Amide Mimics

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Braces for the Peptide Backbone: Insights into Structure–Activity Relationships of Protease Inhibitor Mimics with Locked Amide Conformations**

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The architecture of protein macromolecules fundamentally depends on the sequential arrangement of peptide backbone bonds in defined conformations. Among the three torsion angles (φ, ψ, and ω) present at each amino acid, it is the amide bond (ω) which is intrinsically hindered as a result of its partial double-bond character and it is thus more or less restricted to either a *trans* or a *cis* conformation (Figure 1).[1] Generally, amide bonds occur predominantly in the *trans* conformation as it minimizes unfavorable contacts between adjacent amino acids.[1d] Nevertheless, *cis* amides, if present, are usually an imperative for bioactivity.[2]

The distribution of *cis*/*trans* conformations drastically changes from about 0.03% for non-prolyl peptide bonds to about 6% for Xaa-Pro motifs.[1a–c,e] This is directly linked to the propensity of proline with its cyclic side chain to form a sterically hindered backbone motif which strongly affects the preceding peptide bond.[3]

Beyond the structural features that *cis* amide bonds induce in proteins, *cis*–*trans* isomerization (CTI) adds a kinetic dimension to biomolecular systems. In fact, it is not only one of the rate-determining steps in protein folding,[4] but CTI also provides the possibility for a time-dependent conformational switch, thus allowing for a dynamic modulation of structure and activity.[3]

During the last decade, a few structural mimics of *cis* amides in peptides and proteins have been reported, among them pseudo-prolines,[2f,5] disubstituted tetrazoles,[6] and triazoles.[2a,e,7] Thus, an Asn-*cis*-Pro bond in bovine pancreatic ribonuclease was successfully replaced by a triazolyl unit through expressed protein ligation[8] without loss of catalytic activity.[2e] In fact, disubstituted 1,2,3-triazoles have been shown to be viable surrogates for *cis* and *trans*- amide bonds, depending on their substitution pattern (Figure 1).[2e,9] To our knowledge, no detailed insights into the molecular architecture and structural requirements of a bioactive *cis* amide mimic containing a 1,5-disubstituted 1,2,3-triazole upon binding to its target have been reported.

Herein, we demonstrate the applicability of modular triazole-based backbone elements for locking *cis* and *trans* amides within the functional loop of a Bowman–Birk protease inhibitor (BBI).[10] High-resolution crystal structures revealed the detailed structural features of both *cis* and *trans* triazolyl amide surrogates in highly potent peptidomimetics bound to bovine trypsin. Our results provide new information on the behavior of triazolyl units within active biomolecules,

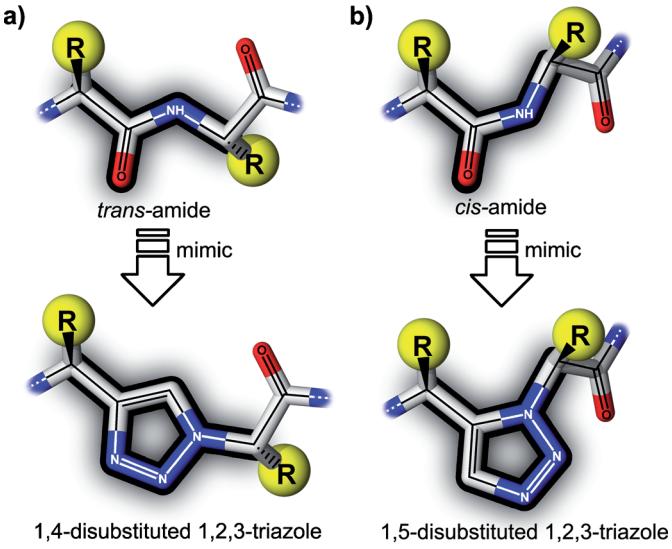

Figure 1. Representation of triazolyl amide mimics incorporated into a generic peptide backbone. a) *trans* amide mimic based on a 1,4-disubstituted 1,2,3-triazole, b) *cis* amide mimic based on a 1,5-disubstituted 1,2,3-triazole. Backbone atoms are depicted as sticks and side-chain moieties as balls. An overlay of the Lewis structure with stereo information is given. N blue, C light gray, O red, side chains yellow; hydrogen atoms omitted for clarity. The dihedral angles defining the *trans* and *cis* amide bonds (ω torsion angles) and the respective triazole-based mimics are highlighted by black outlines.

