Selective folding and stabilization of a cysteine rich conotoxin, MrIA as a 1,2,3-triazole-disulfide bond mimetic**

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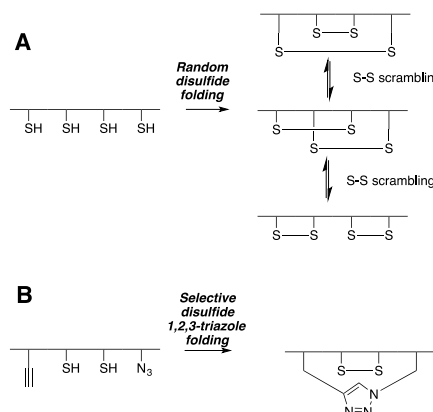
Dedication((optional))

Abstract: *Disulfide bond mimetics are an important strategy in optimising cysteine-rich peptides in drug development. We describe the synthesis and characterisation of peptide mimetics of the drug lead conotoxin MrIA by selective replacement of one disulfide bond by a 1,4-disubstituted-1,2,3-triazole bridge. Sequential copper catalyzed azide-alkyne click reaction (CuAAC) followed by disulfide formation resulted in regioselective syntheses of triazole-disulfide hybrid MrIA analogs. Mimetics with triazole replacing Cys4-Cys13 disulfide retained tertiary structure and full activity as norepinephrine reuptake inhibitors* in vitro *and* in vivo *rat pain models. Importantly, these mimetics were resistant to reduction in the presence of glutathione, resulting in improved plasma stability thus increasing their suitability for drug development.*

Many *v*enom peptides target receptors such as GPCRs, transporters and ion channels,[1] with several candidates entering clinical trials.[2] These peptides are comprised of disulfide bond frameworks, scaffolding well-defined structures and topological orientation of their pharmacophores leading to stable, potent and selective peptides.[3]

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

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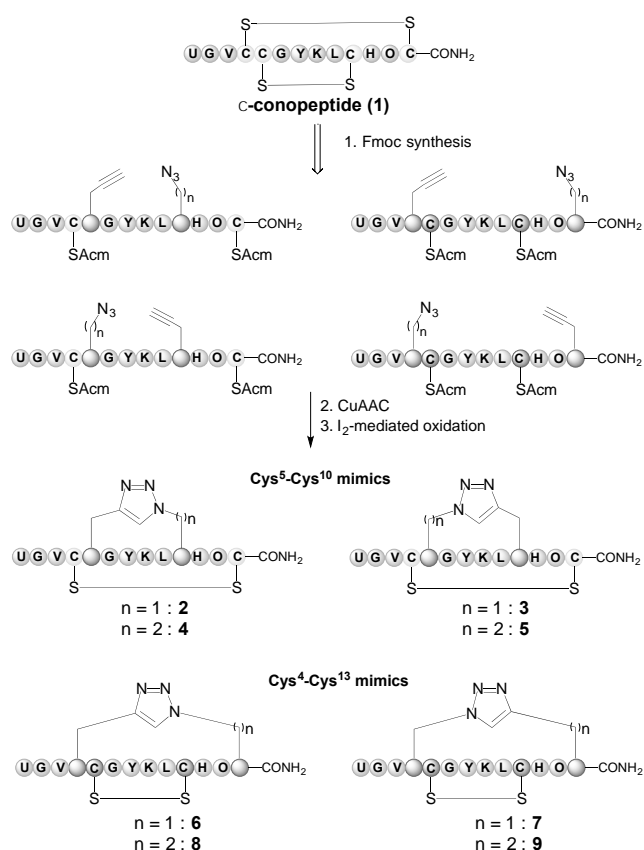   E-mail: a.brust@imb.uq.edu.au

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Scheme 1. A: Disulfide folding may result in multiple disulfide isomers, Disulfide rich peptides can be susceptible to S-S scrambling B: the disulfide/1,2,3-triazole folding strategy delivers only one possible fold.

Alternatively, disulfide bond replacement by 1,4-disubstituted 1,2,3-triazoles[13] obtained by CuAAC[14] may be useful given their stability towards protolytic hydrolysis *in vivo*[15] and their incorporation would both avoid disulfide scrambling and accelerate the folding of remaining cysteine residues as observed with the α-selenoconotoxins.[12a]

Here we introduce a strategy (Scheme 1B), combining disulfide bond formation with orthogonal CuAAC[14] that enables the regioselective synthesis of triazole mimetics of the norepinephrine reuptake inhibitor χ-conotoxins **MrIa** and **1** with full bioactivity and improved stability in thiol-containing environments. (Fig 7S-10S).

