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Stereoelectronic and Steric Effects in the Collagen Triple Helix: Toward a Code for Strand Association

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Abstract: Collagen is the most abundant protein in animals. The protein consists of a helix of three strands, each with sequence X−Y−Gly. Natural collagen is most stable when X is (2*S*)-proline (Pro) and Y is (2*S*,4*R*)-4-hydroxyproline (4*R*-Hyp). We had shown previously that triple helices in which X is (2*S*,4*S*)-4-fluoroproline (4*S*-Flp) or Y is (2*S*,4*R*)-4-fluoroproline (4*R*-Flp) display hyperstability. This hyperstability arises from stereoelectronic effects that preorganize the main-chain dihedral angles in the conformation found in the triple helix. Here, we report the synthesis of strands containing both 4*S*-Flp in the X-position and 4*R*-Flp in the Y-position. We find that these strands do not form a stable triple helix, presumably because of an unfavorable steric interaction between fluoro groups on adjacent strands. Density functional theory calculations indicate that (2*S*,3*S*)-3-fluoroproline (3*S*-Flp), like 4*S*-Flp, should preorganize the main chain properly for triple-helix formation but without a steric conflict. Synthetic strands containing 3*S*-Flp in the X-position and 4*R*-Flp in the Y-position do form a triple helix. This helix is, however, less stable than one with Pro in the X-position, presumably because of an unfavorable inductive effect that diminishes the strength of the interstrand 3*S*-FlpC=O···H−NGly hydrogen bond. Thus, other forces can counter the benefits derived from the proper preorganization. Although $(Pro-Pro-Gly)_7$ and $(4S\text{-Flp}-4R\text{-Flp}-Gly)_7$ do not form stable homotrimeric helices, mixtures of these two peptides form stable heterotrimeric helices containing one $(Pro-Pro-Gly)_7$ strand and two $(4S\text{-Flp}-4R\text{-Flp}-Gly)_7$ strands. This stoichiometry can be understood by considering the cross sections of the two possible heterotrimeric helices. This unexpected finding portends the development of a "code" for the self-assembly of determinate triple helices from two or three strands.

Introduction

Collagen consists of three individual peptide strands folded into a right-handed triple helix. Each strand comprises about 300 tripeptide repeats of the sequence X−Y−Gly folded into a polyproline-II helix.[1−4] Nearly all of the 20 proteinogenic amino acids are represented in the X- and Y-positions, but (2*S*)-proline (Pro) and (2*S*,4*R*)-4-hydroxyproline (4*R*-Hyp) are the most prevalent.[5]

The pyrrolidine ring in proline adopts two primary conformations, C^{γ}-exo and C^{γ}-endo (Figure 1).[6] Unsubstituted proline exhibits a slight preference for the C^{γ}-endo conformation, but both conformations are populated at room temperature.[7] The installation of an electronegative substituent on the ring alters the relative energies of the conformers and can lead to the nearly exclusive population of a single conformer.[8−12] For example, 4*R*-Hyp and (2*S*,4*R*)-4-fluoroproline (4*R*-Flp) favor the C^{γ}-exo conformation, but their diastereomers (2*S*,4*S*)-4-hydroxyproline (4*S*-Hyp) and (2*S*,4*S*)-4-fluoroproline (4*S*-Flp) prefer the C^{γ}-endo conformation.[7,13]

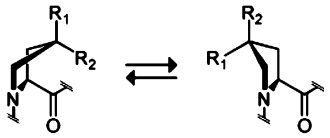

Figure 1. Ring conformations of 4-substituted proline residues. The C^{γ}-endo conformation is favored when R_1 = H and R_2 = H, OH, or F (as in 4*S*-Flp). The C^{γ}-exo conformation is favored when R_1 = OH or F (as in 4*R*-Flp) and R_2 = H.

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These conformational preferences arise from the gauche effect.[7,9,14,15] The electron-withdrawing hydroxyl or fluoro group prefers to orient itself gauche to the amide nitrogen found in the pyrrolidine ring because that orientation maximizes delocalization of electron density through the σ bond framework.[7,14,16,17] In the C$^\gamma$-exo conformation, a 4*S*-substituent is anti to the amide nitrogen, but a 4*R*-substituent is gauche (Figure 1). As a result, 4*R*-Hyp and 4*R*-Flp prefer the C$^\gamma$-exo conformation. The opposite is true for the C$^\gamma$-endo conformation, and 4*S*-Hyp and 4*S*-Flp prefer that conformation.

