Using Chemistry to Reconstruct Evolution: On the Origins of Fish-hunting in Venomous Cone Snails[1]

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CHEMICAL COMPOUNDS derived from living organisms are similar to non-biological substances in that their composition and structure can be characterized, and many can be directly synthesized by standard chemical methods. However, biological compounds are fundamentally different in that each has an underlying evolutionary history. We describe several compounds from an unusual source, the venoms of fish-hunting cone snails. The chemical characterization of some toxins from these venoms has unexpectedly revealed how fish-hunting may have originated in cone snails, and suggests that piscivory may have evolved independently multiple times in these venomous predators.

Fish-hunting marine snails would appear to be a most improbable product of evolution. These snails devour fish as their major prey; a priori, fish would be expected to easily elude capture, simply by swimming out of the reach of the snails. However, a significant fraction of the >500 venomous cone snail species (genus *Conus*) have evolved to become specialized fish-hunters (Röckel et al. 1995).

Cone snails spear their fish prey with a hollow, harpoon-shaped tooth that jets out from a long distensible proboscis; this functions as

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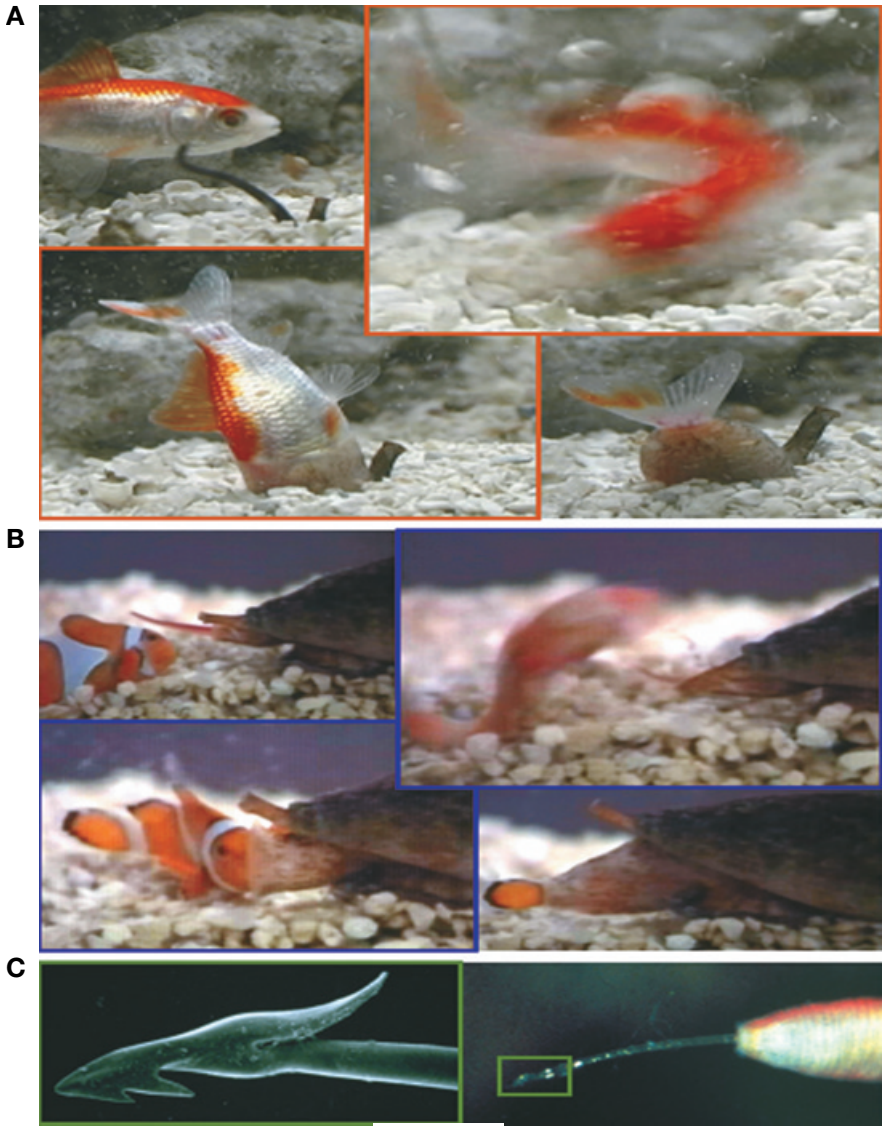

FIGURE 1. (A) *Conus monachus* catching and eating a fish. The snail has buried itself, and in the first panel its black proboscis, extended toward the fish, is visible (the shorter black structure is the snail's siphon). The snail extends its harpoon tooth through the proboscis and spears the fish, simultaneously injecting it with venom; the fish thrashes about wildly (*top right*), after which it is rigidly immobilized, making it possible for the snail to ingest it (*bottom*). The entire sequence took approximately three seconds. (B) A similar sequence with a *Conus purpurascens* attacking a clownfish. In this species, the extended proboscis is red in color. Once again, the entire sequence lasted approximately three seconds. (C) At left is an enlargement of a cone snail's harpoon tooth used to inject venom, and in the case of fish-hunting *Conus*, to tether the prey. The picture to the right shows a tooth in the snail's proboscis. The light green box indicates the section of the tooth that is magnified. The proboscis shown (at the far right) is the tip of the entire proboscis, visible in the top two sections.

