Peptidomimetics

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Stabilization of the Cysteine-Rich Conotoxin MrIA by Using a 1,2,3-Triazole as a Disulfide Bond Mimetic**

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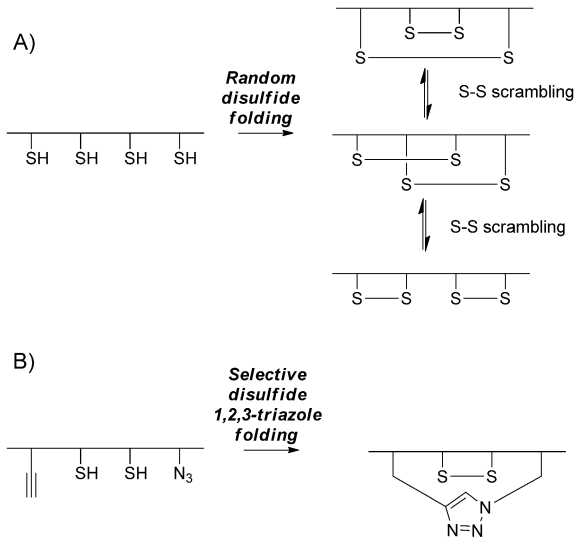
Abstract: *The design of disulfide bond mimetics is an important strategy for optimising cysteine-rich peptides in drug development. Mimetics of the drug lead conotoxin MrIA, in which one disulfide bond is selectively replaced of by a 1,4-disubstituted-1,2,3-triazole bridge, are described. Sequential copper-catalyzed azide–alkyne cycloaddition (CuAAC; click reaction) followed by disulfide formation resulted in the regioselective syntheses of triazole–disulfide hybrid MrIA analogues. Mimetics with a triazole replacing the Cys4–Cys13 disulfide bond retained tertiary structure and full in vitro and in vivo activity as norepinephrine reuptake inhibitors. Importantly, these mimetics are resistant to reduction in the presence of glutathione, thus resulting in improved plasma stability and increased suitability for drug development.*

Venom peptides target receptors such as GPCRs, transporters, and ion channels,[1] and several candidates have entered clinical trials.[2] These peptides are comprised of disulfide bond frameworks, which scaffold well-defined structures and topological orientation of the pharmacophore, thus leading to stable, potent, and selective peptides.[3]

Disulfide-rich peptides are relatively stable to proteases but are susceptible to "S–S scrambling"[4] through thiol–disulfide exchange,[4b,5] which leads to structural rearrangements that lower activity[6] and increasing loop sizes, which facilitates enzymatic degradation in vivo.[6,7] Orthogonal cysteine protection strategies[8] allow regioselective folding although the inherent potential for S–S scrambling remains (Scheme 1 A).[9] Stable disulfide bond mimetics[10] can prevent S–S scrambling, but such strategies lack broad applicability owing to complex chemistry or incompatibility of the mimetic moiety with bioactivity.[10a] Selenocysteine replacement is the most viable strategy[9b,10f,11] and its application has led to regioselective folding combined with increased stability to reduction.[7,12]



Scheme 1. A) Disulfide folding results in multiple isomers. Disulfide-rich peptides are susceptible to S–S scrambling. B) Disulfide/1,2,3-triazole folding delivers only one possible fold.

Alternatively, replacement of disulfide bonds by 1,4-disubstituted 1,2,3-triazoles,[13] obtained through copper-catalyzed azide–alkyne cycloaddition (CuAAC),[14] may be useful given their stability towards protolytic hydrolysis in vivo.[15] Their incorporation would prevent disulfide scrambling and accelerate folding of the remaining cysteine residues as observed with α-selenoconotoxins.[12a]

We introduce a strategy (Scheme 1 B) that combines disulfide bond formation with orthogonal CuAAC,[14] thereby enabling the regioselective synthesis of triazole mimetics of the norepinephrine reuptake inhibitor χ-conotoxins MrIa and

