Conus Peptides as Chemical Probes for Receptors and Ion Channels

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I. Introduction

A. Scope of the Review

Marine snails belonging to the genus *Conus* ("cone snails") are venomous predators that have evolved an unusual biochemical strategy for envenomating their prey.[1] Their venoms contain a large number (40–200) of small, conformationally constrained peptides, each with highly potent and specific biological activity. The size of most peptides present in cone snail venoms is 10–30 amino acids; polypeptide toxins directly encoded by genes from other animal venoms are significantly larger, usually 40–100 amino acids in length (this is true of the polypeptide toxins from snakes, spiders, scorpions, and sea anemones).[2–4] Although small peptide toxins have been reported (e.g., apamin in bee venom, sarafatoxin in snake venom[5,6]), their occurrence is generally sporadic in other taxa. In the cone snails, it is a major feature of the venoms of all species examined (see Figure 1). Furthermore, the number of peptides in each venom is unprecedented both in the great variety of sequences as well as the remarkable pharmacological spectrum encompassed. These peptides are the general subject of the present review.

The *Conus* peptides are currently being used in hundreds of research laboratories for a wide variety of physiological and pharmacological investigations in both vertebrate and invertebrate nervous systems. Some *Conus* peptides (e.g., ω-conotoxin GVIA) have become well-established neurobiological tools, and these applications will no doubt continue to expand. We will *not* provide a comprehensive review of the pharmacological and physiological applications of specific *Conus* peptides here but will concentrate on their chemistry and biochemistry. One focus of this review will be the use of *Conus* peptides as sophisticated ligands for large multisubunit receptor complexes on the surface of cells. Every *Conus* peptide probably targets to a specific receptor protein on the plasma membrane; consequently, these peptides are proving to be refined *chemical* probes of potentially great resolving power. Several nascent developments are rapidly establishing a standard methodology for using *Conus* peptides to investigate their protein targets. As such, these peptides should become an increasingly important set of tools that link chemistry with molecular physiology.

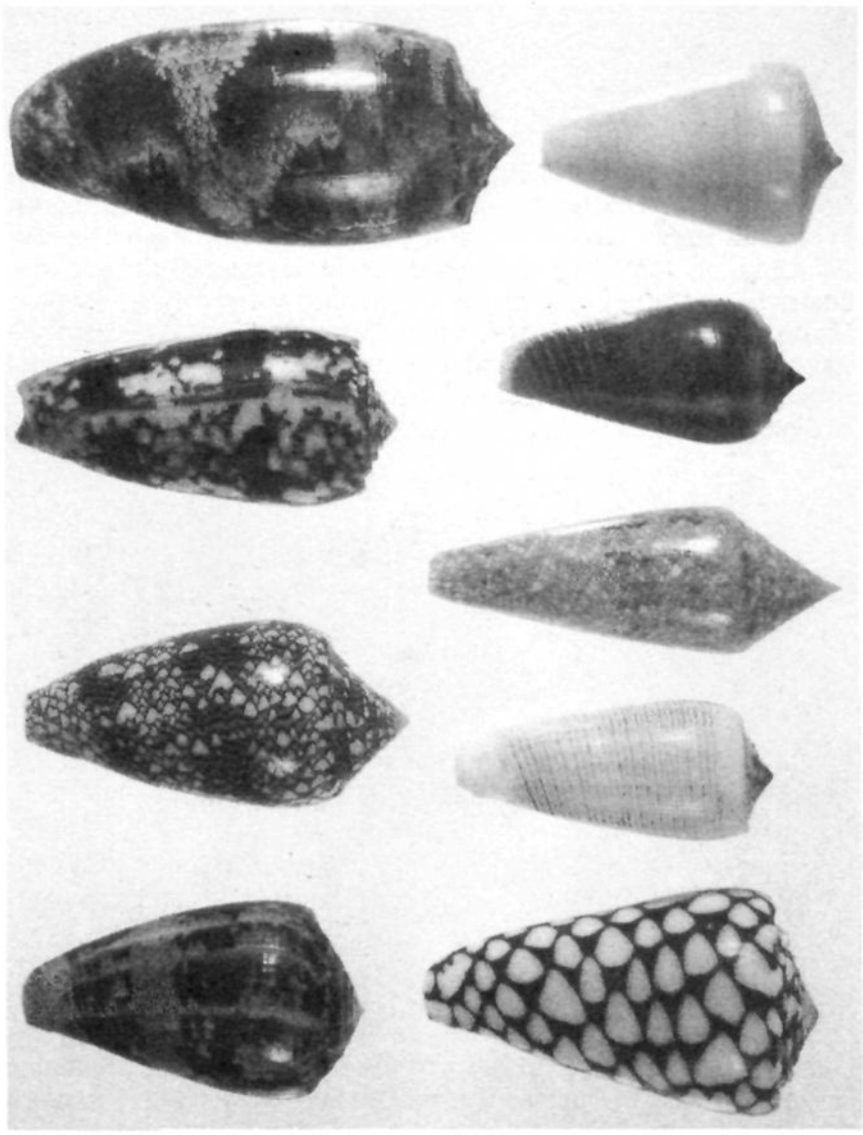

Figure 1. Shells of some *Conus* species, from which venom peptides have been purified and biochemically analyzed. Left row, top to bottom: *Conus geographus* (the geography cone), *C. striatus* (the striated cone), *C. textile* (the cloth-of-gold cone), and *C. purpurascens* (the purple cone). Right row, top to bottom: *C. quercinus* (the oak cone), *C. radiatus* (the radial cone), *C. gloriamaris* (the glory-of-the-sea), *C. magus* (the magus cone), and *C. marmoreus* (the marble cone). The *Conus* peptide sequences which have been published are shown in Table II; although peptides have been characterized from *C. purpurascens*, *C. radiatus*, and *C. gloriamaris*, these have not yet been published. (B. Olivera, L.J. Cruz, J. Rivier, and K.-J. Shon; unpublished results.)

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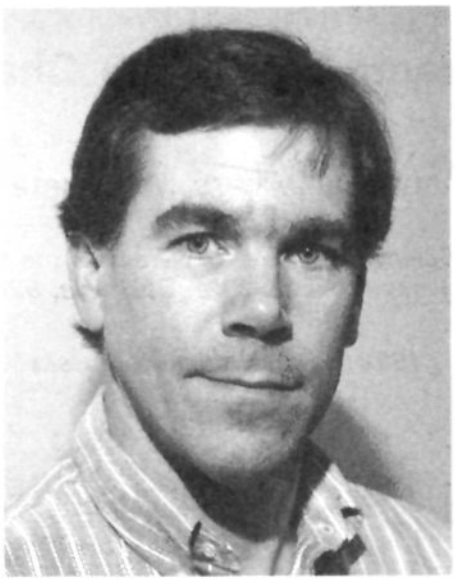


Richard A. Myers holds B.S. and M.S. degrees in Biology from Brigham Young University. In 1989 he received his Ph.D. degree in Biochemistry from the University of Utah where he worked with Bill Gray on the chemical synthesis of α-conotoxins and the interaction of these peptides with the nicotinic acetylcholine receptor. His postdoctoral work was with Toto Olivera studying *Conus* peptides as probes for the characterization and purification of novel neuronal receptor subtypes. He is presently a Research Assistant Professor of Biology at the same institution where he pursues these interests.

Lourdes J. Cruz is a Professor of the Marine Science Institute, University of the Philippines Diliman, and a Research Professor of the Department of Biology, University of Utah. She obtained her B.S. degree in Chemistry from the University of the Philippines and a Ph.D. degree in Biochemistry from the University of Iowa. Her major research interests are the characterization of neuropeptides from *Conus* venom and their action on biological systems. Her collaboration with B. M. Olivera on the *Conus* project, which started in the early 1970s in the University of the Philippines Manila has resulted in numerous publications on conotoxins and other *Conus* peptides. She is a member of the National Academy of Science and Technology of the Philippines.

B. Overview of Relevant Scientific Areas

A fundamental breakthrough in understanding how the different cells in a multicellular organism communicate with each other has occurred, with the elucidation of the general role of receptor proteins, ion channel complexes and signal transduction systems.[7,8] Advances in recombinant DNA technology have permitted an impressive catalog of receptor and ion channel molecules to be assembled,[9] and the number of new receptor proteins and ion channel subunits that are being cloned is increasing at an accelerating pace. This rapid progress has had, as an inadvertent consequence, a widening gap between the number of cell surface proteins cloned, and those for which even a modest amount of structural and/or functional information is available.

