DOI: 10.1002/anie.201008142

Disulfide Mimics

"Triazole Bridge": Disulfide-Bond Replacement by Ruthenium-Catalyzed Formation of 1,5-Disubstituted 1,2,3-Triazoles**

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About one fourth of the peptidic macromolecular structures deposited in the protein data base (PDB) contain at least one disulfide bridge.[1] In nature, disulfide bonds are formed in a milieu where oxidizing conditions prevail, for example, on the cell surface or in the extracellular matrix. Many proteins benefit from disulfide contributions to their conformational stability. In particular, the defined tertiary folding of oligopeptides smaller than 30 residues essentially relies on macrocyclization through the cystine motif because of the restricted number of noncovalent intramolecular interactions available. Moreover, formation of the disulfide pattern results in structural rigidity of the peptidic framework, as for example, in the family of cystine knot miniproteins,[2] leading to conformationally constrained scaffolds with extraordinary thermal stability and resistance against proteolytic degradation.[2a] Hence, the discovery and development of disulfide-bridged peptides suitable for diagnostic and therapeutic applications remains a field of intense research.[3]

The in-vitro generation of disulfide bonds in peptides is usually achieved post-synthetically and mediated by DMSO, air oxygen, or other oxidizing agents. Although this reaction step can be achieved under relatively mild conditions in solution, it remains one of the most demanding obstacles towards high-yield peptide synthesis, especially for disulfide-rich species in which the controlled regiospecific formation of several disulfide bonds is not trivial to control.[4] In addition, to suppress unwanted intermolecular reactions of the thiol groups of individual peptides, oxidative folding usually has to be conducted in highly diluted solutions. In spite of the use of gluthathione-based redox buffers, polymer-supported oxidation systems, macrocyclization on the solid support and/or orthogonal protecting groups, control over the topology of the disulfide bridges formed is still a challenge.[4a,e,f,5]

In view of these difficulties and to improve the redox stability of bridged peptides, several routes towards synthetic disulfide surrogates have been developed.[6] Straightforward approaches usually employ thioether, olefin, or alkane-based isosters.[6a,b,d–f] However, cystathione bridges require multiple synthetic steps and careful choice of orthogonal protection, and dicarba bridges give *cis*/*trans* isomers during ring-closing metathesis (RCM).[6a,b,f] Only an additional purification step or the subsequent palladium-catalyzed hydrogenation of the unsaturated species to the corresponding alkane leads to a construct with defined configuration.[6b,f]

In 2004, Meldal et al. described the utility of copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) for a triazole-based disulfide replacement.[6c] Owing to the compelling characteristics of this prototypic "click" reaction, it has been extensively applied in peptide chemistry exploiting the almost perfect orthogonality to side-chain reactivities.[7] The introduction of 1,4-disubstituted 1,2,3-triazoles into peptides has also been used to mimic and rigidify conformations of the amide backbone.[7d,8] Moreover, a variety of examples of CuAAC-based macrocyclizations of peptides in solution and on solid supports has been reported.[6c,8d,9]

Using the same azide- and alkyne-functionalized buidling blocks, 1,5-disubstituted 1,2,3-triazoles can be generated in the ruthenium(II)-catalyzed variant (RuAAC) of the CuAAC.[10] This reaction expands the range of peptidomimetic structures selectively accessible from the same precursor and having different biological activities governed by the architecture of the incorporated triazole.[10c,f–h]

To our knowledge, 1,5-disubstitiuted 1,2,3-triazoles have not been taken into consideration as disulfide mimics to date. Herein, we report the facile introduction of 1,4- and 1,5-disubstituted 1,2,3-triazoles into a monocyclic variant of the sunflower trypsin inhibitor-I (SFTI-1[*1,14*], **1**;[11] Figure 1) and show that the macrocyclic peptidomimeticum **2** with the "1,5" substitution pattern retains nearly full biological activity in contrast to the "1,4" variants **3** and **4**.

The choice of **1** as the model peptide for the investigation of triazole-based disulfide replacements had several reasons. SFTI-1 is a small, though very potent, inhibitor of trypsin.[11,12] Therefore, the influence of different modes of macrocyclization on the bioactivity of the corresponding synthetic variant can be routinely examined by serine protease inhibition assays.[3e,6e,11,12c,13]

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[**] This research was supported by the Deutsche Forschungsgemeinschaft through grant Ko 1390/9-1, LOEWE—Soft Control and by BMBF.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201008142.

