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Diversity of *Conus* Neuropeptides

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***Conus* venoms contain a remarkable diversity of pharmacologically active small peptides. Their targets are ion channels and receptors in the neuromuscular system. The venom of *Conus geographus* contains high-affinity peptides that act on voltage-sensitive calcium channels, sodium channels, *N*-methyl-D-aspartate (NMDA) receptors, acetylcholine receptors, and vasopressin receptors; many more peptides with still uncharacterized receptor targets are present in this venom. It now seems that the *Conus* species (approximately 500 in number) will each use a distinctive assortment of peptides and that the pharmacological diversity in *Conus* venoms may be ultimately comparable to that of plant alkaloids or secondary metabolites of microorganisms. The cone snails may generate this diverse spectrum of venom peptides by a "fold-lock-cut" synthetic pathway. These peptides are specific enough to discriminate effectively between closely related receptor subtypes and can be used for structure-function correlations.**

CERTAIN TAXONOMIC GROUPS HAVE DEVELOPED CHARACteristic chemical strategies for interacting with other organisms in their environment. Thus, higher plants have evolved alkaloids, presumably as a defense against animals, and microorganisms (such as the *Streptomycetes*) have many secondary metabolites, including antibiotics. These compounds have provided a major source of raw materials for drug development. In these two cases, the agents used by the organisms constitute a large and varied set of molecules that are a characteristic biochemical specialization of the group.

Few interactions between organisms are more striking than those between a venomous animal and its envenomated victim. Venom may be used as a primary weapon to capture prey or as a defense mechanism (*1*). Venoms disrupt essential organ systems in the envenomated animal: many venoms contain molecules directed to receptors and ion channels of neuromuscular systems.

The predatory cone snails (*Conus*) have developed a unique biological strategy. Their venom contains small peptides that are targeted to various neuromuscular receptors and may be equivalent in their pharmacological diversity to the alkaloids of plants or secondary metabolites of microorganisms. These peptides are among the smallest nucleic acid–encoded translation products with defined conformations. Peptides in this size range normally equilibrate among many conformations (in order to have a fixed conformation, proteins generally have to be much larger).

Biology of *Conus*

The cone snails that produce these toxic peptides are a large genus of venomous gastropods comprising approximately 500 species (*2*). Since living *Conus* have inflicted stings fatal to humans, both the popular and scientific literature have alluded to the "beautiful but deadly cones" (*3*). Many times humans have been stung when, as collectors, they picked up cone snails for their striking shells; rarer varieties such as the glory-of-the-sea cone (*Conus gloriamaris*) and the matchless cone (*Conus cedonulli*) were once so highly prized that they were routinely listed at auctions with paintings by the Old Masters (*4*) (Fig. 1).

All cone snail species are predators that inject venom to capture prey. The spectrum of animals that the genus as a whole can envenomate is broad; in addition, a wide variety of hunting strategies is used. However, every *Conus* species uses fundamentally the same basic pattern of envenomation. The prey is harpooned with a dispos-

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able hollow tooth through which venom is injected (*5*) (Fig. 2).

There has been a worldwide expansive dispersal of the genus, much of it occurring relatively recently on a geological time scale. The first radiation of cone snails occurred long after the extinction of dinosaurs and ammonites, in the Eocene period (*6*). At the present time, cones are among the major predators in tropical reef communities and have adapted to almost every type of tropical marine habitat.

Although most *Conus* species prey on only a few species, the prey of the genus as a whole comprises at least five different phyla. One group of *Conus* feeds exclusively on other mollusks; several hundred species envenomate various marine worms (mostly polychaetes, and some echiuroids and hemichordates). Perhaps most remarkable are the fish-hunting *Conus* species (approximately 50); these are the only snails known to overpower and devour vertebrates (*7*). Thus, compared to other major groups of venomous predators, most geologically more ancient, the cone snails have been particularly successful in evolving a spectrum of venoms effective against very diverse types of prey.

Small Rigid Peptides

The major pharmacologically active molecules in all *Conus* venoms are small peptides, 10 to 30 amino acids in length (*8*). Most are exceptionally rich in disulfide bonds (Table 1). Almost invariably, *Conus* toxins are smaller than polypeptide toxins used by other venomous animals. Spider, scorpion, and snake venoms all contain toxins that are typically between 40 and 100 amino acids in length (in certain cases, for instance, β-bungarotoxin, they may be even larger). Thus, although in elapid snakes (such as cobras), paralytic toxins that target to the acetylcholine receptor are polypeptides of 60 to 80 amino acids (*9*), in fish-hunting cone snails the analogous peptides are only 13 to 15 amino acids in length (Table 1).