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and lead to a re-evaluation of their steric and electronic homology to the native peptide bond.

As a model peptide the highly potent sunflower trypsin inhibitor 1 (SFTI-1) from *Helianthus annuus* was chosen.[11] In the native form, its 14 amino acid backbone GRCTKSIPPICFPD is cystine bridged and head-to-tail cyclized, and contains an Ile-*cis*-Pro amide bond as an indispensible prerequisite for bioactivity.[12] Recently we demonstrated the utility of its open-chain variant (SFTI-1[*1,14*]) to study the influence of subtle changes within shape-defining regions, like the disulfide bridge, on structure–activity relationships.[13] Because of to the highly conserved canonical conformation of the functional loop of protease inhibitors, even minor steric alterations on the sub-Ångstrom range have significantly affected the bioactivity of the investigated peptidomimetics.[13]

Besides the cystine motif, the *cis* amide bond between Ile7 and Pro8 is a prominent structural element within the inhibitor loop of SFTI-1[*1,14*] crucial for its bioactivity (Figure 2).[12] Consequently, the replacement of this intriguing conformational archetype by a 1,5-disubstituted 1,2,3-triazole mimic appeared to be a promising concept, although previous attempts to mimic this particular motif with non-natural surrogates were not successful.[14] Thus, a *cis* amide mimic must not only match the steric requirements of the parent perfectly, but also provide a similar chemical environment.

It has been shown that replacement of Pro8 by an alanine residue (Figure 2, compound **1**) led to a drastic decrease of inhibitory activity against trypsin.[12] This was apparently caused by an induced conformational heterogeneity resulting from the loss of the *cis* amide stabilizing effect of proline. To

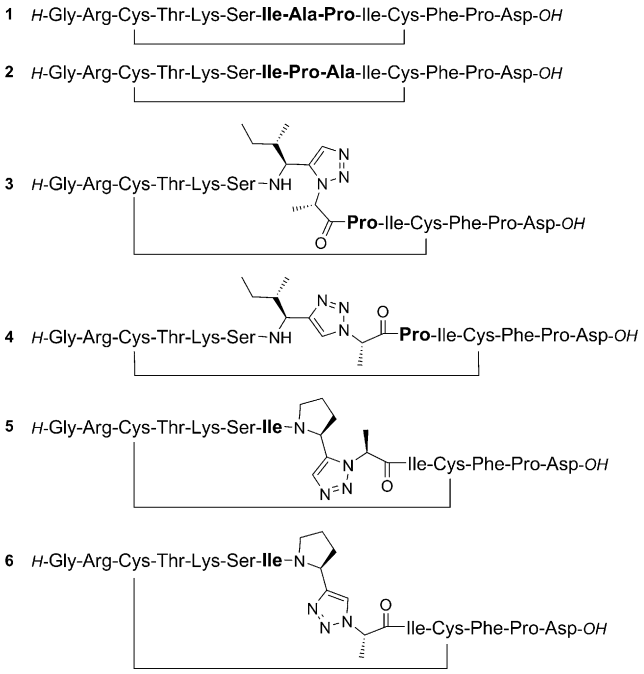


Figure 2. Overview of synthesized compounds: [Ala^{8}]SFTI-1[*1,14*] (**1**), [Ala^{9}]SFTI-1[*1,14*] (**2**), [I*c*A^{7,8}]SFTI-1[*1,14*] (**3**), [I*t*A^{7,8}]SFTI-1[*1,14*] (**4**), [P*c*A^{8,9}]SFTI-1[*1,14*] (**5**), [P*t*A^{8,9}]SFTI-1[*1,14*] (**6**). The investigated *cis* (between Ile7 and Pro8) and *trans* amides (between Pro8 and Pro9) are highlighted in bold.