Jean E. Rivier is currently Professor (since 1989) at the Clayton Foundation Laboratories for Peptide Biology at The Salk Institute for Biological Studies which he joined as research Associate in 1970. He obtained his Ph.D. in Organic Chemistry from the Université de Lausanne in Switzerland in 1968 and spent one year as a post-doctoral fellow at Rice University, Houston, TX. His research at Salk on the structure and function of peptide hormones (most particularly the hypothalamic hormones) was aimed initially at their characterization and total synthesis. Presently he is focusing his research on the understanding at the molecular level of the interactions of these hormones with their respective receptors. From these ongoing studies, several peptides were selected for extensive clinical investigations in the areas of reproduction, growth, and stress. More recently, he has expanded his interests to the study of other neuropeptides and peptide toxins. He pioneered the use of RP-HPLC for peptide/protein isolation and characterization in the mid-1970s. He has published numerous scientific papers and coedited the 1989 and 1991 Proceedings of the American Peptide Symposium. He is number 12 on the list of the 100 most-cited authors ranked by citations to papers indexed in the 1981–1990 *Science Citation Index*. He serves on the Editorial Board of the *Journal of Medicinal Chemistry*. Dr. Rivier was the recipient of the Gordon Peptide Conference 1990 Vincent du Vigneaud Award and is currently President-elect of the American Peptide Society for 1991–1993.

Baldomero M. Olivera, called Toto by his friends, was born in Manila and received his B.S. in Chemistry from the University of the Philippines. He holds a Ph.D. degree in Chemistry from Caltech where he worked with Norman Davidson on the electrophoretic properties of DNA and nucleohistones. His post-doctoral work was carried out at Stanford University, where he worked with I. R. Lehman on the discovery and characterization of *E. coli* DNA ligase. He is a presently a Distinguished Professor of Biology at the University of Utah. His research interests have included discontinuous DNA replication, NAD metabolism, and ADP-ribosylation. During a four-year period where he held a faculty position at the University of the Philippines, he initiated the work on *Conus* peptides with Lourdes J. Cruz. These peptides are the subject of the present review.

Since cell surface receptor proteins or ion channels are often large and multisubunit complexes, they are difficult to analyze directly by either X-ray crystal-

Table I. Known Targets of *Conus* Peptides

conopeptide class	target[a]	subtype specificity	conopeptide examples	ref(s)
α-conotoxin	nicotinic acetylcholine receptor	neuromuscular junctions	GI	21
			SI	22
μ-conotoxin	voltage-sensitive sodium channel	skeletal muscle and post-ganglionic sympathetic neurons	GIII	23,24
ω-conotoxin	voltage-sensitive calcium channel	neuronal (N-type)	GVIA	20
		neuronal (broader specficity)	MVIIC	12
conantokin	glutamate receptor	NMDA[b] subtype	conantokin-G	25,26
conopressin	vasopressin receptor	(not determined)	conopressin-G	27

[a] The sites of action of many *Conus* peptides are still unknown. [b] Glutamate and glycine are coagonists of the *N*-methyl-D-aspartate (NMDA) receptor.

lography or NMR techniques.[10] A *Conus* peptide and its target protein are analogous to a small key and a very large lock. The structure of a *Conus* peptide can serve to define the complementary interacting surface on the cognate target "lock", invariably a functionally important site on the large receptor protein, since peptide binding interferes with target function. The small, rigid *Conus* peptide structures are relatively straightforward to solve by multidimensional NMR techniques,[11] but a direct structural analysis of the large multisubunit membrane-bound protein complexes which they bind will probably remain a formidable task. Thus, elucidation of the structure of a *Conus* peptide, if accompanied by a determination of how the peptide is oriented on the target protein, could provide important information regarding key ligand binding sites of receptor and ion channel complexes. An additional advantage is that because they are the direct translation products of genes, *Conus* peptides can be readily manipulated and analyzed by the most advanced recombinant DNA technologies,[1,12-14] but because they are relatively small, direct chemical synthesis can also be carried out.

II. General Background

A. Biological Considerations

There are ca. 500 living species of cone snails, presently included in a single large genus, *Conus*. All cone snails are venomous predators, and various *Conus* species feed on fish, other gastropod mollusks, or polychaete worms[15] as well as two smaller phyla (hemichordates and echiuroid worms). The cone snails are relatively recently evolved[16] when compared to other larger taxa of venomous animals (i.e., snakes, spiders, scorpions); nevertheless, the genus exhibits the greatest diversity of prey of any generic group of venomous predators. Most venomous animals prey on members of their own phylum, presumably because it is easier to evolve effective toxins to more closely related prey by modifying endogenous ligands to the predators own key physiological components.

In general, each cone snail species is a highly specialized predator; indeed, certain *Conus* will feed on only a single prey species. All species are believed to use a biochemically complex venom, made in a long, tubular venom duct, as the major weapon for prey capture. The venom is injected into the prey through a chitinous, hollow harpoon-like tooth which serves a dual function: first, to mechanically tether the prey, and second, to inject the venom.[15,17,18] The fish-hunting cones are able to achieve remarkably rapid paralysis after striking the prey with their disposable harpoons. In the rich marine environments where cone snails are found, speed of capture and paralysis of prey may be particularly critical. Not only must the snails immobilize their swifter prey, but they are vulnerable to being preyed upon while exposed. Toward these ends, the 500 different cone snail species[19] have evolved their novel biochemical strategies.

B. *Conus* Peptide Chemistry: General Features

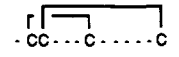
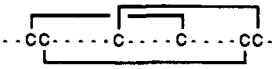
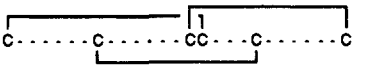
The major biologically active components characterized from *Conus* venoms are small, highly constrained peptides, 10–30 amino acids in length. Each peptide is believed to specifically target a receptor protein on the cell surface. Several families of *Conus* peptides which are specific for the same receptors have been elucidated[12,20-27] (see Table I). Among these are peptides which target to nicotinic acetylcholine receptors, voltage-sensitive calcium channels, sodium channels, and *N*-methyl-D-aspartate (NMDA) receptors (a type of excitatory amino acid receptor). As shown in Table II, the members of a peptide family are recognizably homologous to each other, but there is also surprising hypervariability observed between family members (see next section).

In addition to the *pharmacological families* of *Conus* peptides, there are *structural classes* that can be defined by characteristic patterns of arrangement of either the Cys residues, or of the post-translationally modified amino acid, γ-carboxyglutamate (Gla).[1,46] Both the arrangement of cysteine residues and/or γ-carboxyglutamate residues provide a structural framework that permits these small peptides to assume a specific, relatively rigid conformation. The cysteine residues have the capacity to form disulfide cross-links, while Gla residues have the potential for stabilizing α-helical regions in the presence of Ca^{2+}.

It appears that there are only a few structural classes found among all *Conus* peptides. A structural class comprises multiple pharmacological families of peptides. Thus, more than 30% of all sequenced *Conus* peptides belong to a single structural class, typified by the arrangement of cysteine residues in ω-conotoxins (i.e., C–C–CC–C–C, the "4-loop Cys framework").[13,46] This structural class not only includes the ω-conotoxin family (which targets vertebrate voltage-sensitive calcium channels), but the "King-Kong" family[40] of peptides as well (which does *not* target voltage-sensitive calcium channels). Over two-thirds of the *Conus* peptides sequenced belong to the three most common structural classes.

A structural class of *Conus* peptides also shares extensive sequence homology in the peptide precursor

