REVIEW

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Chemical Modification of Conotoxins to Improve Stability and Activity

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ABSTRACT Conotoxins are small disulfide-rich peptides from the venom of cone snails. Along with other conopeptides, they target a wide range of membrane receptors, ion channels, and transporters, and because of their high potency and selectivity for defined subtypes of these receptors, they have attracted a great deal of attention recently as leads in drug development. However, like most peptides, conopeptides potentially suffer from the disadvantages of poor absorption, poor stability, or short biological half-lives. Recently, various chemical approaches, including residue substitutions, backbone cyclization, and disulfide-bridge modification, have been reported to increase the stability of conopeptides. These manufactured interventions add to the array of post-translational modifications that occur naturally in conopeptides. They enhance the versatility of these peptides as tools in neuroscience and as drug leads.

Predatory marine mollusks of the genus *Conus* produce venom, which is used to capture prey and contains a mixture of constrained peptides called conotoxins. These peptides are typically small and highly constrained, with 10–40 amino acids and up to five disulfide bonds (*1*–*4*), although most conotoxins discovered so far contain two or three disulfide bonds. The peptide cocktail in the venom targets a diverse range of cell membrane receptors and ion channels and leads to rapid and efficient immobilization of the prey. The shell of a cone snail and some representative conotoxin structures are shown (Figure 1).

Because of their high potency and specificity for a range of important physiological targets, conotoxins have attracted much interest as neuropharmacological tools, as potential leads in drug development, or indeed as drugs themselves. One conotoxin, ω-conotoxin MVIIA, with the generic name ziconotide and the trade name Prialt (*5, 6*), is currently marketed in the U.S. and the E.U. for chronic pain, and others are in clinical or preclinical trials, as indicated in Table 1. Given that up to 700 species of cone snails exist and ~100–200 unique peptides are present in each species, it is estimated that up to 100,000 conotoxins may await discovery (*7, 8*). This natural library of bioactive peptides thus provides a rich source of potential drug leads or neurophysiological probes. So far, <0.1% of this diverse library of compounds has been examined.

One potential drawback of conotoxins as drugs is that they may suffer the generic problems of peptides *in vivo*, such as poor absorption, susceptibility to proteolysis, and short biological half-lives. Conotoxins are perhaps potentially less affected by some of these problems than small linear peptides, and extensive pharmacokinetic data are available for ziconotide (*9*, *10*). Nevertheless, significant value is still thought to exist in

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stabilizing conotoxins for therapeutic or diagnostic applications and for improving their route of delivery. For example, Prialt is administered *via* direct spinal infusion, a more invasive approach than the generally preferred oral route for conventional pharmaceuticals.

In this Review, we focus on studies conducted over the last few years on the chemical modification of conotoxins to improve their biological stability or biopharmaceutical properties. Various other aspects of conotoxins have been reviewed previously, including historical overviews of their discovery, their biological activities, and their structures, and the reader is referred to these articles for a broader background on conotoxins (*1–4*, *11–16*).

Sequences, Structures, Targets, and Activities. Peptides from cone snail venom broadly fall into two classes: the disulfide-rich cases (two or more disulfide bonds) and those with none or just one disulfide bond (*4*). The term conotoxin is mainly used in the literature for disulfide-rich peptides from *Conus* venom, whereas the term conopeptide includes all peptidic venom compounds. We focus here on the disulfide-rich peptides. Conopeptides are also categorized in terms of pharmacological families, which are further consolidated into superfamilies based on sequence and framework homology. Individual peptides are named with a Greek letter to indicate the pharmacological activity, one or two letters to indicate the *Conus* species from which the peptide was first isolated, a Roman numeral to indicate the disulfide framework category, and an upper-case letter to denote the order of discovery within that category. For instance, MVIIA, which belongs to the ω-conotoxin family, was isolated from *Conus magus* and was the first peptide in this family discovered with framework VII (*17*).

As is apparent (Figure 1), conotoxins adopt a variety of 3D structures and can be regarded as "mini-proteins" (*18*). They range from simple helical structures for non-disulfide-bonded conopeptides to tightly folded structures with cross-bracing disulfide bonds for the cystine-rich conotoxins. An extreme example of the latter is the cystine knotted structure found in the ω-conotoxins such as MVIIA (*19*, *20*), in which two disulfide bonds and their connecting backbone segments form an embedded ring that is threaded by a third disulfide bond (*21*). In general, this type of structural motif provides conotoxins with high stability compared with non-disulfide-bonded peptides.

Shown are some examples of conopeptides to illustrate the diversity of their sequences and receptor targets (Table 2). From these examples, it is apparent that they often contain unusual amino acids, including hydroxyproline and γ-carboxyglutamic acid (Gla), as well as other modifications such as amidation, glycosylation, and epimerization, further enhancing the molecular diversity of this peptide class (*22*, *23*).