both hypodermic needle and harpoon to simultaneously inject venom and tether the fish (see fig. 1). The fish are rapidly immobilized using a strategy first described for the purple cone, *Conus purpurascens* (Terlau et al. 1996). A combination of toxins in the injected venom causes an effect on the fish equivalent to a powerful electric shock, similar to one delivered when an electric eel stuns its prey. With a good strike, the fish prey is almost instantly unable to swim, and as the snail retracts its distensible proboscis, the immobilized fish, tethered through the harpoon tooth, is effectively reeled into its "false mouth." This sequence, illustrated in figure 1, takes only a few seconds. Almost all fish-hunting *Conus* apparently use this rapid immobilization strategy for prey capture. For a slow-moving snail that is unable to swim, the ability to quickly immobilize fish would seem critical for success as a piscivorous predator. The toxin combination that rapidly stuns the injected fish is called the "lightning-strike cabal" ("toxin cabals" are multiple toxins acting in concert to subvert a normal physiological process in the targeted animal) (Terlau and Olivera 2004).

In the sections that follow, we first establish that three fish-hunting *Conus* species, the striated cone, *Conus striatus*, the purple cone, *Conus purpurascens,* and the radial cone, *Conus radiatus*, are not closely related; each belongs to a different subgenus in *Conus*. We then compare venom components required to rapidly immobilize fish in the three species. For one of the essential "lightning-strike cabal" components, the analogous components identified from the venoms of the three species were all similar, structurally related compounds. In contrast, for the other essential component of the "lightning-strike cabal," a dramatic divergence among the three cone snail species was found. Thus, the three fish-hunting lineages of *Conus* each have their own distinctive "lightning-strike cabals," consistent with fish-hunting's having evolved independently within each lineage.

Molecular Taxonomy of Fish-hunting *Conus*

The cone snails are a large (>500 species) and taxonomically complex group. Because their shells have long been favored objects for natural history cabinets, *Conus* has been one of the most intensively collected and studied groups of marine invertebrates. However, there has been no generally accepted scheme to describe how the various *Conus* species are related to each other. Only recently, by analyzing DNA sequence divergence between species, has progress been made in the evaluation of relationships between *Conus* species. Previous studies using standard DNA sequences as molecular markers had suggested that fish-hunting *Conus* species fall into divergent species clades (Duda Jr. and

Palumbi 2004; Duda Jr. et al. 2001; Espiritu et al. 2001; Monje et al. 1999).

We have used a novel DNA sequence for the analysis of fish-hunting cone snails. This DNA sequence, which is within an intron, is a facile but definitive diagnostic marker for differentiating between groups of fish-hunting *Conus*. The intron is in a gene encoding a highly conserved and very ancient enzyme called γ-glutamyl carboxylase (γ-GC). In the human and *Conus* γ-GC genes, introns were found at exactly the same place in the gene (Bandyopadhyay et al. 2002), with the bordering exons (coding sequences) around each intron being highly conserved. The latter provide convenient primers for the analysis of intron divergence using a standard technique to obtain DNA sequence, the polymerase chain reaction (PCR). One particular intron, γ-GC intron 9, proved to be ideal for the desired analysis of piscivorous *Conus* species.

This intron has a variable length primarily due to a repetitive sequence at one end that diverges extremely rapidly. Within the nonrepetitive regions of the intron, a 120-nucleotide interval was diagnostic for the different groups of *Conus* piscivores (the "diagnostic piscivore intron interval"). This 120-nucleotide sequence is shown for nine fish-hunting species in table 1. If sequences in this interval are aligned, an obvious consensus sequence emerges. The consensus sequence contains the major nucleotide found at each position; all bases deviating from the consensus are shaded. Loci that do not conform to the consensus are strongly correlated between multiple fish-hunting *Conus* species.

Even by inspection of table 1, it is clear that the sequences from the fish-hunting cone snails fall into three groups: species 1–3, 4–5, 6–9. All fish-hunting *Conus* species diverge from the consensus sequence at between 6–11 positions. The single snail-hunting *Conus* species in table 1, *Conus aulicus*, the court cone (#10), also diverges at six positions, but these are uncorrelated to the fish-hunters. The three fish-hunting species groups each have their characteristic "subconsensus": species 1–3, the "*Conus striatus* group" (*Conus striatus*, *magus*, and *consors*); species 4–5, the "*Conus purpurascens* group" (*Conus purpurascens* and *ermineus*); and species 6–9, the "*Conus radiatus* group" (*Conus radiatus*, *flavus*, *lynceus,* and *cinereus*). These data, together with the earlier data using standard molecular markers, establish that *Conus striatus*, *purpurascens,* and *radiatus* belong to different lineages of fish-hunting species within the genus *Conus*, each such group probably deserving of subgeneric rank.[4]

[4]A major taxonomic revision is presently being carried out by Alan Kohn. The most appropriate subgeneric assignments appear to be: Pionoconus Morch, 1852 for the *Conus striatus* group; Chelyconus Morch, 1842 for the *Conus purpurascens* group, and Phasmoconus Morch, 1852 for the *Conus radiatus* group.

TABLE 1. *Conus* Species Within the "Diagnostic Piscivore Interval" (from Intron 9 of γ-glutamyl carboxylase). A 120-nucleotide DNA sequence from nine fish-hunting species and one snail-hunting species (*Conus aulicus*) are compared; differences are summarized through the "subconsensus sequences." All differences from the consensus are shaded.

