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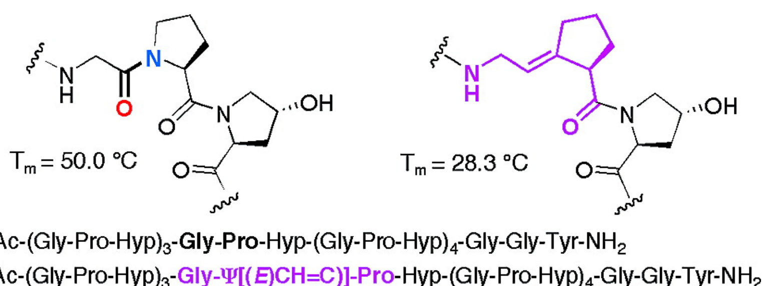

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The Effect of a Trans-Locked Gly−Pro Alkene Isostere on Collagen Triple Helix Stability

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Collagen is one of the most abundant and important proteins in mammals.[1] It acts as a scaffolding material to support cells, and it is responsible for the elasticity and strength of the body.[2] The primary structure of type I collagen can be described as a polymer with tripeptide repeating units (Gly−Xaa−Yaa)$_n$ in which about 10% of the Xaa residue is proline (Pro) and 10–12% of the Yaa residue is 4(*R*)-hydroxyproline (Hyp).[3] Glycine is required at the first position; it lacks a side chain, so it fits into the compact core of the triple helix.[4,5] Pro at Xaa and Hyp at Yaa gave the most stable collagen triple helixes in a host–guest system.[6,7]

The peptide amide bond has partial double bond character and is planar.[8] The dialkylated amino acid, proline, is unique because the preceding amide bond exists in 10−30% cis conformation in peptides and unfolded proteins.[9] In collagen protein folding, the *cis*-amide bonds must all be converted to *trans*-amide bonds. The *cis–trans* proline isomerization is responsible for the slow step in collagen folding and can be catalyzed by peptidyl-prolyl isomerases.[10,11] Raines and co-workers found that 4(*S*)-fluoroproline and 4(*S*)-methylproline both form stable triple helical structures, leading to the conclusion that the *exo*-conformation of the Hyp five-membered ring increases the trans content that is essential for triple helix formation.[12,13] We hypothesized that a Gly–*trans*-Pro conformationally locked isostere would decrease the entropic barrier to folding and stabilize collagen peptides.

The interchain hydrogen bond between the C═O of Xaa (often Pro) and the N−H of Gly stabilizes the collagen triple helix.[5] Replacement of one Pro−Gly (a 2° amide that does not isomerize) with a *trans*-alkene isostere showed severe destabilization of the triple helix, with a T_m value of –24.7 °C, because of the missing interchain hydrogen bond.[14]

We designed a collagen Gly−Pro−Hyp tripeptide isostere **1**, in which the Gly−Pro amide bond was replaced with an (*E*)-alkene in a host–guest peptide **2** to stabilize the collagen triple helix (Figure 1). The alkene bond locks Gly−Pro in the trans conformation to prevent cis–trans isomerization, which should reduce the conformational entropy in collagen peptide folding. By replacing the Gly−Pro amide, the N−H of Gly and the C═O of Pro were left intact to maintain the interchain hydrogen bonding. The C═O of Gly and the N of Pro involved in the amide bond that we replaced do not participate in direct hydrogen bonding.[15,16] We expected this substitution to lead to overall stabilization of the collagen peptide triple helix.

Our published method for the synthesis of a Ser−Ψ-[(*E*)CH═C]−Pro isostere was modified to synthesize the Gly−Ψ[(*E*)CH═C]−Pro isostere.[17] Because of the lack of a side chain, Gly has no stereogenic center, and there was no stereocontrol in the reduction of ketone **3** to alcohol **4** nor in the Ireland−Claisen rearrangement of **5** to **6** (Scheme 1). The mixture of two enantiomers was carried through the synthesis until diastereomers **9** could be separated. The diastereomers were separated by normal phase HPLC with 4% isopropanol in hexanes. To synthesize the

