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Effect of Sterically Demanding Substituents on the Conformational Stability of the Collagen Triple Helix

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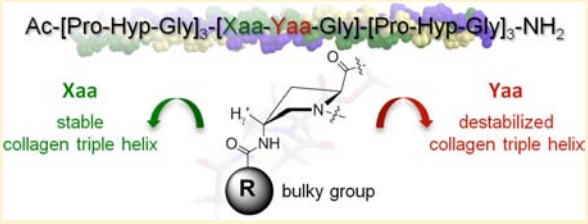
Supporting Information

ABSTRACT: The effect of sterically demanding groups at proline residues on the conformational stability of the collagen triple helix was examined. The thermal stabilities (T_m and ΔG) of eight different triple helices derived from collagen model peptides with (4*R*)- or (4*S*)-configured amidoprolines bearing either methyl or bulkier *tert*-butyl groups in the Xaa or Yaa position were determined and served as a relative measure for the conformational stability of the corresponding collagen triple helices. The results show that sterically demanding substituents are tolerated in the collagen triple helix when they are attached to (4*R*)-configured amidoprolines in the Xaa position or to (4*S*)-configured amidoprolines in the Yaa position. Structural studies in which the preferred conformation of (4*R*)- or (4*S*)-configured amidoproline were overlaid with the Pro and Hyp residues within a crystal structure of collagen revealed that the sterically demanding groups point to the outside of these two triple helices and thereby do not interfere with the formation of the triple helix. In all of the other examined collagen derivatives with lower stability of the triple helices, the acetyl or pivaloyl residues point toward the inside of the triple helix and clash with a residue of the neighboring strand. The results also revealed that unfavorable steric dispositions affect the conformational stability of the collagen triple helix more than unfavorable ring puckers of the proline residues. The results are useful for the design of functionalized collagen based materials.



INTRODUCTION

Collagen, the most abundant protein in mammals, is crucial for the structure of connective tissue such as skin and bones and is also involved in numerous cellular activities.[1] Understanding the relationship between the molecular structure and the conformational stability of collagen is therefore important. For applications in e.g. tissue engineering and wound healing, the design of biocompatible collagen based synthetic materials is attractive.[2,3] Toward this goal, synthetically accessible collagen peptides bearing functional moieties are promising tools.[3−6] Thus, for both the fundamental understanding of the conformational stability of collagen and the design of collagen based synthetic materials, it is important to probe in which positions functionalization affects the conformational stability of collagen.

Collagen is built up of three polyproline II (PPII)-like helices that are coiled around each other to form triple helices that further assemble into fibers and bundles.[1] The single strands consist of tripeptidic repeating units Xaa-Yaa-Gly. The glycin (Gly) residues are critical since they reside in the center of the triple helix where there is no space to accommodate sterically more demanding amino acids.[7−9] In the Xaa position proline (Pro) is the most common amino acid, and (4*R*)-hydroxyproline (Hyp) is most abundant in the Yaa position (Figure 1). Crystal structures show C(4)-*endo* ring puckering of the Pro residues in the Xaa positions and C(4)-*exo* ring puckering of the Hyp residues in the Yaa positions.[7,10] For the dihedral angles Ψ (N_i−C_{ia}−C_i−N_{i+1}) that are responsible for the

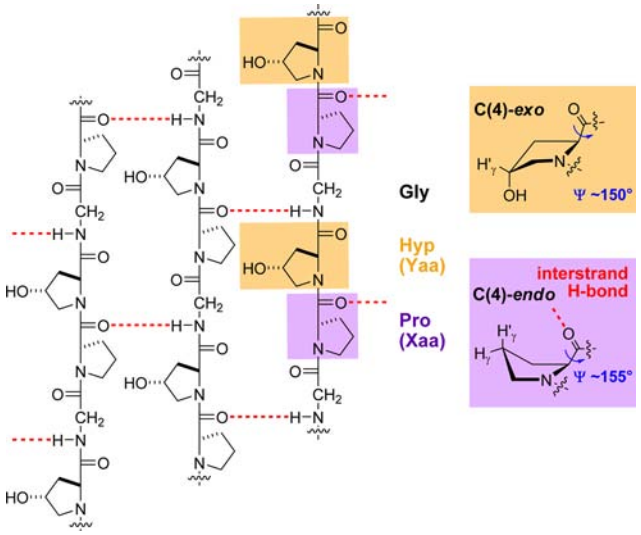


Figure 1. General structure of the collagen triple helix (left), ring puckering of the residues in the Xaa and Yaa position (right).

directionality of the collagen strands average values around 150° are observed along the entire strand.[7,10] Within the collagen triple helix the single strands are held together by H-bonds between the NH groups of glycine of one strand and the

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C═O groups of the residue in the Xaa position in an adjacent strand (Figure 1).[1,7,10]

Studies with collagen model peptides (CMPs) showed that replacement of the natural Pro or Hyp residues by non-imino acids causes a destabilization of the triple helix.[11] Pro and Hyp can, however, be replaced by other proline derivatives without a significant destabilization of the collagen triple helix provided that they have conformational properties (ring pucker and *trans*/*cis* amide bond ratio) similar to those of the natural residues[5,12−18] and do not interfere with the interstrand H-bonds.[6,19] The ring puckering and the *trans*/*cis* amide conformer ratio are influenced by stereoelectronic and/or steric effects exerted by functional groups attached to the pyrrolidine ring of the proline residues.[12−17,20,21] These studies have been performed with proline residues bearing substituents that do not have large steric demands (e.g., F,[12] Cl,[13] Me,[14] OAc,[15] OMe,[16] SH,[17] NH_2,[18] NHCHO[6]). Little is known, however, about the effect of sterically demanding substituents in the Xaa and Yaa positions on the conformational properties of the collagen triple helix.

For the development of functionalized collagen, residues such as lysine (Lys) bearing an amino group as an attachment site for the functional moieties have so far been most commonly used.[4b,d] However, as with any other non-imino acid in the Xaa or Yaa position the incorporation of Lys causes a destabilization of the PPII helix and also the collagen triple helix.[11,22] Thus, alternative methods for the incorporation of functional moieties that preserve the conformational properties of the collagen triple helix are desirable.