The receptor targets for conopeptides regulate a wide range of crucial physiological functions. The voltage-gated ion channel superfamily comprises structurally similar membrane-bound proteins activated by a change in the transmembrane voltage (*24*). These proteins exhibit different selectivity for cations and are conventionally divided into Na^+, K^+, and Ca^{2+} channels. Their most important physiological role is to generate, shape, and transduce the electrical signals of cells. Upon activation, voltage-gated ion channels undergo a conformational change, which under physiological conditions results in the selective permeation of cations through the pore. From this open state, the channels can be either inactivated by an additional conformational change, thereby entering a nonconducting state, or they may be deactivated, returning to a closed state.

Three different *Conus* peptide families target voltage-gated Na^+ channels: the μ-conotoxins are channel pore blockers, the μO-conotoxins inhibit Na^+ channel conductance by hindering channel gating, and the δ-conotoxins delay or inhibit fast inactivation. The ω-conotoxin family blocks voltage-gated Ca^{2+} channels and is the most widely used *Conus* peptide family in neuroscience, primarily as a pharmacological tool to inhibit synaptic transmission. Two *Conus* peptide families that specifically target K^+ channels have been characterized: κ-conotoxins (*25*, *26*) and κM-conotoxins (*27*). A number of other *Conus* peptides also target K^+ channels, but the molecular specificity of most of these has not yet been defined. A striking contrast between peptides that target K^+ channels and those that target Na^+ channels is that the latter appear to be widely conserved over a broad range of *Conus* species. In contrast, structurally and genetically divergent conopeptides target K^+ channels in different groups of *Conus* species (*28–30*).

KEYWORDS
Conotoxins: Small disulfide-rich peptides from the venom of marine *Conus* snails. These peptides are used by the snails to capture prey. They also make valuable neuroscience probes and drug leads because of their exquisite selectivity for ion channels and membrane receptors.
Ion channels: Transmembrane proteins gated by either voltage or ligand (neurotransmitter) to permit the selective permeation of either cations or anions across the cell membrane through the open pore.

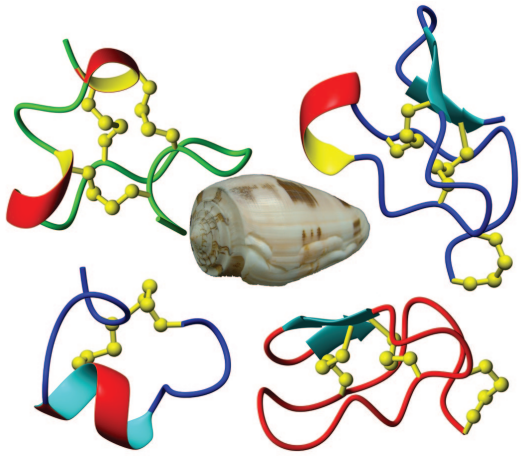

Figure 1. Image of the cone shell *C. striatus* surrounded by the structures of several conotoxins representative of different classes. The structures represented are μ-conotoxin GIIIB from *C. geographus* (Protein Data Bank (PDB) code 1GIB, top left) (*99*), κ-conotoxin PVIIA from *C. purpurascens* (PDB code 1AV3, top right) (*25*), α-conotoxin MII from *C. magus* (PDB code 1MII, bottom left) (*100*), and ω-conotoxin MVIIA from *C. magus* (PDB code 1MVI, bottom right) (*20*). The β-strands are shown as arrows, the helical/turn regions appear as thickened ribbons, and the disulfide bonds are shown in ball-and-stick format.

In the venom of the fish-hunting *Conus*, μ-conotoxins target the muscle subtype of Na^+ channels, whereas the ω-conotoxins block the presynaptic Ca^{2+} channels at the neuromuscular junction, the combination of which causes an irreversible block of neuromuscular transmission. The κ-conotoxins act in a synergistic manner with the δ-conotoxins that inhibit Na^+ channel inactivation. This leads to massive hyperexcitation of the injected animal and results in an almost instant tetanic paralysis. Thus, prey immobilization and paralysis are likely functions of many of these peptides.

Ligand-gated (ionotropic) ion channels mediate rapid synaptic transmission. Many of these proteins are grouped according to their structural and functional similarities. One major group belonging to the same gene superfamily, the cysteine-loop superfamily, comprises those activated by acetylcholine (ACh), serotonin, γ-aminobutyric acid, or glycine (*31*). A second gene superfamily of ligand-gated ion channels is the glutamate receptors (*32*), usually subdivided into *N*-methyl-D-aspartate (NMDA) and non-NMDA (kainite and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors. A third family of ligand-gated ion channels involved in synaptic transmission at certain central and peripheral synapses is the ATP-activated (P2X) purinergic receptors (*33*).