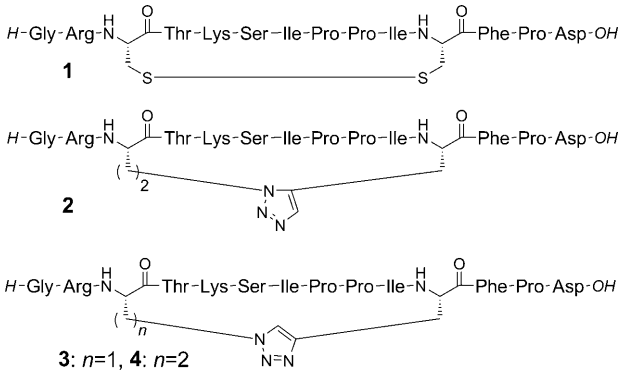


Figure 1. Chemical formula of monocyclic SFTI-1[*1,14*] (**1**),[11] [Ala^3(&^{1}),Ala^{11}(&^{2})]SFTI-1[*1,14*][(&^{1}-CH$_2$-1,5-[1,2,3]triazolyl-&^{2})] (**2**), [Ala^3(&^{1}),Ala^{11}(&^{2})]SFTI-1[*1,14*][(&^{1}-1,4-[1,2,3]triazolyl-&^{2})] (**3**), and [Ala^3(&^{1}),Ala^{11}(&^{2})]SFTI-1[*1,14*][(&^{1}-CH$_2$-1,4-[1,2,3]triazolyl-&^{2})] (**4**). & = Conjunction of peptide backbone and the corresponding macrocyclization motif.

Craik and co-workers intensely investigated the structure and proteolytic stabilities of monocyclic and linear variants of SFTI-1 and demonstrated the importance of the disulfide bridge in maintaining the inhibitory activity of open-chain species.[12a,b,14] Furthermore, Roller et al. and Rolka et al. have shown that disulfide replacements are tolerated depending on the steric demand at residue 3 (Figure 1).[6e,12c,15]

A solution structure of SFTI-1[*1,14*] (PDB code: 1JBN)[12a] enabled possible triazole-based linkages within the peptide chain to be modeled and to evaluate their fit into the parent disulfide-bridged template **1** (Figure 2).

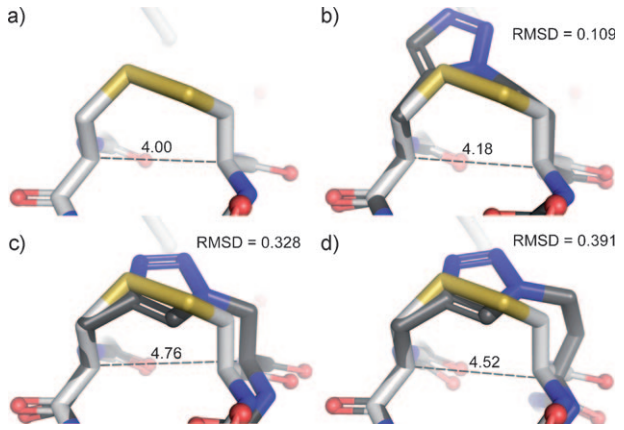


Figure 2. Structure and overlays of energy-minimized 3D models of SFTI-1 variants **1**–**4** showing the region of the disulfide bridge and the corresponding triazole-based substitutes. a) **1**, b) **1** and **2**, c) **1** and **3**, d) **1** and **4**.[12a] Models were aligned at the respective carbonyl, C_α, C_β, and amide nitrogen atoms of residue 11. The root mean square deviations (RMSD) calculated for the respective carbonyl, C_α, C_β, and amide nitrogen atoms of residues 3 and 11 at the compared structures are given in Å. Measured distances between the C_α atoms of residues 3 and 11 of the corresponding model are shown as dashed lines and values are given in Å. Blue nitrogen, light gray carbon atoms of **1**, dark gray carbon atoms of **2**–**4**, red oxygen, yellow sulfur, hydrogen atoms are omitted for clarity (for details see the Supporting Information).

