δ-Conotoxins Synthesized Using an Acid-cleavable Solubility Tag Approach Reveal Key Structural Determinants for NaV Subtype Selectivity*

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Background: Conotoxins are marine snail venom peptides of interest as pharmacological tools and potential medicines. Conotoxins are classified into superfamilies according to the signal sequence of their protein precursors and their disulfide bridge pattern, then subdivided into families according to their pharmacological activity. The δ-conotoxin family, which is part of the O-superfamily, acts on voltage-gated sodium (Nav) channels, inhibiting fast inactivation and modulating voltage dependence to more negative potentials, leading to propagation of action potentials and persistent neuronal firing (2). Nine different mammalian Nav channel subtypes (Nav 1.1 through 1.9) have been described to date (6). These show differential tissue distribution, different electrophysiological and pharmacological properties, and are linked to different physiological roles and pathologies (7, 8). Drugs that selectively target Nav channel subtypes represent valuable pharmacological tools and have potential for development as medicines (7, 8).

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Relatively little is currently known about the structure-activity relationships for δ-conotoxins (2). It has been proposed that hydrophobic residues exposed on the surface of the folded molecule may be important for interaction with Nav channels (9), potentially through interaction with a corresponding hydrophobic site on domain IV of the channels (10). Little detailed study has been carried out on the Nav subtype selectivity of δ-conotoxins, but the characterization of one example, δ-EVIA, suggests that they have the capacity to distinguish between different subtypes (11).

Conotoxins are venom peptides from cone snails with multiple disulfide bridges that provide a rigid structural scaffold. Typically acting on ion channels implicated in neurotransmission, conotoxins are of interest both as tools for pharmacological studies and as potential new medicines. δ-Conotoxins act by inhibiting inactivation of voltage-gated sodium channels (Nav). Their pharmacology has not been extensively studied because their highly hydrophobic character makes them difficult targets for chemical synthesis. Here we adopted an acid-cleavable solubility tag strategy that facilitated synthesis, purification, and directed disulfide bridge formation. Using this approach we readily produced three δ-conotoxins and two analogs, obtaining new pharmacological insights.

Results: δ-Conotoxins are poorly understood because they are difficult to synthesize. Using a new synthesis approach, we readily produced three δ-conotoxins and two analogs, obtaining new pharmacological insights.

Conclusion: Key structures on δ-conotoxins determine NaV subtype selectivity.

Significance: Our synthesis approach is applicable to other similarly challenging peptides.

Conotoxins are venom peptides from predatory cone snails with multiple disulfide bridges that provide a rigid structural scaffold. Typically acting on ion channels implicated in neurotransmission, conotoxins are of interest both as tools for pharmacological studies and as potential new medicines. δ-Conotoxins act by inhibiting inactivation of voltage-gated sodium channels (Nav). Their pharmacology has not been extensively studied because their highly hydrophobic character makes them difficult targets for chemical synthesis. Here we adopted an acid-cleavable solubility tag strategy that facilitated synthesis, purification, and directed disulfide bridge formation. Using this approach we readily produced three native δ-conotoxins from Conus consors plus two rationally designed hybrid peptides. We observed striking differences in Nav subtype selectivity across this group of compounds, which differ in primary structure at only three positions: 12, 23, and 25. Our results provide new insights into the structure-activity relationships underlying the NaV subtype selectivity of δ-conotoxins. Use of the acid-cleavable solubility tag strategy should facilitate synthesis of other hydrophobic peptides with complex disulfide bridge patterns.

Venom peptides from predatory cone snails (conotoxins), which have natural roles in prey capture and defense, act by interfering with the activity of ion channels and receptors (reviewed in Refs. 1–4). The majority of conotoxins are cysteine-rich and adopt rigid folded structures stabilized by multiple disulfide bridges (1–4). Their relatively small size, high stability, and typically high affinity and selectivity for their targets make conotoxins not only useful tools to study the pharmacology of their molecular targets, but also a rich potential source of medicines, with one molecule already licensed for clinical use in pain management and several others in clinical development (5).
The major hurdle to studying the structure-activity relationships of δ-conotoxins has been the difficulty of obtaining them in a pure and correctly folded form by chemical synthesis (2). In this study we developed a new acid-cleavable solubility tag approach to facilitate handling and folding of cysteine-rich hydrophobic peptides and used it to synthesize three previously identified (12, 13) but uncharacterized δ-conotoxins from Conus consors, plus two rationally chosen analogs. We analyzed pharmacological activity of these molecules on a range of Na₅ channel subtypes.