Table II. Amino Acid Sequences of Naturally Occurring *Conus* Peptides

family	peptide	sequence[c]	ref(s)
α-conotoxin	GI	ECCNPACGRHYSC*	21
	GIA	ECCNPACGRHYSCGK*	21
	GII	ECCHPACGKHFSC*	21
	MI	GRCCHPACGKNYSC*	28
	SI	ICCNPACGPKYSC*	22
	SIA	YCCHPACGKNFDC*	29
	SII	GCCCNPACGPNYGCGTSCS^	30
	disulfide linkages	 (2-loop structural class)	31,32
μ-conotoxin[a]	GIIIA	RDCCTOOKKCKDRQCKOQRCCA*	23,24
	GIIIB	RDCCTOORKCKDRRCKOMKCCA*	23,24
	GIIIC	RDCCTOOKKCKDRRCKOLKCCA*	24
	disulfide linkages	 (3-loop structural class)	33
ω-conotoxin	GVIA	CKSOGSSCSOTSYNCCR-SCNOYTKRCY*	20
	GVIB	CKSOGSSCSOTSYNCCR-SCNOYTKRCYG*	17
	GVIC	CKSOGSSCSOTSYNCCR-SCNOYTKRC*	17
	SVIA	CRSSGSOCGVTSI-CC-GRC--YRGKCT*	30
	SVIB	CKLKGQSCRKTSYDCCSGSCGRS-GKC*	30
	GVIIA	CKSOGTOCSRGMRDCCT-SCLLYSNKCRRY*	17
	GVIIB	CKSOGTOCSRGMRDCCT-SCLSYSNKCRRY*	17
	MVIIA	CKGKGAKCSRLMYDCCTGSCRS--GKC*	34
	MVIIB	CKGKGASCHRTSYDCCTGSCNR--GKC*	34
	MVIIC	CKGKGAPCRKTMYDCCSGSCGRR-GKC*	12
	disulfide linkages	 (4-loop structural class)	31,35
conopressin	Arg-conopressin-S	CIIRNCPRG*	27
	Lys-conopressin-G	CFIRNCPKG*	27
conantokin (sleeper peptides)	conantokin-G	GEγγLQγNQγLIRγKSN*	36
	conantokin-T	GEγγYQKMLγNLRγAEVKKNA*	37
miscellaneous[b]			
C. geographus peptides	conotoxin GS*	ACSGRGSRCOOQCCMGLRCGRGNPQKCIGAHγDV^	38,39
	scratcher	KFLSGGFKγIVCHRYCAKGIAKEFCNCPD*	1,39
C. textile	King Kong (KK-O,TxIA)*	WCKQSGEMCNLLDQNCCDGYCIVLVCT^	40,41
peptides	TxIB	WCKQSGEMCNLLDQNCCDGYCIVFVCT^	41
(mollusk hunter)	KK-1	CIEQFDPCEMIRHTCCVGVCFLMACI	40
	KK-2	CAPFLHPCTFFFPNCCNSYCVQFICL	40
	TxIIA	WGGYSTYCγVDSγCCSDNCVRSYCT	41
	convulsant	NCPYCVVYCCPPAYCEASGCRPP*	42
	scratcher	CCRTCFGCTOCC*	1
C. quercinus	QcIIIA	CCSQDCLVCIOCCPN*	1,43
peptides	QcIIIB	CCSRHCWVCIOCCPN	43
(worm hunter)	QcVIA	DQSCOWCGFTCCLPNYCQGLTC(T,V,I)^	1,43

[a] Four underhydroxylated forms of GIII have been isolated: [Pro[6]]GIIIA, [Pro[7]]GIIIA, [Pro[6]]GIIIB, and [Pro[7]]GIIIB. [b] The receptor targets for these peptides have not yet been definitively elucidated. Conotoxin GS has been reported to bind Na channels.[38] The KK-O peptide from *Conus textile* has been reported to affect a number of ionic currents.[44,45] [c] An asterisk or hat (^) indicates that the α-carboxyl group is *known* to be either amidated or the free acid, respectively. Except for γ (γ-carboxyglutamate) and O (*trans*-4-hydroxyproline), the standard one-letter amino acid code is used. Disulfide bridges are indicated by solid lines connecting cysteine residues.

structure; the prepropeptides have a high degree of identity in the signal sequence region and strong homology in the "pro" region of the precursor.[1,13] In addition, the members of a pharmacological family exhibit more closely identical precursor sequences than do members of different pharmacological families within the same structural class.[14] Thus, the true relationships between different *Conus* peptides become much more apparent if the sequences of the precursor prepropeptides are compared, rather than the final mature toxins.

Most of the generalizations about conotoxins and other *Conus* peptides are derived primarily from studies of fish-hunting species; much less work has been done on *Conus* species of other feeding types. However, the results to date establish that their venoms are also complex mixtures of peptides, with the same major structural classes. In the mollusk-hunting and worm-hunting species (such as *Conus textile* and *C. quercinus*, respectively), several peptide sequences have been found, such as KK-O[13] and peptide QcVIA[1,43] (see Table II) which belong to the same structural class as the ω-conotoxins from fish-hunting cones (the "4-loop Cys framework"). A 3-loop Cys framework is found in the "scratcher" peptide of *C. textile*[1,39] and in QcIIIA and QcIIIB of *C. quercinus*.[1,43] Furthermore, similar post-

translational modifications such as C-terminal amidation, hydroxylation of proline, and γ-carboxylation are found in peptides from these nonpiscivorous species.

The worm hunters are believed to be the stem group of the genus. Cones are hypothesized to have evolved from an older group of gastropod mollusks, the turrids,[16] which are also worm hunters. Thus, the basic strategy for generating a structurally and pharmacologically diverse set of peptides may have been established early in the evolution of the genus (with some features possibly predating the emergence of the genus *Conus*). The peptides recently characterized from venoms of worm-hunting and snail-hunting *Conus* species strongly suggest that the generalizations deduced from the more extensive analysis of fish-hunting *Conus* venoms will likely be true for the whole genus *Conus*.

C. *Conus* Peptides as Marine Natural Products

Conus peptides are derived from venoms and are therefore used in a different biological context from most marine natural products that have been investigated. The latter are usually highly potent defensive chemicals which sessile animals use to protect themselves against predators; studies of these have dominated the marine chemistry field. Although some components in *Conus* venoms may serve defensive purposes, the majority likely function to facilitate the capture of prey.

Furthermore, the *Conus* peptides are direct translation products of genes, and their amino acid sequences are directly encoded by nucleic acid sequence. In contrast, most marine natural products are complex organic compounds produced by the action of enzymes; these are consequently one step further removed from the information specified in the DNA of the organism (see other chapters in this volume). Thus, the more direct line to encoding DNA may be one determinant in the ability of the cone snails to rapidly generate novel structures in a particular window of evolutionary time.

However, many *Conus* peptides are heavily post-translationally modified (see Table II). In this respect, they have some aspects of a more conventional marine natural products strategy. Among the post-translational modifications found in *Conus* peptides are C-terminal amidation, proline hydroxylation, O-glycosylation, glutamate γ-carboxylation, and N-terminal glutaminyl ring closure to pyroglutamate. Thus, the normal "maturation" of *Conus* peptides requires the activity of modification enzymes such as proline hydroxylase or a γ-glutamyl carboxylase. Indeed, the formation of multiple disulfide bonds is likely to be kinetically facilitated by such factors as a peptide disulfide isomerase.

The basic plan for making *Conus* peptides, in which the underlying amino acid sequence is specified by conventional translation and then followed by extensive post-translational modification, seems a novel evolutionary strategy for generating a pharmacologically diverse set of marine natural products. Because these peptides are encoded by DNA, very rapid changes in sequence can occur. Hypermutation yields a large number of directly translated structures which can be explored in a short period of evolutionary time. However, by overlaying conventional peptide structures with numerous post-translational modifications, the cone snails are presumably able to thus optimize the specificity and affinity of the unmodified peptides for their respective receptor targets, and/or expand the repertoire of mechanistic possibilities. The use of γ-carboxylation of glutamate residues to stabilize α-helical structures in conantokins (see section V) is one example of facilitating biological activity of *Conus* peptides by post-translational modification.

III. Natural and Synthetic Conus Peptide Homologs. Interactions with Receptor Sites

A. Sequence Diversity of *Conus* Peptides

Sequence analysis of paralytic conotoxins has revealed a hypervariability in primary structure which is unprecedented.[1] Normally, functionally homologous peptides produced by different species of the same genus would be expected to be highly conserved in amino acid sequence. The published conotoxin sequences are shown in Table II. In each homologous family of *Conus* peptides (such as the ω-conotoxin series), only the cysteine residues are highly conserved. Although all of the ω-conotoxin peptides are paralytic to fish and targeted to presynaptic calcium channels, there can be as much as 70% nonidentity in the non-cysteine amino acid residues if ω-conotoxins from two different *Conus* venoms are compared. Indeed, the rather limited sample already collected indicates that the majority of fish-hunting cone snails will contain α-conotoxins and ω-conotoxins, with homologous peptides from different species showing very significant sequence differences.

The data in Table II might be interpreted to mean that the nonconserved amino acids are unimportant for function, and that only the cysteine residues and the few additional conserved residues are important for ω-conotoxin specificity for calcium channels; this does not appear to be the case. For example, the King Kong peptide from *Conus textile* which is *not* a calcium channel antagonist,[44,45] and has no detectable activity at the ω-conotoxin site in vertebrate calcium channels, has exactly the same set of cysteine residues and conserved amino acids, and yet it has entirely different biological activity (see Table II). The hypervariable regions are in fact critical for binding specificity.

Thus, the cone snails can evolve a very large number of sequence solutions for binding a single specific ligand site, such as the one targeted by the ω-conotoxins on voltage-sensitive calcium channels. How is such extensive sequence degeneracy possible? It has been postulated[46] that the underlying explanation is that these peptides interact with a target "macrosite" on a particular receptor or ion channel, comprising a significantly larger surface area than the binding site of a typical small molecule ligand. Given a number of potential contact "microsites" within the "macrosite", a given peptide ligand might bind only a small subset of all of the potential microsites available. Sequence degeneracy in *Conus* peptide ligands would be made possible by their being many ways in which a ligand can bind the macrosite to effectively block the biological activity of the receptor or ion channel. A specific (but hypothetical) representation of this hypothesis is shown in Figure 2.