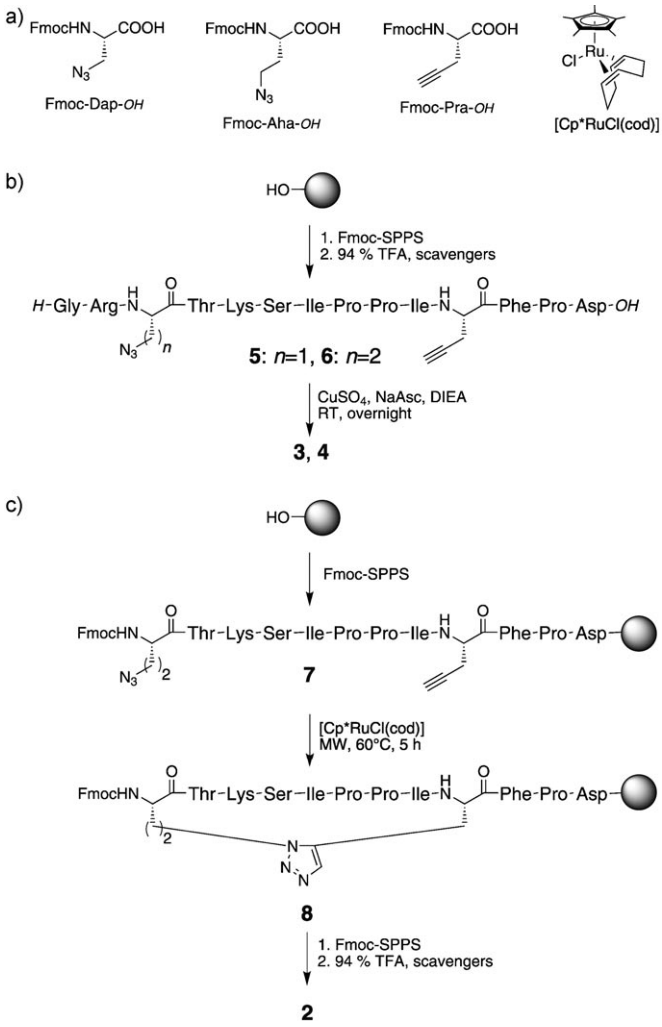
The alignment of the deduced structures of peptidomimetics **2**–**4** with disulfide-bridged peptide **1** gave insight into the steric requirements of the proposed triazole bridges and allowed their tendency to disturb the parent backbone conformation to be investigated (Figure 2b–d). The incorporation of 1,4-disubstituted 1,2,3-triazoles led to increased distances between the C_α atoms of residues 3 and 11 compared to those of the corresponding cysteines in **1** (Figure 2c,d). In the case of the 1,5-disubstituted species the spacing remained essentially the same (Figure 2a,b). Owing to the planar and rigid architecture resulting in low degrees of structural freedom for the aromatic heterocycle, the "1,4" substitution pattern renders compounds **3** and **4** unable to adopt the native conformation of **1** properly, thereby forcing the residues 3 and 11 into more remote positions. In contrast, the "1,5" pattern of **2** seems to be compatible with the intrinsic geometry of **1**. Therefore, a significant difference between the inhibitory activities of compounds **2**, **3**, and **4** depending on the mode of macrocyclization was expected.

SFTI-1[*1,14*] (**1**) was synthesized by microwave-assisted Fmoc-SPPS with subsequent DMSO mediated oxidation (Supporting Information). Compounds **3** and **4** were synthesized in a similar way using commercially available SPPS building blocks Fmoc-L-propargylglycine (Fmoc-Pra-*OH*) and Fmoc-L-azidoalanine (Fmoc-Aza-*OH*) or Fmoc-L-azidohomoalanine (Fmoc-Aha-*OH*) yielding the linear precursors **5** ([Aza^3,Pra^{11}]SFTI-1[*1,14*]) and **6** ([Aha^3,Pra^{11}]SFTI-1-[*1,14*]), respectively (Scheme 1a,b). CuAAC-mediated macrocyclization of unprotected peptides **5** and **6** was performed in diluted solution after acidic cleavage from the support.

As expected, RuAAC conditions appeared incompatible to solution-phase macrocylization of unprotected peptide **6** leading to an undefined mixture of side products. Instead, the 1,5-disubstituted 1,2,3-triazole was successfully installed during SPPS using [Cp*RuCl(cod)] as the catalyst and microwave irradiation to give compound **2**, though in low yield (2.1% according to initial resin load, Scheme 1c). Purified peptides **1**–**6** were characterized by RP-HPLC, ESI-MS, IR-, and NMR-spectroscopy.

