Peptide Folding

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Total Synthesis of the Analgesic Conotoxin MrVIB through Selenocysteine-Assisted Folding**

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The well-being of millions of people worldwide is negatively affected by chronic pain. In particular, neuropathic pain is difficult to treat, and there is an urgent need to find new and better therapeutic targets. Different subtypes of voltage-gated sodium channels (VGSCs) play specialized roles in the transmission of pain and other pathological processes of the central nervous system.[1,2] One of nine known VGSC subtypes, the tetrodoxin-resistant (TTX-R) sodium channel $Na_v1.8$ has attracted significant interest as a strategic therapeutic target for pain relief, as it is predominantly expressed in dorsal root ganglion (DRG) neurons and is involved in the development and maintenance of persistent pain states.[2,3]

Within the large repertoire of peptide toxins isolated from the venom of marine cone snails, the μO-conotoxin MrVIB (Figure 1 a) is the first peptide to show analgesic properties that acts on VGSCs.[4–7] Interestingly, when tested on individual VGSC subtypes expressed in *Xenopus* oocytes, MrVIB exhibited a minimum of 10-fold higher potency for the blocking of $Na_v1.8$ over other VGSC subtypes. To date, this toxin and a small molecule, A-803467,[8] are the only known VGSC inhibitors to display selectivity for $Na_v1.8$. Studies on chronic-pain models in rats have also highlighted the potential therapeutic use of MrVIB. Allodynia- and hyperalgesia-induced pain sensations were reduced by infusions of MrVIB at doses that did not compromise motor behavior.[4,5]

Since the initial isolation of MrVIB in the mid-1990s,[9,10] further investigations on this potential drug lead have been hampered by its limited availability. Only restricted amounts of the toxin can be isolated from the cone snail venom, and its hydrophobic nature has long hindered the development of an efficient chemical synthesis. With a high content of hydrophobic residues and only one localized concentration of charged residues, MrVIB tends to aggregate and precipitate in aqueous media, which makes it difficult to fold and to handle throughout standard peptide purification procedures.[5,6]

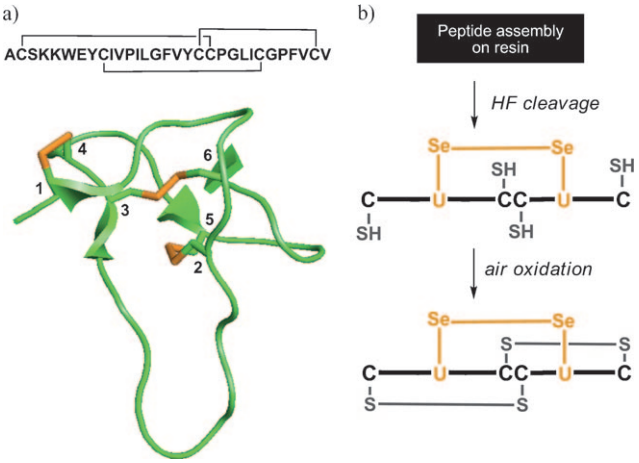

Figure 1. a) MrVIB sequence and structure showing the position of the three disulfide bridges (orange): Cys2–Cys20 (1–4), Cys9–Cys25 (2–5), and Cys19–Cys30 (3–6). b) Overview of MrVIB folding directed by the replacement of a native disulfide with a diselenide bond (shown for the [C9U,C25U]-MrVIB analogue).

The first chemical synthesis of MrVIB was accomplished in very low yield by a two-step oxidative folding strategy, for which the native cysteine pairs were orthogonally protected to enable control over the formation of one disulfide bridge.[10] Importantly, all our attempts to convert linear fully reduced MrVIB into the native folded peptide through oxidative folding failed (data not shown).