The peptides in Table 1 are from one *Conus* species of each major feeding type. Sequences of five peptides from a mollusk-hunting cone (*Conus textile*) and a worm-hunting species (*Conus quercinus*) are included. All of these peptides are disulfide-rich, with a cysteine content between 22 and 50%; most have three disulfide bonds. Although one of these peptides (the King Kong peptide) (*10*) has no detectable biological activity in vertebrates, all others are active in the vertebrate central nervous system. The symptomatologies that are induced by the peptides from nonpiscivorous venoms are generally different from those elicited by *Conus geographus* venom peptides.

Although these peptides can elicit diverse biological activities, a relatively conservative arrangement of cysteine residues is observed. The distribution of cysteine residues in the peptides of *C. textile*, *quercinus*, and *geographus* is nonrandom; two basic patterns emerge in peptides with very different biological activities (C . . . C . . . CC . . . C . . . C and CC . . . C . . . C . . . CC; Table 1) (*10*).

The major paralytic peptides of fish-hunting cone venoms were the first to be identified and are the most well characterized (*8*). In *C. geographus* venom, three classes of disulfide-rich peptides were found (Table 1): the α-conotoxins (target, acetylcholine receptors) (*11*); the μ-conotoxins (target, skeletal muscle Na^+ channels) (*12*);

Fig. 1. The cone shell and the Vermeer painting. Two legendary *Conus* species with striking shell patterns. (**Top**) *Conus gloriamaris*, the glory-of-the-sea cone, the most highly prized of all collectors' shells. (**Left bottom**) *Conus cedonulli*, the matchless cone. A specimen of this species was offered for sale at the Lyonet sale of 1796 along with Vermeer's painting, Woman in Blue Reading a Letter (**bottom right**). At this auction, two paintings by Franz Hals made just over 1 and 10 guilders, respectively. Vermeer's masterpiece sold for 43 guilders, and the *C. cedonulli* shell, only 5 cm in length, brought 273 guilders (*4*). Photographs of shells by K. Matz; the painting is reproduced with permission of the Rijksmuseum, Amsterdam.

and the ω-conotoxins (target, presynaptic neuronal Ca^{2+} channels) (*13*). There are multiple homologs in each toxin class; at least five different ω-conotoxins are present in *C. geographus* venom alone. Considerable variation in sequence is evident. When all available ω-conotoxin sequences are compared, only the cysteine residues involved in disulfide bonding, and one glycine residue are invariant. Indeed, no ω-conotoxin sequence (nor any other peptide of known sequence in *Conus* venoms) has been found in more than one venom. Each cone venom appears to have its own distinctive signature of conotoxin sequences.

These peptides are now standard research tools in neuroscience. The μ-conotoxins, because of their ability to preferentially block muscle but not axonal Na^+ channels, are convenient tools for immobilizing skeletal muscle without affecting axonal or synaptic events (*14*). The ω-conotoxins have become standard pharmacological reagents for investigating voltage-sensitive Ca^{2+} channels and are used to block presynaptic termini and neurotransmitter release (*15*). Conotoxins are also used in medical diagnosis; an immunoprecipitation assay with radiolabeled ω-conotoxin can be used to diagnose the Lambert-Eaton myasthenic syndrome (*16*), a disease in which autoimmune antibodies targeted to endogenous Ca^{2+} channels are inappropriately elicited, thereby causing muscle weakness and autonomic dysfunction.

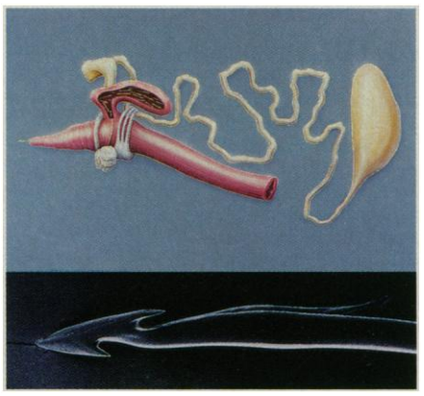

Fig. 2. (**Top panel**) The venom apparatus of *Conus*. The venom, which is made in the venom duct (long tube), is expelled by contraction of the muscular venom bulb (extreme right). The harpoon-like teeth are stored in the radular sac, and, by an unknown mechanism, one tooth is moved through the pharynx and into the proboscis (a single tooth shown in place at extreme left). Over 50 teeth may be stored in the radula sac. (**Bottom panel**) A scanning electron micrograph of the harpoon-like tooth from *Conus purpurascens*. Drawing by K. Matz.