Conus peptides targeted to two of these families of ligand-gated ion channels have been identified. Many target nicotinic ACh receptors (nAChRs) and are competitive antagonists, although noncompetitive nAChR antagonists have also been characterized (*34, 35*). The

TABLE 1. Examples of conopeptides currently in clinical and preclinical trials for treatment of chronic and neuropathic pain[a]

Toxin	Target	*Conus* species	Clinical stage	Company
ω-Conotoxin MVIIA (ziconotide, Prialt)	N-type Ca^{2+} channels	*C. magus*	Marketed in U.S./E.U.	Elan (www.elan.com)
ω-Conotoxin CVID (AM336)	N-type Ca^{2+} channels	*C. catus*	Phase I/IIa Preclinical	Zenyth (formerly Amrad)[b] CNSBio Pty Ltd. (www.monashinstitute.org/cnsbio.html)
Contulakin-G (CGX-1160)	Neurotensin receptor	*C. geographus*	Phase II	Cognetix, Inc.[c]
Conantokin-G (CGX-1007)	NR2B/NMDA receptors	*C. geographus*	Phase II	Cognetix, Inc.[c]
χ-Conotoxin MrIA (Xen2174)	NET	*C. marmoreus*	Phase I/IIa	Xenome (www.xenome.com)
α-Conotoxin Vc1.1	Neuronal nAChRs	*C. victoriae*	Phase IIa	Metabolic Pharmaceuticals (www.metabolic.com)

[a]Adapted from Hogg (*88*). [b]Development as an intrathecal analgesic was discontinued, and the rights for further development are now held by CNSBio. [c]Status unclear.

TABLE 2. Selected sequences that highlight the diversity of sequences, post-translational modifications, and targets of conopeptides[a,b]

Superfamily/ family	Peptide	Sequence	Nominal charge	Target	Reference
		Disulfide-rich			
A/α	PnIA	G**CC**SLPP**C**AANNPDY^{SO_4}**C***	0	nAChR	*89*
T/χ	MrIA	NGV**CC**GYKL**C**HO**C**	+2	NET	*62*
M/μ	GIIIA	RD**CC**TPPKK**C**KDRQ**C**RPQR**CC**A*	+6	Na^+ channel	*90*
O/ω	MVIIA	**C**KGKGAK**C**SRLMYD**CC**TGS**C**RSGK**C***	+6	Ca^{2+} channel	*17*
I	ViTx	SR**C**FPPGIY**C**TSYLP**CC**WGI**CC**ST**C**RNV**C**HLRIGK	+4	K^+ channel	*91*
P	tx9a	G**C**NNS**C**QγHSD**C**γSH**C**I**C**TFRG**C**GAVN	−3	?	*92*
S/σ	GVIIIA	G**C**TRT**C**GGOK**C**TGT**C**T**C**TNSSK**C**G**C**RYNVHPSGW^{Br}G**C**G**C**A**C**S*	+6	5-HT_3 receptor	*39*
		Nondisulfide-rich			
Conopressin	γConopressin-vil	**C**LIQD**C**PγG*	+2	Vasopressin receptor	*93*
Contryphan	Contryphan-Sm	G**C**OwQPW**C***	+1	?	*94*
Contulakin	Contulakin-G	ZSEEGGSNAT^{G}KKPYIL	−1	Neurotensin receptor	*95*
Conantokin	Conantokin-G	GEγγLWVNQγLIRγKSN*	−6	NMDA receptor	*96*, *97*
Conorfamide	Conorfamide-Sr1	GPMGWVPVFYRF*	+2	RFamide receptor	*98*

[a]Modifications: Gla (γ), hydroxyproline (O), 4-sulfato-tyrosine (Y^{SO_4}), 6-Br-tryptophan (W^{Br}), D-tryptophan (w), glycosylation (T^{G}), pyroglutamic acid (Z), amidated C terminus (*). [b]Table adapted from Marx *et al.* (*23*).

most widely distributed of the nAChR antagonists are the α-conotoxins, multiple representatives of which are expressed in the venom ducts of most, if not all, *Conus* species. α-Conotoxins are highly subtype-selective nAChR antagonists and are proving to be valuable pharmacological reagents for discriminating between closely related neuronal nAChR isoforms (*36*). The major nAChR antagonists found in venom target the nAChR subtype(s) present at the neuromuscular junction, disrupting neuromuscular transmission to paralyze prey. This is clearly the case for fish-hunting cone snails, where the most abundant α-conotoxins in the venom are paralytic to fish (and other vertebrates). Several neuronal nAChR subtypes are present in the vertebrate autonomic nervous system and may be involved in suppressing the fight-or-flight response of the prey (*37*).