Nature invokes the gauche effect by the post-translational hydroxylation of proline residues in the Y-position of collagen strands. Using synthetic (Pro−Pro−Gly)$_{10}$ and (Pro−4*R*-Hyp−Gly)$_{10}$ peptides, Prockop and co-workers showed that proline hydroxylation greatly enhances triple-helix stability.[18] Subsequently, we showed that the enzyme that carries out this transformation, prolyl 4-hydroxylase, is essential for animal life.[19] The stability endowed by 4-hydroxyproline is dependent on its stereochemistry and location in the peptide, as (Pro−4*S*-Hyp−Gly)$_{10}$, (4*R*-Hyp−Pro−Gly)$_{10}$, and (4*S*-Hyp−Pro−Gly)$_{10}$ do not fold into a triple helix.[20,21]

For decades, the marked contribution of 4*R*-Hyp to triple-helix stability was attributed solely to the ability of its hydroxyl group to form hydrogen bonds.[22−25] Specifically, bridges of two water molecules observed in collagen crystals[24] were thought to bind the strands together. We rejected this argument for several reasons. For example, the construction of these water-molecule bridges should incur a substantial, perhaps prohibitive, entropic penalty. Moreover, 4*R*-Hyp in the Y-position was known to stabilize the triple helix in nonaqueous solvents.[26]

Studies with 4-fluoroproline led us to an alternative explanation. Although organic fluorine is a poor hydrogen-bond acceptor, replacement of 4*R*-Hyp in the Y-position with 4*R*-Flp caused a further increase in triple-helix stability.[10,11] As a fluoro group is more electron-withdrawing than a hydroxyl group but forms only weak hydrogen bonds, this result demonstrated that the stabilization imparted by the substituent arises from inductive effects. We then found that replacing 4*R*-Flp in the Y-position with its diastereomer, 4*S*-Flp, precludes triple-helix formation.[27] Thus, collagen stability relies on a stereoelectronic effect.

High-resolution structures of collagen triple helices have revealed that proline residues adopt a C$^\gamma$-endo conformation in the X-position and a C$^\gamma$-exo conformation in the Y-position.[24,28,29] Notably, these two conformers have values for the φ ($C'_{i-1}-N_i-C^\alpha_i-C'_i$) and ψ ($N_i-C^\alpha_i-C'_i-N_{i+1}$) dihedral angles that correspond to those preferred by residues in the X-position (where φ and ψ are $-72.6 \pm 7.6°$ and $163.8 \pm 8.8°$, respectively[24]) and Y-position (where φ and ψ are $-59.6 \pm 7.3°$ and $149.8 \pm 8.8°$, respectively[24]) of a triple helix of (Pro-4*R*-Hyp-Gly)$_{10}$ strands.[30,31,7] Hence, according to the principle of preorganization,[32] nonnatural proline derivatives that prefer a C$^\gamma$-endo or C$^\gamma$-exo conformation could produce more stable triple helices. Indeed, the C$^\gamma$-exo conformation of 4*R*-Flp (but not the C$^\gamma$-endo conformation of 4*S*-Flp) manifests φ and ψ dihedral angles requisite for the Y-position[8,7] and there enhances triple-helix stability.[27,33]

The preorganization of proline residues in the C$^\gamma$-endo conformation can also increase triple-helix stability. Replacing Pro with 4*S*-Flp in the X-position leads to the increased stability of triple-helical (4*S*-Flp−Pro−Gly)$_7$[33] and (4*S*-Flp−Pro−Gly)$_{10}$,[34] but replacing Pro with 4*R*-Flp decreases stability. These results indicate, once again, that a 4-fluoroproline diastereomer can enhance triple-helix stability by preorganization.[7] Recently, this interpretation was supported by calorimetric analyses.[35]