### EXPERIMENTAL PROCEDURES

**Venom Extraction and Fractionation**—C. consors specimen collection, maintenance, and venom gland extraction was performed as described previously (12), and the δ-CnVID-containing fraction was further purified by size exclusion chromatography using an Alliance HT 2795 (Waters, Milford, MA) separation module fitted with a Waters 487 dual wavelength detector and operated with Waters Millenium 4.0 software. The column used was a BioSep-SEC-S 2000 (300 7.8 mm, Phenomenex, Torrance, CA) with a linear gradient from 10 to 30% buffer B in buffer A over 59 min.

![UV-HPLC chromatogram](image.png)

**FIGURE 1. Purification of δ-CnVID from C. consors venom.** Shown is a UV-HPLC chromatogram of the dissected venom from C. consors. Manually collected fractions identified as containing δ-CnVIB, δ-CnVIC, and δ-CnVID (12) are indicated.

**TABLE 1**

Amino acid sequence comparison of δ-conotoxins purified from the venom of piscivorous cone snails

| Peptide   | Primary structure | Reference |
|-----------|-------------------|-----------|
| δ-EVIA    | DDCIKPYFCSLPILKNGLCCSGACV-GVCADL-- | (11)     |
| δ-CnVIA   | YEYSTGTFCG-------INGGLCCSNLCFLFFVCLTFSC | (15)     |
| δ-SVIE    | DGCSSGTFCG-------IKOGCCSEFCFLWCTIFID | (10)     |
| δ-PVIA    | EACYAOGTFCG-------IKOGCCSEFCLPGVCFG-- | (21)     |
| δ-NgVIA   | SKCSGTFCG-------IKOGCCSVRCFSLFCISFEE | (30)     |
| δ-CnVIB   | DEFSOGTFCG-------TKOGCCSARGFSCFISCLEF | This work |
| δ-CnVIC   | DEFSOGTFCG-------IKOGCCSARCFSFFCISLEF | This work |
| δ-CnVID   | DEFSOGTFCG-------FKOGCCSARCFSFFCISLEF | This work |
| Hybrid 1  | DEFSOGTFCG-------TKOGCCSARCFSLFCISLEF |           |
| Hybrid 2  | DEFSOGTFCG-------FKOGCCSARCFSLFCISLEF |           |
The flow rate was set to 0.5 ml/min with an isocratic composition of 30% acetonitrile and 0.1% trifluoroacetic acid. δ-CnVID identity was confirmed by ESI-MS. Samples were then freeze-dried and kept frozen until use.

Edman Degradation—Edman sequencing was performed using a Procise 491LC sequencer (Applied Biosystems). Approximately 1 nmol of the fraction of interest was deposited on a Biobrene-fixed filter and 32 sequencing cycles were carried out for N-terminal sequence identification.

Synthesis of Peptide Fragments—Peptide synthesis was carried out on Rink amide resin (0.49 mmol/g (Novabiochem)) with a Prelude peptide synthesizer (Protein Technologies, Inc.) using standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and standard side chain protection (Protein Technologies) except where indicated below.

Incorporation of Acid-cleavable Solubility Tag—A series of four lysine residues was coupled to the resin, then the N-terminal lysine residue was acetylated with phenylacetamido (PAM)-substituted phenylalanine (corresponding to the C-terminal Phe residue of the conotoxins). Fmoc-Phe-PAM, which was generated by substituting the t-butoxycarbonyl group on t-butoxycarbonyl-Phe-PAM (Polypeptide Laboratories) for Fmoc, was incorporated manually over a 3-fold excess of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate and a 6-fold excess of N,N-disopropylethylamine.

Incorporation of Orthogonally Protected Pairs of Cysteine Residues—Cysteine residues were protected with S-acetamidomethyl (Acn) for Cys10 and Cys22, S-trityl (Trt) for Cys3 and Cys18, and tert-butyl (tBu) for Cys17 and Cys27. For incorporation of these residues, a 10-fold excess of amino acid was used with a coupling time of 30 min, followed by a 10-min capping step with a 30-fold excess of acetic anhydride.