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                          10        20        30        40
1. C. striatus            AAATTAAAATCCTTAATACAGGAGTAATGCCTGAAGAAATGGCA
2. C. magus               AAATTAAAATCCTTAATACAGGAGTAATGCCTGAAGAAATGGCA
3. C. consors             AAATTAAAATCCTTAATACAGGAGTAATGCCTGAAGAAATGGCA
4. C. purpurascens        AAATCAAAATTCCTAATACAGGAGTAGNACCTAAAGAAATGGCA
5. C. ermineus            AAATCAAAATTCTTAATACAGGAGTAGTACCTAAAGAAACGGCA
6. C. radiatus            GAATTAAAATTCTTAATACAGGAGTAATACCTGAAAAAATGGCA
7. C. flavus              AAATTAAAATTCTTAATACAGGAGTAATACCTGAAGAAATGGCA
8. C. lynceus             AAATTAAAATTCTTAATACAGGAGTAATACCTGAAGAAATGGCA
9. C. cinereus            AAATTAAAATTCTTAATACAGGAGTAATACCTGAAAAAATGGCA
10. C. aulicus            AAATTAAGATTCTTAATACAGGAGCAATACCTGAAGAAATGGCA
    Consensus             AAATTAAAATTCTTAATACAGGAGTAATACCTGAAGAAATGGCA

                          50        60        70        80
1. C. striatus            GTAGGACTCGCTTAGCTGTAATATTGGAAAGGTACCAAAACTG
2. C. magus               GCAGGACTCGCTTAGCTGTAATATTGGAAAGGTACCAAAACTG
3. C. consors             GTAGGACTCGCTTAGCTGTAATATTGGAAAGGTACCAAAACTG
4. C. purpurascens        GTAGGACTCACTTAGCTGTAATATTGGAAAGGTCACAAAACTA
5. C. ermineus            GTAGGACTCACTTAGCTGTAATATTGGAAAGGTCACAAAACTA
6. C. radiatus            GTAGGACTCGCTTAGCTGTAATATTGGAAAGGTCACAAAACTA
7. C. flavus              GTAGGACTCGCTTAGCTGTAATATTGGAAAGGTCACAAAACTA
8. C. lynceus             GTAGGACTCGCTTAGCTGTAATATTGGAAAGGTCACAAAACTA
9. C. cinereus            GTAGGACTCGCTTAGCTGTAATATTGGAAAGGTCACAAAACTA
10. C. aulicus            GTAGGACTCGCTTAGCTGTAACATTGAAAAGGTCACAAAACTA
    Consensus             GTAGGACTCGCTTAGCTGTAATATTGGAAAGGTCACAAAACTA

                          90        100       110       120
1. C. striatus            ACTACATCAGCATTGGGATATGTCAATGCTACG
2. C. magus               ACTACATCAGCATTGGGATATGTCAATGCTATG
3. C. consors             ACTACATCAGCATTGGGATATGTCAATGCTACG
4. C. purpurascens        ACTACATCAGCATTGGGATATATCAATGTTAAG
5. C. ermineus            ACTACATCAGCATTGGGATATATCAATGTTAAG
6. C. radiatus            AC----------TGGGATATATCAATGCTACG
7. C. flavus              AC----------TGGGATATATCAATGCTGCG
8. C. lynceus             AC----------TGGGATATATCAATGCTACG
9. C. cinereus            AC----------TGGGATATATCAATGCTACG
10. C. aulicus            ACTACATCAGCACTGGGATATATCAATACTACG
    Consensus             ACTACATCAGCATTGGGATATATCAATGCTACG
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"Subconsensus" sequences

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Consensus       AAATTAAAATTCTTAATACAGGAGTAATACCTGAAGAAATGGCAGTAGGACTCGCTTAGCTG
Species 1–3[a]  AAATTAAAATCCTTAATACAGGAGTAATGCCTGAAGAAATGGCAGTAGGACTCGCTTAGCTG
Species 4–5[b]  AAATCAAAATTCCTAATACAGGAGTAGNACCTAAAGAAATGGCAGTAGGACTCACTTAGCTG
Species 6–9[c]  GAATTAAAATTCTTAATACAGGAGTAATACCTGAAGAAATGGCAGTAGGACTCGCTTAGCTG

                TAATATTGGAAAGGTCACAAAACTAACTACATCAGCATTGGGATATATCAATGCTACG
                TAATATTGGAAAGGTACCAAAACTGACTACATCAGCATTGGGATATGTCAATGCTACG
                TAATATTGGAAAGGTCACAAAACTAACTACATCAGCATTGGGATATATCAATGTTAAG
                TAATATTGGAAAGGTCACAAAACTAAC----------TGGGATATATCAATGCTACG
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[a] *C. striatus*, *magus*, *consors;* [b] *C. purpurascens*, *emineus;* [c] *C. radiatus*, *flavus*, *lynceus*, *cinereus*).