We have recently introduced azidoproline (Azp) containing CMPs and demonstrated that click chemistry allows for functionalization with triazoles bearing, for example, monosaccharides.[5] These functionalized CMPs are easily accessible and form triple helices that have, however, lower thermal stability compared to triple helices derived from CMPs consisting of the natural residues. We also investigated the impact of (4*S*)-configured acetylamidoproline (Acp) and formamidoproline (Fmp) residues on the thermal stability of the collagen triple helix.[6] These studies showed that their incorporation in the Yaa position affects the stability of the collagen triple helix only to a minor extent. In contrast, incorporation of (4*S*)Acp or (4*S*)Fmp in the Xaa position destabilizes the collagen triple helix significantly due to a competition between intra- and interstrand H-bonds.[6]

Herein we present a systematic investigation on the influence of acyl groups with different steric demands attached to (4*R*)- and (4*S*)-configured aminoproline (Amp) moieties in either the Xaa or Yaa position on the conformational stability of the collagen triple helix. We demonstrate that bulky substituents are tolerated in the side chain of (4*R*)-configured proline derivatives in the Xaa position and also in the side chain of (4*S*)-configured proline derivatives in the Yaa position. In contrast, steric repulsion with neighboring strands causes significant destabilization of the collagen triple helix in the other variations. Furthermore, the results show that unfavorable ring puckering in the Xaa or Yaa position is less important for the conformational stability of the collagen triple helix compared to an unfavorable steric disposition. The insights are important guidelines for the design of functional collagen based materials.

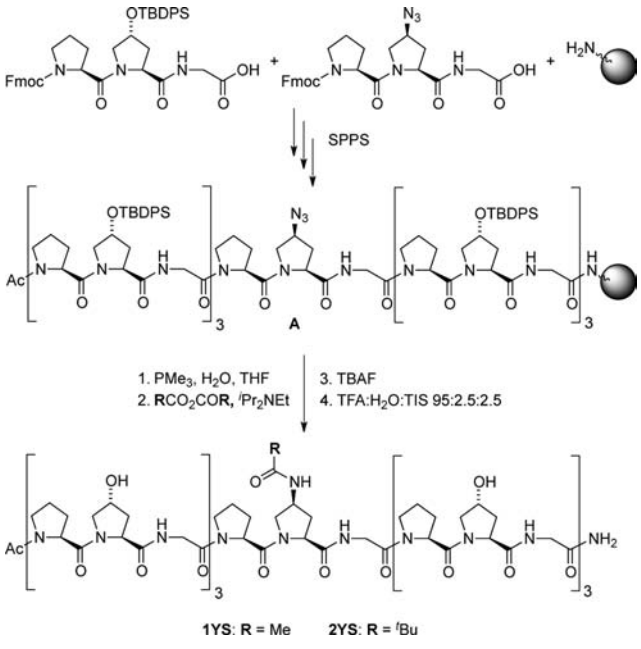
RESULTS AND DISCUSSIONS

Design of Collagen Model Peptides. To analyze in which positions within the collagen triple helix sterically demanding moieties are tolerated, we designed a series of collagen model peptides that differ from each other in the Xaa or Yaa position of the middle repeat unit within a 21-mer. Such so-called "host-guest-CMPs" have proven to be valuable for monitoring the effect of a single residue on the conformational stability of collagen.[11] To probe the effect of sterically demanding moieties, the CMPs were functionalized either with an acetylamido (**1**) or the significantly bulkier pivaloylamido (**2**) group in the Xaa (**X**) or the Yaa (**Y**) position (pivaloylamidoproline, Pvp). Both (4*R*)- and (4*S*)-configured residues were installed to evaluate which stereoisomer (**R** or **S**) is better suited for functionalization in the two possible positions. CMPs **3** and **4** bearing Pro and (4*R*)Hyp residues, respectively, in the Yaa position were prepared as reference peptides.

Ac-(Pro-Hyp-Gly)$_3$-((**4*R*)Acp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**1XR**)
Ac-(Pro-Hyp-Gly)$_3$-((**4*R*)Pvp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**2XR**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-(**4*R*)Acp**-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**1YR**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-(**4*R*)Pvp**-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**2YR**)
Ac-(Pro-Hyp-Gly)$_3$-((**4*S*)Acp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**1XS**)
Ac-(Pro-Hyp-Gly)$_3$-((**4*S*)Pvp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**2XS**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-(**4*S*)Acp**-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**1YS**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-(**4*S*)Pvp**-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**2YS**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-**Pro**-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**3**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-(**4*R*)Hyp**-Gly)-(Pro-Hyp-Gly)$_3$-NH_2 (**4**)

The synthesis of these CMPs was straightforward and is exemplified for CMPs **1YS** and **2YS** in Scheme 1. Peptides with (4*S*)Azp or (4*R*)Azp residues in either the Xaa or Yaa position (CMP **A**) of the middle repeat unit were used as common precursors. They were easily prepared by successive couplings

Scheme 1. Synthesis of CMPs Exemplified for 1YS and 2YS[a]



[a]TBAF = tetrabutyl ammonium fluoride. TIS = triisopropyl silane.

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of the building block Fmoc-Pro-Hyp(TBDPS)-Gly-OH and the respective trimeric Azp-containing building block using standard solid phase peptide synthesis.[5,6,23]

Staudinger reduction of the azido group of the solid phase bound CMP (**A**) followed by reaction with either acetic anhydride or pivaloyl anhydride yielded the desired acylated peptide. Removal of the silyl protecting groups and cleavage from the resin provided the functionalized CMPs that were purified by preparative HPLC (Scheme 1).

Conformational Properties of (4*R*)- and (4*S*)-Configured Ac-Acp-OMe and Ac-Pvp-OMe. For understanding the effect of proline derivatives on the conformational stability of the collagen triple helix, the according acetylated methylesters (Ac-Aaa-OMe) have proven to be valuable model compounds.[1d,12−18] Since the conformations of the acetylated model amino acids Ac-(4*S*)Acp-OMe (**5S**) and Ac-(4*R*)Acp-OMe (**5R**) had been analyzed before[21] we started the present studies by synthesizing and examining the preferred conformations of the related (4*R*)- and (4*S*)-configured pivaloylated analogues **6R** and **6S** (Figure 2) and compared them to