Cleavage/Deprotection and Purification—Linear peptide cleavage and deprotection was accomplished with treatment with 86% trifluoroacetic acid, 5% phenol, 5% thioanisole, 4% triisopropylsilane for 2 h at room temperature. Soluble crude product was precipitated with cold diethyl ether, washed with diethyl ether, dried, redissolved in aqueous acetonitrile, and freeze-dried. The linear peptide was then purified to 90% purity by preparative HPLC. Purified peptides were characterized by analytical HPLC and mass spectrometry (ESI-MS or MALDI-TOF).

Directed Disulfide Bridge Formation—To generate the first disulfide bridge (Cys18-Cys27), the purified linear peptide was dissolved in 50% (v/v) acetonitrile at 10 mg/ml and slowly added to a 0.1 M Tris/HCl buffer containing 4 M guanidine chloride and 0.5 mM, 0.1 mM reduced/oxidized glutathione, pH 7.9, at a 50 µg/ml final concentration and stirred overnight at room temperature. Once analytical HPLC and MS analysis confirmed that oxidation was complete, the solution was acidified to pH 2–3 with TFA and the peptide was isolated by preparative HPLC.

The second disulfide bridge (Cys3-Cys18) was introduced by iodine oxidation of Acn groups. The peptide was dissolved in acetic acid:acetonitrile:water (2:1:1, v/v; 5 mg/ml peptide concentration) and 1.5 eq of HCl (0.1 M solution) per Acn group were added to the reaction medium, followed by 5 eq of I2 (50 mM solution in acetic acid) per Acn group. The reaction mixture was stirred vigorously for 30 min then quenched by the addition of a 10-fold excess of ascorbic acid (1 M in water) over I2. The product was purified by preparative HPLC.

The final disulfide bond (Cys17-Cys22) was formed by dissolving the peptide in TFA/DMSO (95:5, v/v) to a concentration of 0.1 mg/ml and stirring for 1 h at room temperature. The solution was then concentrated on a rotary evaporator and purified by preparative HPLC. Fractions containing the desired product, as confirmed by analytical HPLC and ESI-MS analysis, were pooled and lyophilized.

Cleavage of the Solubility Tag—The solubility tag was cleaved by treatment with anhydrous HF for 1 h at 0 °C with 2% p-creosol. After removal of HF under vacuum, the peptide was dissolved in aqueous acetonitrile, lyophilized, and purified by semi-preparative HPLC.

Na+ Channel Electrophysiology—Heterologous expression of Na+ channels in Xenopus laevis oocytes and electrophysiological experiments were performed as described previously (14).

RESULTS

Purification of 3 Novel δ-Conotoxins from Crude Venom—Reverse-phase chromatography fractionation and proteomics analysis of dissected C. consors venom glands (Fig. 1) led to the
isolation of fractions containing previously identified δ-conotoxins: δ-CnVIB, δ-CnVIC, and δ-CnVID (12, 13).

**Mass Spectrometry and Sequence Determination**—ESI-MS analysis of these fractions yielded monoisotopic masses of 3527.41, 3505.46, and 3539.45 Da for δ-CnVIB, δ-CnVIC, and δ-CnVID, respectively. 32-Residue primary structures (Table 1) were obtained for each conotoxin by Edman degradation. These amino acid sequences were consistent with the measured monoisotopic masses, assuming the presence of three disulfide bridges. We noted that the sequences of δ-CnVIB, δ-CnVIC, and δ-CnVID only differed at three positions: 12 (Thr in δ-CnVIB, Ile in δ-CnVIC, and Phe in δ-CnVID), 23 (Phe in δ-CnVIB and δ-CnVID, Leu in δ-CnVIC), and 25 (Phe in δ-CnVIB and δ-CnVIC, Leu in δ-CnVID). Sequence alignment with δ-conotoxins from other piscivorous snails indicates that these three positions are generally occupied by hydrophobic residues.
chemical synthesis strategy. In addition to the three native quantities for pharmacological profiling, we opted to develop a civorus snails (15): Phe9 and Leu16. The third site, position 12, is occupied by hydrophobic residues at two of the three positions previously identified as highly conserved hydrophobic sites in Drosophila melanogaster (15); Phe9 and Leu16. The third site, position 12, is occupied by Thr.

Chemical Synthesis of δ-Conotoxins—To obtain sufficient quantities for pharmacological profiling, we opted to develop a chemical synthesis strategy. In addition to the three native δ-conotoxins, we set out to synthesize two rationally designed hybrids in which residues from CnVIB it is occupied by a polar residue, Thr.