An unusual family of *Conus* peptides, the conantokins, are antagonists of NMDA receptors, a subclass of the glutamate receptor superfamily of ligand-gated ion channels (*38*). These peptides are biochemically distinctive in their high content of the modified amino acid γ-Gla and lack of cysteine residues. One peptide, σ-conotoxin GVIIIA, has also been shown to target the 5-hydroxytryptamine (5-HT_3) receptor (*39*). It has been suggested that the cone snails from which these have been isolated use the peptides to deaden the sensory circuitry of the prey, making it easier to capture and digest (*40*).

With this brief background on the sequences, structures, molecular targets, and activities of conotoxins, we turn to the main focus of this Review: the approaches to their chemical modification to modulate activity or stability.

Chemical Modifications of Conotoxins. Because of their small size, conotoxins are eminently suitable for chemical synthesis in the laboratory. Indeed, it is common practice in conotoxin discovery programs to determine the sequence of new peptides directly from native material extracted from venom ducts and to then chemically synthesize the peptides for structural or pharmacological studies. This reflects the typical availability of only microgram quantities of purified peptides from native venom, but milligram quantities may be synthesized. The suitability of conotoxins for chemical synthesis means that an array of medicinal chemistry approaches is potentially available for modifying them, including residue substitutions, capping or truncation of the termini, disulfide bond engineering, or cyclization. Solid-phase methods that employ either di-*tert*-butyl dicarbonate or 9-fluorenylmethyl protecting groups (*41*) are typically used to carry out synthesis. Although the assembly of the peptide sequence is generally not problematic, the oxidation step to form disulfide bonds tends to be the most time consuming and least predictable aspect of their synthesis. Nevertheless, folding conditions can generally be found that lead to good yields of native-like synthetic peptides. In some cases, selective protection and directed formation of individual disulfide bonds may be necessary (*e.g.*, as recently applied for μO-conotoxin MrVIA) (*42*). Modified conotoxins are made *via* the same approach but are based on assembly of the modified peptide sequence. In the following sections, the focus is on modifications that have been used to improve stability. An overview of the range of approaches that have been taken is provided (Figure 2).

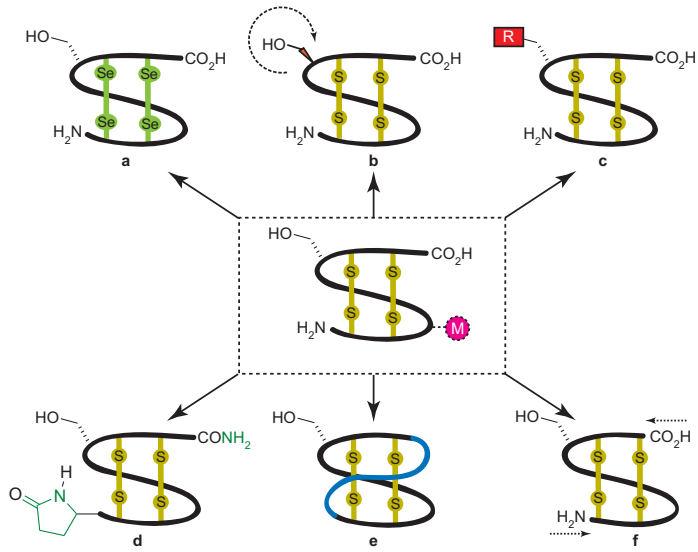

Figure 2. Overview of the types of chemical modifications that have been applied to conotoxins to improve their activity or stability. The central structure represents a prototypical native conotoxin, with a methionine indicated in pink as an example of a chemically unstable residue. Methionine is readily oxidized and is substituted in the modified peptides (a–f). a) Substitution of cystine with selenocystine provides greater resistance to reducing conditions. b) Stereochemical inversion of a selected residue reduces recognition by proteases. c) Conjugation of a side chain, such as glycosylation or lipidation, changes pharmacological properties. d) The addition of N- and C-terminal capping reduces susceptibility to exo-proteases. e) Backbone cyclization stabilizes the entire framework and eliminates endo- and exo-protease digestion. f) Truncation of the N- and C-termini removes flexible regions susceptible to proteolysis.

Sequence Modifications. In general for proteins, several amino acids are susceptible to chemical degradation. If these can be replaced in peptides intended for pharmaceutical applications, then potential stability problems can be avoided, provided that substituting these residues does not lead to a loss of biological activity or to unwanted side effects. In particular, methionine residues are susceptible to

KEYWORDS

Cystine framework: The covalent cross-links between cysteine residues in conotoxins help to stabilize their structure. Different families of conotoxins have different numbers and spacings of their cysteine residues, which define their cystine frameworks. Conotoxin folding refers to the oxidation of the cysteine residues to form the correct disulfide connectivity and native 3D fold.

