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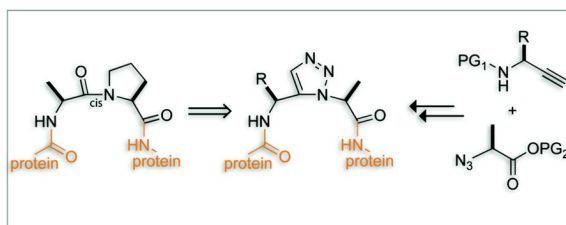
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Protein Prosthesis: 1,5-Disubstituted-[1,2,3]-triazoles as cis-Peptide Bond Surrogates

Annie Tam[†], Ulrich Arnold[‡], Matthew B. Soellner^{†,§}, and Ronald T. Raines^{*,†,||}

Department of Chemistry and Biochemistry, University of Wisconsin, Madison, Wisconsin 53706, and Department of Biochemistry and Biotechnology, Martin-Luther University, 06099 Halle, Germany

Abstract



Proline is unique among the natural amino acids in the similar propensity of its peptide bond to be in the cis or trans conformation. This attribute affects many processes, including the rate at which proteins fold, their structures, and their activities. Other aliphatic amino acids can serve as mimics for proline residues with trans peptide bonds. In contrast, chemical synthesis is needed to create surrogates for cis prolyl peptide bonds. Here, 1,5-disubstituted-[1,2,3]-triazoles were assessed as cis-peptide bond surrogates. Huisgen's 1,3-dipolar cycloaddition reaction of amino alkynes and azido acids and a Ru(II) catalyst were used to synthesize a variety of Xaa-1,5-triazole-Ala modules in moderate-to-high yields. Two of these modules, along with their 1,4-triazole regioisomers, were installed in a turn region of bovine pancreatic ribonuclease by using expressed protein ligation. The resulting semisynthetic enzymes displayed full enzymatic activity, indicating the maintenance of native structure. The 1,5-triazole surrogates instilled conformational stability that was comparable to that of Xaa-cis-Pro segments, whereas the 1,4-triazoles conferred markedly less stability. The stability conferred by both surrogates was independent of the Xaa residue, eliminating an uncertainty in protein design. We conclude that Xaa-1,5-triazole-Ala modules can serve as viable mimics of Xaa-cis-Pro segments. The possibility of synthesizing this surrogate by the ligation of fragments in situ and the emergence of biocompatible catalysts for that process portends its widespread use.

Proline is unique among the natural amino acids in the similar propensity of its peptide bond to be in the cis or trans conformation.¹ This attribute affects many processes, including the rate at which proteins fold,² their structures,³ and their activities.⁴ Other aliphatic amino acids can serve as mimics for proline residues with trans peptide bonds. In contrast, chemical synthesis is needed to create surrogates for cis prolyl peptide bonds.^{1,5–7}

*Corresponding author. E-mail: raines@biochem.wisc.edu.

[†]Department of Chemistry, University of Wisconsin–Madison.

[‡]Department of Biochemistry and Biotechnology, Martin-Luther University.

[§]Current address: Department of Chemistry, University of California, Berkeley.

^{||}Department of Biochemistry, University of Wisconsin–Madison.

An effective surrogate for a *cis*-peptide bond must meet certain criteria. The surrogate must be similar in size to proline, so as not to disturb secondary and tertiary interactions within the folded protein. The surrogate must be able to accommodate adjacent residues, which could be important in protein structure and function. Finally, it is desirable if the surrogate is accessible via a facile synthetic route and amenable to solid-phase peptide synthesis.

We reasoned that 1,5-disubstituted-[1,2,3]-triazoles could meet all of these criteria. In particular, we noted that an Xaa-1,5-triazole-Ala module is a remarkable isostere of an Xaa-*cis*-Pro dipeptide, retaining its stereochemistry and number of non-hydrogen atoms, and maintaining similar hybridization (Scheme 1). A regioisomer, 1,4-disubstituted triazoles, had been incorporated into small peptides,^{7–9} and a simple 1,5-disubstituted triazole induced peptoid oligomers to adopt a turn.¹⁰ The synthesis and use of 1,5-disubstituted triazoles as *cis*-peptide bond mimics are, however, unknown. Likewise, neither 1,5- nor 1,4-disubstituted triazoles has been incorporated into a protein. Herein, we report on the attainment of these goals.