Hybrid 1
Obs: 3495.53
Exp: 3496.05
YL: 30 mg
YF: 2.0 mg

Hybrid 2
Obs: 3576.57
Exp: 3576.14
YL: 30 mg
YF: 0.6 mg

FIGURE 4. Reverse phase chromatography profiles of conotoxins synthesized in this study. Following the final purification step (see Fig. 2 and main text), samples were separated by reverse phase HPLC using Macherey Nagel C8 column (4.6 × 250 mm, 5 μm, 300 Å) at a flow rate of 0.7 ml/min. Solvents used were A (0.1% TFA in H2O) and B (0.1% TFA in acetonitrile/H2O 90:10), with an elution gradient (indicated on each profile) of 10–80% of solvent B over 35 min. Peaks were detected by absorbance at 214 nm. Mass spectrometry was carried out using MALDI in linear mode.

(underlined in Table 1). All three sequences feature hydrophobic residues at two of the three positions previously identified as highly conserved hydrophobic sites in δ-conotoxins from piscivorus snails (15): Phe9 and Leu16. The third site, position 12, is occupied by hydrophobic residues in δ-CnVIC and δ-CnVIB and δ-CnVID at the three divergent positions in the primary structure (Table 1).

In accord with the previously documented difficulty in obtaining synthetic δ-conotoxins (2), we were unsuccessful in our initial attempts to obtain purified linear peptides using Boc chemistry with in situ neutralization (16), either as a single fragment or by using native chemical ligation (17) to join two fragments corresponding to residues 1–16 and 17–32 (data not shown). Further attempts using Fmoc chemistry incorporating pseudoproline dipeptides and 2-hydroxy-4-methoxy-benzyl-protected amino acids, which have previously been shown to improve synthesis efficiency and subsequent handling by impeding the formation of inter- and intramolecular hydrogen bonds (18), were also unsuccessful (data not shown).

Directed Disulfide Bridge Formation Coupled with an Acid-cleavable Solubility Tag Approach—Working on the assumption that the problems with synthesis and purification were due to the strongly hydrophobic nature of these conotoxins, we devised an acid-cleavable solubility tag approach strategy in which the linear peptide is appended with a hydrophilic (Lys4) C-terminal adapter via a PAM moiety, which can be cleaved from the rest of the peptide by treatment with HF at the end of the synthesis and folding procedures (Fig. 2). This approach enabled us to produce 20–50 mg yields of purified linear δ-conotoxins.

The ease of handling afforded by the solubility tag enabled us to adopt a fully directed disulfide bridge strategy, using the two unprotected cysteines in the linear peptide, Cys10 and Cys22 to form a first bridge, then the Acm-protected cysteines to generate the Cys5–Cys18 disulfide bridge, and then the tBu-protected cysteines to generate the Cys17–Cys27 disulfide bridge (Figs. 2 and 3). At the end of refolding and removal of the solubility tag, final yields of pure, folded material ranged from 0.6 to 3.8 mg, representing overall yields of 1–10% with respect to the starting linear peptide (Fig. 4).

Validating the Authenticity of Chemically Synthesized δ-CnVID—We performed coelution experiments to demonstrate that synthetic δ-CnVID has an identical retention time as native δ-CnVID on reverse-phase HPLC (Fig. 5A), indicating that the synthetic material is conformationally identical to the native material. Furthermore, the pharmacological activity of the synthetic sample (5 μM) on four different mammalian Nav subtypes was very similar to that obtained with the native sample (1 μM) that had been isolated from the original fraction by size exclusion chromatography (Fig. 6). Both samples completely abolished Na1.6 inactivation, neither sample showed any detectable effect on Na1.4 and Na1.5, and whereas the synthetic sample showed greater inhibition of inactivation on Na1.2 than the natural sample, this is likely to be due to the higher concentration of synthetic sample that was used than to any difference in potency (Fig. 7).

Because of the limited availability of native material we were unable to perform coelution experiments comparing native and synthetic δ-CnVIB and δ-CnVIC, but we note that the elution behavior of these two conotoxins relative to δ-CnVID (Fig. 5B) closely matches (i) the pattern obtained when they were purified from a venom sample (13) and (ii) the predicted behavior based on differences in amino acid sequence using the method of Tripet et al. (19) (data not shown). Based on these results we believe it is reasonable to conclude that in addition to δ-CnVID, δ-CnVIB and δ-CnVIC are authentically folded.