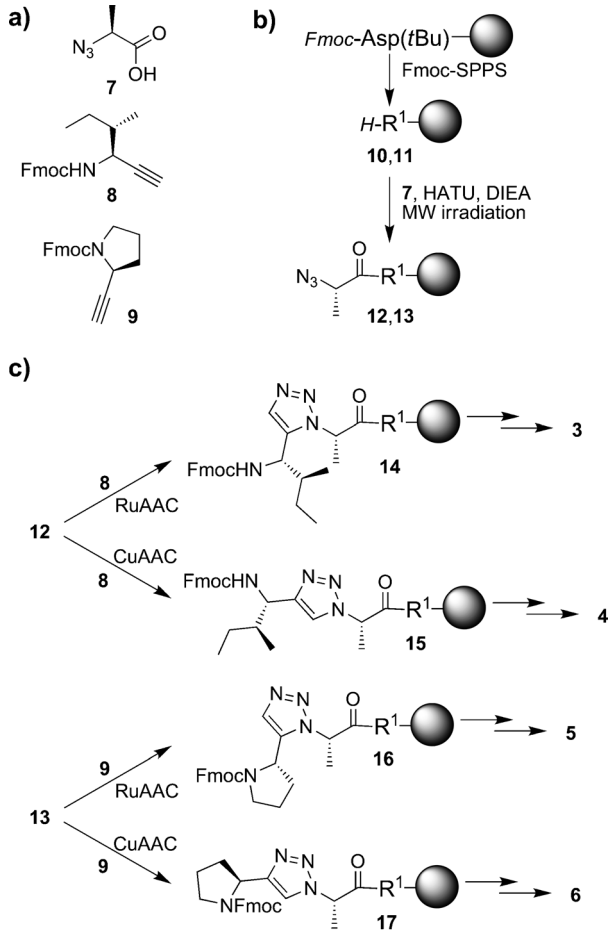
validate our results and to assure consistency within the test system used, we included the corresponding Pro→Ala mutants **1** and **2** in this work. Efforts were made to exchange the subsequent *trans* amide bond with a corresponding 1,4-disubstituted counterpart **6** and to synthesize SFTI-1[*1,14*] derivatives bearing the respective mismatched substitution patterns of the triazolyl moiety at each position (**4** and **5**). To precisely refer to each peptidomimetic compound, we assigned a three-letter code for the introduced dipeptide surrogates. Thus, the one-letter code was used to reflect the mimicked amino acid sequence. A "*c*" or "*t*" indicates the conformation of the amide bond locked by the triazole counterfeit.

Based on 1,5-disubstituted 1,2,3-triazoles, conformationally defined non-prolyl backbone motifs are easily accessible by solid-phase peptide synthesis. All peptides were assembled on a solid support either using commercially available building blocks or synthetic precursors (Scheme 1 and the Supporting Information). (*S*)-2-Azidopropanoic acid (**7**) and the Fmoc-protected alkyne components **8** and **9** were synthesized according to previously reported procedures.[15]

Azide **7** was introduced at position 8 or 9 in the growing peptide chain using in situ activation under gentle microwave irradiation. The subsequent generation of 1,4- or 1,5-disubstituted 1,2,3-triazoles **14**–**17** was achieved by Cu^{I}- or Ru^{II}-catalyzed azide–alkyne cycloaddition (CuAAC or RuAAC, respectively) on the solid support.[2a,7b,13,16] Microwave-assisted Fmoc-SPPS was continued until the peptide chain was assembled. After acidolytic cleavage, precipitation, and DMSO- or air-mediated oxidation of crude products, chromatographic purification yielded the cyclic target compounds **1**–**6** on a multi-milligram scale.