Specific Conformations in Small Peptides

All major venomous taxa appear to have evolved polypeptide toxins in their venoms: snakes, scorpions, spiders, sea anemones, and cone snails (*1*). The molecules are mostly small- to intermediate-sized polypeptides that achieve significantly enhanced conformational stability through disulfide bonding.

Except for the cone snails, the toxins in most venoms are small proteins with cysteine residues involved in disulfide bonding distributed throughout the length of the peptide. This general motif is shared by extracellular blocking ligands of nonvenomous origin as well. One of the best studied of these is bovine pancreatic trypsin inhibitor (BPTI), a molecule of 58 amino acids that has three disulfide bonds (*17*). This molecule can be denatured in vitro but is readily refolded into a rigid structure that then binds tightly to its receptor target, trypsin, thereby inhibiting function. Although there are 15 ways to arrange the six cysteine residues of BPTI into three disulfide bonds, the biologically active disulfide configuration forms as the molecule folds into its final conformation (*17*). Polypeptide molecules in the size range of BPTI, therefore, contain sufficient information to fold in a specific manner. Subsequently, disulfide bonds covalently lock in this conformation. X-ray studies of the trypsin-BPTI complex have shown that only a small fraction of the amino acids in BPTI are in direct contact with the receptor target (*18*). The detailed structural data for BPTI can serve as a prototype of extracellular blocking ligands. We contrast this structure with that of the conotoxins (Fig. 3).

The peptides from cone snail venoms appear to be made by an adaptation of the strategy typified by BPTI. Most native toxin peptides in *Conus* venoms have three disulfide bonds, as does BPTI, but instead of being 58 amino acids in length, they vary in size from 12 to 27 amino acids. *Conus* peptides are assembled from intermediate-sized propeptide molecules approximately the size of BPTI (*19*). However, the conotoxin propeptides that have been sequenced contain the disulfide-locking groups only at the COOH-terminal end rather than distributed throughout the length of the polypeptide. Furthermore, once secreted and folded, the disulfide-rich COOH-terminal end of these molecules is proteolytically cleaved from the rest of the peptide to form the small, mature toxin that is densely cross-linked by disulfide bonds (Table 1 and Fig. 3). Thus, the amino acids directly involved in receptor interaction of the mature toxin are separated from those required to orchestrate the folding of the propeptide.

The final result is an exceptionally small blocking ligand greatly enriched in the amino acids that directly contact the receptor target

Table 1. Disulfide-rich peptides from *Conus* venom. Sequences are given in the standard one-letter amino acid code (*38*); P, hydroxyproline. The order of the last three amino acids in the second *C. quercinus* peptide has not yet been definitively established. Note that all the *C. textile* and *C. quercinus* peptides have the same cysteine residue grouping as either μ-conotoxin (CC...C...C...CC) or ω-conotoxin (C...C...CC...C...C); intracranial, ic.

Conus species	Sequence	Name of peptide	Effect (target)	Reference
C. geographus (fish hunter)	E**CC**NPA**C**GRHYS**C***	α-Conotoxin GI	Paralysis in vertebrates (acetylcholine receptor at neuromuscular junction)	(*11*)
	RD**CC**TPPKK**C**KDRQ**C**KPQR**CC**A*	μ-Conotoxin GIIIA	Paralysis in vertebrates (skeletal muscle Na^+ channel)	(*12*)
	CKSPGSS**C**SPTSYN**CC**RS**C**NPYTKR**C**Y*	ω-Conotoxin GVIA	Paralysis in lower vertebrates; shaking symptons upon ic injection in mice (presynaptic Ca^{2+} channel)	(*13*)
C. textile (mollusk hunter)	W**C**KQSGEM**C**NLLDQN**CC**DGY**C**IVLV**C**T	"King Kong" peptide	Makes lobsters assume dominant posture contractions in snails; no symptoms in mice	(*9*)
	CCRT**C**FG**C**TP**CC***	"Scratcher" peptide	Scratching in mice	(*39*)
	N**C**PY**C**VVY**CC**PPAY**C**EASG**C**RPP*	"Convulsant" peptide	Convulsions in mice	(*39*)
C. quercinus (worm eater)	**CC**SQD**C**LV**C**IP**CC**PN*		Scratching in mice	(*28*)
	DQS**C**PW**C**GFT**CC**LPNY**C**QGLT**C**(T,V,I)		Scratching and restlessness in mice	(*28*)

*Amidated COOH-terminus.