Positions 12, 23, and 25 Strongly Affect Nav Channel Subtype Selectivity—We next assayed the pharmacological activity of δ-CnVIB, δ-CnVIC, δ-CnVID, and the two hybrid δ-conotoxins on a panel of Nav channels (Fig. 8). At 5 μM all of the δ-conotoxins showed strong activity on Na1.6, and none showed any activity on Na1.7, Na1.8, or the Drosophila melanogaster...
sodium channel DmA1. The absence of activity of these δ-conotoxins on the insect Nav channel contrasts with activity seen with Na channel-targeting toxins from other venomous animals such as scorpions or spiders, which typically display higher potencies against insect channels than against the mammalian Na channel isoforms (20). No activity for δ-CnVIB, δ-CnVIC, or δ-CnVID (1 μM) was observed on the potassium channels Kv1.1, Kv1.3, Kv1.4, Kv1.5, Shaker IR, and hERG (data not shown).

Notably, striking differences in subtype selectivity were observed across the other mammalian Nav channel isoforms tested: Na1.2, Na1.3, Na1.4, and Na1.5. On Na1.2, δ-CnVIC, δ-CnVID, and Hybrid 2 exhibited moderate levels of inhibition of inactivation, in contrast to δ-CnVIB and Hybrid 1, where inhibition of inactivation was absent or barely detectable. For Na1.3, δ-CnVID showed robust inhibition of inactivation in contrast to the other δ-conotoxins, which showed no detectable activity. For Na1.4, δ-CnVIB, δ-CnVIC, and Hybrid 2 generate modest to high levels of inhibition of inactivation, whereas activity of δ-CnVID and Hybrid 1 is undetectable and strongly reduced, respectively. For Na1.5, δ-CnVIC and Hybrid 2 showed low levels of inhibition of inactivation, in contrast to the other δ-conotoxins, which did not exhibit any detectable activity. In terms of channel subtype selectivity, δ-CnVIC and Hybrid 2 showed the broadest range of activity, strongly inhibiting inactivation on Na1.6 with no activity.
on any other subtype tested except on Na\(_{v}\) 1.4, where inhibition of inactivation was barely detectable at 5 \(\mu\)M.

Dose-response curves were performed on Na\(_{v}\) 1.6, the channel on which the whole panel of compounds showed strong activity at 5 \(\mu\)M, and on Na\(_{v}\) 1.4, the channel on which the most striking differences in activity were observed (Fig. 9). All conotoxins showed similar potency on Na\(_{v}\) 1.6, with EC\(_{50}\) values of 2.3 \(\pm\) 0.2 \(\mu\)M for \(\delta\)-CnVIB, 2.5 \(\pm\) 0.3 \(\mu\)M for \(\delta\)-CnVIC, 1.7 \(\pm\) 0.4 \(\mu\)M for \(\delta\)-CnVID, 1.4 \(\pm\) 0.4 \(\mu\)M for Hybrid 1, and 1.7 \(\pm\) 0.2 \(\mu\)M for Hybrid 2. Although potencies (EC\(_{50}\)) on Na\(_{v}\) 1.4, where they could reliably be measured, were similarly in the 1–2 \(\mu\)M range, efficacies (plateau levels of activity) were strikingly different, consistent with the different activities seen on Na\(_{v}\) 1.4 when the different conotoxins were used at a single concentration (Fig. 8). I\(_{30\ ms}\)/I\(_{\text{peak}}\) values of 0.8, 0.6, and 0.4 were determined for \(\delta\)-CnVIB, Hybrid 2, and \(\delta\)-CnVIC, respectively, whereas the
corresponding value for Hybrid 1 was ~0.2. No detectable efficacy on Na$_{1.4}$ was observed for -CnVID.