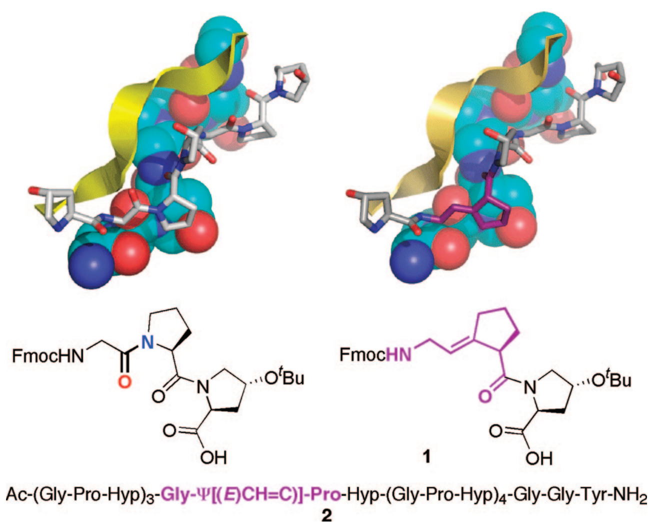

Figure 1. Top left: X-ray crystal structure of a collagen peptide showing the three strands as yellow ribbon, spheres, and sticks.[18] Top right: Same image replacing one Gly−Pro with a Gly-*trans*-Pro alkene isostere in purple. Images were created with MacPyMOL v. 0.99.[19] Middle left: Collagen Fmoc-Gly−Pro−Hyp−OH tripeptide repeat.[20] Middle right: Synthetic tripeptide isostere, Fmoc−Gly−Ψ[(*E*)CH═C)]−Pro−Hyp(^{t}Bu)−OH **1**. Bottom: Host–guest peptide **2** with Gly–*trans*-Pro isostere close to the middle.

tripeptide diastereomers (*R*)-**9** and (*S*)-**9**, no base was used to avoid alkene isomerization into the ring, which is known to occur instead of racemization for these isosteres.[21] A mild hydrogenation method was used to remove the benzyl group to make Fmoc−Gly−Ψ[(*E*)CH═C]−Pro−Hyp(^{t}Bu)−OH **1**. The double bond was not reduced as shown by ^{1}H NMR and HSQC (see Supporting Information).

A host–guest peptide **2** with eight tripeptide repeating units was designed to study the stability of the collagen triple helix. A control peptide with all natural amino acids and two peptides with the diastereomeric tripeptide isosteres in the middle were synthesized on solid-phase Rink amide MBHA resin. Two Gly residues were put at the *C*-terminus as a spacer to facilitate synthesis, and one Tyr residue was inserted to permit determination of the peptide concentrations by UV. The tripeptide building block Fmoc−Gly−Pro−Hyp(^{t}Bu)−OH was synthesized by the method reported by Ottl et al.[20] *tert*-Butyl protection of the hydroxyl group of Hyp was used to avoid side chain acylation during solid-phase peptide synthesis. The crude peptides were purified by HPLC with a protein C4 column. The identities of the peptide were confirmed by LC-MS and MALDI-TOF.

The stability of the collagen peptides was determined by measuring the melting temperature (T_m) using the ellipticity at 226 nm by circular dichroism (CD) (Figure 2). Values of T_m were determined by fitting the data into a two-state model. The control