Homologous peptides with divergent sequences that the different *Conus* species evolve likely represent

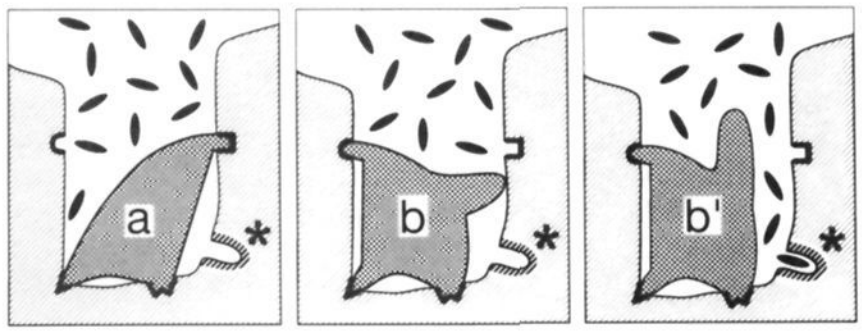


Figure 2. Degenerate *Conus* peptide conformations, toxins and antitoxins. A diagramatic representation of a ligand binding site on a receptor for a small molecule such as acetylcholine (represented by small ellipsoids). The agonist binding site is indicated by the asterisk; both a and b are toxins which inhibit agonist binding. Both toxins a and b bind the macrosite and share two microsites, with a third microsite unique to each toxin. Antitoxin b (derived from and closely related to b) would prevent either toxin a or b from binding to the macrosite, but does *not* prevent agonist binding. Thus, b would have no toxic activity and would serve as an antitoxin.

different conformational solutions to target the same macrosite. Consequently, the different peptides in a family would not be expected to have equivalent affinity for related receptors or ion channels that were not the specific biological target of that peptide class. (In the case of the ω-conotoxins, the relevant biological target would be the presynaptic calcium channels at the neuromuscular junction of teleost fish.) Indeed, differences in both phylogenetic specificity and receptor target subtype specificity are observed when homologous *Conus* peptides from different species are compared. Thus, α-conotoxin MI potently inhibits the acetylcholine receptor at the neuromuscular junction of mammals,[22,28] α-conotoxin SI is orders of magnitude less effective in mammalian systems.[22] Similarly, ω-conotoxin GVIA is ineffective on P-type voltage-gated calcium channels in the mammalian CNS, while ω-conotoxin MVIIC inhibits this subtype.[12] However, all of these peptides are very potent when applied to their "relevant targets" at the neuromuscular junctions of teleosts.

B. Synthetic Variants of α-Conotoxins: Toxins and Antitoxins

α-Conotoxins antagonize acetylcholine binding to the acetylcholine receptor of the neuromuscular junction.[21,47] When administered intraperitoneally (ip) in vertebrates, the outcome is general paralysis—including that of the diaphragm—and then death.

In the last few years, there has been an increasingly large number of *Conus* peptide variants not found in nature which have been chemically synthesized. A number of sequence variants of both α-conotoxins MI and GI have been made, and the biological activity of each have been assessed.[48,49] A single substitution in α-conotoxins at most positions does not abolish biological activity, excepting those in which major conformational changes would be expected to occur. Thus, substitution of Pro^6 by Gly, or Tyr^{12} by D-Tyr in α-conotoxin MI (or at the homologous positions of GI) result in loss of activity. Substitution of Gly^9 by Ala in MI led to a 30-fold reduction in activity.

A recent study of Almquist et al.[50] has demonstrated that a series of synthetic variants of des-Gly^1-α-conotoxin GI have strikingly different biological activities. Certain changes in amino acid sequence completely abolish biological activity (e.g., see analog 8, Table III). However, some nontoxic analogs proved, in fact, to be antitoxins (such as analog 15). This analog exhibited no toxicity even when injected at doses several hundred-fold higher than that of the natural toxin. However, when coinjected with a normally lethal dose of the toxin, it prevented death in mice, i.e., had antitoxin activity (in contrast, the inactive analog 8 exhibited no antitoxin activity). The antitoxin is effectively the natural sequence lacking one disulfide bond—the terminal Cys residue deleted and its Cys partner substituted by Ala. Surprisingly, by changing a single amino acid in the antitoxin, a toxic peptide which had only one disulfide linkage was produced (see analog 16, Table III). Thus, synthetic variants of α-conotoxins include a set which are completely inactive, some of which are nontoxic but have antitoxin activity, as well as a new toxin with only one disulfide bond (in contrast, at least two disulfide crosslinks are present in all natural α-conotoxins).

These results can be adequately explained by a macrosite hypothesis[46] as shown in Figure 2. Presumably, antitoxins occupy the same macrosite as the parent toxin. Binding of antitoxin to the macrosite would prevent the toxin from binding. However, in contrast to the natural toxin, binding by the antitoxic analog would not prevent access of acetylcholine to the agonist site. Thus the antitoxin has no direct inhibitory activity on the acetylcholine receptor.

The substitution of D-alanine for glycine at position 7 of the antitoxin (analog 15)[50] to give the new toxin (analog 16) would make the C-terminus less flexible. It seems likely that in the new toxic variant, the C-terminal amino acids probably assume a conformation significantly different from that in the original natural peptide, but one which blocks acetylcholine from

Table III. Synthetic Variants of α-Conotoxins[a]

	amino acid sequence	toxicity
natural sequence	Cys-Cys-Asn-Pro-Ala-Cys-Gly-Arg-His-Tyr-Ser-Cys-NH_2	(++)
analog 8	Cys-Cys-Asn-Pro-Ala-Cys-DPhe-Arg-His-Tyr-Ser-Cys-NH_2	(–) (not antitoxin)
analog 15	Cys-Ala-Asn-Pro-Ala-Cys-Gly-Arg-His-Tyr-Ser-NH_2	(–) (antitoxin)
analog 16	Cys-Ala-Asn-Pro-Ala-Cys-DAla-Arg-His-Tyr-Ser-NH_2	(+)

[a] Adapted from Almquist et al.[50] Amino acids underlined in the natural sequence indicate sites of alteration or deletion in the analogs.

binding to the agonist site. These possibilities need to be definitively settled by a multidimensional NMR analysis of a series of synthetic variants that exhibit different biological activity, such as the set in Table III.

C. μ-Conotoxins and Na Channel Subtypes

μ-Conotoxins are paralytic peptides which selectively block currents of voltage-gated sodium channels in muscle.[24] Blockage at either neuronal or cardiac subtypes requires toxin concentrations many orders of magnitude higher.

μ-Conotoxins are the most selective ligands for the neurotoxin site 1 of skeletal muscle sodium channels.[51] μ-GIIIA and μ-GIIIB (see Table II) have been shown to discriminate between the skeletal muscle and neuronal subtypes by about 1000-fold, whereas tetrodotoxin (TTX) and saxitoxin do not strongly discriminate between the two subtypes.[24] A distinct subtype of voltage-sensitive sodium channel with low affinity for TTX ($K_D \approx 1$ μM) has been described by several workers[52-54] in fetal rat muscle and in adult muscle after denervation. Using colchicine-treated muscle cell culture from embryonic rats, Gonoi et al.[51] showed μ-conotoxin GIIIB to be a much better tool for distinguishing the TTX-insensitive from the TTX-sensitive channel than TTX itself with a discrimination factor of at least 10 000-fold (compared to 200-fold for TTX).

Structure–function studies using various synthetic analogs of μ-conotoxin GIIIA[55,56] indicate that Arg^{13} is a key residue for biological activity. Ala substitution for Arg at position 13 increased the ED_{50} ~200-fold and at position 19 ~100-fold in assays of twitch contraction in rat diaphragm. No effect on activity was observed when Arg^{19} was replaced by Lys, indicating the importance of a positive charge at this locus. On the other hand, [Lys^{13}]GIIIA had ~6-fold higher ED_{50} than GIIIA. Replacement of Arg^1 by Ala, Lys, or Gln did not affect the activity much. Attachment of an iodinatable group at the amino terminus ([4-azidosalicyl]μ-GIIIA) also had little effect and the radioiodinated form was shown to label a 260 kDa protein, which is presumably the sodium channel.[57] In the 3-D structures obtained by NMR,[58,59] the amino terminus is distantly located relative to Arg^{13}.