Conkunitzins, A New Family of "Lightning-strike Cabal" Toxins

In a successful strike, most fish-hunting cone snails cause instant tetanic paralysis in the prey using the "lightning-strike cabal" toxin combination. The physiological state that is the basis for rapid prey immobilization is massive hyperexcitability: nerves at the injection site are induced to fire uncontrollably; as a consequence, trains of action potentials hit the central nervous system (these action potentials are traveling along nerves in the opposite direction from normal). It has been suggested that venom injection creates the equivalent of an epileptic focus at the periphery, and that when struck the fish, in effect, undergoes a tonic/clonic seizure with very stiff fins. The basic molecular events required to induce the nerves to fire in such an uncontrolled fashion are a greatly elevated sodium ion flux *into* the nerve, concomitant with a block of potassium ion flux *out* of the nerve. Thus, to elicit this state of massive hyperexcitability, several classes of toxins are essential: the first class keeps sodium (Na) channels open, allowing more sodium to flow in, and a second class of toxins blocks potassium (K) channels, preventing potassium ions from flowing out. This combination massively changes the nerve membrane potential to a more positive value, causing action potentials to be generated.

In all species examined, the conotoxins that keep Na channels open belong to the same group of peptides, the δ-conotoxin family. These δ-conotoxins are found not only in fish-hunting *Conus*, but in non-fish-hunting *Conus* species as well (Bulaj et al. 2001; Espiritu et al. 2001; Terlau et al. 1996). δ-Conotoxins are a well-defined, extremely hydrophobic family of peptides that target a specific site on Na channels, designated site 6 (Fainzilber et al. 1994; Leipold et al. 2005). Some examples of the striking sequence similarity between δ-conotoxins from species belonging to divergent classes of fish-hunting *Conus* are shown in table 3.

The second essential element for rapid immobilization of fish prey is toxins that block K channels. Although a variety of different K channels appear to be targeted, a subclass of K channels (belonging to the *Shaker* or Kv1 subfamily) are always specifically targeted, since these are major molecular components that control excitability of peripheral nerves. The first characterized K-channel blocker was from *Conus purpurascens*, κ-conotoxin PVIIA (κ-PVIIA) (Shon et al. 1998; Terlau et al. 1996). An analogous venom component that inhibits K channels was later characterized from *Conus radiatus*, κM-conotoxin RIIIK (κM-RIIIK) (Al-Sabi et al. 2004; Ferber et al. 2003). An unexpected and surprising result was that these two peptides were chemically unrelated,

even though they had overlapping pharmacological specificity (both block the *Shaker* K channel), and both interact with the same region of the ion channel (the extracellular vestibule of the ion channel pore).

Thus, two unrelated *Conus* peptides targeted to K channels of the *Shaker* subfamily were identified from *Conus purpurascens* and *Conus radiatus*; however, no such peptide toxins from a major lineage of fish-hunting *Conus* that includes *Conus striatus* (the subgenus *Pionoconus*) had been characterized (the shells of all three species are illustrated in fig. 3). We have therefore purified and characterized toxins with this mechanism from crude *Conus striatus* venom. Two toxins that inhibited K channels of the *Shaker* subfamily have been identified.

Upon purification and sequencing, the most active factor from *Conus striatus* that inhibited *Shaker* K channels proved to be a 60-amino-acid toxin that had no homology to any previously characterized cone snail toxin; the sequence assignment was consistent with the value obtained by mass spectrometry (M = 6931). Upon inspection, the sequence of the toxin revealed that it has a well-characterized motif known as a "Kunitz domain" (Pritchard and Dafton 1999), and defines a novel

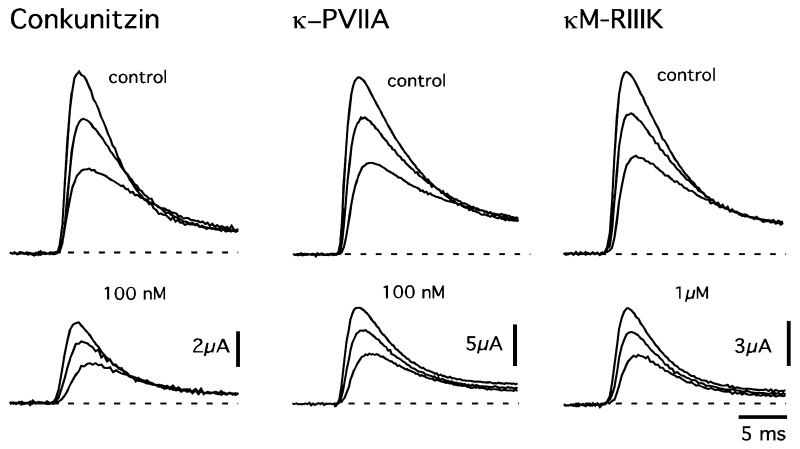

FIGURE 2. Block of potassium currents through the *Shaker* K channel by various toxins from different fish-hunting cone snail venoms. Shown are electrophysiological recordings from *Xenopus* oocytes. The upper panels show control currents elicited by changing the voltage across the oocyte membrane. Currents in the presence of the individual toxins are shown in the lower panels, using the same changes in voltage across the membrane as in the control. Conkunitzin-S1 and κ-PVIIA (from *Conus striatus* and *Conus purpurascens*, respectively) effectively block the current at a concentration of 100 nM, whereas κM-RIIIK (from *Conus radiatus*) is less potent; a toxin concentration of 1 μM is required to achieve a similar block. For conkunitzin-S1, block of the *Shaker* K channel, the mean inhibitory concentration (IC_{50}) is 66 ± 21 nM (n = 4).