In order to gain insights into the spatial aspects of the active loop conformation upon binding to a serine protease, in silico calculations (Supporting Information) and crystallographic analysis were conducted using a modified procedure,[17] which resulted in high-resolution crystal structures (1.45–1.55 Å) for the complex of bovine trypsin with [I*c*A^{7,8}]SFTI-1[*1,14*] **4** and [P*t*A^{8,9}]SFTI-1[*1,14*] **6** (Figure 3). The inhibitory potency of all synthesized SFTI derivatives was evaluated in enzyme kinetic studies with active-site-titrated trypsin using the chromogenic substrate *Boc*-QAR-*p*NA, and the apparent and substrate-independent inhibitory constants (K_i^{app} and K_i) were determined as previously reported.[10b,13]

As seen from Table 1, the presence of the undesirable conformation at each of the two examined positions led to a drastic decrease of inhibitory activity. Thus, the alanine exchange at position 8 (SFTI derivative **1**) resulted in the loss of the *cis*-stabilizing effect of proline, hence, dynamic disorder of the inhibitory loop by CTI. Locking the *trans* conformation at this position by a 1,4-disubstituted 1,2,3-triazole had an even more dramatic effect: peptidomimetic **4** possessed the lowest activity of all studied compounds. Vice versa, the variant with a locked *cis* conformation through the use of a 1,5-disubstituted triazolyl building block between Ile7 and Ala8 (compound **3**) showed a significant improvement in bioactivity over compound **1**, although not in the range of the parent peptide SFTI-1[*1,14*]. This can be explained considering the crystal structures of the wild-type inhibitor and **3** in



10,**12**,**14**,**15**: R^1= Pro-Ile-Cys(Trt)-Phe-Pro-Asp(*t*Bu)
11,**13**,**16**,**17**: R^1= Ile-Cys(Trt)-Phe-Pro-Asp(*t*Bu)

Scheme 1. a) Azide- and alkyne-bearing building blocks (2*S*)-2-azidopropanoic acid (**7**), (2*S*,3*S*)-*N*-(9-fluorenylmethyloxycarbonyl)-1-ethynyl-2-methylbutylamine (**8**), and (2*S*)-*N*-(9-fluorenylmethyloxycarbonyl)-2-ethynylpyrrolidine (**9**). b) Synthesis of azide-bearing peptide resins **12** and **13**. c) Synthesis of oxidized peptidomimetics **3**–**6**. Fmoc-SPPS: Fmoc-assisted solid-phase peptide synthesis; HATU: 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIEA: *N*,*N*-diisopropylethylamine; MW: microwave; RuAAC: ruthenium(II)-catalyzed azide–alkyne cycloaddition using [Cp*Ru(cod)Cl] (Cp* = C_5Me_5, cod = cyclooctadiene) as catalyst; CuAAC: copper(I)-catalyzed azide–alkyne cycloaddition using $CuSO_4$, sodium ascorbate, and DIEA for catalysis; multiple reaction arrows at the end of the synthetic sequence: Fmoc-SPPS, acidolytic cleavage, precipitation, DMSO- or air-mediated oxidation, and chromatographic isolation.

