Peptidomimetics

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Maintaining Biological Activity by Using Triazoles as Disulfide Bond Mimetics**

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Disulfide bonds constitute an abundant and important structural element in the folding of proteins and peptides. By forming macrocycles, the three-dimensional structure of proteins and peptides can be stabilized and rigidified. For peptides in particular, the biological activity of these compounds is closely associated with the correct folding of the structure.[1] Despite its stabilizing effect in peptides and proteins, the disulfide bridge itself is rather unstable. Disulfide isomerases, as well as reducing agents and thiols, can affect this covalent bond and can lead to structural rearrangement with a complete loss of activity. The replacement of this essential structural element by bioisosteric, and hydrolytically and reductively stable substitutes is therefore of great interest.

Several approaches to substitution of the disulfide bridge by more stable covalent connections such as amides, thiolethers, diselenides, or carbon-based bridges, have been reported.[2,3] In the present study we explore the potential of using triazoles as functional mimetics of multiple naturally occurring disulfide bonds in biologically active peptides. Triazoles exhibit chemical orthogonality and provide excellent stability against isomerases and proteases.[4] Moreover, triazoles can be formed in a two-component approach, which is comparable to that of disulfide bond formation from two cysteine residues. The Huisgen cycloaddition of an alkyne and an azide readily generates the triazole. The introduction of copper(I) catalysis in this reaction has accelerated the reaction rate by seven to eight orders of magnitude, therefore the reaction can now be performed under very mild conditions and has been applied in numerous assemblies of complex molecular architectures.[5] The two-component approach allows directional formation of disulfide bond mimetics by the substitution of two or more cysteine residues in a peptide with alkyno- and azido-functionalized amino acids. On-resin cyclization during solid-phase peptide synthesis (SPPS) provides triazoles as disulfide mimetics.

Tachyplesin I (TP-I), which is a 17-residue bicyclic peptide that contains two disulfide bonds, maintains a β-hairpin ribbon structure (Figure 1) in its bioactive form, as shown by NMR spectroscopy studies.[6,7] When the four cysteine groups involved in disulfide bridging are replaced with functionalized amino acids for the "click strategy", the intrinsic fold of the linear TP-I should facilitate the correct positioning of the reactive groups to provide the TP-I connection pattern and produce bioactive TP-I analogues.

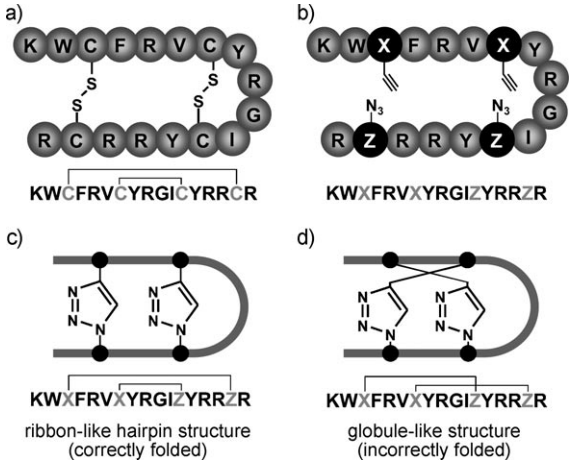

Figure 1. Mimicking of disulfide bridges by triazoles in tachyplesin-I by the replacement of cysteine residues by alkyno (X) and azido amino acids (Z), which form triazoles upon the copper-catalyzed cycloaddition of the azides and alkynes ("click" reaction): a) tachyplesin-I and b) linear analogues. Cyclization leads to c) the correctly folded hairpin structure or d) incorrectly folded globule-like structure.

Propargylglycine (Pra) was selected as the alkyno-functionalized amino acid to replace Cys-3 and Cys-7; the azido-functionalized amino acids, 2-amino-4-azido-butyric acid (2Abu(γ-N_3)) and 5-azido-norvaline (Nva(δ-N_3)) were used to replace Cys-12 and Cys-16 in TP-I. 2Abu(γ-N_3) and Nva(δ-N_3) were produced in a good yield by using the diazotransfer method reported by Lundquist and Pelletier.[8]

In order to maintain the correct structural folding of TP-I upon cyclization, the peptide synthesis was performed on poly(acryloyl-bis(aminopropyl)polyethylene glycol) (PEGA) resins, which enables the cyclization to occur under aqueous reaction conditions.[9] Additionally, the hydroxymethylbenzoic acid (HMBA) linker facilitates on-resin deprotection prior to the cyclization. The alkyne and azide building blocks were easily incorporated into the peptide chain to generate

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two different linear tachyplesin analogues: [^{3,7}Pra,^{12,16}2Abu(γ-N_3)]-TP-I and [^{3,7}Pra,^{12,16}Nva(δ-N_3)]-TP-I.

