. and Piller F. (2001) Enzymatic glycosylation of contulakin-G, a glycopeptide isolated from *Conus* venom, with a mammalian ppGalNAc-transferase. Toxicon **39:** 809–815
51 Lirazan M. B., Hooper D., Corpuz G. P., Ramilo C. A., Bandyopadhyay P., Cruz L. J. et al. (2000) The spasmodic peptide defines a new conotoxin superfamily. Biochemistry **39:** 1583–1588
52 Van den Steen P., Rudd P. M., Dwek R. A. and Opdenakker G. (1998) Concepts and principles of O-linked glycosylation. Crit. Rev. Biochem. Mol. Biol. **33:** 151–208

53 Craig A. G., Norberg T., Griffin D., Hoeger C., Akhtar M., Schmidt K. et al. (1999) Contulakin-G, an O-glycosylated invertebrate neurotensin. J. Biol. Chem. **274:** 13752–13759

54 Kindahl L., Sandström C., Craig A. G., Norberg T. and Kenne L. (2002) ^{1}H NMR studies on the solution conformation of contulakin-G and analogues. Can. J. Chem. **80:** 1022–1031

55 Kindahl L., Kenne L. and Sandström C. (2005) ^{1}H NMR studies on the solution conformation of the [L-Ser^{10}] and [D-Ser^{10}] analogues of contulakin-G. Can. J. Chem. **83:** 156–165

56 Wagstaff J. D., Layer R. T., Smith L. B., Bulaj G., Wei S., Nielsen J. et al. (2000) Glycosylation is critical for the pharmacologic properties of contulakin, a potent, broad-spectrum analgesic conopeptide. Soc. Neurosci. Abst. **26:** 936

57 Price-Carter M., Gray W. M. and Goldenberg D. P. (1996) Folding of w-conotoxins. 1. Efficient disulfide-coupled folding of mature sequences in vitro. Biochemistry **35:** 15537–15546

58 Fuller E., Green B. R., Catlin P., Buczek O., Nielsen J. S., Olivera B. M. et al. (2005) Oxidative folding of conotoxins sharing an identical disulfide bridging framework. FEBS J. **272:** 1727–1738

59 Garrett J. E., Buczek O., Watkins M., Olivera B. M. and Bulaj G. (2005) Biochemical and gene expression analyses of conotoxins in *Conus textile* venom ducts. Biochem. Biophys. Res. Commun. **328:** 362–367

60 Balaji R. A., Ohtake A., Sato K., Gopalakrishnakone P., Kini R. M., Seow K. T. et al. (2000) lambda-Conotoxins, a new family of conotoxins with unique disulfide pattern and protein folding. Isolation and characterization from the venom of *Conus marmoreus*. J. Biol. Chem. **275:** 39516–39522

61 McIntosh J. M., Corpuz G. P., Layer R. T., Garrett J. E., Wagstaff J. D., Bulaj G. et al. (2000) Isolation and characerization of a novel *Conus* peptide with apparent antinociceptive activity. J. Biol. Chem. **275:** 32391–32397

62 McDougal O. M. and Poulter C. D. (2004) Three-dimensional structure of the mini-M conotoxin mr3a. Biochemistry **43:** 425–429

63 Teichert R. W., Rivier J., Dykert J., Cervini L., Gulyas J., Bulaj G. et al. (2004) aA-Conotoxin OIVA defines a new aA-conotoxin subfamily of nicotinic acetylcholine receptor inhibitors. Toxicon **44:** 207–214

64 Corpuz G. P., Jacobsen R. B., Jimenez E. C., Watkins M., Walker C., Colledge C. et al. (2005) Definition of the M-conotoxin superfamily: characterization of novel peptides from molluscivorous *Conus* venoms. Biochemistry **44:** 8176–8186

65 Kauferstein S., Huys I., Lamthanh H., Stöcklin R., Sotto F., Ménez A. et al. (2003) A novel conotoxin inhibiting vertebrate voltage-sensitive potassium channels. Toxicon **42:** 43–52

66 Milne T. J., Abbenante G., Tyndall J. D., Halliday J. and Lewis R. J. (2003) Isolation and characterization of a cone snail protease with homology to CRISP proteins of the pathogenesis-related protein superfamily. J. Biol. Chem. **278:** 31105–31110

67 Newcomb R., Gaur S., Bell J. R. and Cruz L. (1995) Structural and biosynthetic properties of peptides in cone snail venoms. Peptides **16:** 1007–1017

68 Seidah N. G., Day R., Marcinkiewicz M. and Chretien M. (1998) Precursor convertases: an evolutionary ancient, cell-specific, combinatorial mechanism yielding diverse bioactive peptides and proteins. Ann. N. Y. Acad. Sci. **839:** 9–24