The preorganization of *improper* φ and ψ dihedral angles does not necessarily preclude triple-helix formation. Bächinger and co-workers have observed that stable triple helices can form with 4*R*-Hyp in the X-position, even though 4*R*-Hyp adopts the C$^\gamma$-exo conformation with accordant φ and ψ dihedral angles that are unfavorable for that position.[36−39] They explained this apparent anomaly by invoking the preorganization that arises from a water-mediated hydrogen bond between the 4*R*-Hyp hydroxyl group and a main-chain oxygen in the same strand.[39] It is noteworthy that an additional contribution likely arises from the intrinsic preference of 4*R*-Hyp for a trans peptide bonds (Table 1),[9−11,27] which is essential for triple-helix formation. This preference derives from yet another stereoelectronic effect.[7,41]

Here, we explore further the ability of preorganization to enhance triple-helix stability. Specifically, we incorporate three

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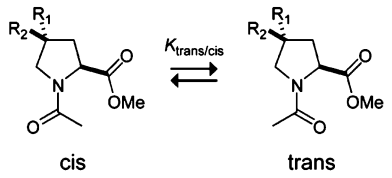
Table 1. Values of $K_{trans/cis}$ and T_m for 4-Substituted Proline Residues



X/Y	R_1	R_2	$K_{trans/cis}$ of Ac-X/Y-OMe[a]	T_m (°C) of triple-helical (X–Pro–Gly)$_7$[b]	T_m (°C) of triple-helical (Pro–Y–Gly)$_7$[c]
4*R*-Flp	F	H	6.7	no helix[d]	45
4*R*-Hyp	OH	H	6.1	no helix	36
Pro	H	H	4.6	no helix	no helix
4*S*-Hyp	H	OH	2.4	no helix	no helix
4*S*-Flp	H	F	2.5	33	no helix

[a] Values are from ref 27 and were measured in D_2O at 25 °C by integration of ^{1}H NMR spectra. [b] T_m refers to the temperature at the midpoint of the thermal transition between folded and unfolded forms as measured by circular dichroism spectroscopy. Values are from refs 33 and 40 (X/Y = Pro). [c] Values are from ref 27. [d] "No helix" refers to T_m < 10 °C.

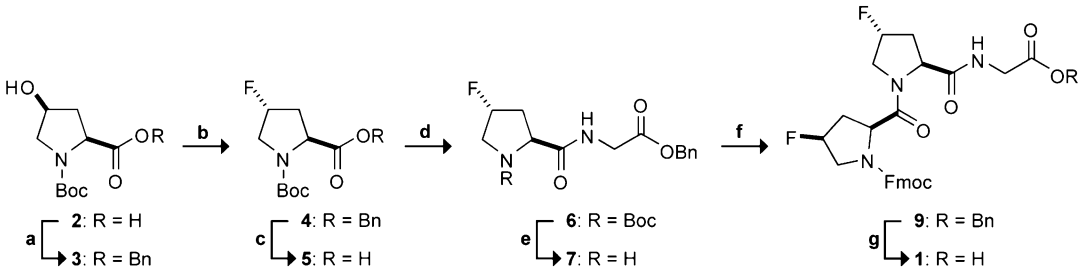
distinct fluoroproline residues into collagen-like peptides to control the geometry of the main chain at the X- and Y-positions simultaneously. We find that other forces can counter the benefits derived from the proper preorganization of φ and ψ dihedral angles. This finding leads to comprehensive conclusions regarding the basis for collagen stability, as well as a new means to envisage strand association.

Results and Discussion

Synthesis of (4*S*-Flp−4*R*-Flp−Gly)$_7$. (4*S*-Flp−4*R*-Flp−Gly)$_7$ was synthesized by the condensation of units of Fmoc-4*S*-Flp−4*R*-Flp−Gly (**1**). Tripeptide **1** was synthesized by the route shown in Scheme 1. Briefly, Boc-4*S*-HypOH (**2**) was protected as its benzyl ester **3**, and then fluorinated with morpholinosulfur trifluoride (morph-DAST) to yield **4** as a single diastereomer. Hydrogenation to the free acid **5** followed by PyBOP-mediated coupling to the tosylate salt of glycine benzyl ester yielded dipeptide **6**. Dipeptide **6** was converted to its hydrochloride salt **7**, and then coupled to Fmoc-4*S*-FlpOH (**8**)[42] to provide tripeptide **9**. Higher yields were obtained with PyBroP (79%) than PyBOP (∼30%) as the coupling reagent. Hydrogenation of tripeptide **9** provided tripeptide **1**. The low yield (38%) of this last reaction was largely the result of poor recovery during recrystallization. Tripeptide **1** was used to produce (4*S*-Flp−4*R*-Flp−Gly)$_7$ by solid-phase peptide synthesis.