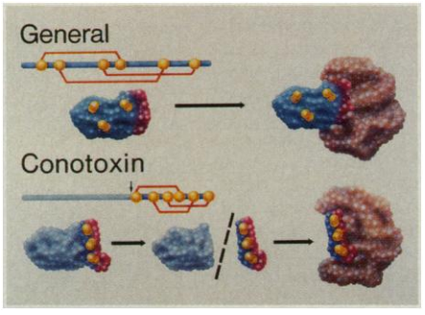

Fig. 3. A representation of the "fold-lock-cut" strategy for synthesis of *Conus* peptides. The generation of *Conus* peptides is contrasted with the more general strategy for most polypeptide toxins in other venoms. (**Top**) A common motif of polypeptide toxins in venoms and other extracellular blocking ligands such as bovine plasma trypsin inhibitor (BPTI). These are polypeptides of 40 to 100 amino acids, with multiple disulfide bonds (in yellow), distributed throughout the polypeptide. When the blocking ligand binds to its receptor target, only a small fraction of amino acids directly interact with the receptor (in red). (**Bottom**) The conotoxin propeptides are approximately the same size as most extracellular blocking ligands, between 40 and 100 amino acids. However, the disulfide bonds are concentrated at the COOH-terminus. Once the conotoxin propeptide folds and specific disulfide bonds form, a proteolytic cleavage occurs such that the highly disulfide-bonded COOH-terminus is released as the mature toxin. This disulfide-rich mature peptide, only 12 to 30 amino acids in length, binds its receptor target with high affinity and specificity.

(Fig. 3). Without "locking in" the disulfide-bonded framework by this "fold-lock-cut" strategy, peptides as small as mature conotoxins would not be expected to assume a defined conformation. The mature "scratcher" conotoxin from *C. textile* (Table 1) is an example of this specialized strategy. This 12–amino acid peptide contains three disulfide bonds; half of all amino acids are thus cysteine residues involved in disulfide bonding. The evolution of such small blocking ligands in *Conus* venoms may be a response to strong selection for rapid prey paralysis, resulting in venom components that diffuse rapidly upon injection.

Targeting to Receptor Subtypes

A potentially useful characteristic of conotoxins is that they combine a high affinity for their macromolecular receptors with a narrow receptor-target specificity. A major problem in medicine is the side effects of drugs, some of which result from drug binding not only to the particular receptor subtype that provides therapeutic value, but to closely related, therapeutically irrelevant receptor subtypes as well. Binding to these irrelevant receptors may cause undesirable physiological effects. In contrast to most drugs, the conotoxins can discriminate among closely related receptor subtypes.

The ω-conotoxin GVIA from *C. geographus* venom (Table 1), which binds to neuronal voltage-sensitive Ca^{2+} channels (*20*), is a relevant example. The affinity (K_d) of ω-conotoxin GVIA for its high-affinity targets is sub-picomolar; it takes more than 7 hours for 50% of the peptide to dissociate. Thus the peptide can be used to block synaptic transmission virtually irreversibly (*21*) because it inhibits presynaptic Ca^{2+} channels. However, ω-conotoxin is highly tissue-specific. In contrast to the standard Ca^{2+} channel–blocking drugs (the dihydropyridines such as nifedipene and nitrendipene, widely used for angina and cardiac problems), which can bind Ca^{2+} channels in smooth, skeletal, and cardiac muscle as well as neuronal tissue, ω-conotoxin binds only to a subset of neuronal Ca^{2+} channels, primarily of the N subtype (*15, 21*). The discrimination ratio for ω-conotoxin binding to voltage-sensitive Ca^{2+} channels in neuronal versus nonneuronal tissue (skeletal or cardiac muscle) is greater than 10^8 in certain cases.