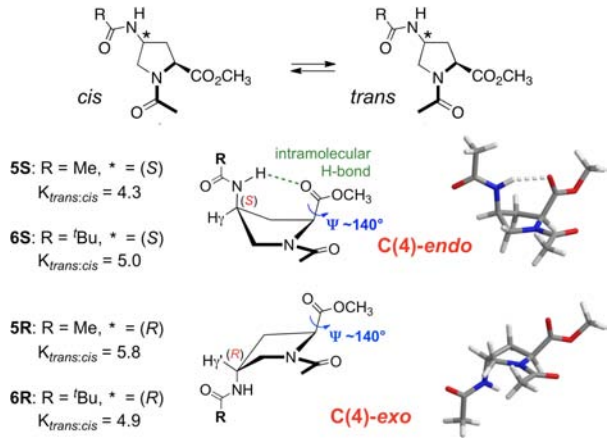

Figure 2. Conformational properties of the model compounds **5S**, **5R**, **6S**, and **6R** determined by NMR spectroscopy using 80 mM solutions in D_2O and lowest energy structuresof **5S** and **5R**.

those of the acetylated derivatives. As expected for proline derivatives, the ^1H and ^{13}C NMR spectra of **6S** and **6R** in D_2O show two sets of signals corresponding to the *cis*- and *trans*-amide conformers. Analyses of the $^1H,^1H$-vicinal coupling constants allowed for the assignment of the preferred ring pucker. The comparison of the spectra with those of the acetylated compounds revealed that the type of acyl group does not influence the conformational properties to a significant extent.

Both Ac-(4*R*)Acp-OMe (**5R**) and Ac-(4*R*)Pvp-OMe (**6R**) adopt preferentially C(4)-*exo* ring puckers and have *trans*/*cis* conformer ratios of 5.8:1 and 4.9:1, respectively (Figure 2).[24] These conformational properties are typical for (4*R*)-configured proline derivatives with an electron-withdrawing group at C(4) and comparable to those of, for example, Ac-(4*R*)Hyp-OMe (C(4)-*exo* pucker, *cis*/*trans* 1:6.1 in D_2O).[1d,12d] They are also in agreement with the preferred conformation of Ac-(4*R*)Acp-OMe as predicted by previously performed quantum chemical calculations.[21] This predicted lowest energy conformation exhibits a C(4)-*exo* ring pucker, a *trans* amide bond, and a Ψ-angle (N−C_α−C−OMe) of 140° (Figure 2, bottom).

The (4*S*)-configured amidoproline derivatives **5S** and **6S** prefer C(4)-*endo* ring puckers and have *trans*/*cis* conformer ratios of 4.3:1 and 5.0:1, respectively.[25] Whereas the C(4)-*endo* ring puckering is typical for (4*S*)-configured proline derivatives with an electron-withdrawing group at C(4), the *trans*/*cis* ratios are unusually high. The reason for this high content of the *trans*-amide conformer is the formation of a transannular H-bond between the NH of the amide at C(4) and the carbonyl oxygen of the ester moiety (Figure 2).[21] This intramolecular H-bond leads to a Ψ-angle (N−C_α−C−OMe) of approximately 140° which is optimal for an n→π* interaction between the acetyl group and the methyl ester that stabilizes the *trans* conformer.[19,21,26]

Thus, whereas the absolute configuration at C(4) is critical for the conformational properties of amidated proline derivatives, the steric bulk of the substituent at C(4) influences the ring puckering and the *trans*/*cis* conformer ratio of the model compounds to a comparatively small extent.

Thermal Denaturation Studies. CD spectra of solutions of the amidated CMPs revealed that all of the acetylated and pivaloylated CMPs form triple helices as indicated by the observed maximum at 225 nm that is typical for the collagen triple helix and is also observed in the spectra of the reference CMPs **3** and **4** (Figure 3).[1a] To evaluate the relative stabilities of the collagen triple helices derived from the CMPs, thermal denaturation studies were performed using CD spectroscopy as a monitoring tool. Sigmoidal curves with midpoints of thermal transition (T_m) in the range of 26−44 °C were observed for the examined triple helices (Figure 3, Table 1).

Since the unfolding rates of collagen triple helices are slow and are furthermore not necessarily identical for triple helices

Table 1. Thermodynamic Parameters Obtained from Thermal Denaturation Studies Monitored by CD Spectroscopy of the Triple Helices Derived from the CMPs

entry	CMP [middle triplet]	T_m[a] (°C)	ΔH^b kcal·mol^{-1}	$-T\Delta S^b$ kcal·mol^{-1}	$\Delta G^{b,c}$ kcal·mol^{-1}
1	**1XR** [(4*R*)Acp-Pro-Gly]	43	−74.1	62.2	−11.8
2	**2XR** [(4*R*)Pvp-Pro-Gly]	44	−72.2	60.5	−11.7
3	**1YR** [Pro-(4*R*)Acp-Gly]	36	−73.2	62.7	−10.5
4	**2YR** [Pro-(4*R*)Pvp-Gly]	32	−72.8	63.6	−9.2
5[d]	**1XS** [(4*S*)Acp-Pro-Gly]	32	−68.4	59.5	−8.9
6	**2XS** [(4*S*)Pvp-Pro-Gly]	26	−67.3	60.4	−6.9
7[d]	**1YS** [Pro-(4*S*)Acp-Gly]	40	−69.3	58.3	−11.0
8	**2YS** [Pro-(4*S*)Pvp-Gly]	39	−70.4	59.1	−11.3
9[d]	**3** [Pro-Pro-Gly]	40	−71.6	60.4	−11.1
10[d]	**4** [Pro-(4*R*)Hyp-Gly]	43	−74.5	62.1	−12.4

[a] T_m at a heating rate of 1 °C/100 s (±1 °C). [b] Data at a heating rate of 0.1 °C/72 s. [c] ΔG at 25 °C (±0.2 kcalmol^{-1}). [d] Data taken from ref 6.