Cyclization: The process of joining the N- and C-termini of a peptide either directly or with an amino acid linker to produce a cyclic peptide backbone. This can result in the stabilization of peptides and has recently been applied to conotoxins.

Stability needs to be considered early in the candidate selection process for future conotoxin-based leads.

oxidation (*43*), asparagine residues to deamidation, and Asp–Pro peptide bonds to isomerization or cleavage (*44*). It is interesting to note that for ziconotide (MVIIA), the only conotoxin-based drug so far on the market, the decision was made not to substitute any of these residues, even though hundreds of analogues of the native peptide were evaluated before a development candidate was selected (*5*). This decision led to the need for U.S. Food and Drug Administration (FDA)-mandated characterization and toxicity studies on the methionine and asparagine degradation products that might have otherwise been avoided and presumably added to the development costs.

In the case of MVIIA, logistical or commercial reasons probably existed for proceeding with the native peptide sequence in the final product, but the implications of this decision had some significant downstream consequences. Because the oxidation of methionine in MVIIA can be slowed at low temperature, the final product needs to be refrigerated (www.fda.gov/cder/foi/label/2004/021060lbl.pdf lists storage requirements). This in itself may not be a major inconvenience, but FDA regulations also require low-temperature storage in the entire supply chain, and this increases the cost of goods. Perhaps a lesson to be learned from this example is that stability needs to be considered early in the candidate selection process for future conotoxin-based leads. This appears to have been done with the χ-conotoxin drug lead based on MrIA described later in this Review.

So far, relatively little use has been made in the conotoxin field of the otherwise relatively standard approach in peptide chemistry of substituting selected L-amino acids for D-amino acids to improve stability by reducing susceptibility to proteolytic breakdown. Some native conotoxins incorporate such a modification, including contryphans (*45*) and conomap-Vt (*46*). In other cases, this approach has been used to probe the structural and functional importance of key residues. For example, replacement of Tyr13 with D-tyrosine in MVIIA leads to a 4-fold loss of activity and confirms the importance of Tyr13 for binding to N-type Ca^{2+} channels (*47*).

The use of D-amino acids is particularly common in peptide-based drug design of cyclic peptides and has been applied in the design of small-molecule mimics of one of the exposed loops of CVID (*48, 49*). CVID is member of the ω-conotoxin family and, like MVIIA, has been proposed as a lead for the treatment of neuropathic pain *via* blockade of N-type Ca^{2+} channels (see Table 1). Unfortunately, the approach of minimizing conotoxin structures has not yet led to molecules of high potency (*48, 50*).

Although our focus here is on modifications to improve stability, the common occurrence of post-translationally modified residues in natural conotoxins has led to substitution efforts for a range of other reasons, including reducing the cost of synthesis. In this context, a common residue-specific modification of synthetic conotoxins is the replacement of Gla (*39*) with glutamic acid. Gla is very expensive, and its replacement with the unmodified amino acid glutamic acid often does not lead to any significant loss of activity. However, this approach needs to be taken with caution, because a loss of activity can occur upon substitution of some post-translational modifications. For example, conotoxins sulfated at tyrosine have been isolated (*51*) with synthetic unsulfated analogues shown to have a 2–10-fold loss of activity at various neuronal nAChR subtypes. Nevertheless, unsulfated analogues are synthetically easier to make, and if the focus is on structural rather than activity studies, presence or absence of the sulfate is thought to not perturb the structure (*52*).

It is interesting that in some cases, the naturally occurring post-translationally modified conotoxin is inactive in a particular assay, whereas the synthetic unmodified peptide is active. This applies, for example, to α-conotoxin Vc1.1 (*53, 54*). In this case, the natural peptide is inactive in an animal pain model, but the synthetic peptide in which hydroxyproline and Gla residues are replaced with proline and glutamic acid, respectively, has potent analgesic activity and is currently in phase IIa clinical trials (see Table 1) (*55*). Thus, in summary, cases exist where post-translational modifications increase, decrease, or have no effect on activity. This opens the door to the use of such modifications to optimize the biopharmaceutical properties of conotoxins.

In contrast to the widespread occurrence of N-methylated amino acids in natural peptides from bacterial and fungal sources, this modification has not been reported in native conotoxins and has not been applied in synthetic variants. This is probably because conotoxins are generally such well-structured molecules and have an extensive internal hydrogen-bonding network. Their structures and activities would

likely be perturbed by substitution of backbone amide protons.