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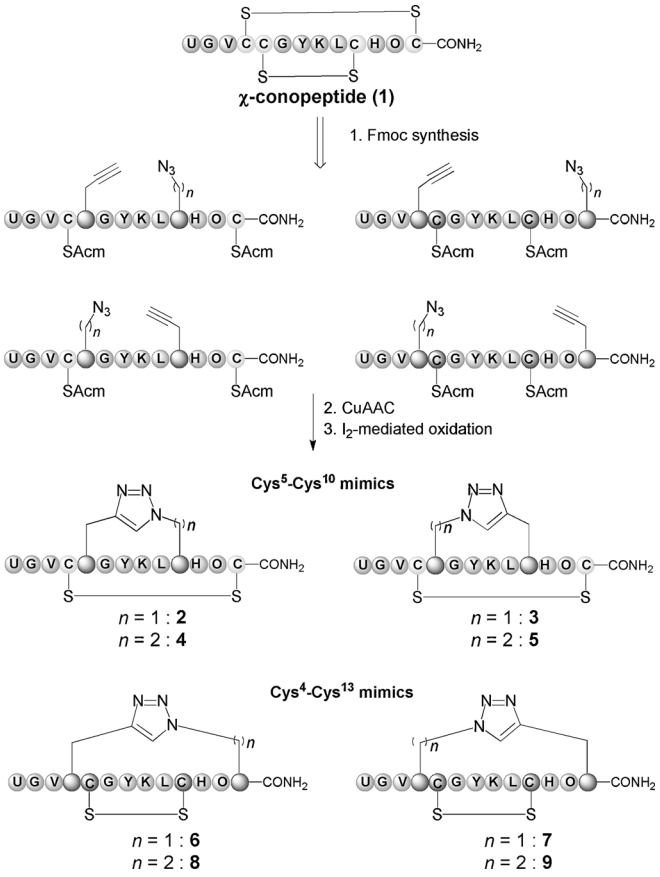

Scheme 2. Selective replacement of the disulfide bond in **1** by 1,4-disubstituted 1,2,3-triazole to give analogues **2**–**9**. Cysteine was substituted by azido alanine [Aza]/homoalanine [Aha] ($n=1/2$) and propargylglycine as the alkyne counterpart. Precursors underwent CuAAC followed by I_2-mediated Acm removal/oxidation to yield **2**–**9**.

1 with improved stability in thiol-containing environments. (Figures S7–S10 in the Supporting Information).

Eight analogues (**2**–**9**) of **1** were synthesized by using Fmoc-chemistry. The Cys5–Cys10 or the Cys4–Cys13 S–S bonds were individually replaced with a triazole bridge while maintaining the second S–S bond (Scheme 2). The 1,2,3-triazoles were introduced with directionality depending on placement of the azide/alkyne moieties [Scheme 2 and Table 1: azide towards the C terminal (**2**,**4**,**6**,**8**) versus azide towards the N terminal (**3**,**5**,**7**,**9**)]. Bridge flexibility was introduced by incorporating either (*S*)-2-amino-3-azidopropanoic acid (**2**,**3**,**6**,**7**) or (*S*)-2-amino-4-azidobutanoic acid (**4**,**5**,**8**,**9**). Propargylglycine [Prg] was the alkyne moiety employed for triazole formation.

Cysteine residues were incorporated with S-acetamidomethyl (Acm) protection and folding was performed by forming the 1,2,3-triazole by CuAAC ($CuSO_4$/ascorbic acid to generate the Cu^I catalyst in situ),[14] followed by disulfide bond closure by using iodine in methanolic water[16] to yield heterodetic peptides (**2**–**9**) in good yields (Table S2 in the Supporting Information). Peptides were evaluated in a functional norepinephrine reuptake assay and compared to peptide **1** and the equipotent MrIa. Table 1 shows that the strategy can deliver fully functional mimetics of parent peptide **1** when the triazole moiety is appropriately positioned.