Though a formation of an intramolecular triazole bridge proceeds without change of the molecular mass and cannot be detected by standard mass spectrometry, the azide group gives a prominent IR absorption band around 2100 cm^{-1}.[16] This band is sufficiently separated from the main IR signals commonly found in peptides, and it absence products **2**, **3**, and **4** thus indicates the generation of triazoles (Figure 3a). 2D HSQC NMR spectroscopy studies enabled to discriminate 1,4- from 1,5-disubstituted 1,2,3-triazoles by measuring the chemical shifts of the ^{1}H,^{13}C coupling signal assigned to the unique carbon-bound proton found in both heterocycles (Figure 3b).

The signals correlating to the single proton and the corresponding carbon nucleus at position 5 or 4 in the 1,4- or 1,5-disubstituted 1,2,3-triazole bridges of compounds **2**, **3**, and **4** were found in the aromatic region between $\delta = 7.8$–7.3 ppm (^{1}H) and $\delta = 135$–120 ppm (^{13}C). The 2D NMR spectroscopy procedure enabled them to be distinguished from protons not bound to carbon and from the intrinsic phenyl multiplet arising from the side chain of residue 12. The measured ^{1}H



Scheme 1. a) Non-natural amino acid building blocks Fmoc-L-azidoalanine (Fmoc-Aza-*OH*), Fmoc-L-azidohomoalanine (Fmoc-Aha-*OH*), Fmoc-L-propargylglycine (Fmoc-Pra-*OH*) and the catalyst for RuAAC mediated macrocyclization. b) Synthesis of peptidomimetics **3** and **4** bridged by 1,4-disubstituted 1,2,3-triazole. c) Synthesis of peptidomimetic **2** bridged by 1,5-disubstituted 1,2,3-triazole. DIEA = *N*,*N*-diisopropylethylamine, Fmoc = Fluorenylmethoxycarbonyl, NaAsc = sodium ascorbate, TFA = trifluoroscetic acid, Cp* = C_5Me_5, cod = cyclooctadiene, SPPS = solid-phase peptide synthesis.

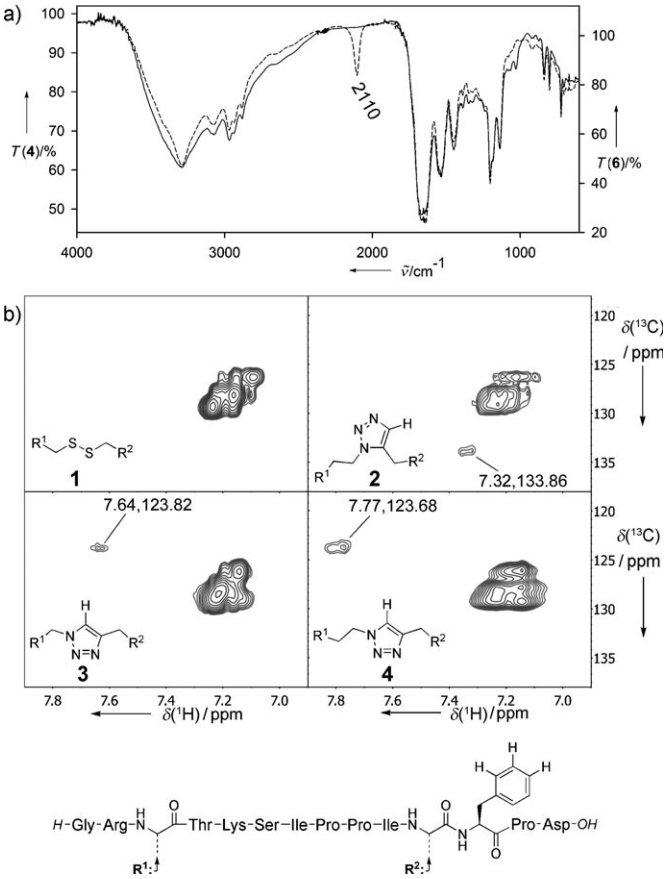


Figure 3. a) Superimposed IR transmission spectra of peptides **4** (——) and **6** (-----), respectively, and assigned asymmetric NNN stretch band of the azide group of **6** at 2110 cm^{-1}. b) 2D HSQC NMR spectra (^1H,^{13}C heteronuclear correlations) showing the aromatic region of the peptides **1**–**4**. Corresponding structural formula and ^1H,^{13}C chemical shifts for signals assigned to the unique carbon-bound proton of 1,4- and 1,5-disubstituted 1,2,3-triazoles are shown.

and ^{13}C chemical shifts were in good agreement with reported data[10d] and displayed a significant difference between the "1,4" and "1,5" substitution pattern confirming the proposed constitution of RuAAC and CuAAC products **2**, **3**, and **4** (Figure 3b).