Such narrow specificity must be important to the snail to achieve efficient envenomation. If a ligand were only designed to exhibit as high an affinity as possible to presynaptic Ca^{2+} channels, it would also bind significantly to related Ca^{2+} channel subtypes with homologous binding sites. However, after injection, a Ca^{2+} channel ligand in a *Conus* venom would enter the circulatory system before gaining access to the neuromuscular junction. If an ω-conotoxin could not discriminate among Ca^{2+} channel subtypes, it would bind the Ca^{2+} channel of blood vessel smooth muscle, a target physiologically irrelevant to paralysis. These Ca^{2+} channels would have to be saturated before an effective toxin dose for the presynaptic channels could be achieved. Clearly, there is a selective advantage to toxins that can effectively discriminate among closely related receptor subtypes. The ω-conotoxins have determinants that confer high affinity for presynaptic Ca^{2+} channels, but also have structural features that discriminate against muscle Ca^{2+} channel subtypes. Because these peptide toxins are relatively small, it should be possible to precisely identify the discriminatory structural elements.

Other conotoxins also show greater receptor target discrimination than analogous organic molecule ligands that bind the same macromolecular receptors (*22, 23*). The μ-conotoxins discriminate between muscle and axonal Na^+ channels, while the classical Na^+ channel toxins tetrodotoxin and saxitoxin do not. Similarly, α-conotoxin GI discriminates between neuromuscular and neuronal acetylcholine receptors, while curare and carbamylcholine do not. Toxins with such discriminatory properties may occur in other venoms as well.

Diversity of Peptides in *Conus* Venoms

The major toxins we described above from the venom of a fish-hunting *Conus* (such as *C. geographus*) constitute a potent pharmacological brew: the α-, ω-, and μ-conotoxins block presynaptic and postsynaptic termini of the neuromuscular junction, as well as inhibit electrical conduction in muscle. To match this, a potion would have to contain curare, the poison arrow ingredient of the Amazonian Indians; tetrodotoxin, the deadly toxin in fugu puffer fish; and a presynaptic blocker such as botulinum toxin. However, these three potent conotoxins are only a few of the multitude of venom neuropeptides.

The large variety of peptides in *Conus* venoms is illustrated in Fig. 4, which shows the effect on mice of components from one of the four size fractions of *C. geographus* venom. Most components in this crude fraction are not directly paralytic either to fish or mammals; biological activity can often only be revealed upon injection into the mammalian central nervous system. Clearly, there are many active components, which cause a wide spectrum of different behavioral effects from convulsions, to sleep, to scratching.

Most of the biologically active components in venom have proven to be peptides. Some of these peptides have been purified to homogeneity (*11–13*); the primary amino acid sequences of three peptides from peaks in Fig. 4, each of which elicits different symptoms in mice, are included in Table 2. Apart from the three major classes of paralytic conotoxins (α, μ, ω), most other biologically active peptides in *Conus* venoms that have been sequenced appear unrelated to each other and are likely to bind to different macromolecular receptors (*24–27*). The receptors at which most of these peptides act have yet to be determined.

Conus geographus is not unique among *Conus* venoms in containing a broad spectrum of biologically active peptides. Other fish-hunting cone venoms (such as the one from *Conus magus*) are comparably diverse. Even venoms of worm-hunting cones (such as *C. quercinus*) show remarkably complex peptide profiles (*28*).

In addition to peptide diversity within a single venom, there is also an impressive interspecies diversity. The peptides from a snail-

Table 2. *Conus* venom peptides containing γ-carboxyglutamate. *Conantokin* comes from the Filipino word antokin, meaning sleepy. Sequences are given in the standard one-letter amino acid code (*38*); γ, γ-carboxyglutamate.

Species	Sequence	Name of peptide	Effect (target)	Reference
C. tulipa	GEγγYQKMLγNLRγAEVKKNA*	Conantokin-T	Sleeping in young mice; hyperactivity in older mice (NMDA receptor)	(*30*)
C. geographus	GEγγLQγNQγLIRγKSN*	Conantokin-G	Sleeping in young mice; hyperactivity in older mice (NMDA receptor)	(*25*)
C. geographus	**AC**SGRGSR**C**PPQ**CC**MGLR**C**GRGNPQK**C**IGAHγDV	Conotoxin-GS	Lethal upon ic injection (Na^+ channel)	(*27, 40*)
C. geographus	KFLSGGFKγI$\overline{\text{V}}$**C**HRY**C**AKGIAKEF**C**N**C**PD*		Scratching (unknown target)	(*26, 40*)

*These peptides were isolated and their structures determined by procedures described by Olivera and co-workers (*12, 25, 30*). Sequences were obtained with standard gas-phase sequencing methods.

hunting *Conus* (*C. textile*) venom contrast markedly with those from a fish-hunting *Conus* venom (Table 1). Among venoms of more closely related species, some physiological overlap occurs, although each venom possesses novel components. Thus, the fish-hunting *Conus striatus* venom appears to lack the μ-conotoxins found in *C. geographus* venom, whereas a major *C. striatus* toxin causing excitatory symptoms (such as spastic paralysis) is not found in *C. geographus*. Homologous peptides also occur: all fish-hunting *Conus* venoms examined contain ω-conotoxins, but each venom displays its distinct complement of molecular forms.