**DISCUSSION**

**Overcoming the Challenge of Conotoxin Synthesis**—Study of the pharmacology of δ-conotoxins has been hampered by the challenges of producing the molecules in a correctly folded form by chemical synthesis. This is because the highly hydrophobic nature of the majority of δ-conotoxins makes them not only difficult targets for solid-phase peptide synthesis but also difficult to handle during refolding steps (2). To address this problem we developed a strategy centered on the use of an acid-cleavable solubility tag together with a fully directed disulfide bridge formation procedure (Fig. 2). The solubility tag was readily removed by HF treatment, yielding fully active, authentically folded material (Figs. 5–7). The robustness, simplicity, and yield provided by this strategy compares favorably with previous approaches used to produce δ-conotoxins using partly directed disulfide bridge formation (21), spontaneous disulfide bridge formation (15, 22), and appending a conotoxin propeptide (23). Several other removable solubility tag approaches involving short stretches of positively charged amino acids have been proposed for the synthesis of hydrophobic peptides (24–28). However, use of the C-terminal thioester tag (27) is restricted to the assembly of linear peptides by native chemical ligation, and the other approaches (24–26, 28), which involve cleavage of the linker under strongly basic conditions are not compatible with directed disulfide bridge approaches, because basic conditions favor disulfide bridge rearrangement (29). In contrast, disulfide bridges remain stable under the acid conditions used for tag cleavage in our strategy. Our acid-cleavable solubility tag approach may therefore facilitate the chemical synthesis of other hydrophobic conotoxin families that are currently challenging to produce (2), as well as being of broader applicability to the synthesis of strongly hydrophobic peptides with complex disulfide bridge patterns. Our approach may also be of utility in the context of syntheses involving non-directed disulfide bridge formation, but the beneficial effect of the tag on handling and purification would have to be weighed against any influence its presence might have on the formation of non-native disulfide bridge patterns.

**Native δ-Conotoxins from C. consors Show Na$_{v}$ Subtype Selectivity**—Successful synthesis of the three native δ-conotoxins plus two analogs enabled us to conduct a deeper exploration of Na$_{v}$ channel pharmacology (Fig. 8). Our results demonstrate considerable differences in channel subtype selectivity across the native conotoxins, with δ-CnVIB active on Na$_{1.4}$ and Na$_{1.6}$, δ-CnVIC active on Na$_{1.2}$, Na$_{1.4}$, Na$_{1.5}$, and Na$_{1.6}$, and δ-CnVID active on Na$_{1.2}$, Na$_{1.3}$, and Na$_{1.6}$. Together with the previous characterization of δ-EVIA (11), this observation provides further exemplification of the capacity of native δ-conotoxins to interact selectively with Na$_{v}$ subtypes.

**Structure-Activity Relationships**—Because the primary structures of the three native δ-conotoxins are identical except at three positions (Table 1) and are likely to share similar backbone structures, differences in channel subtype selectivity are likely to be due to amino acid combinations in these positions. By matching amino acid combinations and subtype selectivity at these positions across the panel of δ-conotoxin hybrids that we synthesized and tested, it is possible to make some preliminary observations about structure-activity relationships for Na$_{v}$ channel selectivity (Fig. 10). Activity on Na$_{1.2}$ is abolished in the two conotoxins, δ-CnVIB and Hybrid 1, that fea-
ture a polar residue (Thr) rather than a hydrophobic residue (Ile, Leu, or Phe) at position 12. For Na\textsubscript{v}1.3, the Phe\textsuperscript{12}–Phe\textsuperscript{23}–Leu\textsuperscript{25} motif present in δ-CNVID provides strong activity, whereas the similar permutations present in the other δ-conotoxins (Thr\textsuperscript{12}–Phe\textsuperscript{23}–Phe\textsuperscript{25}, Ile\textsuperscript{12}–Leu\textsuperscript{23}–Phe\textsuperscript{25}, Phe\textsuperscript{12}–Phe\textsuperscript{23}–Phe\textsuperscript{25} or Thr\textsuperscript{12}–Phe\textsuperscript{23}–Leu\textsuperscript{25}) provide no detectable activity. Finally, for Na\textsubscript{v}1.4, we note that activity is abolished in the δ-conotoxins featuring Leu rather than Phe at position 25.

Dose-response experiments carried out on Na\textsubscript{v}1.4 indicated that the differences in activity on this channel are reflected as differences in efficacy $I_{30}$ (peak ratio) rather than potency (EC\textsubscript{50}). We would therefore speculate that modifications at positions 12, 23, and/or 25 have little effect on Na\textsubscript{v}1.4 binding affinity but instead strongly influence effector function.

Conclusion—Using an acid-cleavable solubility tag approach we were able to overcome obstacles in the synthesis of hydrophobic conotoxins that have up until now limited pharmacological investigation of their molecular targets. This approach may be of broader use in the synthesis of other difficult hydrophobic cysteine-rich peptides. Successful synthesis of a panel of structurally similar δ-conotoxins enabled us to begin to probe the structure-activity relationships governing Na\textsubscript{v} channel modulation by δ-conotoxins. These observations could be used as a starting point for further investigation, which could more completely explain the structure-activity relationship for δ-conotoxin Na\textsubscript{v} subtype selectivity and lead to the discovery of highly selective δ-conotoxins.

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