Herein we describe a different approach based on the regioselective formation of a single diselenide bond to assist the folding of MrVIB. Selenocysteine (Sec) is an uncommon naturally occurring amino acid that can be employed as an isosteric replacement for cysteine.[11] The chemical substitution of a disulfide bond by a diselenide bond has been shown for a variety of peptides, including oxytocin,[12] endothelin,[13] apamin,[14] a trypsin inhibitor,[15] and various conotoxins, including ImI,[16] AuIB,[17] MI,[17] PnIA,[17] and SIIIA.[18] Recently, the ω-conotoxin GVIA[19] was also shown to fold efficiently. In these cases, the diselenide-bond analogues maintained full biological activity without any significant change in the conformational structure of the peptide. Cys-to-Sec replacements have also been described for more complex

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systems, such as the spider toxin κ-HXTX-Hv1c[20] and proteins glutaredoxin[21] and thioredoxin.[22]

Despite the similarity between Sec and Cys, a diselenide bond has a lower redox potential than a disulfide bond (−381 mV for Se–Se and −181 mV for S–S).[11,23] There is also a difference in nucleophilicity between the selenolate (Se^-) and thiolate (S^-) anions (pK_a(Sec) = 5.24–5.63, pK_a(Cys) = 8.25); hence, reactions with Sec occur much faster at lower pH values than reactions with Cys.[11] These properties suggest that, under acidic conditions, diselenide-bond formation is favored over disulfide-bond formation. Such regioselectivity can be exploited to direct the folding of peptides containing multiple disulfide bridges. This strategy was initially demonstrated by Moroder et al.[13,14a] for the folding of peptide frameworks containing two disulfide bonds and later applied to the synthesis of α-conotoxins.[17] The method was recently extended by Bulaj and co-workers to more complex toxins containing three disulfide bonds.[15,18,19]

Herein we report an efficient synthesis of MrVIB analogues containing one diselenide isosteric bridge. The incorporation of a diselenide bond was anticipated to both decrease the number of possible misfolded disulfide-bond connectivities and possibly direct correct folding via the partially folded species. To establish the position in which the diselenide bridge would better direct correct folding of the conotoxin MrVIB, we prepared three different analogues ([C2U,C20U]-, [C9U,C25U]-, and [C19U,C30U]-MrVIB) by standard solid-phase peptide synthesis based on temporary protection of the α-amino groups with a *tert*-butoxycarbonyl (Boc) group.[24] The Boc strategy is particularly advantageous for the synthesis of Sec-containing peptides owing to minimization of the formation of side products during peptide-chain elongation and ease of diselenide-bond formation.[16]

After assembly on a solid cross-linked polystyrene support, the MrVIB analogues were cleaved by acidolysis with HF (Figure 1b). Under these conditions, the 4-methylbenzyl protecting groups of Sec and Cys residues were cleanly removed, and simultaneous formation of the diselenide bond took place; the four Cys residues remained as unprotected thiols (see Figure S1 in the Supporting Information). The diselenide-bridged peptides showed the expected molecular weight by MALDI-TOF mass spectrometry (3503.1 Da $[M+H]^+$; expected: 3502.6 Da), and the formation of the diselenide bond was further confirmed by alkylation experiments (see Figure S2).

The final folding of the three seleno-MrVIB analogues was carried out by air oxidation of the four remaining cysteine residues in a glutathione buffer (Figure 1b). The addition of up to 50% isopropanol to the buffer improved the accumulation of the correctly folded isomer and assisted the solubilization of the peptide. Distinct oxidative-folding profiles were observed for different placements of the diselenide cross-link in the peptide sequence (Figure 2). Anchoring of the Sec9–Sec25 bridge better directed folding, with up to 61% formation of the correct isomer, whereas locking of the Sec2–Sec20 and Sec19–Sec30 bridges led to 42 and 47% conversion, respectively, into the desired product. The fully folded seleno-MrVIB analogues were purified by HPLC and analyzed by mass spectrometry. All products showed the expected molecular weight (3499.1 Da $[M+H]^+$; expected: 3498.6 Da) and were isolated in good purity (see Figure S3).

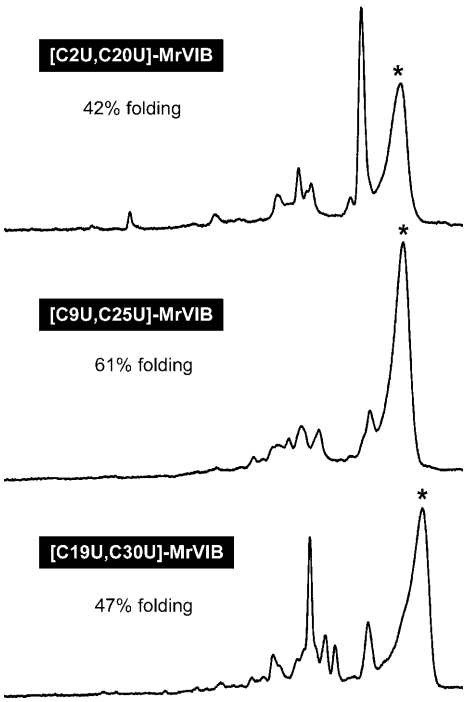

Figure 2. HPLC traces of the oxidative folding for the seleno-MrVIB analogues and accumulation (%) of the correctly folded peptide in each case. The seleno products were eluted as a broad peak, as typical for MrVIB (asterisk).