Single-channel recordings using artifical bilayers obtained for the [Gln^{13}]GIIIA analog were particularly intriguing. Like other synthetic analogs with substitutions at position 13, [Gln^{13}]GIIIA had very low activity; no displacement of [^{3}H]saxitoxin binding was observed even at very high concentration (1 mM) of the peptide.[55] However, in contrast to saxitoxin, tetrodotoxin, and the other μ-conotoxin analogs, which cause an all-or-none block, 435 nM [Gln^{13}]GIIIA was found to block the channel to a none–zero subconductance state in recordings from single batrachotoxin-activated rat skeletal muscle sodium channels.[55] As underscored by Becker et al.,[55] the partial occlusion of individual sodium channels is a unique action of [Gln^{13}]-GIIIA.

All three neurotoxic ligands for site 1 of the sodium channel possess a guanidino group, which may be involved in blocking the channel. The three-dimensional structure obtained from NMR data[56] indicates Arg^{13} to be located at the flexible segment of the toxin. As suggested by Sato et al.,[56] the flexibility may be important in facilitating the subtle fit of Arg^{13} to the relevant site of the sodium channel.

The four amino acids that are most affected by replacement with Ala (Arg^{13}, Arg^{19}, Hyp^{17}, and Lys^{16}) are on one side of the proposed structure for GIIIA and it has been suggested that this portion of the molecule interacts with the sodium channel.[59] The hydroxyl group of Hyp^{17} has also been postulated to correspond to the essential hydroxyl groups of saxitoxin and tetrodotoxin.[55,59] Since the channel is permeable to guanidinium ions,[60] it is presumed that the guanidinium group of STX, TTX, and perhaps Arg^{13} of GIIIA may protrude partially into the pore as the toxins sit on the cavity on the extracellular surface of the sodium channel.

The basic amino acids of the toxin presumably interact with acidic groups of the "anion-lined funnel" on the extracellular side of the sodium channel.[59] The invariant glutamic and aspartic acid residues, which have been demonstrated to be important in tetrodotoxin and saxitoxin binding are contributed by the short segments (SS2) of the region connecting the S5 and S6 transmembrane segments of the four domains of the protein.[61-64] The primary structures of the TTX-sensitive[65] and TTX-resistant[66] sodium channels from rat skeletal muscle differ in the SS2 segment of domain I, where Tyr^{401} of the TTX-S channel is replaced by Cys and Asn^{404} by Arg at the corresponding positions in the TTX-R channel. The rat muscle TTX-R isoform, which is identical to the cardiac TTX-R isoform is also resistant to μ-conotoxin GIIIA.[66] Mutation experiments with the cardiac sodium channel has indicated a greater contribution of the Tyr → Cys (as compared to the Asn → Arg) replacement in bestowing TTX resistance.[67] The aromatic residue, Tyr, may either be necessary for binding of the toxins or the replacement with Cys may alter the conformation greatly to prevent toxin binding to the channel.

Thus, multiple groups in μ-conotoxin GIIIA presumably direct binding to the "anion-lined funnel" of the sodium channel. This may provide an example of ligand binding to a receptor as proposed by the macrosite model[46] where key functional groups on the toxin such as the guanidino moiety of Arg^{13} may interact with critically important microsites within the ion channel macrosite.

IV. Structural Studies of Multiply Disulfide Cross-Linked Conus Peptides

A. General Considerations

There are several factors that should be taken into account when considering the structures of *Conus* peptides. First, without additional stabilizing features, many peptides the size of cone snail toxins usually equilibrate between multiple conformations without a single conformational minimum being dominant at physiological temperatures. Peptides, under 30 amino acids in length, that have fixed conformations are mostly stabilized by multiple disulfide cross-links; most *Conus* peptides belong to this class. In addition, the principal factors governing larger protein structure—sequestration of hydrophobic side chains to the interior and solvation

of polar side chains at the exterior—might be expected to play less of a role in determining most *Conus* peptide structures.[68]

Early attempts were made to predict the structures of *Conus* peptides[69,70] as the first amino acid sequences were published. At the present time, several *Conus* peptides have been analyzed by multidimensional NMR techniques, and a number of solution structures have been solved. In the sections that follow, we describe the multiply disulfide cross-linked *Conus* peptides which have been solved.

B. α-Conotoxins

The solution structure of a 13 amino acid α-conotoxin, α-conotoxin GI, was determined independently by two different groups.[71,72] The initial work was performed in dimethyl sulfoxide.[71] Furthermore, the various structures calculated by distance geometry did not converge to a single structure. Instead, minimization revealed two different conformations—the major difference being the orientation of the Tyr^{11} side chain. Nevertheless, the global backbone structures were essentially convergent and are largely in agreement with the structure of Pardi et al.,[72] which was determined in water. However, since no distance constraints were reported for the DMSO structure, no further comparison with the water structure can be made.

Both groups reported that the structure of GI is highly organized as evidenced by analysis of chemical shifts. For example, the NH chemical shifts in GI range from 9.2 to 7.7 ppm which is much greater than the range of shifts observed in random-coil peptides;[73] similar trends are seen for the C^{α} proton chemical shifts. Further evidence that GI forms a non-random-coil structure in solution is that 14 of the 17 methylene carbon centers have resolvable chemical shifts of both prochiral protons, indicating nonequivalent chemical environments.

The GI backbone, as determined from 10 distance geometry calculations,[72] follows a course that roughly approximates a "ω", but with the two loops orthogonal to one another. Each loop represents a reverse turn, the first between Asn^4 and Cys^7 and the second between Gly^8 and Tyr^{11}. In most of the structures calculated, both turns are regular β-turns, but in a few structures the second turn was better approximated by two γ-turns. The evidence for the 1,4-β-turn is that the Asn^4 carbonyl is generally within H-bonding distance (~2 Å) of the Cys^7 amide proton in the 10 energy-minimized structures. The unexpected γ-turns are rare and their existence is less well supported, but they have been reported in a few proteins including thermolysin.[72] Minimization resulted in just a few structures with these two adjacent γ-turns centered on Arg^9. Whichever structure is correct, the net effect is that the arginyl side chain is pinched off between the two loops of the "ω".

The overall structure is that of a rigid, prolate ellipsoid of ~8 × 12 Å (for the backbone), which is in general agreement with that proposed by Gray et al.,[69] i.e., two turns starting at Asn^4 and Gly^8. It is also consonant with the structural requirements for paralytic properties of curare-like acetylcholine antagonists, viz., the presence of two "acetylcholine-like" units about 11 Å apart[74] as elaborated by Gray et al.[69] Each unit is composed of a cationic center (quaternary nitrogen) separated by approximately 5 Å from an electronegative group (carbonyl oxygen). These positive charges must originate from the Arg^9 guanidyl side chain and the amino terminus. The average distance between the positive charge on Glu^1 and Arg^9 is 15.5 Å, but small rotations about torsional angles on the Arg^9 side chain can easily move the cationic sites close to the ideal distance without large changes in energy. Docking of the toxin to the receptor could facilitate such rotation.

For many of the α-conotoxin homologs thus far described, no significant phylogenetic discrimination between the various vertebrate receptors analyzed has been observed. This is also true of other cholinergic antagonists, e.g., *d*-tubocurarine and α-bungarotoxin. However α-conotoxin SI from the venom of *Conus striatus*, which is highly potent on receptors of the lower vertebrates (viz., fish), is unique among cholinergic ligands in that it is orders of magnitude less potent on the mammalian receptor.[22] Since α-conotoxins GI and SI share considerable homology and elicit similar biological responses, it might be anticipated that their structures would be very similar, but with important differences to account for the phylogenetic selectivity of receptor binding. The structure of SI has only been preliminarily described[75] (a detailed report is presently being prepared). In short, α-conotoxin SI, also with 13 amino acids, has the same general structural elements as α-conotoxin GI but with a tighter turn at the additional Pro residue at position 9. It is notable that the only other *Conus* peptide with a Pro residue at this position, α-conotoxin SII, also shows the phylogenetic selectivity against the mammalian receptor.[30]

The structure of a novel acetylcholine receptor-targeted *Conus* peptide, which does not bind at the agonist binding site, is presently being determined. (M. Foster, K.-J. Shon, and C. Ireland, unpublished results.)

C. μ-Conotoxins

The aqueous solution structure of μ-conotoxin GIIIA has also been determined by NMR.[58,59] Similar to that of the α-conotoxins, considerable chemical shift dispersion of the otherwise chemically similar protons indicates a rigid and well-organized structure. The general folding of the 22 amino acid peptide is composed of the successive secondary structures: β turn (Asp^2 to Thr^5), β turn (Thr^5 to Lys^8), linear extension (Lys^8 to Asp^{12}), non-H-bonded loop (Asp^{12} to Cys^{15}), a single right-handed helical turn (Cys^{15} to Gln^{18}), with the carboxyl terminus protruding away from the core by a final loop in the opposite direction relative to the amino terminus. This ensemble is supported by a sulfur cage that derives from the three disulfide bridges organized at the center of the molecule. The overall structure is that of an oblate ellipsoid of ~6 × 15 Å (for the backbone) with the basic side chains radiating outward from the center. Studies on synthetic GIIIA analogs by replacement of each amino acid with Ala and Lys to identify key residues responsible for sodium channel blockade of skeletal muscle revealed that Arg^{13} was crucial for biological activity (see preceding section). Since tetrodotoxin and other sodium channel ligands possess guanidino groups as a putative binding moiety, it was postulated that Arg is necessary for interaction with the sodium channel.[55,56,59] Another NMR structure

of GIIIA that exhibits a slight variation in the backbone conformation has also appeared in a preliminary report,[76] but is pending further refinement.