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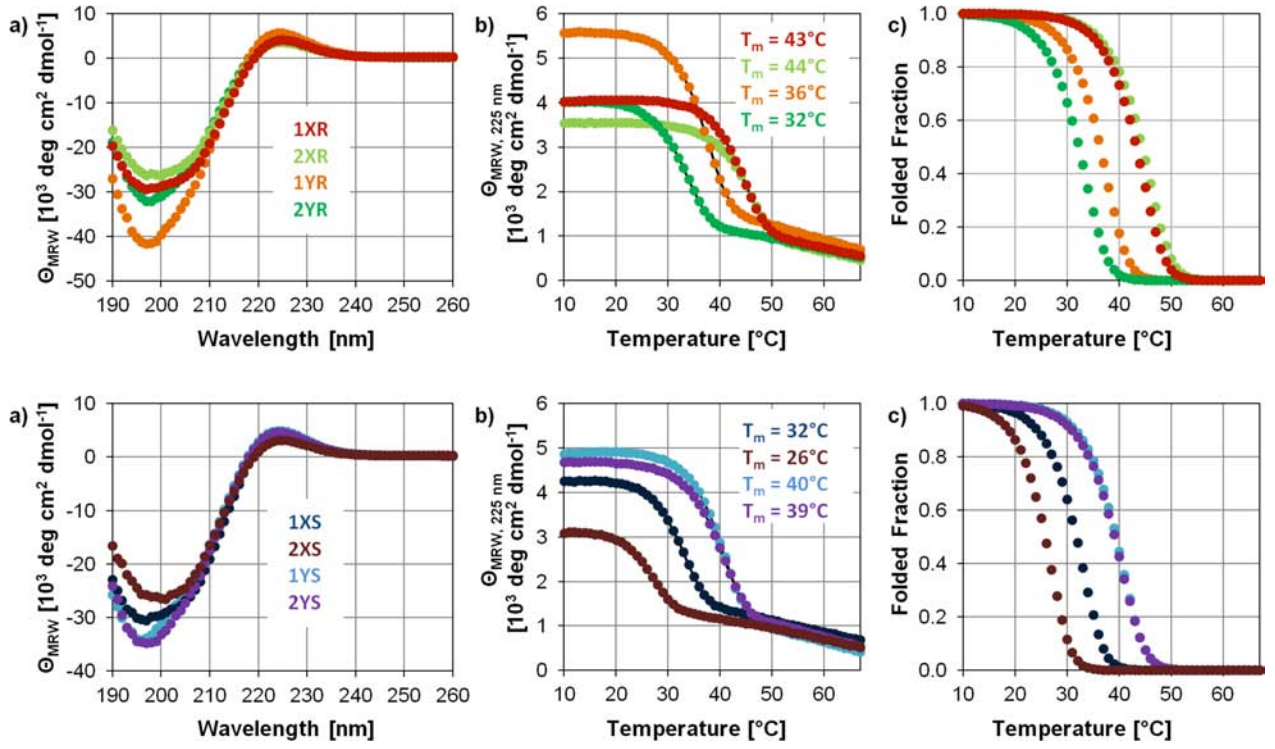

Figure 3. (a) CD spectra of CMPs **1XR**, **2XR**, **1YR**, **2YR**, **1SX**, **2SX**, **1SY**, and **2SY** in aqueous 50 mM AcOH. (b) Thermal denaturation curves of the according CMPs, fitted curves as black lines. (c) Folded fractions of the collagen triple helices formed by the CMPs.

composed of different amino acids, the T_m values often depend on the heating rate.[1b,27,28] To gain a deeper insight into the thermal stabilities of the triple helices derived from the amidated CMPs, we therefore performed additional thermal denaturation experiments and also monitored the refolding process by cooling the sample. The resulting hysteresis curves were fitted to a model introduced by Engel and Bächinger and provided the thermodynamic parameters ΔH, ΔS, and ΔG (Table 1).[27,28] Some of the ΔG values that would have been expected to be identical based on the T_m values deviate slightly from each other (e.g., entries 1 and 10, T_m = 43 °C, ΔG = −11.8 kcal·mol^{-1} and −12.4 kcal·mol^{-1}) indicating that kinetic effects indeed play a role. Reassuringly the determined free energies (ΔG) of the triple helices derived from the CMPs reflect the order of the relative thermal stabilities established by the melting temperatures.

Effect of (4*R*)Acp and (4*R*)Pvp Residues in the Xaa Position on the Stability of the Collagen Triple Helix. For the triple helices derived from the CMPs **1XR** and **2XR** bearing the acetylated or pivaloylated proline residues with the (4*R*)-configuration in the Xaa position, T_m values of 43 and 44 °C and ΔG values of −11.8 and −11.7 kcal·mol^{-1}, respectively, were observed (Table 1, entries 1 and 2). Their stabilities are slightly lower compared to that of the triple helix derived from CMP **4** (Table 1, entry 10) with the natural Pro-Hyp-Gly motif (T_m = 43 °C, ΔG = −12.4 kcal·mol^{-1}) and slightly higher compared to that derived from the CMP **3** (Table 1, entry 9) with the Pro-Pro-Gly motif (T_m = 40 °C, ΔG = −11.1 kcalmol^{-1}) in the middle repeat unit. Thus, both (4*R*)Acp and the sterically more demanding (4*R*)Pvp residue are readily tolerated in the Xaa position of the collagen triple helix. These results are surprising when the ring pucker that was previously shown to be important for the stability of the collagen triple is taken into account.[1d,12−19] Whereas, in natural collagen, the proline residue in the Xaa position adopts preferentially a C(4)-*endo* pucker, the amidated (4*R*)-configured Acp and Pvp residues prefer C(4)-*exo* ring puckering as described above. An overlay of the lowest energy structure of Ac-(4*R*)Acp-OMe[29] with a Pro residue in the Xaa position of a crystal structure of collagen (PDB 1 V7H)[10] shows not only this mismatch in the ring pucker (Figure 4) but also that the torsion angles along the

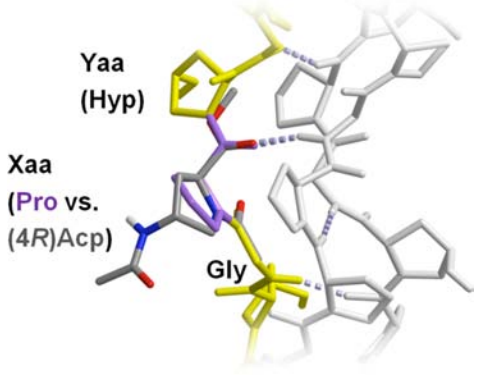

Figure 4. Overlay of the lowest energy structure of Ac-(4*R*)Acp-OMe[21] with a Pro residue in the Xaa position of a CMP crystal structure (PDB 1V7H[10]).

backbone of collagen match very well with those of the (4*R*)Acp residue. In particular the Ψ-angles within collagen that are important for the directionality of the strands are in good agreement with that predicted for Ac-(4*R*)Acp-OMe. Thus, the results show that a C(4)-*endo* ring pucker is not strictly required in the Xaa position of the collagen triple helix. The overlay also illustrates why the substituent at C(4) does not have a significant destabilizing effect: It points to the outside of

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the triple helix (Figure 4) and, as a result, does not affect the conformational stability of the collagen triple helix regardless of the size of the functional group.