How have the cone snails generated so many diverse peptides in their venoms? Recently, evidence suggesting an origin of this prodigious capacity of the cone snails for peptide innovation has been obtained (*19*). This peptide diversification scheme is an outgrowth of the specialized fold-lock-cut pathway for generating small peptides (Fig. 3) and could account for conotoxin diversification.

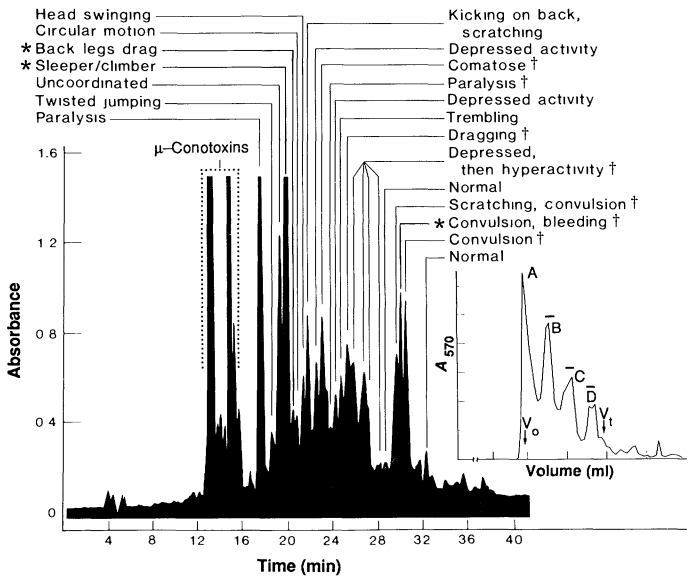

Fig. 4. Multiple biologically active components in a single *Conus* venom. Injection of ~0.5 to 2 nmol of each fraction intracranially into mice elicits the symptoms shown above each peak. (**Inset**) A profile of the elution pattern of whole *C. geographus* venom from Sephadex G-25. The column was run as described (*12*). The four major peaks contain μ-conotoxins (peak B), ω-conotoxins (peak C), and α-conotoxins (peak D). If the components in peak B are further separated by high-performance liquid chromatography (HPLC) with a VYDAC C18 column and a trifluoroacetic acid–acetonitrile gradient, the elution profile shown in the main figure is obtained. The absorbance at 210 nm of each fraction is plotted as a function of the elution time. In many cases, a peak in the figure contains a mixture of several biologically active peptides, and the activity observed is either the symptomatology induced by the most potent component or a composite of symptoms from several peptides. *The peaks from which the Gla-containing peptides in Table 2 were purified: conantokin-G, conotoxin-GS, and "scratcher" peptide (left to right). †The fraction was lethal in at least one injected animal.

We characterized a family of propeptide molecules (the King Kong family from *C. textile*) (*10, 19*). When the propeptide sequences are aligned, obvious constant and hypervariable regions are defined. The constant regions comprise the excised NH_2-terminus of the propeptides (containing presumptive folding determinants), as well as the cysteine residues in the mature toxin (which are directly involved in the disulfide configuration). The cysteine residues are totally conserved and the amino acids in the pre-propeptide that signal secretion and direct folding are more than 90% identical. In contrast, the presumptive ligand-specificity determinants (all amino acids other than cysteine in the mature toxin region) are hypervariable; none of the 20 amino acids (non-cysteine) of the mature toxins is conserved in all three sequences. Thus, hypervariability is focused on the intercysteine regions of the mature peptide. A diagram of the relation between these propeptide molecules and an actual sequence is shown in Fig. 5. By using this scheme, peptides that act at different receptors can be generated, but the folding pathway is conserved. This is a consequence of hypervariation of contact determinants at the COOH-terminus accompanied by concomitant conservation of the NH_2-terminal folding determinants. The presence of the three peptides in a single *Conus* venom with such strikingly divergent contact determinant sequences is indicative that cone snails may use specialized genetic mechanisms (such as cassette switching or site-specific recombination) to focus hypervariability to regions enriched in the contact amino acids determining receptor specificity. Conotoxin propeptides, with constant and hypervariable domains, are in this respect somewhat analogous to antibody molecules. In both cases, the constant regions specify a structural framework, whereas hypervariability exists to generate ligands of diverse target specificity.