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Scheme 1

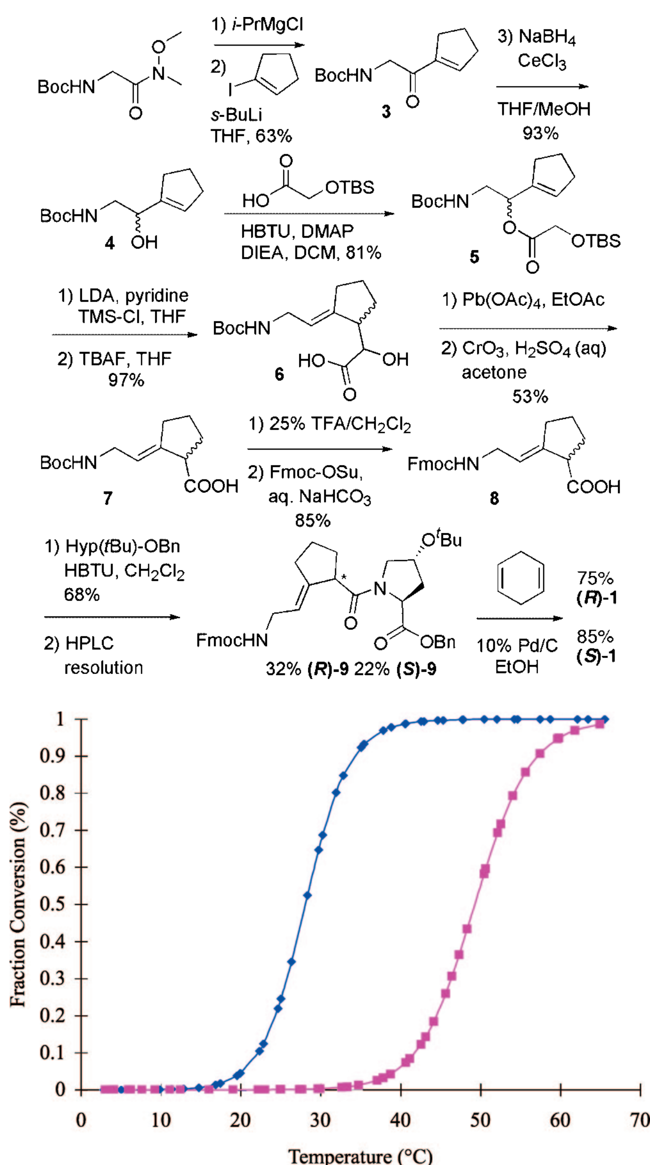

Figure 2. CD T_m curves of peptide (*R*)-**2**, Ac–(Gly–Pro–Hyp)$_3$–Gly–Ψ[(*E*)-CH═C)]-L-Pro–Hyp–(Gly–Pro–Hyp)$_4$–Gly–Gly–Tyr–NH$_2$ (♦), and control peptide **10**, Ac-(Gly–Pro–Hyp)$_8$–Gly–Gly–Tyr–NH$_2$ (■).

peptide Ac–(Gly–Pro–Hyp)$_8$–Gly–Gly–Tyr–NH$_2$ **10** had a T_m value of 50.0 °C, which is close to the literature value (47.3 °C) of Ac–(Gly–Pro–Hyp)$_8$–Gly–Gly–NH$_2$.[6] One peptide with an alkene isostere in the sequence, Ac–(Gly–Pro–Hyp)$_3$–Gly–Ψ[(*E*)CH═C]–Pro–Hyp–(Gly–Pro–Hyp)$_4$–Gly–Gly–Tyr–NH$_2$, (*R*)-**2**, had a T_m value of 28.3 °C. The other peptide with an alkene isostere in the sequence, (*S*)-**2**, showed a linear decrease of ellipticity with increasing temperature as expected, which meant that no collagen triple helical structure was formed.

The absolute stereochemical assignments of the intermediates (*R*)-**1** and (*S*)-**1** were inferred from the results of the T_m measurements by CD. Collagen contains all L-amino acids; one D-amino acid in the sequence prevents the formation of the triple helix in aqueous solution.[22,23] The diastereomer of **9** with the T_m value of 28.3 °C was assumed to have the *R*-stereochemistry at the α-carbon position mimicking L-Pro. The other diastereomer of **9** did not show a cooperative melting transition; therefore, it was assumed to have the *S*-stereochemistry mimicking D-Pro.

From the T_m values, the peptide with alkene isostere (*R*)-**2** formed a stable triple helix in aqueous phosphate buffered saline (PBS). From the work of Jenkins et al., the T_m value of a Pro–*trans*-Gly isostere in the host–guest peptide, (Pro–Pro–Gly)$_{10}$, was –24.7 °C, compared with the control at 32.8 °C, a ΔT_m of –57.5 °C.[14] On the other hand, our Gly–*trans*-Pro isostere peptide showed a ΔT_m of only –21.7 °C from its control **10** (Figure 2). Although the *trans*-alkene should lock the structure and reduce the *cis*–*trans* proline isomerization entropically, it does not have the dipole moment of the original amide bond. Favorable dipole–dipole interactions are one of the factors that stabilize the α-helix.[24,25] We believe that either intra- or intermolecular dipole–dipole interactions of amide bonds may be a factor in formation of the collagen triple helix. The destabilization of peptides containing alkene isosteres may also be attributed in part to the lack of two stabilizing n-to-π* interactions within the amide backbone of polyproline helices.[14,26] Experiments are underway to investigate these hypotheses.

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Supporting Information Available: Experimental procedures for the synthesis of **1** and **2**, NMR spectra, HPLC chromatograms, and CD data. This material is available free of charge via the Internet at http://pubs.acs.org.

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