Effect of (4*R*)Acp and (4*R*)Pvp Residues in the Yaa Position on the Stability of the Collagen Triple Helix. For the triple helices derived from the CMPs bearing the (4*R*)-configured amidoprolines in the Yaa position (**1YR** and **2YR**), significantly lower T_m (36 and 32 °C) and ΔG values (−10.5 and −9.2 kcalmol^{-1}) compared to those derived from the reference CMPs **3** and **4** were observed (Table 1, entries 3 and 4 versus 9 and 10). Based on the ring puckering and the *cis*/*trans* conformer ratio of (4*R*)Acp and (4*R*)Pvp that are comparable to those of the natural (4*R*)Hyp residue, these findings are unexpected. The observation that the collagen triple helix derived from the *tert*-butyl group bearing CMP **2YR** has an even lower stability compared to that derived from the methyl group bearing CMP **1YR** suggests that steric effects are important for the observed destabilization. This hypothesis is supported by an overlay of the lowest energy structure of Ac-(4*R*)Acp-OMe[29] with a Hyp residue in the Yaa position within the crystal structure (PDB 1 V7H)[10] of a collagen triple helix (Figure 5).

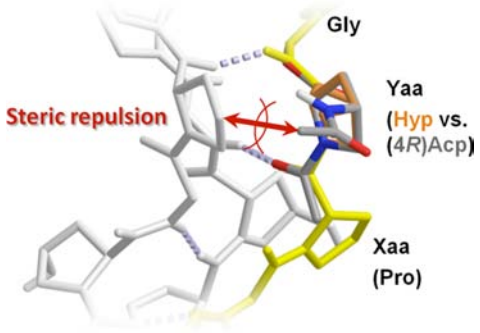

Figure 5. Overlay of the lowest energy structure of Ac-(4*R*)Acp-OMe[21] with a Hyp residue in the Yaa position of a CMP crystal structure (PDB 1V7H[10]).

The acetyl group points toward a proline residue of the neighboring strand which leads to steric repulsion that will be even higher in the case of the pivaloyl moiety.[30] These results show that favorable ring puckering and *cis*/*trans* amide bond preferences can be overwritten by unfavorable steric constraints. They are in agreement with studies on acetylated (4*R*)Hyp residues in the Yaa position of CMPs that were found to destabilize the collagen triple helix.[15] They also suggest that only (4*R*)-configured proline residues bearing substituents with a steric demand that is not significantly higher compared to that of the natural hydroxy group (e.g., OMe)[16] are tolerated in the Yaa position of collagen.

Effect of (4*S*)Acp and (4*S*)Pvp Residues in the Xaa Position on the Stability of the Collagen Triple Helix. Our previous studies had shown that incorporation of (4*S*)Acp in the Xaa position causes a significant destabilization of the collagen triple helix (T_m = 32 °C, ΔG = −8.9 kcalmol^{-1}, Table 1, entry 5).[6] A reason for this low thermal stability of the triple helix derived from CMP **1XS** is a weakening of the interstrand H-bond that holds the three collagen single strands together by a competing transannular H-bond within the (4*S*)Acp residue. This conflict between the H-bonds is seen in an overlay of the lowest energy structure of Ac-(4*S*)Acp-OMe[29] with a Pro residue in the Xaa position within the crystal structure (PDB 1 V7H)[10] of a collagen triple helix (Figure 6). Based on this

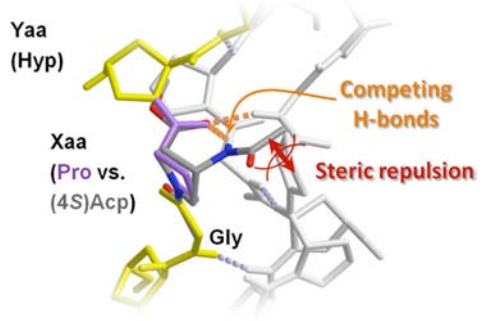

Figure 6. Overlay of the lowest energy structure of Ac-(4*S*)Acp-OMe[21] with a Pro residue in the Xaa position of a CMP crystal structure (PDB 1V7H[10]).

finding, also the collagen triple helix derived from the CMP **2XS** bearing a (4*S*)Pvp residue in the Xaa position was expected to be comparatively low. In fact, this triple helix has T_m and ΔG values as low as 26 °C and −6.9 kcal·mol^{-1}, respectively, values that are even lower compared to those of the acetylated analogue. Compared to the triple helix derived from the reference CMP **4** consisting of the naturally most abundant residues, the difference in the T_m value is 17 °C ($\Delta\Delta G$ = −5.5 kcal·mol^{-1}). This is indicative of a high destabilization, particularly considering that only 1 out of 21 residues was exchanged. The significant difference in stabilities between the acetylated and pivaloylated triple helices suggests that in addition to the competing H-bonds also steric constraints are responsible for this high destabilization. This is again supported by the overlay of Ac-(4*S*)Acp-OMe with a Pro residue in the Xaa position of the collagen triple helix (Figure 6). The substituent of the amide points to the inside of the triple helix and clashes sterically with the residue in the Yaa position of the neighboring strand. Combined with the weakened interstrand H-bond, this steric repulsion explains the observed dramatic destabilizing effect of (4*S*)-configured amidoproline derivatives in the Xaa position.

This result demonstrates that a high *trans*/*cis* amide bond ratio, a matching ring pucker, and a matching Ψ-angle are not sufficient for the formation of a highly stable triple helix if steric constraints and competing H-bonds are hindering the triple helix formation.