D. ω-Conotoxins

ω-Conotoxins selectively inhibit the neuronal subtypes of voltage-gated calcium channels that mediate synaptic release of neurotransmitter. Intracerebral (ic) injection in mice of most of these peptides is manifested by a characteristic tremor. Some of these peptides discriminate between related receptor subtypes by many orders of magnitude.[12,34]

Preliminary multidimensional NMR data of the solution structure has been reported[77] for ω-conotoxin GVIA; a high-resolution solution structure determination has recently been completed,[78] using two-dimensional NMR spectroscopy and the full-relaxation matrix analysis program MARDIGRAS.[79] The most striking feature of the ω-conotoxin GVIA structure is the presence of a short segment of triple-stranded β-sheet, making ω-conotoxin GVIA the smallest peptide known to contain a triple-stranded β-sheet. As might be predicted, there are numerous turns: a hairpin turn is present in the β-sheet region (from Hyp^{21} to Lys^{24}), a type II turn from Ser^{3} to Ser^{6}, a type I turn from Ser^{9} to Ser^{12}, and a type VII turn from Cys^{15} to Ser^{18}.

V. γ-Carboxyglutamate (Gla) in Conus Peptides

A. Background

γ-Carboxyglutamate (Gla) is a post-translationally modified amino acid, first detected in prothrombin[80-82] in 1974. Since then, it has been found in several mammalian proteins involved in the blood clotting cascade (see refs 83–87 for reviews) and in a few bone proteins including osteocalcin (also called the "bone Gla-protein").[88] There is a report that free Gla circulates in serum.[89] The discovery of Gla in several *Conus* peptides (see Table II) established that this post-translational modification had a much wider phylogenetic distribution than previously thought. This amino acid has also been identified in hermatypic corals.[90]

A large and diverse set of *Conus* peptides contain this post-translational modification. In certain *Conus* venoms, more than 20% of all peptides appear to contain at least one Gla residue. However, to date, only in one Gla-containing peptide family, the conantokins, has the role of Gla been studied at a mechanistic level. Thus, the *Conus* peptide data discussed here are restricted to the conantokins. However, we have attempted to present the role of Gla in *Conus* peptides in the general context of other chemical and biochemical studies on this post-translational modification. Since chemical methods for the detection and synthesis of Gla have not been reviewed recently, a general overview of these methodologies is included here. Since the presence of Gla in small natural peptides was only established with the discoveries in *Conus*, we also summarize alternatives for synthesizing Gla-containing peptides.

B. Identification of Gla

While Nishimoto[91] and Danielson et al.[92] described a colorimetric determination of Gla in protein hydrolyzates without a separation step, (the latter used 4-diazobenzensulfonic acid which yields a highly colored red product with a molar absorptivity of 3510 at 530 nm), the most common method used to identify Gla in a peptide/protein is by amino acid analysis on anion[93,94] or cation[95-98] exchange resins, with post-column derivatization after hydrolysis under basic conditions. Under conventional acid hydrolysis conditions, Gla is converted quantitatively to Glu. In principle, the separation of Gla from other amino acids using anion or cation exchange columns is feasible as demonstrated by a large number of investigators. The method of choice will depend on the investigator's need for sensitivity and on the equipment available. However, most of the references cited above give experimental details that have become largely obsolete (since the advent of new ion exchange resins with small particle size, packed in microcolumns and used under high pressure), although extrapolation from the older protocols is feasible.

Precolumn derivatization [*o*-phthaladehyde[89] or phenylisothiocyanate to give the phenylthiocarbamyl derivatives[99]] and analysis of amino acid mixtures on reverse-phase supports is also practical. Other sophisticated methods such as isotachophoresis[100] and mass spectrometry after derivatization and gas chromatographic separation of the different amino acids[101] have also been described.

Under the conditions used for automated sequence analysis, Gla is not converted to Glu and has not been detected under the routine HPLC conditions used for the separation of the PTH amino acids. A recent paper by Cairns et al.[102] suggested treatment with methanolic HCl to convert the γ-carboxyls (among others) in the peptide/protein to the corresponding methyl esters. This procedure significantly reduces the polarity of the corresponding ATZ derivative of Gla and greatly improves its extraction from the polybrene-treated glass fiber filter. After conversion to the PTH derivative in methanolic HCl, the dimethyl ester of Gla can be identified directly by HPLC.

C. Conantokins

The conantokins are a family of *Conus* peptides which contain 4–5 residues of Gla; the most well-characterized is conantokin-G, which has five Gla residues (see Table II). Conantokins block glutamate receptors of the NMDA (*N*-methyl-D-aspartate) subtype, that are found largely in the brain.[25,37] Intracranial injections induce either sleep or hyperactivity in young (<14 day) or mature (>3 week) mice, respectively. These seemingly anomalous behaviors are possible due to switching from expression of fetal to adult NMDA receptors subtypes—a common feature in neuronal development.[103] The structures of α-, μ-, and ω-conotoxins are largely constrained by the multiple disulfide bonds. In contrast, most conantokin peptides have no disulfides to constrain conformation.[25,37]

Although no precise structural information has yet been obtained for these peptides using NMR or X-ray techniques, spectroscopic measurements strongly indicate that these peptides are folded into a stable α-helical conformation under physiological conditions. The γ-carboxyglutamate residues appear to be the key elements that stabilize the helical structure in these peptides.[103,104]

A strong preference toward α-helicity of conantokins is predicted by standard analyses of amino acids.[105] When modeled, the γ-carboxyglutamate (Gla) residues align along one side of such helices and the resulting negative charge density would be seriously destabilizing to helicity. It has been demonstrated by both Raman spectroscopy and circular dichroism[103,104] that in the presence of Ca^{2+}, which is chelated by Gla residues, conantokin-G adopts a tight α-helical structure. In the absence of Ca^{2+}, the Raman spectrum revealed broad amide I and III bands characteristic of an unfolded peptide. In the presence of excess Ca^{2+}, the sharp amide bands were fitted to a linear combination of amide spectra from a reference set of proteins of crystallographically-determined secondary structures. These calculations produced values of 80–90% α-helix.[103] Similarly with conantokin-T in the presence of 5 mM Ca^{2+}, CD produced a corrected $[\theta]_{222\ nm}$ of ~29 000 deg cm^2 dmol^{-1}, equivalent to 90% α-helicity.[103] This proportion of α-helicity indicates that for peptides of this size, the Ca^{2+}–conantokin chelates are among the most α-helical ever described.

D. Biosynthesis and Chemical Synthesis

The biosynthesis of Gla is vitamin K dependent. The mechanism by which carboxylation of certain glutamic acid residues within a mammalian protein sequence occurs has been intensively investigated; this has been described by Suttie in a comprehensive review.[106]

Although detailed information regarding the biosynthesis of γ-carboxyglutamate-containing peptides in *Conus* venom ducts has not yet been obtained, it has been established that Gla-containing peptides in *Conus* are translated as larger prepropeptide precursors, which are subsequently proteolytically cleaved to give the mature peptide. (D. Hillyard, C. Colledge, C. Walker, and B. Olivera, unpublished results.) Presumably, it is in the precursor form that the glutamate residues are post-translationally modified to Gla.

The precedents provided by mammalian systems strongly suggest that the production of γ-carboxyglutamate in *Conus* should be vitamin K requiring, and that a target-recognition sequence will be required in the precursor to instruct the modification enzyme to convert Glu to Gla. However, it is possible that the *Conus* recognition sequences are different, and possibly more complex. In mammalian systems, all glutamate residues within a long segment after the recognition sequence are carboxylated. In the case of the conantokins, there are three glutamate residues after the N-terminal Gly in the mature peptides (see Table II). The most N-terminal glutamate, Glu^2, is unmodified, while the immediately adjacent pair are quantitatively carboxylated to Gla. How such specificity is achieved is yet to be determined; nevertheless, the instructions for γ-carboxyglutamate modification in *Conus* encoded in recognition sequences, or in the intrinsic specificity of the modification enzyme, may be different from mammalian systems.