To minimize the use of HPLC purification and increase peptide recovery, we subjected the [C9U,C25U]-MrVIB analogue to direct oxidization after cleavage with HF from the resin. The desired product was obtained in 4% overall yield, which is a considerable enhancement in comparison with the yield of the reported optimized two-step oxidation (approximately 1%).[5]

We used ^{1}H NMR spectroscopy to compare the structure of the Sec-MrVIB toxins. The similar chemical shifts observed for the α hydrogen atoms indicated that the tertiary fold of the native peptide is maintained throughout each analogue structure (see Figure S4).

We subsequently investigated the effect of the selenium substitution on the potency and selectivity of each MrVIB analogue in the blocking of different VGSCs. The concentration-dependent inhibition of $Na_v1.8$ channels in *Xenopus* oocytes yielded comparable IC_{50} values for wild-type MrVIB and the seleno analogues (Table 1). When tested on TTX-R and TTX-S sodium channels in rat DRG neurons, [C2U,C20U]-MrVIB displayed improved selectivity in comparison with wild-type MrVIB (Figure 3). Furthermore, [C9U,C25U]-MrVIB showed similar potency to that of wild-type MrVIB, whereas [C19U,C30U]-MrVIB showed a four-fold reduction in potency (Table 1; see also Figure S5).

To examine the analgesic potential of the seleno-MrVIB analogues, the peptides were injected intrathecally into rats in which chronic pain symptoms had been induced. The signs of chronic pain relief produced by synthetic wild-type MrVB

Table 1: IC_{50} values for the inhibition of TTX-R and TTX-S (tetrodotoxin-sensitive) sodium channels in DRG neurons and $Na_v1.8$ expressed in *Xenopus* oocytes.

MrVIB	IC_{50} (TTX-R) [nM]	IC_{50} (TTX-S) [μM]	IC_{50} ($Na_v1.8$) [nM]
wild type	22	>1	326
[C2U,C20U]	12	>1	253
[C9U,C25U]	26	>1	310
[C19U,C30U]	100	>1	340

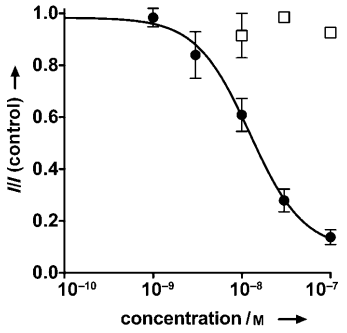

Figure 3. Concentration–response curve for the inhibition of TTX-R (●) versus TTX-S (□) sodium currents by [C2U,C20U]-MrVIB in DRG neurons. The analogue showed improved potency and selectivity over wild-type MrVIB.

and the two seleno analogues that were most active in DRG neurons, [C2U,C20U]- and [C9U,C25U]-MrVIB, were indistinguishable. At 1 nmol doses, the seleno analogues produced significant reversal of allodynia. The reversal of allodynia was similar in magnitude and duration to the pain relief produced by authentic MrVIB.[4] Furthermore, all MrVIB analogues produced similar levels of modest impairment of motor function. The degree of impairment was similar to that previously reported for native MrVIB (see Figure S6).

In conclusion, we have described a novel chemical strategy for the correct folding of MrVIB into its native structure. The folding was driven by regioselective diselenide-bond formation. In this approach, the replacement of a single disulfide bond with a diselenide bond in the previously "unfoldable" linear reduced conotoxin MrVIB yielded correctly folded seleno-MrVIB analogues of identical structure and potency. This method is much faster than the use of orthogonally protected cysteine residues for selective disulfide-bond formation and opens up the possibility of further optimization of selectivity for a particular VGSC. Interestingly, [C2U,C20U]-MrVIB displayed slightly higher potency at TTX-R sodium channels than the native peptide but considerably greater TTX-R/TTX-S selectivity.

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