Effect of (4*S*)Acp and (4*S*)Pvp Residues in the Yaa Position on the Stability of the Collagen Triple Helix. A (4*S*)Acp residue in the Yaa position (CMP **1YS**) had been found to have only a minor effect on the conformational stability of the triple helix (T_m = 40 °C, ΔG = −11.0 kcal·mol^{-1}, Table 1, entry 7).[6] The overlay of the lowest energy structure of Ac-(4*S*)Acp-OMe[29] with a Hyp residue in the Yaa position of the collagen triple helix (Figure 7) shows that the acetyl group is not interfering with a residue in a neighboring strand but points to the outside of the triple helix. This model suggests that there is enough space even for more sterically demanding residues such as a *tert*-butyl moiety. Thus, also a (4*S*)Pvp residue in the Yaa position should not destabilize the collagen triple helix significantly. Indeed, the collagen triple helices derived from CMPs **1YS** and **2YS** proved to have similar thermal stabilities as judged by the observed T_m (40 and 39 °C) and ΔG values (−11.0 and −11.3 kcal·mol^{-1}, respectively) that

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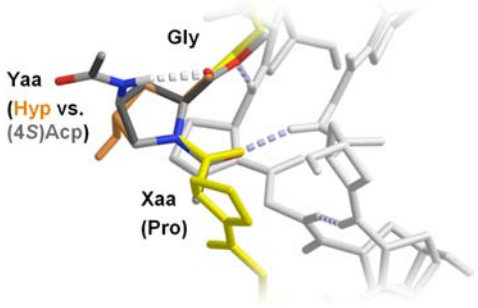

Figure 7. Overlay of the lowest energy structure of Ac-(4*S*)Acp-OMe[21] with a Hyp residue in the Yaa position of a CMP crystal structure (PDB 1V7H[10]).

are comparable with those of the triple helices derived from CMPs **3** and **4** (Table 1, entries 7−10). Since both (4*S*)Acp and (4*S*)Pvp prefer a C(4)-*endo* pucker whereas the (4*R*)Hyp residue in natural collagen adopts a C(4)-*exo* pucker, these findings underline that a mismatch in the ring puckering is not as critical for the conformational stability of the collagen triple helix as the interstrand H-bonds and steric constraints.

CONCLUSIONS

Our results show that sterically demanding groups are tolerated within the collagen triple helix when they are installed either in the Xaa position at a (4*R*)-configured amidoproline or in the Yaa position at a (4*S*)-configured amidoproline residue. In both cases, the bulky moieties point to the outside of the collagen triple helix and therefore do not interfere with the supramolecular assembly. Thus, substituents do not necessarily hinder triple helix formation when they are installed in the Xaa or Yaa position on proline residues. This is in contrast to modifications of the Gly residues within collagen that cannot be functionalized without a significant destabilization of the collagen triple helix.[7−9] The results also revealed that sterically unfavorable dispositions affect the conformational stability of the collagen triple helix more than unfavorable ring puckering of the proline residues. These insights provide a guide for the design of functionalized collagen model peptides that are becoming increasingly important for the development of biocompatible functional materials.[1−4]

EXPERIMENTAL SECTION

General Aspects. Materials and reagents were of the highest commercially available grade and used without further purification. For solid phase peptide synthesis, Rink Amide-ChemMatrix resin from pcas BioMatrix (Saint-Jean-sur-Richelieu, Canada) was used. Reactions were monitored by thin layer chromatography using Merck silica gel 60 F_{254} plates. Compounds were visualized by UV, ninhydrin, and 4,4′-tetramethyldiaminodiphenylmethane (TDM). Flash chromatography was performed using Merck silica gel 60, particle size 40−63 μm. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX 500 and DPX 400 spectrometers. Chemical shifts are reported in ppm using TMS as a reference. A Bruker Esquire 3000plus instrument was used for electrospray ionization (ESI) mass spectrometry measurements. High resolution mass spectra were recorded on a Bruker maXis ESI-Q-TOF. Analytical HPLC was performed using a LiChrospher 100 RP-18e 5 μm (250 mm × 4 mm) column from Merck. Preparative HPLC was carried out on a LiChrospher RP-18e 5 μm (250 mm × 10 mm) column from Merck. A Chirascan (Applied Photophysics Ltd., Leatherhead, UK) was used for CD measurements. The solutions were measured in a quartz cell with a path length of 1 mm (Hellma 110-QS). For automated peptide synthesis, a Syro I Peptide Synthesizer (MultiSynTech GmbH, Witten, Germany) was employed.

Ac-(4*S*)Pvp-OMe (6S). Ac-(4*S*)Azp-OMe (100 mg, 471 μmol, 1.0 equiv) was added to a suspension of Pd/C (10 mg, 10% w/w) in MeOH (7.6 mL), and Piv_2O (191 μL, 942 μmol, 2.0 equiv) was added. The black suspension was stirred under a H_2 atmosphere for 3 h and filtered over Celite, and all volatiles were then removed under reduced pressure. After flash chromatography on silica (10% MeOH in CH_2Cl_2) a colorless oil was isolated. The oil was then redissolved in CH_2Cl_2 (50 mL), and the solution was extracted with sat. $NaHCO_3$ (3 × 10 mL) to hydrolyze and remove remainders of Piv_2O. The combined organic layers were dried over $MgSO_4$, and all volatiles were then removed under reduced pressure to obtain Ac-(4*S*)Pvp-OMe (**6S**, 127 mg, >98%) as a colorless oil. TLC: R_f = 0.54 (10% MeOH in CH_2Cl_2, TDM). ^{1}H NMR (500 MHz, D_2O, 25 °C): (Two conformers due to *cis*−*trans* isomerism around the amide bond are visible in a ratio of ∼5:1) *trans*-conformer: δ = 4.45 (dd, *J* = 9.4, *J* = 4.7 Hz, 1H; Hα), 4.41 (tt, *J* = 6.2, *J* = 4.6 Hz, 1H; Hγ), 3.87 (dd, *J* = 11.2, *J* = 6.3 Hz, 1H; Hδ), 3.71 (s, 3H; OMe), 3.58 (dd, *J* = 11.2, *J* = 4.2 Hz, 1H; Hδ), 2.53 (ddd, *J* = 13.8, *J* = 9.4, 6.2 Hz, 1H), 2.08−2.02 (m, 1H; Hβ), 2.05 (s, 3H; Ac), 1.08 (s, 9H, Piv); isolated signals of the *cis*-conformer: δ = 4.73 (dd, *J* = 9.5, *J* = 2.4 Hz, 1H; Hα), 4.27 (tt, *J* = 5.6, *J* = 2.6 Hz, 1H; Hγ), 3.75 (s, 3H; OMe), 3.52 (dd, *J* = 12.9, *J* = 1.1 Hz, 1H; Hδ), 2.32 (ddd, *J* = 14.1, *J* = 3.5, *J* = 2.4 Hz, 1H; Hβ), 1.96 (s, 3H; Ac), 1.06 (s, 9H; Piv). ^{13}C NMR (126 MHz, D_2O, 25 °C): δ = 182.2 (Piv), 174.7, 173.2 (ester, amide), 57.8 (Cα), 53.2 (OMe), 52.8 (Cγ), 49.3 (Cδ), 38.3 (Piv), 33.9 (Cβ), 26.3 (Piv), 21.2 (Ac). HRMS (ESI): *m*/*z*: calcd for $C_{13}H_{22}N_2NaO_4$: 293.1472; found: 293.1464 $[M + Na]^+$.