The total chemical synthesis of Gla and appropriate derivatives for peptide synthesis was first described by Maerki[107] and later refined.[108] In both cases, the D and L isomers were obtained and separated through the tedious use of crystallization of optically active salts. Several other syntheses attempted to render the process more efficient by starting with the optically active glutamate,[109] L-5-oxoproline esters[110] and proline.[111] All three chiral syntheses are certainly elegant and attractive yet do not lend themselves to the large-scale syntheses (>10 gm) of the desired derivatives such as the Fmoc-Gla(di-tBut ester) currently used for the routine synthesis of numerous analogs by the Fmoc strategy.[108] The chemical resolution of D- and L-Gla derivatives appear to be the most feasible alternative for large-scale synthesis.

Finally, while it was known that Gla was unstable to strong acids, it was also assumed that a Gla-containing peptide would not be stable to HF. A recent report by Nishiuchi et al.[112] demonstrated that Gla-containing peptides could be synthesized by the Boc strategy (the γ-carboxyls being protected with the cyclohexyl ester which is stable to TFA) with final HF deprotection and cleavage from the resin, thus fully opening one's armamentarium of synthetic strategies.

Because of the high cost associated with the synthesis of the Gla building block, and for a long time the unavailability of properly derivatized resins to generate peptide amides after TFA cleavage, synthetic Gla-containing peptides were limited in number and originated mostly from three different groups with varied interests. Hiskey and collaborators concentrated their efforts over the years on the study of metal binding to Gla containing peptides.[113–115] Birr and Krueck[116] studied the calcium-chelating properties of [Gla^{24},Gla^{25}]-thymosin-a1 while our group (see Table II) and that of Sakakibara[112] synthesized Gla-containing *Conus* peptides and their derivatives for the purpose of studying their unique biological properties and structure–activity relationships.

Some Gla-containing peptides are difficult to chromatograph under very acidic conditions; the peptide may be difficult to solubilize even in the presence of high concentrations of organic modifier. Artifacts, such as split absorbances, can arise if mixed salt forms of these peptides are analyzed. It has been suggested that the synthetic peptides be analyzed/chromatographed as a single salt form[95] to avoid such artifacts.

VI. Conus Peptides as Advanced Probes for Receptors and Ion Channels

Conus peptides have been used in several studies as probes for their receptor targets. A number of features make them intrinsically attractive as probes: the rigid structure and relatively small size provide a framework for attaching different reporter groups (such as photoactivatable cross-linking moieties). Because of their peptidic nature, there are several functional groups which can be modified using different chemical strategies.[117–119] Thus, the number of potential derivatives that can be made from one peptide without significantly affecting biological activity is generally large. When a *Conus* peptide is bound to its receptor target, the orientation of any reporter groups introduced by chemical modification would be dependent on where they are attached to the peptide (see Figure 3).

The major groups of derivatives that have been made are radiolabeled peptides (generally [^{125}I]tyrosyl derivatives of the peptides), photoactivatable derivatives (which when irradiated generate a reactive nitrene that covalently insert into receptors), and derivatives that

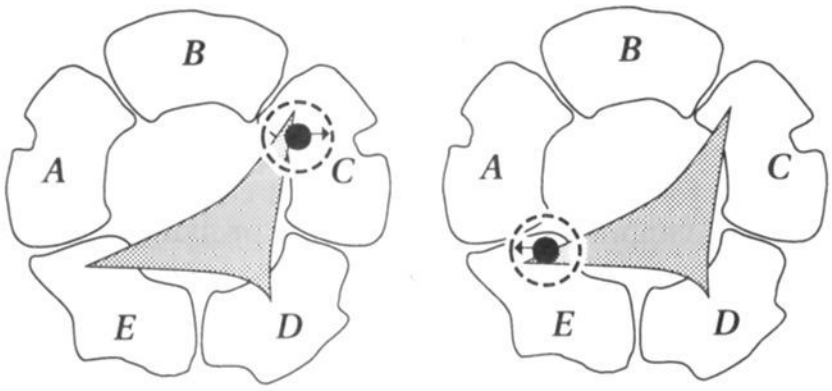


Figure 3. Schematized interaction of two conopeptide derivatives (indicated by gray area) with their heteromultimeric receptors (shown with five subunits). When the reporter group of known placement (depicted by black dots with their interactive radii) is anchored to different sites on a conopeptide, the region of the receptor potentially interacting with the reporter group will be different. For example, a reporter group placed at one position on the probe selectively interacts with subunit C (left panel), whereas a different probe placement would have the reporter group primarily interacting with subunit E and to a lesser extent, A (right panel). From the known conopeptide structure and reporter group placement, topological information regarding the receptor surface can be then deduced. The unique advantages of the conopeptides as receptor probes are: (1) reporter groups can be differentially placed (see text). (2) The small size allows only limited interaction with the receptor. Other peptide toxins are much larger, while small organic ligands often suffer from inactivation upon reporter group introduction. (3) As small peptides, they are readily available by chemical synthesis.

can be used for various applications (microscopy, fluorescence assays, etc.), such as biotinylated peptides. In general, chemical modification reactions lead to a mixture of different derivatives being formed; the relative biological activity of each derivative can then be monitored. The different derivatives can readily be separated from each other—usually in a single reverse-phase HPLC run—and an analysis of which residues on the peptide are derivatized is generally straightforward. Recent advances in mass spectrometry should make such analyses even more facile. In addition, it is usually possible to design the modification reactions to favor the formation of particular derivatives.

Studies using different radiolabeled forms of ω-conotoxins which are iodinated at different tyrosine residues have revealed that most iodinated forms retain significant biological activity, despite the addition of the bulky and hydrophobic iodine moiety. Similarly, derivatization of amino groups of ω-conotoxins using activated esters to introduce biotin,[120] a fluorophore,[121] or a photoactivatable reporter group[122] has also been carried out.

In the α-conotoxin series, several such derivatives have proven to be biologically active, still capable of binding (and hence chemically cross-linking) the nicotinic acetylcholine receptor. A recent study using different derivatives of α-conotoxin GIA had demonstrated that *Conus* peptides, derivatized so that a photoactivatable moiety is radioiodinated and attached to different amino groups of the peptide, give a strikingly different cross-linking pattern when the nicotinic acetylcholine receptor from the electric organ of *Torpedo* was used.[29] The two different radiolabeled derivatives were isomeric; one with a reporter group at the N-terminal amine and the second with a reporter group at the ε-amino group of Lys[15]. The data strongly