γ-Carboxyglutamate in *Conus* Peptides

In addition to a high density of disulfide cross-links, a second general strategy may be used by the genus to make small, effective ligands. *Conus* peptides have a high frequency of the unusual amino acid, γ-carboxyglutamate (Gla) (*29*). Approximately 20% of all peptides sequenced contain Gla (*26*). Some Gla-containing peptides from *Conus* venoms are illustrated in Table 2. These include three peptides purified from fractions in the chromatogram in Fig. 4. In addition to the peptides in Table 2, sequences have been established for eight other Gla-containing *Conus* peptides (*26*). All the Gla-containing sequences in Table 2 are significantly less disulfide-rich than the peptide sequences in Table 1 (<15% Cys). Conantokin-G and conantokin-T have no disulfide bonds at all (*25, 30*). Nevertheless, these peptides may be conformationally rigid under physiological conditions (*31*).

The number of Gla-containing peptides detected and sequenced from *Conus* already exceeds the number of biochemically characterized Gla-containing proteins in all other systems combined. In fact, the concentration of Gla in some crude *Conus* venoms is greater than in pure mammalian Gla-containing proteins (*32*). In mammalian systems, the formation of Gla occurs via vitamin K–dependent posttranslational γ-carboxylation of glutamate residues in polypeptides (*29*). γ-Carboxyglutamate is found in several proteins of the blood-clotting cascade (such as prothrombin) and in two mammalian bone proteins; the conversion of prothrombin to thrombin is dependent on Gla. γ-Carboxyglutamate may play either a structural or a targeting role in *Conus* peptides. In the conantokins, the Ca^{2+} chelate of Gla facilitates α helix formation. It has also been suggested that the presence of Gla may target Gla-containing *Conus* peptides to receptors on acidic membranes (*31*).

The physiological mechanism through which most of the Gla-containing *Conus* peptides elicit biological activity is unknown. Most are compositionally dissimilar and, in fact, cause different behavioral symptoms when tested in vivo. It is probable, therefore, that these peptides bind to a variety of receptors and ion channels. Conotoxin-GS, for instance, targets voltage-sensitive Na^+ channels (*27*).

The two conantokins (formerly called the "sleeper" peptides) (*5*) target glutamate receptors of the *N*-methyl-D-aspartate (NMDA) subtype (*29, 33*). The most well characterized of these is conantokin-G, a 17–amino acid peptide from *C. geographus* venom, with five residues of Gla and no disulfides. Symptoms induced by conantokins in live mice show a characteristic developmental switch (*25*); injection into animals that are less than 2 weeks old induces a sleeplike syndrome. However, in mice 3 weeks old or older, a similar injection elicits a hyperactive state, often characterized by climbing of walls or continuous darting between the corners of the cage. Mice of transitional age will often cycle between these two behaviors. It is not clear why a fish-hunting snail such as *C. geographus* should produce a neuropeptide with NMDA antagonist activity. The relevant physiological targets of these peptides may be glutamate-mediated synapses in peripheral sensory circuits of fish (*34*).