Full in vitro activity was retained for peptides with the Cys4–Cys13 disulfide bond replacement (**6**–**9**), whereas modifications to the Cys5–Cys10 disulfide gave analogues (**2**–**5**) with reduced ability to inhibit the norepinephrine transporter (NET). The most active analogues **7** and **9** were as active as MrIA in a neuropathic pain study in a rat model sciatic nerve injury assay (Figure S5, S6).[17] The activity loss in **2**–**5** is due to the sensitivity of the turn region to topological changes,[17,18] which are likely to occur when introducing a 1,2,3-triazole ring in place of the Cys4–Cys10 disulfide bond. The triazole orientation is important for NET activity (compare peptides **7**,**9** with **6**,**8**). By contrast, the length of the bridge, which differed by one CH_2 group, plays a minor role (**7** vs. **9**). These structural effects were studied by secondary Hα NMR shift comparison[17] of MrIA and analogues **2**–**9**. The results revealed that the less active analogues **2**–**5** suffer from strong perturbation of the overall backbone structure (Figure 1 A). By contrast, the active analogues **6**–**9** showed good Hα shift correlation compared to MrIA, particularly in the critical GYKL turn region[18] (Figure 1 B). The most potent analogues (**7**, **9**) are highly consistent with MrIA, while the slightly less active isomers (**6**, **8**), which have a retro-triazole structure, show perturbation of the backbone. A 3D NMR structure for the most potent peptide **9** was compared with that of MrIA (Figure 1 C). An overlay of their sheet regions (RMSD, 0.3 Å) and the side chains of the pharmacophore (Tyr7, Lys8, and Leu9) showed high conservation (RMSD of 0.24 Å across backbone and β carbons), with side-chain orientation as described for MrIA and **1**.[17,19] Disulfide-folded peptides have rigid structures, which limits access to cleavage sites, thus resulting in improved proteolytic stability.[20] This suggests that degradation likely involves partial disulfide reduction/disulfide scrambling.[4a] Indeed, peptide **1** undergoes isomerisation, partial reduction, adduct formation, and dimerization in the presence of thiols (GSH) and it is unstable in plasma (Figure 2 and Figure S7).[21] In hybrids **2**–**9**, the incorporation of a stable

Table 1: Norepinephrine reuptake inhibition by the triazole-disulfide hybrid analogues (**2**–**9**) compared to the parent peptide (**1**).

Entry	Sequence[a]	IC_{50} [μM][f]
MrIA	NGVCCGYKLCHOC[b]	2.45 ± 0.07
1	UGVCCGYKLCHOC[b,c]	2.55 ± 0.18
2	UGVC[Prg]GYKL[Aza]HOC[d,f]	52.40 ± 5.50
3	UGVC[Aza]GYKL[Prg]HOC[e,f]	60.60 ± 14.8
4	UGVC[Prg]GYKL[Aha]HOC	41.20 ± 5.80
5	UGVC[Aha]GYKL[Prg]HOC	30.00 ± 2.00
6	UGV[Prg]CGYKLCHO[Aza]	3.84 ± 0.50
7	UGV[Aza]CGYKLCHO[Prg]	1.73 ± 0.30
8	UGV[Prg]CGYKLCHO[Aha]	8.90 ± 1.00
9	UGV[Aha]CGYKLCHO[Prg]	1.10 ± 0.25

[a] C-terminal amidated; [b] disulfide connectivity (1–4, 2–3), O = hydroxyproline, [c] U = pyroglutamic acid, [d] [Prg] = propargylglycine, [Aza] = azidoalanine; [e] [Aha] = azidohomoalanine; [f] in **2**–**9**, a 1,2,3-triazole moiety is formed between [Aza]/[Aha] and [Prg] residue and S–S bond between remaining cysteine. IC_{50} = half maximal inhibitory concentration.