TABLE 2. Sequence of conkunitzin-S1, comparison with other Kunitz domain proteins. The final assignment for conkunitzin-S1 is shown. Conkunitzin-S1 is aligned with δ-dendrotoxin and BPTI. Note the absence of two Cys residues in the conkunitzin. The amino acids that are identical in all sequences are shaded.

Sequence of conkunitzin-S1

KDRPSLCDLPADSGSGTKAEKRIYYNSARKQCLRFDYTGQGGNENNFRRTYDCQRTCLYT

Comparison of conkunitzin to other Kunitz domain proteins

Conkunitzin-S1	KDRPSLCDLPADSGSGTKAEKRIYYNSARKQCLRFDYTGQGGNENNFRRTYDCQRTCLYT
δ-Dendrotoxin[a]	AAKYCKLPVRYGPCKKKIPSFYYKWKAKQCLPFDYSGCGGNANRFKTIEECRRTCVG
BPTI	RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA

[a] Joubert and Taljaard 1980.

group of *Conus* toxins, the conkunitzin family. We designate the specific toxin purified from *Conus striatus* conkunitzin-S1 ("con*kunitz*in" for a Kunitz domain; "S" indicating that the peptide comes from *Conus striatus* venom). The sequence of conkunitzin-S1 is shown in table 2.

The second *Shaker* K-channel inhibitor from *Conus striatus*, designated conkunitzin-S2, was similarly purified from venom and sequenced; it proved to be homologous to conkunitzin-S1, but 30% of amino acids are not identical. Both sequences were verified by isolating cDNA clones from *Conus striatus* venom ducts (J. Garrett, unpublished results). Conkunitzins have extensive amino acid sequence similarity to other classes of Kunitz domain proteins, such as protease inhibitors (e.g., bovine pancreatic trypsin inhibitor, BPTI), as well as certain snake toxins (e.g., the dendrotoxins from African mambas; [Harvey 2001]), which target voltage-gated K^+ channels (see table 2). However, there is one disulfide bond less in conkunitzin-S1 than in the other Kunitz domain proteins; the four other cysteine residues are well aligned.

The possibility that a contaminant in the venom fraction was responsible for the K-channel-blocking activity observed (and not conkunitzin) was eliminated using both chemical synthesis and gene expression. Conkunitzin-S1 was chemically synthesized by native chemical ligation, as well as expression of a cDNA clone encoding conkunitzin-S1 in an *E. coli* vector (to be described in detail elsewhere). Both the chemically synthesized conkunitzin-S1 and the product of expression of the cDNA clone were tested for the ability to block the Shaker K channel; both potently inhibited the K channel (results not shown). These results demonstrate that the K-channel-blocking activity is due to the toxin that was biochemically characterized from *Conus striatus* venom, conkunitzin-S1.

A functional comparison of conkunitzin-S1 from *Conus striatus* to

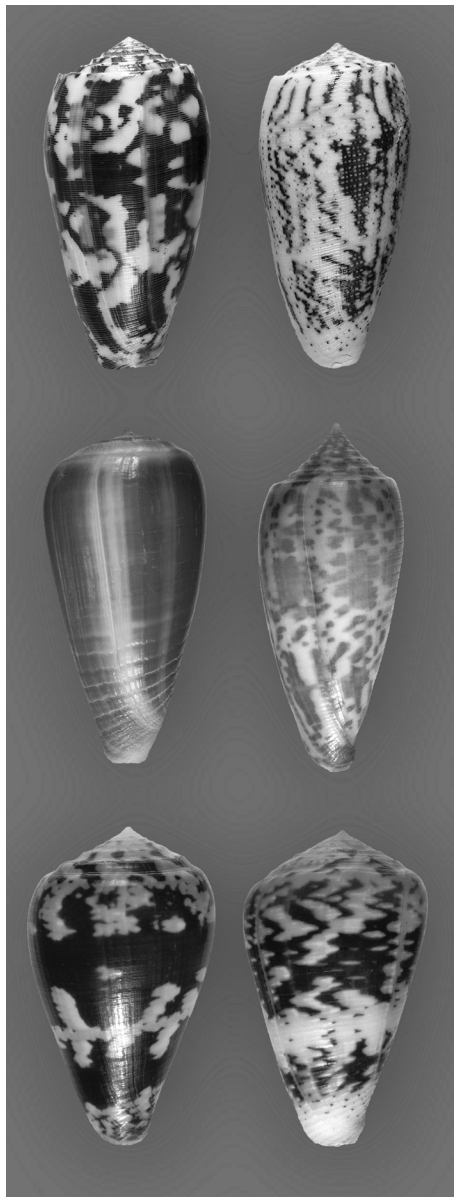

FIGURE 3. Fish-hunting cone snails. Shown are shells of *Conus* species from the three different lineages of fish-hunting *Conus* described in this article. *Top row* (*left to right*): the striated cone, *Conus (Pionoconus) striatus*, and the magician's cone, *Conus (Pionoconus) magus*. *Middle row*: the radial cone, *Conus (Phasmoconus) radiatus*, and *Conus (Phasmoconus) lynceus*. *Bottom row*: the purple cone, *Conus (Chelyconus) purpurascens*, and the turtle cone, *Conus (Chelyconus) ermineus*. The first four species are from the tropical Indo-Pacific and were collected from the Philippines, *Conus purpurascens* is from Clipperton Island in the Eastern Pacific, and *Conus ermineus* is from Cape Verde Island in the Atlantic Ocean.

the analogous K-channel-targeted conotoxins from *Conus purpurascens* and *Conus radiatus* is illustrated in figure 2. The striking result is that all three peptides inhibit the same K channel (the *Shaker* channel), although they are completely unrelated in their amino acid sequences (as shown in table 3) and their structures (see fig. 5), and are members of entirely different gene superfamilies.