Another approach that has been attempted to improve the biopharmaceutical properties of conotoxins is the use of lipid tags on selected residues. This was recently exemplified for α-conotoxin MII (*56*). MII is a potent inhibitor of neuronal nAChRs of the subtype α3β2 and also binds to the α6 nAChR. To improve the bioavailability of this peptide, scientists synthesized two lipidic analogues of MII, the first by coupling 2-amino-D,L-dodecanoic acid (Laa) to the N terminus (LaaMII) and the second by replacing Asn5 in the MII sequence with this lipoamino acid (5LaaMII). ^{1}H NMR chemical shift analysis showed that the tertiary structure of the N-conjugated analogue was the same as the native conotoxin, whereas the 5LaaMII analogue formed the correct disulfide bridges but failed to adopt the native helical structure. The N-terminal conjugate was found to inhibit α3β2 nAChRs with equal potency to the parent peptide, whereas the 5LaaMII analogue had no inhibitory activity. The active LaaMII analogue had significantly improved permeability across Caco-2 cell monolayers compared with the native MII, and both peptides showed negligible toxicity.

Another recently applied approach is the incorporation of non-natural (*i.e.*, nonpeptidic) backbone spaces within conotoxins to improve their activity. Green *et al.* (*57*) reported the use of flexible spacers such as amino-3-oxypentanoic and 6-aminohexanoic acids to replace conformationally constrained parts of the three-disulfide conotoxin SIIIA. The polymer–conotoxin hybrids, referred to as polytides, appeared to be better inhibitors of sodium currents in dorsal root ganglion neurons and sciatic nerves in mice than the native peptide. Furthermore, the polytides were significantly more potent and longer lasting analgesics in an inflammatory pain model in mice compared with the native peptide.

In summary, a wide range of approaches based on residue substitutions has been explored in attempts to transform conotoxins into therapeutics.

Terminal Capping, Truncation, or Both. Many naturally occurring conotoxins contain an amidated C-terminus that, in principle, reduces their susceptibility to proteolysis by carboxypeptidases. In cases where synthetic derivatives are made with a free carboxyl terminus, an associated loss of activity often occurs. The C-terminal amide may play roles in addition to improving stability and activity. For example, the presence of a C-terminal amide, along with the presence of a conserved proline residue, is critical in directing the disulfide bond formation of some α-conotoxins, and in the absence of these two structural features a non-native disulfide-bonding pattern is formed (*58*). Interestingly, a C-terminal glycine residue, which is the biosynthetic precursor of the C-terminal amide, enhances the oxidative folding efficiency of ω-conotoxin MVIIA even more than the amide alone (*59*).

The χ-conotoxins (*60*–*62*) are examples of a free C-terminus occurring naturally and provide a convenient framework in which to describe the effects of amidation and terminal truncation. Members of this family include MrIA and MrIB, two 13-residue peptides from the venom of *C. marmoreus* that have activity at the neuronal norepinephrine transporter (NET) (*62*). These conotoxins are of interest because drugs that inhibit the NET may be used to treat a number of neurological disorders, including depression (*63*), schizophrenia (*64*), and anxiety (*65*), and they may possibly modulate pain pathways (*66*). It is interesting that the charge state of the C-terminus of MrIA does not appear to play a significant role in activity, because the non-native, amidated C-terminal form of MrIA, MrIA-NH_2, is only 1.5-fold more potent than MrIA at inhibiting the NET (*62*).

In contrast to the widespread capping of the C-terminus of conotoxins, in general few natural modifications exist of the N-terminus of conotoxins. The most common is pyroglutamic acid formation, which occurs in several diverse classes of conotoxins (*67*–*69*). In the case of μ-conotoxin PIIIA, only minimal differences in activity were observed between the native peptide and PIIIA-(2–22) in which the terminal pyroglutamic acid is removed (*70*). It is presumed, however, that the pyroglutamic acid form is thermodynamically and biologically more stable and may have advantages *in vivo*. N-terminal acetylation of α-conotoxin MI led to a small loss in activity and a shift in its subunit selectivity, an indication that the N-terminal amine forms specific interactions with the receptor (*71*).

As has been applied in peptide-based drug design in general, when the natural occurrence of pyroglutamic acid is mimicked, the stability of conotoxins has been improved by the synthetic use of pyroglutamic acid formation. For example, Xen2174 (Table 1) is a modified version of MrIA in which this change has been made (*72*) and has led to improved chemical stability (*73*).