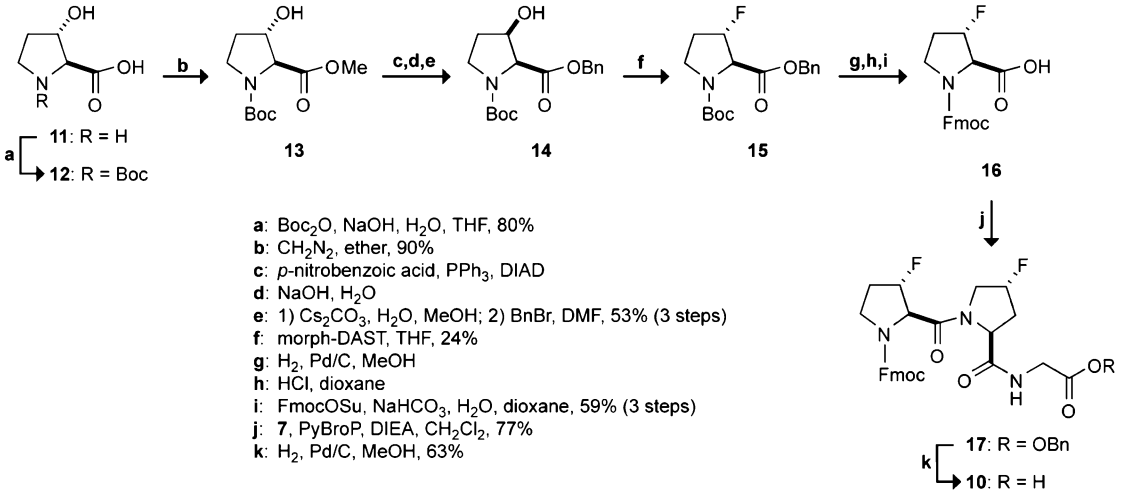
Synthesis of (3*S*-Flp−4*R*-Flp−Gly)$_7$. (3*S*-Flp−4*R*-Flp−Gly)$_7$ was synthesized by the condensation of units of Fmoc-3*S*-Flp−4*R*-Flp−Gly (**10**). Tripeptide **10** was synthesized by the route shown in Scheme 2. Briefly, (2*S*,3*S*)-3-hydroxyproline (**11**) was *N*-acylated to yield free acid **12**, which was then converted into methyl ester **13**. The Mitsunobu reaction with *p*-nitrobenzoic acid was used to invert the stereochemistry of the hydroxyl group. (The use of formic acid instead of *p*-nitrobenzoic acid led to substantial retention of stereochem-

Scheme 1



a: 1) Cs_2CO_3, H_2O, MeOH; 2) BnBr, DMF, 95%
b: morph-DAST, THF, 36%
c: H_2, Pd/C, MeOH, 100%
d: TsOH·GlyOBn, PyBOP, DIEA, CH_2Cl_2, 79%
e: HCl/dioxane, 100%
f: **8**, PyBroP, DIEA, CH_2Cl_2, 79%
g: H_2, Pd/C, AcOH, MeOH, 38%

Scheme 2



a: Boc_2O, NaOH, H_2O, THF, 80%
b: CH_2N_2, ether, 90%
c: *p*-nitrobenzoic acid, PPh_3, DIAD
d: NaOH, H_2O
e: 1) Cs_2CO_3, H_2O, MeOH; 2) BnBr, DMF, 53% (3 steps)
f: morph-DAST, THF, 24%
g: H_2, Pd/C, MeOH
h: HCl, dioxane
i: FmocOSu, $NaHCO_3$, H_2O, dioxane, 59% (3 steps)
j: **7**, PyBroP, DIEA, CH_2Cl_2, 77%
k: H_2, Pd/C, MeOH, 63%