Scheme 2. Selective disulfide bond replacement in **1**, by 1,4-disubstituted 1,2,3-triazole, leading to analogues **2-9**. Azidoalanine (n=1) or azidohomoalanine (n=2) were incorporated as cysteine substitutes, and propargylglycine was the alkyne counterpart. Linear precursors underwent CuAAC, then I_2-mediated Acm removal / oxidation yielding **2-9**.

Eight analogues (**2-9**) of peptide **1** were synthesized by Fmoc-chemistry by individually replacing the Cys5-Cys10 or the Cys4-Cys13 disulfide bonds with a triazole bridge while maintaining the second disulfide bond (Scheme 2). The 1,2,3-triazoles were introduced with different directionality depending where the azide or alkyne moieties were placed (see Scheme 2 and Table 1: azide towards C-terminal (**2,4,6,8**) *versus* azide towards N-terminal (**3,5,7,9**)). Bridge flexibility was introduced by incorporating either azido alanine (**2,3,6,7**) or azido homoalanine (**4,5,8,9**) to ensure conformational freedom. Propargylglycine [Prg] was the alkyne moiety employed for triazole formation.

Cysteine residues were incorporated with S-acetamidomethyl (Acm) protection to avoid possible thiol-yne addition[16] as well as azide reduction triggered by thiols during resin cleavage.[17] Peptide folding was performed by forming the 1,2,3-triazole by CuAAC (CuSO4 /ascorbic acid generating *in situ* the Cu^I catalyst[14]) followed by disulfide bond closure using iodine in methanolic water[18] yielding heterodetic peptides (**2-9**) in good yields (Table 2S). Peptides were evaluated in a functional norepinephrine reuptake assay and compared to peptide **1** and equipotent **MrIa**.

Table 1 shows that the strategy could deliver fully functional mimetics of parent peptide **1** when the triazole moiety was appropriately positioned.
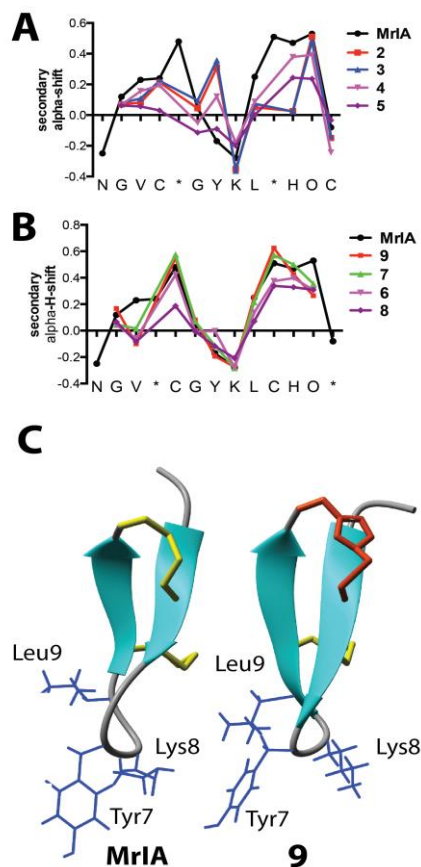
Table 1. Norepinephrine reuptake inhibition of triazole-disulfide hybrid analogues (**2-9**) compared to parent peptide (**1**)

| 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] Peptides were synthesized as C-terminal amide; [b] disulfide bond connectivity (1-4, 2-3), O= hydroxyproline, [c] U= pyroglutamic acid, [d] [Prg]= propargylglycine, [Aza]= azidoalanine; [e] [Aha]= azidohomoalanine; [f] in analogues (**2-9**) a 1,2,3-triazole moiety is formed between [Aza]/[Azh] and [Prg] residue (See Scheme 2). A disulfide bond is between remaining cysteine residues.

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 inactive analogues at NET (→**2-5**). The most active analogues **7** and **9** were also equally active compared with **MrIA** in a neuropathic pain study using a rat model sciatic nerve injury assay (Fig 5,6S).[19] The activity loss in **2-5** is due to the sensitivity of the turn region to topological changes,[19-20] likely to occur when introducing a 1,2,3-triazole ring in place of the Cys4-Cys10 disulfide bond. The triazole orientation appears to be of importance for NET activity (compare the more potent peptides **7, 9** with **6, 8**). In contrast, the length of the bridge, which differed by one CH$_2$ group, plays a minor role (compare **7** vs. **9**). These structural effects, were studied by secondary Hα NMR shift comparison[19] of **MrIA** and analogues **2-9**; this demonstrated that inactive analogues **2-5** suffer from strong perturbation of the overall backbone structure (Figure 1A). In contrast, active analogues **6-9** showed good Hα shift correlation compared to **MrIA**, particularly in the critical GYKL turn region[20] (Figure 1B). On closer inspection, the secondary Hα shifts of the most potent analogues (**7, 9**) are highly consistent with **MrIA**, while the slightly less active isomers (**6**, **8**) with a retro-triazole structure show a small perturbation of the backbone. A 3D NMR structure for the most potent peptide **9** was compared with **MrIA** (Figure 1C). An overlay of their sheet regions (RMSD, 0.3Å) 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**.[19, 21] Disulfide folded peptides with rigid structures and herewith limited access to cleavage sites, possess improved proteolytic stability[22] suggesting 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 is unstable in plasma[23] (Fig 2, 7S). In hybrids **2-9**, the incorporation of a stable triazole moiety both avoided scrambling and lowered the tendency for reduction of the remaining disulfide bond. Stability assays comparing **2-9** with the parent molecule **1** revealed that all triazole-containing molecules (**2-9**) displayed improved rat plasma stability, with extrapolated half-life