Ac-(4*R*)Pvp-OMe (6R). Ac-(4*R*)Pvp-OMe was synthesized according to the same procedure as described for **6S** and was obtained in ≥98% yield (129 mg) as a colorless oil. TLC R_f = 0.28 (3% MeOH in CH_2Cl_2, TDM). ^{1}H NMR (400 MHz, D_2O, 25 °C): (Two conformers due to *cis*−*trans* isomerism around the amide bond are visible in a ratio of ∼5:1) *trans*-conformer: δ = 4.48 (dd, *J* = 7.8 Hz, 6.0 Hz, 1H, Hα), 4.40 (p, *J* = 6.7 Hz, 1H; Hγ), 3.89 (dd, *J* = 10.9 Hz, 6.9 Hz, 1H; Hδ), 3.66 (s, 3H; OMe), 3.42 (dd, *J* = 10.9 Hz, 6.1 Hz, 1H; Hδ), 2.25−2.20 (m, 2H; Hβ), 2.00 (s, 3H; Ac), 1.05 (s, 9H; Piv). Isolated signals of the *cis*-conformer: δ = 4.74 (dd, *J* = 8.8 Hz, 3.0 Hz, 1H; Hα), 3.71 (s, 3H; OMe), 3.31 (dd, *J* = 12.0 Hz, 7.1 Hz, 1H; Hδ), 2.40 (ddd, *J* = 13.1 Hz, 6.7 Hz, 3.1 Hz, 1H; Hβ), 1.91 (s, 3H; Ac), 1.05 (s, 9H; Piv). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 178.8 (Piv), 172.0, 169.7 (ester, amide) 57.2 (Cα), 53.3 (OMe), 52.5 (Cγ), 48.9 (δ), 38.7 (Piv), 34.9 (Cβ), 27.5 (Piv), 22.3 (Ac). HRMS (ESI): *m*/*z*: calcd for $C_{13}H_{22}N_2NaO_4$: 293.1472; found: 293.1471 $[M + Na]^+$.

Fmoc-(4*R*)Azp-Pro-Gly-OH was prepared by solution phase synthesis starting from the amino acids Boc-(4*R*)Azp-OH, H-Pro-OMe·HCl, and H-Gly-O^{t}Bu·HCl in an overall yield of 27%. For details, see the Supporting Information.

^{1}H NMR (500 MHz, DMSO-d_6, 25 °C): (Four conformers due to *cis*−*trans* isomerism around the carbamide and amide bonds are visible in a ratio of ∼12:10:1:1) δ = 12.55 (s, 1H; COOH), 8.12 (t, *J* = 5.9 Hz, 1H; NHGly), 7.92−7.88 (m, 2H; Fmoc),7.64 (d, *J* = 7.6 Hz, 1H; Fmoc), 7.54 (d, *J* = 7.5 Hz, 1H; Fmoc), 7.45−7.39 (m, 2H; Fmoc), 7.36−7.31 (m, 6H; Fmoc), 4.45−4.40 (m, 2H; HαAzp, Fmoc), 4.36−4.29 (m, 1H; HαPro), 4.35−4.22 (m, 1H; Fmoc), 4.17 (t, *J* = 6.1 Hz, 1H; Fmoc); 3.80 (dd, *J* = 7.9 Hz, 6.3 Hz, 1H; HαGly), 3.77 (dd, *J* = 7.8 Hz, 6.2 Hz, 1H; HαGly), 3.68−3.57 (m, 1H; HδPro), 3.59 (dd, *J* = 11.3 Hz, 5.0 Hz, 1H; HδAzp), 3.53−3.41 (m, 1H; HδAzp), 3.48−3.40 (m, 1H; HδPro), 2.43−2.34 (m, 1H; HβAzp), 2.15−2.06 (m, 1H; HβAzp), 2.05−1.80 (m, 2H; HβPro), 1.97−1.75 (m, 2H; HγPro). Isolated signals of the minor conformers: 8.48 (t, *J* = 5.9 Hz, 1H; NHGly), 8.27 (t, *J* = 6.0 Hz, 1H; NHGly), 8.09 (t, *J* = 5.9 Hz, 1H; NHGly), 7.66 (d, *J* = 7.6 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 1H), 4.56 (t, *J* = 7.4 Hz, 1H; HαAzp), 3.66 (dd, *J* = 10.1 Hz, 5.6 Hz, 1H; HαGly), 3.70 (dd, *J* = 10.1 Hz, 5.7 Hz, 1H; HαGly), 3.27−3.21 (m, 1H; HδPro). ^{13}C NMR (101 MHz, DMSO-d_6, 25 °C) δ/ppm = 171.6 (amide Pro), 171.2 (acid Gly), 169.5 (amide Azp), 153.7 (Boc), 143.9 (Fmoc), 143.7 (Fmoc), 140.7 (Fmoc), 127.7 (Fmoc), 127.1 (Fmoc), 125.1 (Fmoc), 120.1 (Fmoc), 66.8 (Fmoc), 59.5 (Fmoc), 58.5 (CαPro), 56.0 (CαAzp), 52.0 (CδAzp), 46.8 (Fmoc), 46.5 (CδPro), 40.5 (CαGly), 34.1 (CAzp), 29.0 (CβPro), 24.3 (CγPro). Isolated signals of minor conformers: 171.7 (amide Pro), 169.6 (amide Azp), 153.7

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(Boc), 143.8 (Fmoc), 143.7 (Fmoc), 140.7 (Fmoc), 127.7 (Fmoc), 127.2 (Fmoc), 127.7 (Fmoc), 127.2 (Fmoc), 124.8 (Fmoc), 120.2 (Fmoc), 59.4 (CαPro), 59.2 (Fmoc), 56.4 (CαPro), 51.6 (CδAzp), 46.6 (Fmoc), 46.2 (CδPro), 35.2 (CβAzp), 29.1 (CβPro), 24.3 (CγPro). Signals were assigned using COSY, HMBC, and HMQC spectra. HRMS (ESI): m/z: calcd for $C_{27}H_{29}N_6O_6$: 533.2143; found 533.2147 $[M + H]^+$.