The inhibitory activity of peptides **1**–**6** was determined by kinetic studies using active-site titrated trypsin (see Figure 4 and the Supporting Information).[17]

The determined K_i^{app} values for compounds **1**–**6** are summarized in Table 1. Peptidomimetic **2** with the "1,5" substitution pattern displayed an inhibitory potency in nanomolar range, which is comparable to that of the disulfide-bridged parent peptide **1**. In contrast, monocyclic SFTI-1 variants **3** and **4** produced by CuAAC macrocyclization showed a dramatic decline in inhibitory activity against trypsin. Two main issues could provide a plausible explanation for this finding. First, the rigidity of the 1,2,3-triazole formed prevents the "1,4" pattern from having a proper fit into the parent structure, thereby increasing the bridging distance of the peptide backbone. Though significant tolerance of the monocyclic SFTI-1 backbone towards the length of the connecting element has been recently reported for non-rigid, flexible linkers,[15] the impact of structurally constrained

Table 1: Summary of K_i^{app} values determined for compounds **1**–**6**.

Entry	Bridging motif	K_i^{app} [nM][a]	Relative activity[b]
1	cystine	1.48 ± 0.1	1
2	1,5-disubstituted 1,2,3-triazole	2.4 ± 0.14	1.6
3	1,4-disubstituted 1,2,3-triazole	1908 ± 261	1288
4	1,4-disubstituted 1,2,3-triazole	742 ± 88	501
5	–	1916 ± 205	1293
6	–	13845 ± 1835	9347

[a] Standard error of the nonlinear regression is given. [b] Relative activities are calculated as the ratios of the K_i^{app} values for the respective compound to that of peptide **1**.

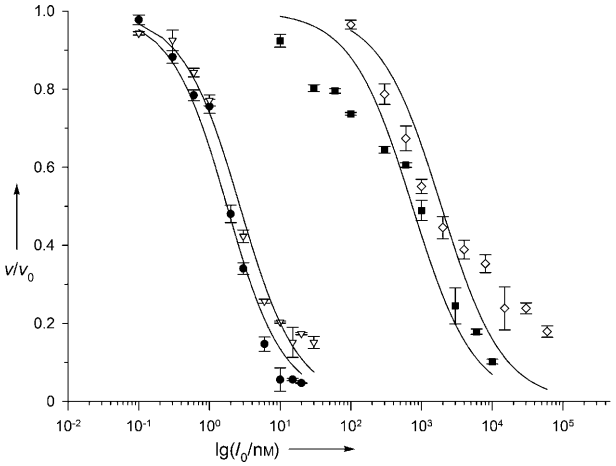


Figure 4. Kinetic data for the inhibition of trypsin-catalyzed proteolysis of chromogenic substrate Boc-QAR-*p*NA (Bachem) by SFTI-1 analogues **1** (●), **2** (▽), **3** (◇), and **4** (■) and corresponding curves of the Morrison equation (fitted by the Marquardt–Levenberg algorithm of SigmaPlot 11). Error bars indicating the standard deviation of each data point (triple determination).

disulfide substitutes could be dramatic, as shown herein. As another reason for the reduced activity of compounds **3** and **4** the steric demand in proximity to or at the C_β atom of residue 3 can be considered, since it has been shown that bulky moieties at this position cause a drastic decrease of the inhibitory effect.[15a]

In conclusion, we demonstrated the utility of a 1,5-disubstituted 1,2,3-triazole bridge as a disulfide replacement. Owing to its redox stability and dissimilarity to common building blocks of nature, improved pharmacokinetic properties can be expected for this disulfide surrogate. Since, for a range of bioactive molecules the preference of 1,5-disubstituted 1,2,3-triazoles over the 1,4 species may turn out to be not so explicit as for the structures described, a modular approach towards tailor-made heterodetic compounds can be achieved through the variation of RuAAC and CuAAC macrocyclization strategies using commercially available building blocks. Experiments towards generation of peptides containing both a triazole bridge and disulfide bonds are currently under way. This strategy may help to overcome the difficulties that often arise during oxidative folding of cystine-rich peptides in vitro.

Received: December 23, 2010
Revised: February 21, 2011
Published online: May 4, 2011

Keywords: click chemistry · disulfide bridges · peptidomimetics · SFTI-1 · trypsin inhibition

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