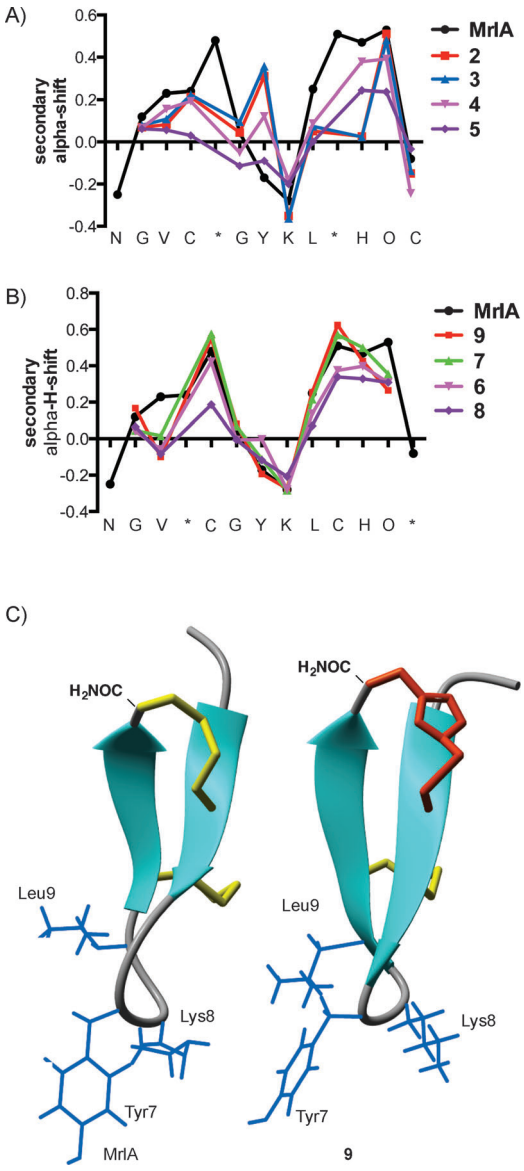

Figure 1. NMR analysis of the 1,2,3-triazole/disulfide hybrid peptides compared to MrIA. Secondary Hα shifts are shown for A) the inactive analogues **2–5** and B) the active analogues 6–9. C) NMR structure of MrIA (left) and 1,2,3-triazole analogue **9** (right). Disulfides are shown in yellow and the 1,2,3-triazole in red.

triazole moiety both prevented scrambling and lowered the susceptibility of the remaining disulfide bond to reduction. Stability assays in which **2–9** were compared with the parent molecule **1** revealed that all of the triazole-containing molecules (**2–9**) displayed improved stability in rat plasma, with extrapolated half-lives in excess of 2 days, whereas **1** was degraded in less than 2 days (Figure 2). Improved stability to disulfide bond exchange in the presence of glutathione was observed for the peptides with a triazole in the place Cys4–Cys13 (**6–9**). The Cys5–Cys10 triazole-substituted peptides (**2–5**) were more plasma stable than **1** although they were still susceptible to disulfide scrambling in a high thiol environment. (Figure S8–S10).

A strategy for the selective folding of disulfide-rich peptides through the combination of CuAAC and disulfide

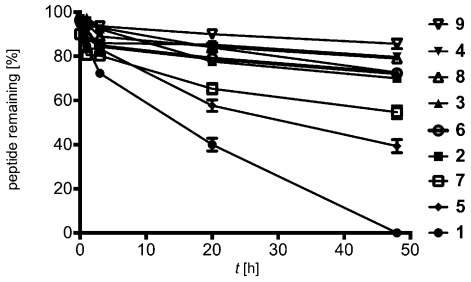

Figure 2. Stability in rat plasma of peptide **1** and the triazole–disulfide hybrid analogues (**2–9**).

formation was investigated with the drug candidate conopeptide **1**. Peptidomimetics **6–9**, in which Cys4–Cys13 disulfide bond is replaced by 1,2,3-triazole, showed improved stability to reduction and maintained native structure and both in vitro and in vivo activity, whereas replacing the Cys5–Cys10 disulfide bond in **2–5** failed owing to structural sensitivity of the pharmacophore region.[17] Considerable plasma stability improvements were found for hybrid peptides **2–9**, thus suggesting that this strategy is broadly applicable to the improvement of cysteine-rich peptides.

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