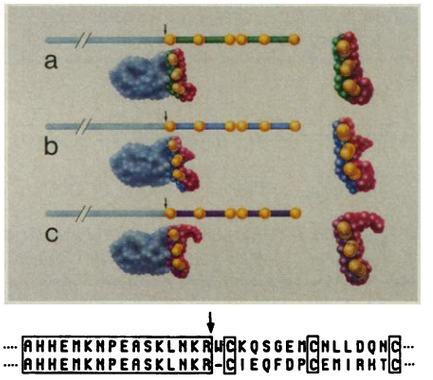

Fig. 5. Constant and hypervariable regions in conotoxin propeptides. A family of conotoxin propeptides, the King Kong peptides from *C. textile*, is shown as an example. (**Top**) When the sequences of the three members of the family are compared, constant and hypervariable regions are defined. Constant regions comprise the NH_2-terminal 50 amino acids of the prepropeptides (in purple), as well as the cysteine residues of the mature toxin (in yellow). The hypervariable regions include all intercysteine amino acids in the mature toxins (other colors). Arrow, the site of proteolytic cleavage. Below each linear diagram of the propeptide is a three-dimensional representation of the propeptide. After proteolytic cleavage, the three mature peptides generated have a conserved disulfide framework. However, the amino acids that putatively interact with the receptor target are different in each peptide; thus, each mature peptide would target a different receptor target binding site. Consequently, the conserved regions generate a conservative structural framework of disulfide bonds, while the hypervariable regions create a set of small peptides with different ligand specificity. The mature toxins generated are small, typically 12 to 30 amino acids. (**Bottom**) The actual amino acid sequences of two members of the King Kong family of peptides at the boundary between the constant excised NH_2-terminal region and the mature peptide region. Proteolytic cleavage occurs at the site indicated by the arrow to generate the mature King Kong peptide.

Perspectives

Conus venoms and their constituent peptides are so diverse that they could prove to have a pharmaceutical potential comparable to plant alkaloids or the fermentation products of microorganisms. It may prove productive to screen *Conus* venoms whenever a ligand targeted to a particular receptor or ion channel is desired. Indeed, such an approach has been validated. Czerwiec and co-workers (*35*) surveyed 22 *Conus* venoms for ligands specific for the α_2-adrenergic receptor; most venoms exhibited either very low or no binding activity. However, in five venoms there was a clear high-affinity α_2-adrenergic binding activity. The five venoms that had the α_2-adrenergic–specific agent were not derived from closely related species; presumably some of these α_2-adrenergic–specific agents evolved independently to act on relevant receptors in different species of prey. Both the venoms (from 17 species) that are negative on the α_2-adrenergic ligand screen and the five that were positive were produced by *Conus* of all major feeding types (fish-hunting, snail-hunting, and worm-hunting). Additionally, the five positive venoms differed by 60-fold in specific activity, suggesting an enormous variation in the concentrations of specific α_2-adrenergic–targeting ligands.

Attempts have also been made to identify Na^+ channel–specific ligands (*27, 36*). One of these studies yielded a surprising result. *Conus geographus* venom has one set of major paralytic toxins that act on Na^+ channels (the μ-conotoxins). A second class of apparently unrelated toxins from this venom competes for binding to exactly the same site on Na^+ channels as the μ-conotoxins (*27*). Thus it would appear that in this species, two classes of peptide toxins have independently evolved to target Na^+ channels. Since no other peptides have been described that act on the tetrodotoxin-saxitoxin binding site of Na^+ channels, it is remarkable that both classes so far characterized occur in the same *Conus* venom. In contrast, a variety of other *Conus* venoms have no Na^+ channel–specific ligands that can be detected by this assay (*36*).

A priori, there seems to be no reason why either an NMDA receptor–specific ligand or an α_2-adrenergic–specific ligand should be useful to a cone snail, but these molecules are in fact present in several *Conus* venoms. Thus the biology of envenomation by cone snails is so sophisticated (and our ignorance sufficiently pervasive) that screening *Conus* venoms for specific ligands is an approach with great potential.

The *Conus* peptides may be the smallest genetically encoded polypeptides of defined conformation. Consequently, they have an intriguing duality. As exceptionally small, conformationally stable peptides, they can be analyzed with a variety of physical and chemical methods (specific conformations are much more easily determined in a 13–amino acid, rigid peptide that can be chemically synthesized than in a standard-sized protein of several hundred amino acids). The conformation of conotoxins was recently determined by two-dimensional nuclear magnetic resonance (*37*). Once conformations have been established, straightforward amino acid modification strategies allow the introduction of reporter groups into specific loci in the peptide, which can then be localized in the three-dimensional structure. Thus, *Conus* peptides are defined molecules that can be relatively easily synthesized, chemically modified, analyzed, and manipulated. However, *Conus* peptides are also primary translation products of genes, with potent biological activity, and these peptides can be manipulated by the techniques of modern molecular genetics. This dual quality confers on the *Conus*

peptides an important role in the expanding bridge between chemistry and modern molecular genetics.

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39. The peptides were isolated as described by C. A. Ramilo (thesis, University of the Philippines, Manila, 1986), and the amino acid sequences were determined as in (*10*).
40. B. M. Olivera *et al.*, unpublished data.

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