To synthesize the triazole, we employed Huisgen's 1,3-dipolar cycloaddition reaction.¹¹ Catalysis of this reaction by Cu(I) is known to yield exclusively a 1,4-disubstituted triazole,¹² but we required the 1,5-disubstituted regioisomer. There are reports of regioselective formation of 1,5-disubstituted triazoles being mediated by metals¹³ or stereoelectronic effects,¹⁴ but only under harsh conditions. Recently, Ru(II) catalysts had been shown to yield exclusively the 1,5-disubstituted triazole in a mild reaction,¹⁵ though this methodology had never been applied to substrates containing amino acids.

As a model protein, we chose bovine pancreatic ribonuclease (RNase A; 124 residues), which has been the object of much seminal work in protein chemistry.¹⁶ In the native protein, residues Gly112–Asn113–Pro114–Tyr115 form a Type VIb reverse turn (Scheme 1), in which the Asn113–Pro114 peptide is in the *cis* conformation. We targeted that dipeptide segment for replacement with 1,5-triazole-Ala surrogates.

The chemoselectivity of the 1,3-dipolar cycloaddition enables a convergent synthetic route to the requisite triazole (Table 1). The synthesis of the azido acids was achieved by Cu(II)-catalyzed diazo transfer of commercially available amino acids,¹⁷ followed by protection of the carboxyl functionality with a *t*-butyl or benzyl group to give **1** or **2**, respectively. The route to the amino alkynes was also general, starting from the Weinreb amide of an amino acid, and followed by reduction to the aldehyde and conversion to the alkyne with the Bestmann–Ohira reagent¹⁸ to give **3–8**.¹⁹

The scope of the Ru(II)-mediated cycloaddition with these α -amino acids derivatives is shown in Table 1. The cycloadditions were effected by the catalyst Cp*RuCl(COD), which had been reported to be effective for the cycloaddition of secondary azides.²⁰ For substrates with a diverse set of proteinogenic functionalities, the reaction afforded the desired regioisomers **9–15** under mild conditions and in moderate-to-high yield. No 1,4-disubstituted regioisomers were detected by the comparison of NMR spectra to those of analogous 1,4-disubstituted triazoles synthesized by Cu(I)-mediated cycloaddition.¹² Toluene was used as the solvent, unless the substrate was found to be insoluble (entries 2 and 3). Benzyl protection of the carboxyl group of the azido substrate was necessary for orthogonal deprotection relative to the acid-sensitive trityl group on the asparagine side chain (entry 3). It is noteworthy that this chemistry is compatible with Boc- and Fmoc-protecting group strategies (entries 6–7), both of which are common in solid-phase peptide synthesis. Moreover, subjecting the resulting triazoles to the conditions of peptide synthesis (*e.g.*, 20% v/v piperidine in DMF) produced no epimerization detectable by NMR spectroscopy.

Next, we used expressed protein ligation²¹ to replace Asn113–Pro114 of RNase A with Asn–1,5-triazole-Ala. To probe the generality of our approach, we also replaced the analogous turn in the N113A variant of RNase A with Ala–1,5-triazole-Ala. In addition, we incorporated Asn–1,4-triazole-Ala and Ala–1,4-triazole-Ala into RNase A. To do so, the four triazole surrogates were incorporated into peptides corresponding to residues 95–124 by solid-phase peptide synthesis. RNase A fragment 1–94 with a C-terminal thioester was produced with recombinant DNA methods.^{6,22} After ligation of the two fragments, the semisynthetic enzymes were folded and purified, and their properties were compared to those of the analogous biosynthetic enzymes.

Enzymatic catalysis can report on protein tertiary structure.²³ All of the semisynthetic proteins retained full catalytic activity (Table 2). The maintenance of native secondary structure was likewise supported by circular dichroism spectroscopy.²⁴ The 1,5-triazole variants **17** and **20** have T_m values comparable to those of the wild-type enzyme (**16**) and its N113A variant (**19**), respectively. The slight decrease in the T_m value of the 1,5-triazole variants could be due to relief of the constraint imposed upon the ϕ dihedral angle by the pyrrolidine ring of Pro114. The 1,5-regioisomers (**17** and **20**) are superior to the 1,4-regioisomers (**18** and **21**) in mimicking the cis-prolyl bond, as indicated by the lower T_m values for the latter pair.