TABLE 3. Similarity of Na-channel-targeted (the δ-conotoxins) contrasted to the divergence of potassium-channel-targeted toxins.

δ-Conotoxins (Na-channel-targeted)		
C. striatus[a]	δ-conotoxin SVIE	DG**C**SSGGT**FC**GI---HOGL**CC**SEF**C**-FLW**C**ITFID
C. purpurascens[b]	δ-conotoxin PVIA	EA**C**YAOGT**FC**GI---KOGL**CC**SEF**C**LPGV**C**FG*
C. ermineus[c]	δ-conotoxin EVIA	DD**C**IKDYG**FC**SLOILKNGL**CC**SGA**C**V-GV**C**ADL
C. radiatus	(δ-conotoxin RVIE)[†]	E**C**TTNGE**FC**GISVFASFL**CC**SGL**C**VF-V**C**I
K-channel-targeted toxins		
C. striatus	conkunitzin-S1	KDRPSL**C**DLPADSGSGTKAEKRIYYNSARKQ**C**LRFD YTGQGGNENNFRRTYD**C**QRT**C**LYT
C. purpurascens[d]	κ-conotoxin PVIIA	**C**RIONQK**C**FQHLDD**CC**SRK**C**NRFNK**C**V
C. radiatus[e]	κM-conotoxin RIIK	LOS**CC**SLNLRL**C**OVOA**C**KRNO**CC**T*

NOTE: Three other sequences from a *Conus radiatus* cDNA library have the conserved amino acids and the Cys pattern that are characteristic of the δ-conotoxins. *Conus ermineus* belongs to the same lineage of piscivorous *Conus* as *C. purpurascens*.

[a]Bulaj et al. 2001; [b]Terlau et al. 1996; [c]Barbier et al. 2004; [d]Shon et al. 1998; Terlau et al. 1996; [e]Al-Sabi et al. 2004; Ferber et al. 2003.

[†]Indicates that this sequence is derived from a cDNA clone. All other sequences shown were purified from venom.

ON THE ORIGINS OF FISH-HUNTING

The DNA sequence interval within an intron, shown in table 1, has provided an unambiguous definition of three distinct groups of fish-hunting *Conus* species: a group found in the Caribbean and Eastern Pacific (subgenus *Chelyconus*, including *Conus purpurascens*), a second group of species (subgenus *Phasmoconus*) from deeper waters of the central Indo-Pacific, including *Conus radiatus*, and the large, well-known class (subgenus *Pionoconus*) of shallow-water fish-hunting cone snails of the Indo-Pacific, including *Conus striatus*. Figure 3 shows examples of two species in each of the separate lineages; some toxins and DNA sequences from these species are shown in tables 1 and 3. Previous analyses using standard molecular markers generally support these subgeneric assignments (Duda Jr. and Palumbi 2004; Duda Jr. et al. 2001; Espiritu et al. 2001; Monje et al. 1999), although some previous attempts to subdivide *Conus* do not (da Motta 1991). The DNA sequence analysis we used provides a facile molecular definition of species groups that diverged 20–50 mya both within the 500–700 species constituting the genus *Conus*; this method can be used as well for other taxa that diverged from each other within a similar timeframe.

In this work, we describe a new K-channel blocker from the venom of *Conus* striatus, which is pharmacologically analogous, but chemically and genetically unrelated to previously described K-channel blockers from *Conus purpurascens* and *Conus radiatus*. The new toxin proved to be a member of a well-known class of proteins, the Kunitz-domain-containing polypeptides. Thus, most unexpectedly, the K-channel blocker

from *Conus striatus* is structurally much more similar to K-channel blockers from mamba snakes (and to a large class of protease inhibitors, including BPTI) than to the analogous K-channel-blocking conotoxins from some other fish-hunting *Conus* lineages.

Although the three fish-hunting species, *Conus purpurascens*, *Conus radiatus*, and *Conus striatus* each belong to a different lineage of fish-hunting *Conus*, all three express structurally similar and genetically related toxins that keep Na channels open, the δ-conotoxins. The uniformity of the Na-channel-targeted δ-conotoxins, when juxtaposed with the divergence between K-channel blockers (table 3) is a most striking feature of the lightning-strike cabals of the three divergent groups of fish-hunting *Conus*. However, cone snails that belong to the same piscivore lineage, such as *Conus magus* and *Conus striatus* (both in the subgenus *Pionoconus*) have related K-channel blockers: we characterized a conkunitzin from *Conus magus* venom with considerable homology to the *Conus striatus* peptides (E. Jimenez and B. Olivera, unpublished results). *Conus magus* and *Conus striatus* do not have κM- and κ-conotoxins; conversely, κM-conotoxins can be identified in other *Conus* species related to *Conus radiatus* (in the subgenus *Chelyconus*) but have not been found in fish-hunting *Conus* from the other fish-hunting lineages (M. Watkins and B. Olivera, unpublished results).