CMPs. The collagen model peptides were synthesized on ChemMatrix resin with a Rink amide linker with a loading of 0.46 mmol/g. The trimeric building blocks Fmoc-Pro-Hyp(TBDPS)-Gly-OH, Fmoc-Pro-Azp-Gly-OH, and Fmoc-Azp-Pro-Gly (4 equiv) were coupled by standard SPPS using (6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminiumhexafluorophosphate (HCTU) (4 equiv) and $^{i}Pr_2NEt$ (12 equiv) as coupling reagents and solutions of piperidine/DMF 1:4 for Fmoc deprotections on a peptide synthesizer. The resulting solid phase bound azidoproline containing CMPs were acetylated with a mixture of Ac_2O (30 equiv) and NEt_3 (30 equiv). After Staudinger reduction with 1 M PMe_3 in THF (5 equiv) and H_2O (65 equiv), the peptides were acylated using either Ac_2O or Piv_2O (30 equiv) and NEt_3 (30 equiv). After cleavage off the resin with a solution of TFA/H_2O/TIS (95:2.5:2.5), the peptides were precipitated with ether and purified by preparative HPLC (Eluents: A = MeCN, B = H_2O/MeCN/TFA 100:1:0.1). For further details on the synthesis and the characterization of each peptide, see the Supporting Information.

1XR. HPLC: t_R = 18.0 min; gradient: 91% to 85.9% B over 30 min at 65 °C. MS (ESI): m/z: calcd for $C_{88}H_{127}N_{23}O_{29}$: 1970.9; found: 1972.3 $[M + H]^+$.

2XR. HPLC: t_R = 22.4 min; gradient: 90% to 80% B over 30 min at 65 °C. MS (ESI): m/z: calcd for $C_{91}H_{133}N_{23}O_{29}$: 2013.0; found: 1007.1 $[M + 2H]^{2+}$ (100%).

1YR. HPLC: t_R = 18.3 min; gradient: 91% to 85.5% B over 30 min at 60 °C. MS (ESI): m/z: calcd for $C_{88}H_{127}N_{23}O_{29}$: 1970.9; found: 1972.3 $[M + H]^+$ (100%).

2YR. HPLC: t_R = 21.3 min; gradient: 90% to 80% B over 30 min at 65 °C. MS (ESI): m/z: calcd for $C_{91}H_{133}N_{23}O_{29}$: 2013.0; found: 1007.0 $[M + 2H]^{2+}$ (100%).

2XS. HPLC: t_R = 19.7 min; gradient: 90% to 80% B over 30 min at 65 °C. MS (ESI): m/z: calcd for $C_{91}H_{133}N_{23}O_{29}$: 2013.0; found: 1007.1 $[M + 2H]^{2+}$ (100%).

2YS. HPLC: t_R = 24.9 min; gradient: 90% to 80% B over 30 min at 65 °C. MS (ESI): m/z: calcd for $C_{91}H_{133}N_{23}O_{29}$: 2013.0; found: 1007.0 $[M + 2H]^{2+}$ (100%).

CD Spectroscopic Analysis and Thermal Denaturation of the Collagen Triple Helices. Stock solutions of the lyophilized peptides in 50 mM AcOH were prepared and diluted to a concentration of 0.20 mM. The solutions were equilibrated at 5 °C for >24 h before the measurements. CD spectra were recorded using a spectral bandwidth of 1 nm at 10 °C with an acquisition time of 5 s per point and a step resolution of 1 nm. For the determination of the T_m values, the unfolding processes were followed at the maxima of the previously recorded spectra with a spectral bandwidth of 1 nm and an acquisition time of 12 s per point. The temperature was increased in 1 °C steps, and the data points were recorded after an equilibration time of 100 s (36 °C/h). For the determination of the thermodynamic parameters, the unfolding and refolding processes (hysteresis) were followed at the maxima of the previously recorded spectra with a spectral bandwidth of 1 nm and an acquisition time of 1 s per data point. The temperature was increased in 0.1 °C steps every 72 s (5 °C/h). For the fitting procedures, see the Supporting Information.

Overlays between the Crystal Structure and the Lowest Energy Structures of Ac-(4*R*)Acp-OMe (5R) and Ac-(4*S*)Acp-OMe (5S). The overlays were generated by minimizing the sum of the distances between the atoms $\mathbf{C}(=O)_{i-1}$, N_i, $C_{\alpha i}$, $\mathbf{C}(=O)_i$, $C_{\beta i}$, $C_{\delta i}$ in the lowest energy structures of **5R** and **5S** and the Xaa or Yaa residue within the crystal structure (PDB1 V7H[10]). The Structure-Overlay-Minimize function of ChemBio 3D Ultra was used to perform this task after an approximate manual overlay of the structures.

■ ASSOCIATED CONTENT

Supporting Information

Details on the synthesis and analytical data of the CMPs; ^1H and ^{13}C NMR spectra of **6R** and **6S** and details on the thermal denaturation studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(25) The slightly higher *trans*/*cis* conformer ratio of the pivaloylated compared to the acetylated derivative is likely due to a shielding effect of the amide moiety by the *t*-Bu group from the surrounding water, thereby allowing for an enhanced transannular H-bond and compensating for the enhanced steric compared to stereoelectronic effect of the *t*-Bu group on the ring pucker.
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(28) We used the faster heating rate of 1 °C/100 s for reporting the T_m values since this has been the most commonly used heating rate in the past by researchers in the field. Thus, T_m values determined at this heating rate allow for a direct comparison of the relative stabilities of the collagen triple helixes reported here with those that were previously examined. T_m values derived at the slower heating rate are lower but reassuringly provide the same relative differences in the stabilities of the collagen triple helices.
(29) The lowest energy structures were derived from quantum chemical calculations as described in ref 21.
(30) Aside from these steric effects, also differences in the hydration of the acetyl versus the pivaloyl groups might play a role. For a discussion of the hydration of collagen, see: Okuyama, K.; Hongo, C.; Wu, G. H.; Mizuno, K.; Noguchi, K.; Ebisuzaki, S.; Tanaka, Y.; Nishino, N.; Bachinger, H. P. *Biopolymers* **2009**, *91*, 361–372.