The triazole-Ala surrogates eliminate an uncertainty in protein design. The trans/cis ratio of an Xaa–Pro peptide bond depends on the nature of Xaa. The higher T_m value of wild-type RNase A compared to its N114A variant is likely due in part to the greater propensity of Asn–Pro than Ala–Pro peptide bonds to assume a cis conformation.²⁵ In contrast, the T_m values of the semisynthetic proteins are independent of the Xaa residue. This distinction arises because cis–trans isomerization is not a factor with the two Xaa–triazole-Ala surrogates.

We conclude that Xaa–1,5-triazole-Ala modules can serve as viable mimics of Xaa–cis-Pro segments in a protein. The possibility of synthesizing this surrogate by the ligation of fragments in situ⁸ and the emergence of biocompatible catalysts for that process^{15,20} portends its widespread use.

Supplementary Material

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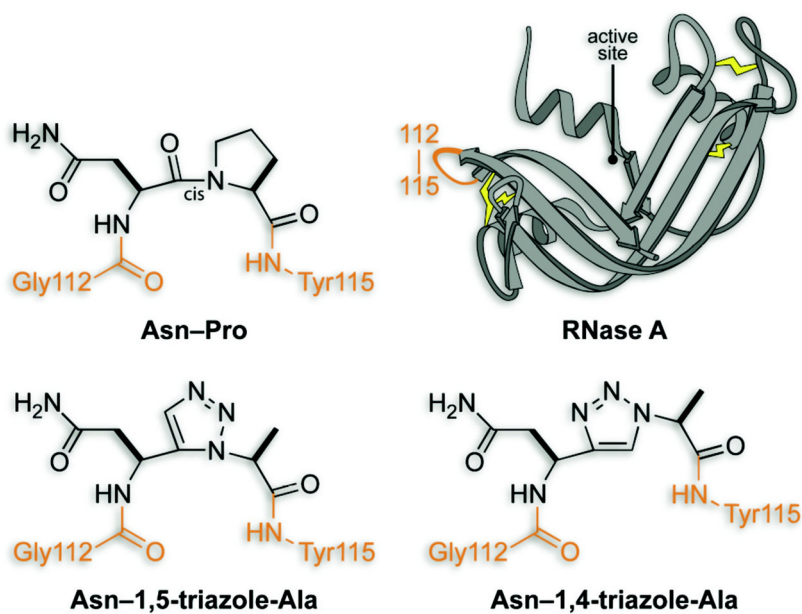
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Scheme 1.

Table 1
Ru(II)-catalyzed 1,3-Dipolar Cycloaddition of Various Amino Alkynes and Azido Acids



entry	R	PG1	PG2	solvent	temp	triazole	yield
1	H (3)	Boc	tBu (1)	toluene	RT	9	45%
2	CH ₂ CONH ₂ (Trt) (4)	Boc	tBu (1)	dioxane	60 °C	10	62%
3	CH ₂ CONH ₂ (Trt) (4)	Boc	Bn (2)	dioxane	60 °C	11	55%
4	CH ₃ (5)	Boc	tBu (1)	toluene	RT	12	67%
5	CH ₂ Ph (6)	Boc	tBu (1)	toluene	RT	13	90%
6	CH(CH ₃) ₂ (7)	Boc	tBu (1)	toluene	RT	14	92%
7	CH(CH ₃) ₂ (8)	Fmoc	tBu (1)	toluene	RT	15	91%

Table 2

Properties of RNase A Variants

residues 113–114	origin ^a	$T_m(^{\circ}\text{C})^b$	$k_{\text{cat}}/K_M(10^7 \text{ M}^{-1}\text{s}^{-1})^c$
Asn-Pro (16)	bovine pancreas	64.0 ± 0.1	5.7
Asn-1,5-triazole-Ala (17)	semisynthesis	60.4 ± 0.2	5.0
Asn-1,4-triazole-Ala (18)	semisynthesis	54.3 ± 0.1	6.1
Ala-Pro (19)	<i>Escherichia coli</i>	61.2 ± 0.2	4.5
Ala-1,5-triazole-Ala (20)	semisynthesis	60.8 ± 0.2	4.2
Ala-1,4-triazole-Ala (21)	semisynthesis	54.6 ± 0.2	5.6

^a All variants except **16** contain an N-terminal methionine residue.

^b Values were determined by CD spectroscopy in 50 mM sodium phosphate buffer (pH 8.0) containing NaCl (25 mM) and protein (0.5–1.0 mg/mL).

^c Values were determined at 25 °C in 0.10 M MES–NaOH buffer (pH 6.0) containing NaCl (0.10 M). Experimental error was ~10%.