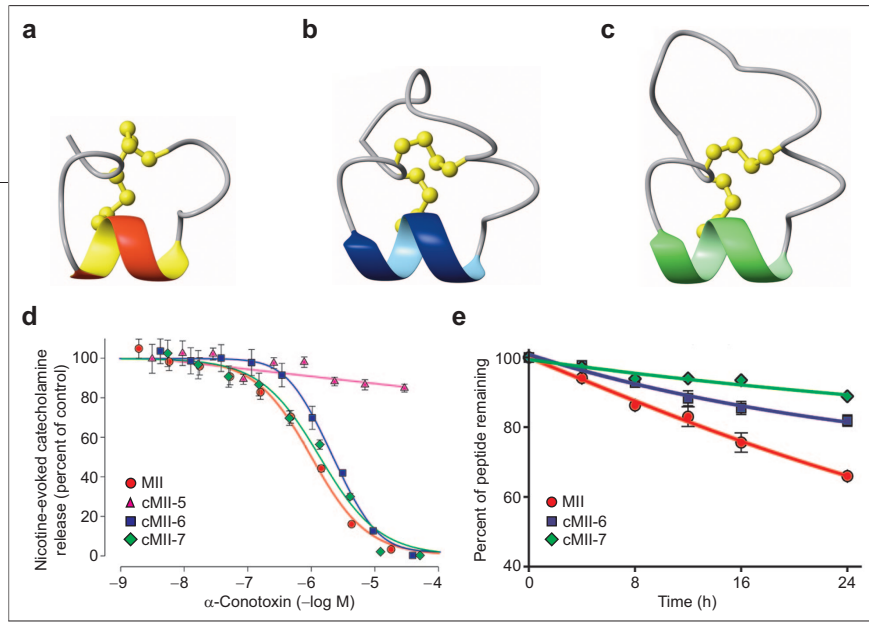

Figure 3. Cyclization as an approach to the stabilization of conotoxins. Use of six- or seven-amino-acid linkers to join the termini of conotoxin MII leads to molecules with similar biological activity to the native peptide but improved stability in human plasma. a) Native peptide. b, c) Structures of the six- and seven-amino-acid linkers used in cyclization do not disrupt the native structure. d) Similar biological activity is demonstrated by the native and cyclized conotoxins. e) Resistance to proteolysis in human plasma is improved in the cyclized conotoxins. Figure adapted from ref 86.

A complementary approach to the protection of N- and C-termini of peptides by the introduction of capping moieties is to truncate residues that lie outside the defined cystine framework; the rationale is that such residues are likely to be more flexible and hence more susceptible to proteolytic or chemical degradation. However, in general, conotoxins have relatively few amino acids outside the cystine framework, thus offering few opportunities to truncate terminal residues. But in a recent example of this approach, truncated analogues of MrIA with residue 1 (MrIA-NH_2[2–13]), residues 1 and 2 (MrIA-NH_2[3–13]), and residues 1–3 (MrIA-NH_2[4–13]) deleted were examined (*74*). An analysis of the NMR secondary shifts of MrIA-NH_2[2–13] and MrIA-NH_2[3–13] showed that the overall native fold is retained in these analogues. Deleting one N-terminal residue resulted in a loss of target specificity, and deleting a second residue resulted in a loss of overall potency. Significant differences in the spectra of MrIA-NH_2[4–13] compared with the native or other truncated forms suggested a critical structural role for Val3 in the structure of MrIA.

Another example of a conotoxin having a significant N-terminal tail is α-conotoxin GID, whose tail comprises four residues preceding the first cysteine residue. Truncation of these residues leads to a significant loss of activity at α4β2 neuronal AChR subtypes but minimal change at α3β2 and α7 subtypes, despite enhancing the off-rates at these receptors (*75*). The N-terminal tail is disordered in solution but may adopt defined conformations at some receptor subtypes and not others, and this accounts for the variation in its relative importance at different subtypes.

Disulfide Bond Engineering. The disulfide bond connectivity is one of the defining features of conotoxin frameworks. In principle, multiple possible variations of connectivity exist for a given conotoxin sequence, but in practice, usually only a single form is seen in native peptides. Conotoxins that contain two disulfide bonds have three potential connectivities, referred to as globular, ribbon, or beads forms, which correspond to (I–III, II–IV), (I–IV, II–III), or (I–II, II–I–IV) linkages, respectively; the globular form is the native connectivity in most cases. A synthetic study of the three possibilities for α-conotoxin GI showed increased conformational heterogeneity of the ribbon and beads forms compared with the native (globular) form (*76*). But in some cases, the ribbon connectivity is the native form. For example, although the length and the number of cysteine residues of the χ-conopeptides resemble the α-conotoxins, the disulfide connectivity and the spacings between the cysteine residues differ (Table 2), with χ-conopeptide MrIA having a ribbon connectivity. The recently determined structure of MrIA (*74*) suggests that it is more flexible than what has been previously reported for most α-conotoxins. The ribbon connectivity of MrIA lacks the cross-bracing present in the globular fold, and this is likely to be the major factor involved in the greater structural disorder.