istry.) Hydrolysis of the newly formed *p*-nitrobenzoate as well as the methyl ester, followed by benzylation of the carboxylic acid, yielded benzyl ester **14**. Fluorination with morph-DAST provided benzyl ester **15** with complete inversion of stereochemistry. 1,2-Elimination of the aminofluorosulfoxyl intermediate to yield an alkene byproduct is a common side reaction during fluorination with DAST and morph-DAST. Although its removal by flash chromatography is often facile, the alkene byproduct here proved to be quite persistent, even after repeated chromatography. Complete purification was performed after shuffling protecting groups to provide free acid **16**. Coupling of free acid **16** to dipeptide **7** with PyBroP afforded tripeptide **17**, which was hydrogenated to provide tripeptide **10**. Tripeptide **10** was used to produce (3*S*-Flp−4*R*-Flp−Gly)$_7$ by solid-phase peptide synthesis.

Conformational Stability of a (4*S*-Flp−4*R*-Flp−Gly)$_7$ Triple Helix. Previous work in our laboratory had shown that either 4*S*-Flp in the X-position or 4*R*-Flp in the Y-position increases the stability of the collagen triple helix (Table 1).[11,27,33] Accordingly, a peptide containing *both* 4*S*-Flp in the X-position *and* 4*R*-Flp in the Y-position could form a triple helix of unprecedented stability. This reasoning is based on the principle of preorganization,[32] as inclusion of both residues in the same peptide would properly preorganize the main chain at the X- and Y-positions.[31]

An earlier experiment invoking a related strategy failed to produce a stable triple helix. Working with templated strands, Moroder and co-workers observed that replacement of Pro in the X-position with 4*S*-Flp prevented triple-helix formation if 4*R*-Hyp was in the Y-position.[43] This result appears to be contrary to the benefits of preorganizing the φ and ψ dihedral angles. Molecular modeling shows, however, the possibility of unfavorable interstrand steric interactions between the pendant hydroxyl and fluoro groups,[44] which could overwhelm the benefit derived from the presence of 4*S*-Flp and 4*R*-Hyp. Similarly, (2*S*,4*R*)-4-acetoxyproline in the Y-position of a collagen triple helix appears to generate a steric clash with a proline residue in the X-position of another strand.[45]

These examples[43,45] were not the first of steric interactions preventing preorganization from increasing triple-helix stability. The gauche effect should endow a 4*S*-Hyp residue with the C$^\gamma$-endo conformation preferred in the X-position. Yet, 4*S*-Hyp in the X-position obviates triple-helix formation (Table 1).[20] A steric clash between the 4*S*-Hyp hydroxyl groups and proline residues on adjacent strands is apparently responsible for the instability.[31] A fluoro group is smaller than a hydroxyl group, and often causes no greater steric perturbation than does a proton.[46−51] Accordingly, 4*S*-Flp in the X-position increases