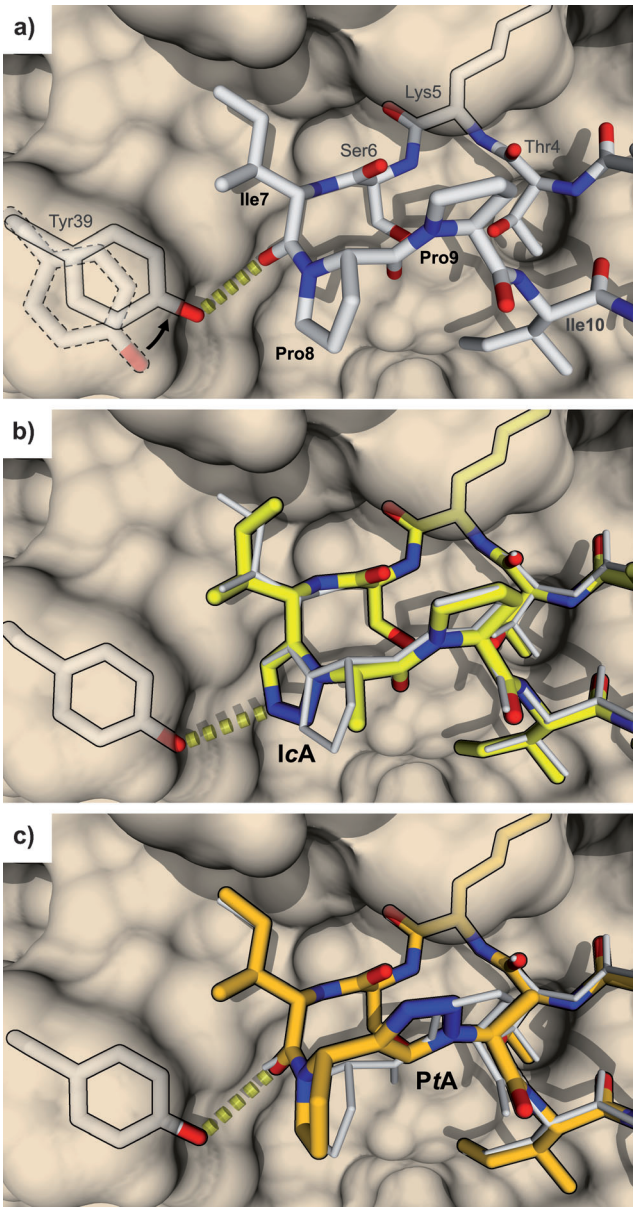


Figure 3. Comparison of the inhibitor loops of SFTI-1 and peptidomimetics **3** and **6** in the corresponing protease/inhibitor complexes. Structures aligned at Lys5 of inhibitors. The orientation of the side chain of Tyr39 from trypsin (surface) before (dashed outline) and after energy minimization (solid outline) and the formed hydrogen bonds are shown (Software: YASARA structure with AMBER03 force field, POVRay). Inhibitors shown as stick representations; N blue, O red; hydrogen atoms omitted for clarity. a) Native bicyclic SFTI-1 (C white, PDB ID code: 1SFI). b) [$IcA^{7,8}$]SFTI-1[*1*,*14*] **3** (solid outlines, C lemon yellow, PDB ID code: 4ABJ) in an overlay with 1SFI (thin white). c) [$PtA^{8,9}$]SFTI-1[*1*,*14*] **6** (solid outlines, C orange, PDB ID code: 4ABI) in an overlay with 1SFI (thin white).

complex with trypsin (Figure 3). It is evident that SFTI-1 is able to form a hydrogen bond with the phenolic hydroxy group of Tyr39 near the binding pocket of the protease through the carbonyl oxygen of Ile7. This finding is supported by in silico experiments, as an energy-minimization procedure (AMBER force field) applied to the crystal structure (1SFI) resulted in formation of the aforementioned hydrogen bond, which may contribute to the binding enthalpy of SFTI-1[*1*,*14*]. Indeed, the N2 and N3 atoms of 1,2,3-triazoles have been reported to possess hydrogen-bond-accepting abilities[18] and, thus, may partly compensate for the missing interactions.