in excess of 2 days whereas **1** was degraded in < 2 days (Figure 2). Improved stability against disulfide bond exchange in the presence of glutathione was observed for peptides with triazole substituting Cys4-Cys13 (**6-9**). The Cys5-Cys10 triazole substituted peptides (**2-5**) were more plasma stable than **1** thought still susceptible to disulfide scrambling in a high thiol environment. (see Fig. 8S, 9S and 10S).

In summary, an alternative strategy for selective folding of disulfide rich peptides by combination of CuAAC and disulfide formation was investigated with the drug candidate conopeptide **1**. Peptidomimetics **6-9** with the 1,2,3-triazole replacing Cys4-Cys13 show improved stability to reduction while maintaining structure and *in vitro* and *in vivo* activity whereas mimicry of the Cys5-Cys10 disulfide bond in **2-5** failed due to structural sensitivity of the pharmacophore region.[19] Considerable plasma stability improvements were found for hybrid peptides **2-9** suggesting this strategy is broadly applicable for cysteine rich peptide improvement.

Figure 1. NMR of 1,2,3-triazole / disulfide hybrid peptides compared to **MrIA**. A) secondary Hα shift of inactive analogues **2-5** and B) active analogues **6-9**. C) NMR structure of **MrIA** (left) and 1,2,3-triazole analogue **9** (right). Disulfides, shown in yellow and 1,2,3-triazole in red.
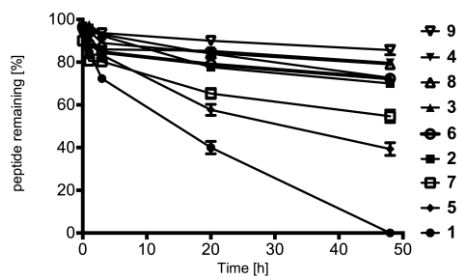
Figure 2. Rat plasma stability comparison of peptide **1** and triazole-disulfide hybrid peptide analogues (**2-9**).

Experimental Section

Experimental details are described in the supporting information

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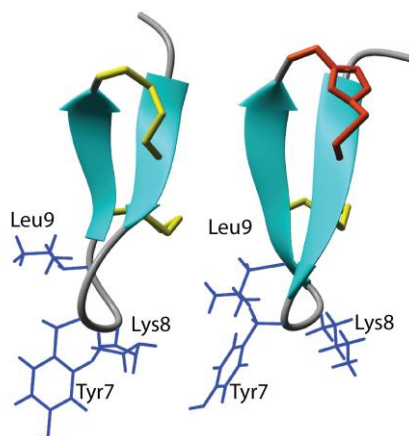
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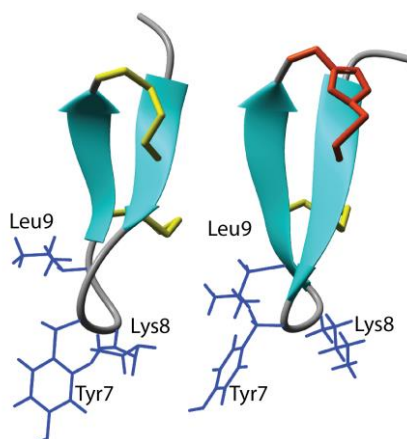
Disulfide bond engineering of a χ-conopeptide delivered selectively formed, bioactive 1,2,3-triazole-disulfide hybrids with vastly improved reductive and plasma stability and function in a neuropathic pain model. We discuss structure implications and the broad impact of this selective folding and disulfide mimetic strategy toward stabilisation of disulfide rich peptides.

Layout 2:

Disulfide bond engineering of a χ-conopeptide delivered selectively formed, bioactive 1,2,3-triazole-disulfide hybrids with vastly improved reductive and plasma stability and function in a neuropathic pain model. We discuss structure implications and the broad impact of this selective folding and disulfide mimetic strategy toward stabilisation of disulfide rich peptides.