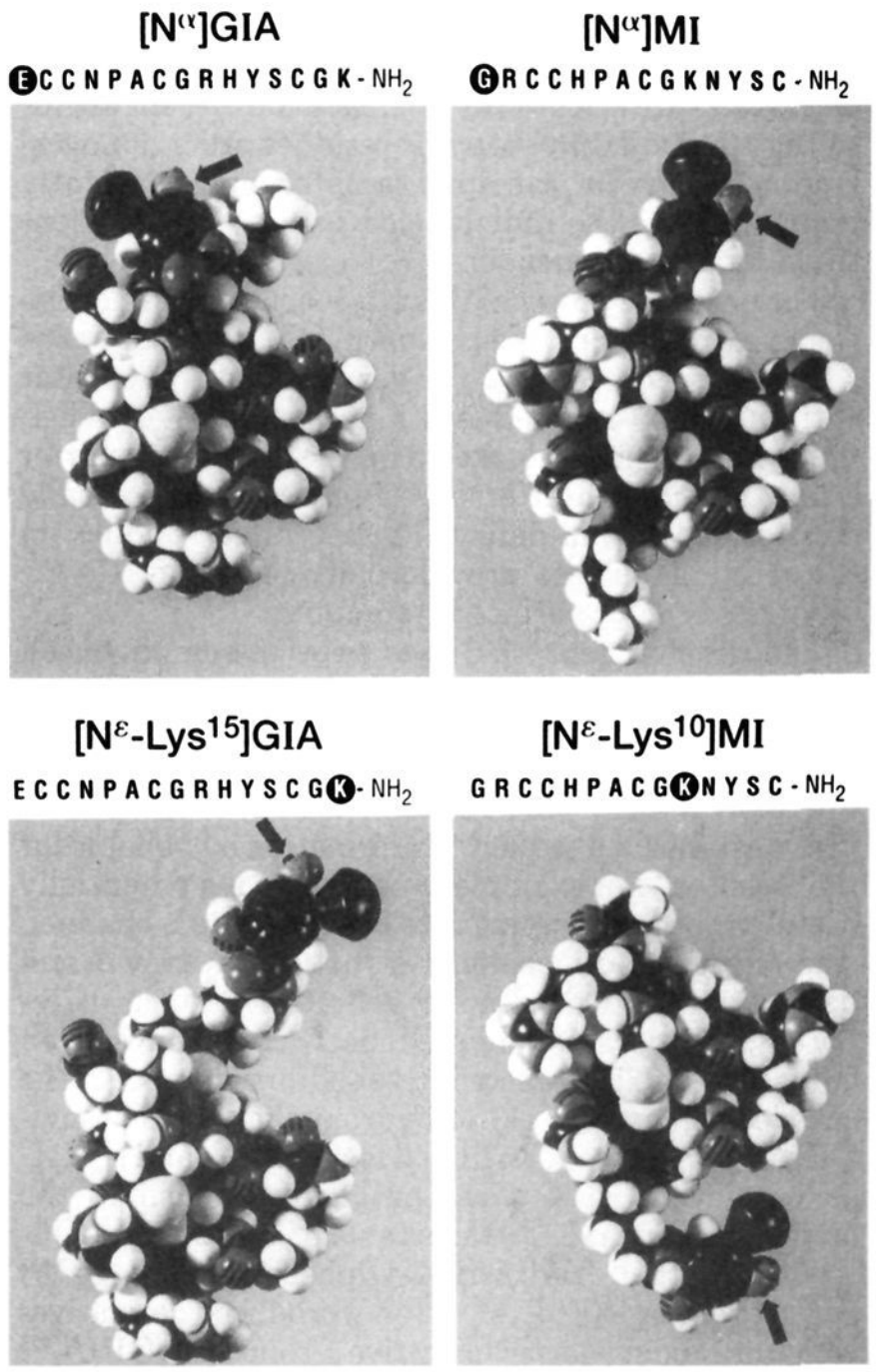


Figure 4. Selective placement of reporter groups on two conotoxins. Peptide models were built on the basis of the published structure of the α-conotoxins. The reporter group, a radioiodinated nitrene precursor is selectively placed at different regions on the surface of two α-conotoxins, GIA and MI. The models show that in the [N$^{\epsilon}$-Lys^{10}]α-conotoxin MI derivative (lower right hand panel), the reporter group has a strikingly different orientation compared to the three other derivatives. These different derivatives were incubated with a receptor preparation and then photoactivated by UV irradiation. The generated nitrene then has a certain probability of covalent attachment to a receptor moiety near the reporter group on the conopeptide (cf. Figure 3). The precise photolabeled amino acid within the subunit can be identified by radiosequence analysis. Analysis of several conopeptide derivatives, with differing reporter group placements, makes possible mapping of receptor sites using groups on the *Conus* peptide as reference coordinates.

suggest that in the two isomeric derivatives, the generated nitrenes associated differently with the receptor surface. Even more striking results were obtained when α-conotoxin MI, similarly derivatized on two different amino groups, was used as a probe. The different orientation of the various receptor groups is shown in Figure 4. Alternative to photoaffinity labeling, similar (although less striking) results were also obtained simply by continued incubation of the radiolabeled peptide/receptor preparations with divalent cross-linking agents of short tether length, e.g., ethylene glycol bis(succinimidyl succinate).[29]

A preliminary analysis of which residues on the nicotinic acetylcholine receptor were actually covalently

cross-linked was carried out. The results strongly suggest that the reporter groups in these different derivatives are oriented at sufficiently different angles so that the adjacent receptor residues are a nonoverlapping set.[123] In principle, receptor sites covalently cross-linked to the radiolabeled peptide can be identified by microsequencing.

The potential power of this approach is that once the structure of the peptide is known, in effect it becomes a framework onto which the complementary receptor surface can be mapped (see Figures 3 and 4). When the peptide ligand is docked onto the receptor, reporter groups attached to different residues can be specifically cross-linked to and define the associated residues on the receptor. These provide pairwise sets of *Conus* peptide–receptor surface interactions.

The development of *Conus* peptides as advanced probes is clearly not restricted to the α-conotoxins and their target acetylcholine receptors. Any *Conus* peptide structure that has been solved can be similarly utilized to explore its target macrosite, especially in those cases where established sequence information is available for the receptor. For example, μ-conotoxin is a potentially useful probe for mapping Site 1 of the sodium channel, since its three-dimensional structure is known and derivatives with nanomolar affinities have been reported. Two radioiodinated derivatives have been cross-linked to the presumptive sodium channel of the eel electroplax: [^{125}I]3-(4-hydroxyphenyl)propionyl-GIIIA where the label is linked to a lysine[124] and [^{125}I]4-azidosalicylyl-GIIIA with the label attached to the amino terminus.[57] Attachment of a [2-nitro-4-[3-(trifluoromethyl)-^{3}H-diazirin-3-yl]phenoxy]acetyl (NDP) group to either Lys^{8}, Lys^{9}, or Lys^{11} produced derivatives which are about as active as native μ-conotoxin GIIIA.[125]

In this review we have mainly discussed experimental results with one reporter group, i.e., photoactivatable cross-linkers. Many other reporter groups can, in principle, be used for probing receptors. For example, energy transfer from tryptophan residues in the receptor can be monitored using appropriate fluorescent groups attached to various residues on the *Conus* peptide. Another approach would be to couple a spin-labeled reporter group (i.e., a nitroxide derivative) at different positions on a *Conus* peptide, allow each derivative to bind the receptor, and then monitor the environment of the spin label of each analog by EPR.[126] The rotational correlation time (τ_R) is determined for each receptor-bound label, either in the presence or absence of freely diffusing oxygen or membrane impermeant chromium oxalate. Protection or exposure to these paramagnetic agents indicates whether the nitroxide of each analog is situated at an aqueous protein or lipid interface. Such experiments may be particularly revealing for unusually hydrophobic *Conus* peptides (e.g., King Kong peptides) in which lipid association could play a role in binding their membrane-bound receptor. An alternative method for defining which residues on a *Conus* peptide directly interact with the receptor is to apply the NMR methodology used by Fesik et al. to study cyclosporin/cyclophilin interactions.[127]

VII. Discussion and Future Directions

As more and more receptor and ion channel subtypes are identified by molecular cloning, there is an increasing need for chemical tools to investigate these large-cell surface proteins that play such important roles in intercellular communication. The *Conus* peptides have the potential to serve as powerful chemical probes for receptor and ion channel protein complexes. The different venoms in the genus *Conus* probably contain many tens of thousands of peptides; each would be expected to have high affinity and specificity for a particular receptor or ion channel subtype. Not only does each venom contain a pharmacologically diverse set of peptides, but there is considerable variation in the pharmacological spectrum of peptides from one *Conus* venom to the next.

One important advantage of using *Conus* peptides as probes is that the genus will generally have a diverse set of homologous peptides which can be isolated, characterized, and synthesized. Furthermore, *Conus* peptides from different species which target to exactly the same site on a particular receptor protein are surprisingly different in sequence, with striking hypervariability observed in the amino acids between cysteine residues.[67] A specific example of this is shown in Table II; ω-conotoxins GVIA and MVIIA compete with each other for binding to the same site on calcium channels, and yet 70% of the non-cysteine amino acids in these peptides are different, a sequence diversity that is even greater than might be expected between homologous peptides in yeast and man. Indeed, within *Conus* venoms, peptides which target to the same site have been found which are completely nonhomologous (examples are the μ-conotoxins and conotoxin GS shown in Table II). An additional example has recently been found for nicotinic acetylcholine receptors, where two nonhomologous sets of peptides, the α-conotoxins (shown in Table II) and the αA-conotoxins which appear to belong to a different structural class[128] (B. Olivera, C. Hopkins, and J. Rivier, unpublished results) both target to the acetylcholine binding sites.

Every *Conus* peptide is thus a promising chemical probe for its cognate receptor, providing a framework upon which reporter groups can be anchored by routine chemical modification reactions. In small organic molecule ligands, such chemical modification often leads to loss of biological activity.

Because of the conformational rigidity of *Conus* peptides, once a reporter group has been anchored at a particular position, and the peptide binds to its receptor, the reporter group will presumably be oriented specifically on the receptor surface (see Figure 2). Both because of the variety of functional groups that can be potentially modified in each *Conus* peptide, and since a large homologous set of *Conus* peptides with considerable sequence variation is generally available, reporter groups on *Conus* peptides can presumably be oriented in a wide variety of ways within a single ligand binding "macrosite".[46] In effect, reporter groups with different orientations, all targeting the same ligand binding pocket on a particular receptor, can probe different surface subsets ("microsites") of the larger binding site.

For these reasons, *Conus* peptides should be particularly useful tools for defining similarities and differences between two homologous macrosites on closely related subtypes of the same receptor type. By having a battery of homologous peptide ligands that target the

same general macrosite, and applying such a battery of ligands to closely related subtypes of one general receptor target, a comparison of how the surface of the macrosite might differ from one subtype to the next, can in principle be probed with great refinement. Understanding differences between ligand binding macrosites on different receptor subtypes is critically important in medicinal chemistry and for the pharmaceutical industry.

VIII. References

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