The resin-bound and deprotected peptides were subjected to different reaction conditions for the copper(I)-mediated [2+3] cycloaddition to produce the triazole-containing bicyclic peptides. Widely used methods for on-resin triazole formation, including CuI or CuBr dissolved in organic solvents, as well as $CuSO_4$/ascorbate under aqueous reaction conditions[5d] resulted in low yields of the cyclic TP-I analogues. However, exposure of the linear TP-I analogues to $CuSO_4$/tris(carboxyethyl)phosphine resulted in a successful cyclization, as shown by HPLC/MS. Two cyclic products were produced for each linear TP-I analogue ([^{3,7}Pra,^{12,16}2Abu(γ-N_3)]-TP-I and [^{3,7}Pra,^{12,16}Nva(δ-N_3)]-TP-I) by using these reaction conditions. Microwave irradiation during the cyclization did not significantly influence the yield of the reaction, but shortened the reaction times and changed the ratio between the two cyclic products formed from 1:7 to 1:1.5 (ribbon-like structure/globule-like structure, Figure 1c,d).

The ESI-MS/MS spectra of the linear and the two cyclic peptides presented completely different fragmentation patterns. The full sequence of ions corresponding to all the amino acids contained in the peptide were present in both the y and b series in the linear product. In contrast, the cyclic TP-I analogues only presented fragment ions corresponding to fragmentation outside the bicyclic region. Additionally, the reduction of the azide by dithiothreitol with concurrent loss of N_2 was carried out to analyze the presence or absence of azido groups.[10] Finally, ^{1}H NMR spectroscopic analysis provided full structure determination of the cyclic products and evidence that the major product had the correctly folded ribbon-like hairpin stucture (see the Supporting Information).

The triazole formation was performed on a solid support in contrast to a previously reported peptide "click" cyclization.[5d] In this on-resin cyclization of the linear TP-I analogues, the cyclodimer and cyclooligomer were not observed, although such products were predominant and, in some cases, the only product in previous studies on cyclization in solution.[11] The absence of these structures here may be partly due to the separation of the peptides during the on-resin cyclization and partly due to the use of aqueous reaction conditions that favour the intrinsic β-hairpin fold of TP-I and minimize the effect of interchain hydrogen bonds. This hypothesis is in agreement with the effect of microwave heating on the unfolding of the linear TP-I analogues, and the formation of incorrectly folded globule-like structures.

Modeling of [^{3,7}Pra,^{12,16}Nva(δ-N_3)]-TP-I and wild-type TP-I based on NOE studies showed there is a high degree of similarity between the wild-type TP-I and the triazole-containing analogue (Figure 2). Similar results were obtained for [^{3,7}Pra,^{12,16}2Abu(γ-N_3)]-TP-I, for which the similarity was optimal for the disulfide bonds but less so for the peptide backbone (see the Supporting Information). The structure of the triazole-containing TP-1 analogues not only imitates the β-hairpin structure of the backbone of the wild-type TP-1, but also positions most of the side chains in the required orientation. Both cyclic TP-I analogues form a symmetrical homodimer in aqueous solution (Figure 3).