69 Duckert P., Brunak S. and Blom N. (2004) Prediction of proprotein convertase cleavage sites. Protein Eng. Des. Sel. **17:** 107–112

70 Schilling S., Manhart S., Hoffmann T., Ludwig H. H., Wasternack C. and Demuth H. U. (2003) Substrate specificity of glutaminyl cyclases from plants and animals. Biol. Chem. **284:** 1583–1592

71 Price P. A., Fraser J. D. and Metz-Virca G. (1987) Molecular cloning of matrix Gla protein: implications for substrate recognition by the vitamin K-dependent carboxylase. Proc. Natl. Acad. Sci. USA **84:** 8335–8339

72 Knobloch J. E. and Suttie J. W. (1987) Vitamin K-dependent carboxylase: control of enzyme activity by the 'propeptide' region of factor X. J. Biol. Chem. **262:** 15334–15337

73 Fan C.-X., Chen X.-K., Zhang C., Wang L.-X., Duan K. L., He L. L. et al. (2003) A novel conotoxin from *Conus betulinus*, k-BtX, unique in cysteine pattern and in function is a specific BK channel modulator. J. Biol. Chem. **278:** 12624–12633

74 Gray W. R., Luque A., Olivera B. M., Barrett J. and Cruz L. J. (1981) Peptide toxins from *Conus geographus* venom. J. Biol. Chem. **256:** 4734–4740

75 Cruz L. J., Gray W. R., Olivera B. M., Zeikus R. D., Kerr L., Yoshikami D. et al. (1985) *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. J. Biol. Chem. **260:** 9280–9288

76 McIntosh J. M., Cruz L. J., Hunkapiller M. W., Gray W. R. and Olivera B. M. (1982) Isolation and structure of a peptide toxin from the marine snail *Conus magus*. Arch. Biochem. Biophys. **218:** 329–334

77 Jimenez E. C., Donevan S. D., Walker C., Zhou L.-M., Nielsen J., Cruz L. J. et al. (2002) Conantokin-L, a new NMDA receptor antagonist: determinants for anticonvulsant potency. Epilepsy Res. **51:** 73–80

78 Jimenez E. C., Olivera B. M., Gray W. R. and Cruz L. J. (1996) Contryphan is a D-tryptophan-containing *Conus* peptide. J. Biol. Chem. **281:** 28002–28005

79 Jacobsen R., Jimenez E. C., de la Cruz R. G. C., Gray W. R., Cruz L. J. and Olivera B. M. (1999) A novel D-leucine-containing *Conus* peptide: diverse conformational dynamics in the contryphan family. J. Peptide Res. **54:** 93–99

80 Loughnan M., Bond T., Atkins A., Cuevas J., Adams D. J., Broxton N. M. et al. (1998) a-Conotoxin EpI, a novel sulfated peptide from *Conus episcopatus* that selectively targets neuronal nicotinic acetylcholine receptors. J. Biol. Chem. **273:** 15667–15674

81 Loughnan M. L., Nicke A., Jones A., Adams D. J., Alewood P. F. and Lewis R. J. (2004) Chemical and functional identification and characterization of novel sulfated a-conotoxins from the cone snail *Conus anemone*. J. Med. Chem. **47:** 1234–1241

82 Jimenez E. C., Shetty R. P., Lirazan M., Rivier J., Walker C., Abogadie F. C. et al. (2003) Novel excitatory *Conus* peptides define a new conotoxin superfamily. J. Neurochem. **85:** 610–621

83 Dutton J. L., Bansal P. S., Hogg R. C., Adams D. J., Alewood P. F. and Craik D. J. (2002) A new level of conotoxin diversity, a non-native disulfide bond connectivity in a-conotoxin AuIB reduces structural definition but increases biological activity. J. Biol. Chem. **277:** 48849–48857

84 Myers R. A., McIntosh J. M., Imperial J., Williams R. W., Oas T., Haack J. A. et al. (1990) Peptides from *Conus* venoms which affect Ca^{++} entry into neurons. J. Toxicol. Toxin Rev. **9:** 179–202

85 Dai Q., Prorok M. and Castellino F. J. (2004) A new mechanism for metal ion-assisted interchain helix assembly in a naturally occurring peptide mediated by optimally spaced g-carboxyglutamic acid residues. J. Mol. Biol. **336:** 731–744

86 Massilia G. R., Schininà M. E., Ascenzi P. and Polticelli F. (2001) Contryphan-Vn: a novel peptide from the venom of the Mediterranean snail *Conus ventricosus*. Biochem. Biophys. Res. Commun. **288:** 908–913