Worm-hunting *Conus*, most of which prey on polychaete worms, are generally regarded as ancestral to other feeding groups (Kohn 1990; Röckel et al. 1995). Our data are consistent with the toxins that keep Na channels open, the δ-conotoxin gene family, being well established in *Conus* before the three piscivorous *Conus* groups diverged. One potential function of toxins that keep sodium channels open in a worm-hunting *Conus* species would be to deter fish competitors from stealing the worm prey of the snail; δ-conotoxins targeted to fish Na channels have the potential to deter marauding fish, since these could activate pain and other sensory fibers. If subsequently such a worm-hunting *Conus* species were to have evolved a blocker of fish K channels, the stage would be set for the immobilization of the envenomed fish. Injection of a combination of a K-channel blocker (presumably low affinity and not very specific at first) with a Na-channel-opening δ-conotoxin already functionally targeted to fish would result in a tetanic paralysis—for a worm-hunting snail, this could initiate using fish as alternative prey and, eventually, an exclusively fish-hunting lifestyle. A cartoon illustrating this hypothesis is shown in figure 4. In such a "founder" worm-hunting species, ancestral to a group of present-day piscivorous cone snails, the gene family of the first K-channel toxin that elicited excitotoxic shock (and consequently, rapid immobilization) in the fish would be expected to be the gene family in which K-channel blockers would continue to evolve in its piscivorous descendants.

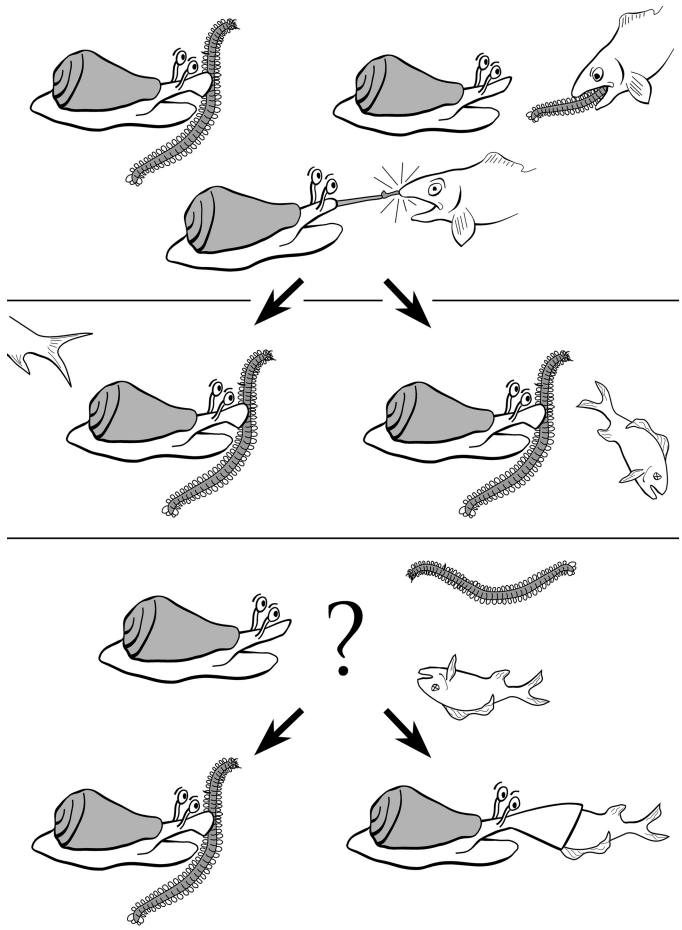

FIGURE 4. Cartoon representing evolution of fish-hunting in cone snails. *Top and middle panels*: An ancestral worm-hunting cone snail would have had to compete with fish for worm prey. There would have been selection for δ-conotoxins, which would have caused pain when injected into a fish, as a deterrent to these fish competitors. Worm-hunting snails with δ-conotoxins would be able to sting and effectively deter the fish (*middle panel*, *left*). *Middle and lower panels*: Rarely, subsequent to δ-conotoxin evolution, if a cone snail evolved a toxin inhibiting K channels, stinging the fish would result not only in pain, but in complete immobilization (*middle panel*, *right*). The immobilized fish would then be a potential alternative prey for the snail, which might eat the fish lying in front of it. This is the proposed evolutionary trajectory toward fish-hunting; as the snails became better adapted to immobilizing fish and devouring them, exclusively fish-hunting species could evolve. The presence of entirely divergent K-channel-targeted toxins suggested that the last steps in this scenario may have occurred at least three times between 20 and 35 million years ago.

Thus, the three different groups of fish-hunting cone snails (as defined by the diagnostic intron sequence in table 1 above) may each have independently evolved the toxin combination responsible for an effective excitotoxic shock mechanism, based on pre-existing δ-conotoxins that open Na channels, and three different primordial K-channel blockers. In each case, descendants that were evolving a fish-hunting lifestyle would have generated in parallel progressively more potent lightning-strike cabals to capture prey, with presumably ever more effective K-channel blockers, but derived from the same conotoxin gene family as in the ancestral founder species. This would explain why *Conus magus* and *Conus striatus* use conkunitzins to block K channels, *Conus* species related to *Conus radiatus* (in the subgenus *Phasmoconus*) use M-superfamily conotoxins, and *Conus purpurascens* uses an O-superfamily conotoxin. The contrasting structures of these K-channel blockers are shown in figure 5. However, all of these species that have structurally divergent K-channel blockers use conotoxins that are all similar structurally to increase sodium flux (the δ-conotoxins).