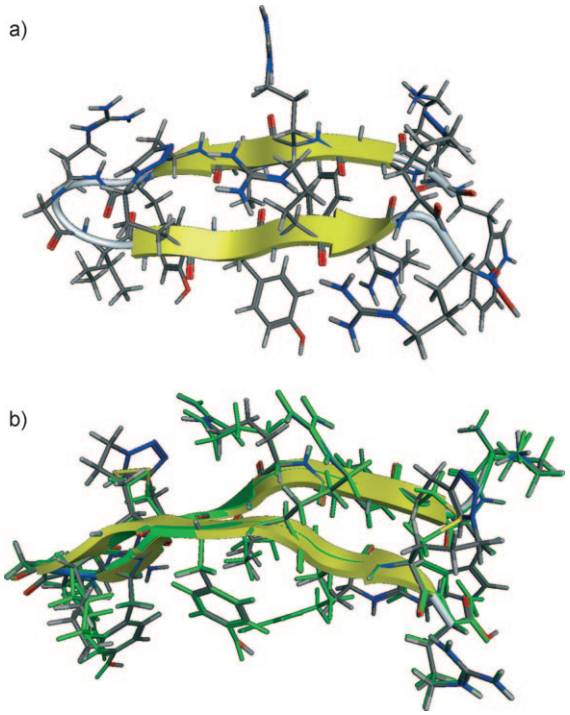

Figure 2. Models of the wild-type TP-I peptide and [^{3,7}Pra,^{12,16}Nva(δ-N_3)]-TP-I based on NMR structure determination: a) [^{3,7}Pra,^{12,16}Nva(δ-N_3)]-TP-I, b) structural alignment of wild type TP-I (green) and [^{3,7}Pra,^{12,16}Nva(δ-N_3)]-TP-I (gray). The yellow arrows represent the peptide backbone.

The almost perfect mimicking of the wild-type TP-I structure indicated that the cyclic analogues should exhibit similar biological activity to the wild-type disulfide-bridged TP-I. The biological activity of the TP1-mimicking peptides was studied in antimicrobial assays. Several bacterial strains

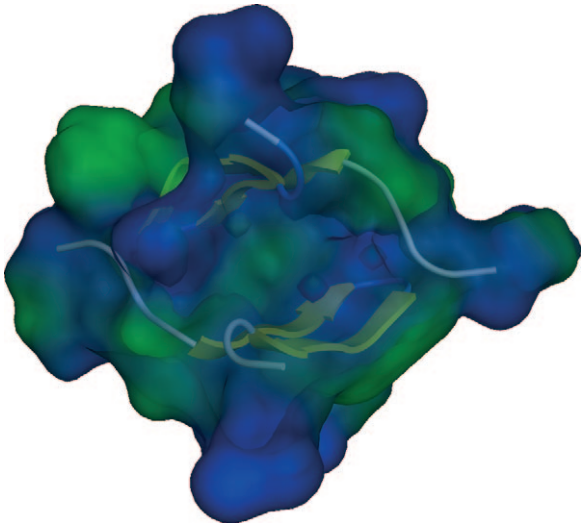

Figure 3. Molecular dynamics calculation of the dimer formation of cyclized [^{3,7}Pra,^{12,16}2-Abu(δ-N_3)]-TP-I according to the NMR structure determination and long-range NOE constraints. The dimerization may be mechanistically important for the high selectivity towards bacterial cell membranes (see the Supporting Information).

were grown in the presence of increasing concentrations of the TP-I analogues and the minimal inhibitor concentration (MIC) was determined (Table 1). Neither the linear nor the misfolded cyclic analogues showed any significant antimicrobial activity. In contrast, the MIC values in the different bacterial strains for the correctly cyclized peptides that have a hairpin structure are comparable or even better than those for the wild-type TP-I.

Table 1: MIC of tachyplesin-I analogues.[a]

	E. coli	*Staphylococcus epdermis*	*Salmonella typhimurium*	*Bacillus subtilis*
TP-I wild-type	11.5[12] 12.5[5]	2.3[13]	6.3[6]	>200[12]
[3,7Pra,12,16 2Abu-(γ-N_3)]-TP-I[b]	10.0	8.0	12.0	5.5
[3,7Pra,12,16 Nva-(δ-N_3)]-TP-I[b]	7.0	10.5	3.0	4.5

[a] MIC values reported in μg mL^{-1}. [b] Tachyplesin-I analogues correctly cyclized in a ribbon-like structure.

In conclusion, we have demonstrated that triazoles can be considered as an appropriate mimetic of disulfide bridges in peptides. The side-chain to side-chain linkage established by "click" chemistry opens a synthetic route for creating even more complex patterns of triazole-bridged peptides. The structural similarity of the novel TP-I analogues is strongly supported by the almost identical biological activity of the analogues and TP-I. The NOE-based structure determination of the triazole analogues predicted a homodimer that completely masks the hydrophobic nature of the molecule and may be important for the design of novel antimicrobiotics based on this scaffold.

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