α-Conotoxin AulB and a disulfide bond variant have been synthesized to determine the role of disulfide bond connectivity on structure and activity (*77*). Both peptides contain the 15-amino-acid sequence GCCSYPPCFATNPDC; the globular (native) isomer has the disulfide connectivity Cys(2–8 and 3–15), and the ribbon isomer has the disulfide connectivity Cys(2–15 and 3–8). The solution structures of the peptides were determined by NMR spectroscopy, and their ability to block the nAChRs on dissociated neurons of the rat parasympathetic ganglia was examined. Although the ribbon disulfide isomer has a less well-defined structure, it surprisingly has ~10× greater potency than the native peptide. To our knowledge, this is the first demonstration of a non-

native disulfide bond isomer of a conotoxin exhibiting greater biological activity than the native isomer.

Despite their importance in stabilizing structure, the disulfide bonds of conotoxins are inherently unstable under reducing conditions. Thus, reduction or scrambling by thiol-containing molecules such as glutathione or serum albumin has the potential to decrease their effectiveness as drugs. An approach to addressing this was recently reported *via* the development of selenoconotoxins, in which cysteine residues were replaced by selenocysteine (Sec) to form isosteric and nonreducible diselenide bonds. Three isoforms of α-conotoxin ImI were synthesized with the systematic replacement of one ([Sec2,8]-ImI, [Sec3,12]-ImI) or both ([Sec2,3,8,12]-ImI) disulfide bonds with a diselenide bond (*78*). These analogues showed a remarkably enhanced stability to scrambling or reduction under a range of chemical or biological reducing conditions. Three-dimensional structure determination by NMR and CD spectroscopy indicated that the conformations of the selenoconotoxins are very similar to native ImI. Furthermore, full bioactivity was maintained at the α7 nAChR, with each seleno-analogue exhibiting a dose–response curve that overlapped with the wild-type ImI, and this further supports an isomorphic structure. Overall, the results demonstrated that selenoconotoxins could be used as highly stable scaffolds. This approach has been applied to the stabilization of a range of small disulfide-rich peptides (*79*).

More radical modifications of disulfide bonds have been attempted. Linkage of a participating cysteine with a chloroacetyl side chain (attached *via* aminoethylglycine) in several locations in the α-conotoxin GI gave thioether analogues with substantially reduced activities (*80*). Another α-conotoxin, SI, had each of its disulfide bonds replaced in turn with a lactam bridge in each orientation (*81*). The four analogues were either inactive or 60–70-fold less active than the native peptide. Although the reduction in activity is disappointing, the potential improvements in stability still make such approaches appealing, and the potential exists for an improved design that will lead to fully active analogues. Finally, ImI was recently modified to give several isosteric hydrocarbon analogues of its disulfide bonds, although activities and stabilities were not reported (*82*).

Cyclization. Cyclization is a widely applied technique in the pharmaceutical industry for stabilizing short linear peptides, and it also occurs in various natural products synthesized by microorganisms. A good example is the immunosuppressive agent cyclosporin, a cyclododecapeptide that has had a major impact in revolutionizing organ transplant therapy (*83*). Such natural products tend to be <12 amino acids in size, and synthetic cyclic peptides produced for drug design applications tend to be even smaller (*84*).

It is surprising that the idea of cyclizing disulfide-rich peptides has attracted so little attention until now. Perhaps this is because in the first reported attempt, for the model protein bovine pancreatic trypsin inhibitor, the termini were joined *via* direct chemical ligation, which actually destabilized the protein (*85*). However, the cyclization of conotoxins has recently been successfully exemplified for the α-conotoxin MII (*86*). Cyclization of this 12-residue peptide with linkers of six or seven amino acids resulted in maintenance of the structure of the native peptide (Figure 3) and essentially equivalent biological activity, but with enhanced resistance to proteolytic breakdown and enhanced stability in human plasma. Cyclization has also been recently reported for the χ-conotoxin MrIA (*87*). In the latter case, linking the termini of the native peptide with an additional two amino acids gave a peptide with an unchanged activity profile but with enhanced stability to degradation by endoproteases.

Outlook. With one conotoxin drug on the market and several others in the pipeline, there is reason for optimism that this class of molecules will have a real impact in medicine in the future. The relatively small size of conotoxins makes them very amenable to chemical synthesis. This opens the possibility of the medicinal chemist improving on nature. So far, relatively unexplored areas include the substitution of D-amino acids, but the myriad of naturally occurring post-translational modifications of conotoxins suggests that a range of other synthetic chemical modifications may be made to improve biopharmaceutical properties. Cyclization appears to be a particularly promising approach. Considerable promise also exists that re-engineering the disulfide bonds of conotoxins may be a useful approach for modulating stability and activity.

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