In full agreement with our expectations, the alanine exchange at residue 9 (**2**) locked the *trans* conformation with a 1,4-disubstituted 1,2,3-triazole between Pro8 and Ala9 (**6**) and also had no pronounced effect on bioactivity. This finding can easily be explained taking into consideration that no alterations have been made relative to the native conforma-

Table 1: Inhibitory activity of compounds **1**–**6** and monocyclic SFTI-1[*1,14*] (wild-type, wt).[13]

Entry	Sequence	K_i [nM][a]	Relative activity
1	GRCTKS**IAP**ICFPD	178 ± 25	1[b]
2	GRCTKS**IPA**ICFPD	3.2 ± 0.5	1[c]
3	GRCTKS**[IcA]P**ICFPD	34 ± 5	0.2[b]
4	GRCTKS**[ItA]P**ICFPD	302 ± 50	1.7[b]
5	GRCTKSI**[PcA]**ICFPD	255 ± 42	80[c]
6	GRCTKSI**[PtA]**ICFPD	6.3 ± 1.8	2[c]
wt	GRCTKS**IPP**ICFPD	0.2 ± 0.03	–

[a] Error calculated by propagation of error for K_i^{app} and K_M (see the Supporting Information); relative activities are calculated as the ratios of the K_i values for the respective compound to that of [b] **1** or [c] **2**.

tion, and no relevant contacts between residue 9 and trypsin were observed in the crystal structure (Figure 3c). In contrast, an induced conformational mismatch (**5**) caused a drastic reduction of binding affinity.

Nevertheless, it must be mentioned that as a result of its intrinsic architecture, the 1,4-disubstituted 1,2,3-triazole unit as a *trans* amide backbone element increases the distance between the α-carbons of adjacent residues by 1.3 Å. In contrast, the 1,5-disubstituted 1,2,3-triazolyl *cis* amide mimic preserves bond length and spatial arrangement almost perfectly (increase of distance between α-carbons: 0.376 Å). Interestingly, the crystal structure of peptidomimetic **6** revealed that the predominant orientation of the incorporated 1,4-disubstituted 1,2,3-triazole significantly differed from the geometry predicted from the hydrogen-bond profile (see the Supporting Information).[9b]

In summary, we have demonstrated that 1,4- and 1,5-disubstituted 1,2,3-triazoles can easily be introduced into the backbone of synthetic peptides as viable amide surrogates for the generation of locked *trans* or *cis* conformations. Only peptidomimetics resembling the native-like amide isomers showed high inhibition potency (two-digit nanomolar K_i values). Our approach allowed for the establishment of the rather uncommon *cis* conformation in sensitive, structure-defining regions of the functional loop of BBI SFTI-1 through the introduction of non-prolyl backbone motifs. The exchange of a proline residue following a *cis* amide bond by any of the other 19 natural amino acids usually leads to a formation of the undesired *trans* isomer. The presented synthetic strategy, in principle, enables the installation of each desired side chain without violating the backbone structure, thus, providing access to tailor-made *cis* amide mimics previously excluded from rational design.

Generally, SFTI-1 is a valuable framework for the development of selective protease inhibitors of diagnostic and therapeutic relevance.[11a,19] Since all BBIs share a conserved *cis*-Pro motif in their functional loop, data and structural insights validated for the SFTI-1 scaffold may be extrapolated to the whole inhibitor family.[20] Nevertheless, as other trypsin-like proteases of pharmaceutical relevance may differ in their amino acid composition close to the enzyme active pocket, the loss of binding affinity for the *cis* amide mimic **3**, presumably caused by changes in the intermolecular hydrogen-bonding pattern, might not be a general problem.

To conclude, our facile preparation of peptidomimetics bearing triazolyl backbone units may find utility in various biochemical applications, as, in contrast to the native amide bond, a triazole does not undergo proteolytic hydrolysis. Therefore, enhanced metabolic stability can be expected.[21] Gathered crystallographic data provide fundamental information important for ongoing research aimed at the improvement of peptidomimetic protease inhibitors through implementation of non-natural (triazole) units in their activity-defining regions.

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