We expect the conkunitzin family of peptides that blocks K channels to be widely distributed across shallow-water Indo-Pacific species of fish-hunting *Conus*, and that many different isoforms of conkunitzins will be found in the ~20 *Conus* species related to *Conus striatus*. *Conus striatus* and *Conus magus* are among the less closely related pairs of species within this group, and the discovery that conkunitzins are present in both leads to the expectation that shallow-water Indo-Pacific piscivores will all have conkunitzins as a component of their lightning-strike cabals. Preliminary molecular evidence consistent with this prediction has already been obtained (J. Garrett, unpublished results).

Although we have established that the conkunitzins are unrelated to pharmacologically analogous conotoxins targeting K channels in other piscivorous *Conus* venoms, it was unexpected for them to be so strikingly similar to the K-channel-targeted dendrotoxins from mamba snakes. Snakes and this particular group of cone snails may have independently recruited the widely distributed Kunitz domain motif as a K-channel-targeted toxin in their venoms, an example of convergence between unrelated venomous animals. The ubiquity of extracellular protease inhibitors containing Kunitz domains could have provided a potential starting point for the evolution of K-channel-targeted ligands in venoms of both the African mambas and the most well-known lineage (*Pionoconus*) of fish-hunting cone snail species. An alternative and intriguing explanation is that endogenous K-channel modulators exist, which are Kunitz domain proteins, and it is from these that toxins like the conkunitzins may be derived.

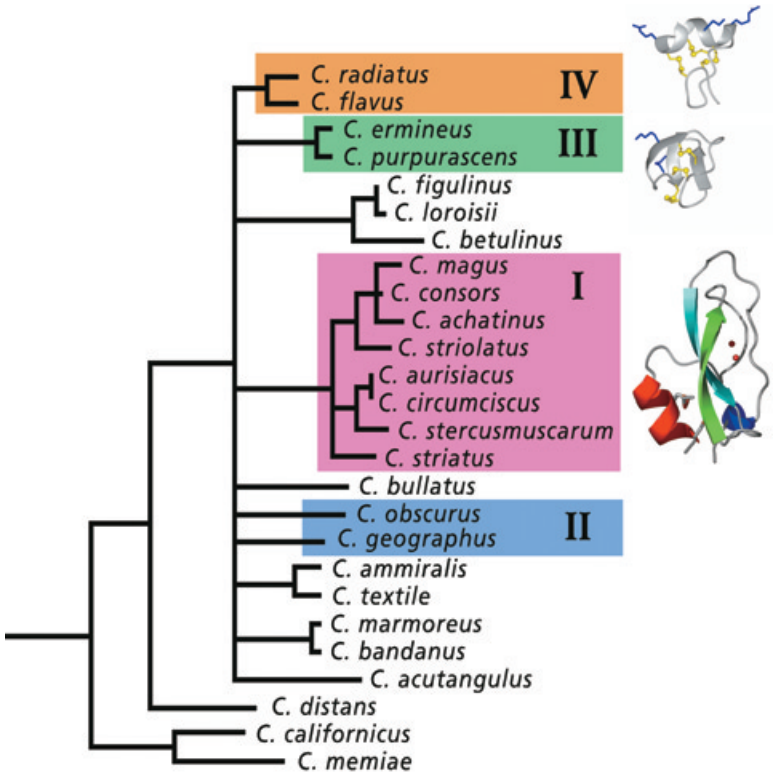

FIGURE 5. Different structures of K-channel-blocking conotoxins are found in divergent groups of fish-hunting *Conus* species. Completely unrelated K-channel blockers were found in the venoms of different fish-hunting *Conus* species. The species that belong to different subgenera are boxed in different colors: pink, subgenus *Pionoconus*, including *Conus striatus*; green, subgenus *Chelyconus*, including *Conus purpurascens*; gold, subgenus *Phasmoconus*, including *Conus radiatus*. Shown next to each subgeneric group is an outline structure of the K-channel-blocking conotoxin isolated from a species in that group (i.e., conkunitzin-S1 from *Conus striatus*, κ-conotoxin PVIIA from *Conus purpurascens*, and kM-conotoxin RIIIK from *Conus radiatus*). It is presumed that all of the species boxed in pink will have a K-channel blocker that has a structure similar to conkunitzin-S1; such components are not present in the other two fish-hunting subgenera. Note the strong divergence between the structures shown. The species boxed in blue, including *Conus geographus*, belong to the subgenus *Gastridium*—some of these use a different strategy for hunting fish, and may not have a conventional lightning-strike cabal, even though they are fish-hunting *Conus* species. The pink box includes fish-hunting species that appear more closely related by this analysis to a group of *Conus* that includes *Conus betulinus*; these are all worm-hunting species that appear to be closely related to *Pionoconus* by this analysis. The phylogenetic tree was adapted from Espiritu et al. (Espiritu et al. 2001). The structures are adapted from Savarin et al. (Savarin et al. 1998) for κ-PVIIA; Al-Sabi et al. (Al-Sabi et al. 2004) for κM-RIIIK and Bayrhuber et al. (Bayrhuber et al. 2005) for conkunitzin-S1.

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