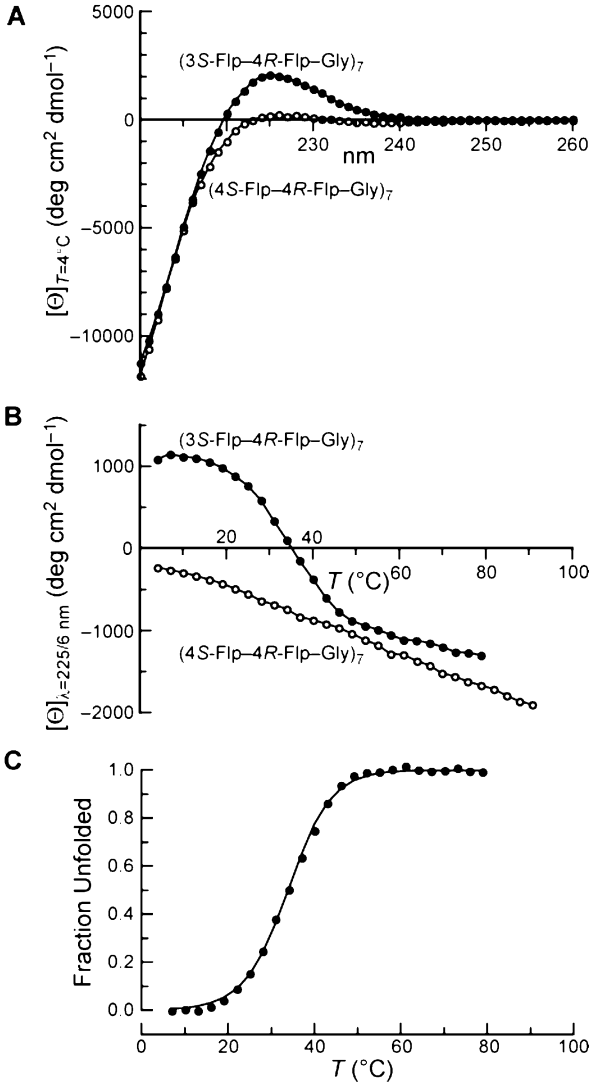


Figure 2. Circular dichroism spectroscopy of (4*S*-Flp−4*R*-Flp−Gly)$_7$ and (3*S*-Flp−4*R*-Flp−Gly)$_7$. All peptides were at 0.2 mM in 50 mM acetic acid. (A) Spectra at 4 °C. (B) Effect of temperature on spectra of (4*S*-Flp−4*R*-Flp−Gly)$_7$ (λ = 226 nm) or (3*S*-Flp−4*R*-Flp−Gly)$_7$ (λ = 225 nm). (C) Transformed data from panel B for (3*S*-Flp−4*R*-Flp−Gly)$_7$.

triple-helix stability, even though 4*S*-Hyp fails to do so (Table 1).[33,34] Hence, we thought that stabilization of the triple helix through preorganization of *both* the X-position and the Y-position could be attained by using only 4-fluoroprolines. If the interstrand steric interactions between fluoro groups is small enough, then the stability of a triple helix containing 4*S*-Flp−4*R*-Flp−Gly repeats should be extraordinary.

We synthesized (4*S*-Flp−4*R*-Flp−Gly)$_7$ and examined its properties. Several pieces of evidence indicated that this peptide does not fold into a triple helix. The CD spectrum at 4 °C has a weak maximum ellipticity at 226 nm and a shallow minimum ellipticity at a slightly longer wavelength (Figure 2A). Such a spectrum is typical of an uncomplexed polyproline-II helix. Triple-helical peptides typically exhibit a stronger maximum near 225 nm and lack the shallow minimum. More diagnostically, heating a solution of the peptide resulted in a linear decrease in ellipticity at 226 nm (Figure 2B), rather than the cooperative transition characteristic of triple-helix denaturation.

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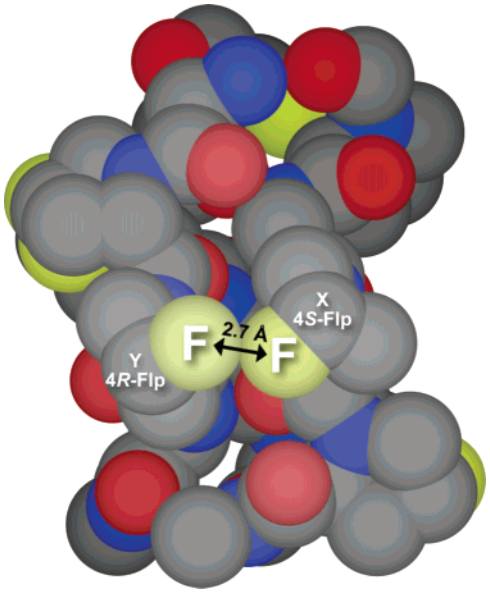

Figure 3. Model of a segment of a (4*S*-Flp−4*R*-Flp−Gly)$_n$ triple helix. The model was constructed from the three-dimensional structure of a (Pro−4*R*-Hyp−Gly)$_n$ triple helix (PDB entry 1CAG[24]) by replacing the appropriate H and OH on Pro and 4*R*-Hyp, respectively, with F. The interstrand F···F distance of 2.7 Å in one cross section is indicated explicitly.

Table 2. Relative Energy (kcal/mol) and Main-Chain Dihedral Angles of Ac-3-Fluoroproline-OMe Diastereomers at the B3LYP/6-311+G(2d,p)//B3LYP/6-31+G(d) Level of Theory

3-Flp diastereomer	*E* (rel)[a]	φ (°)	ψ (°)
3*R* (endo)	0.48	−71.7	147.2
3*R* (exo)	0	−67.9	161.1
3*S* (endo)	0	−68.6	148.3
3*S* (exo)	2.13	−57.3	140.4

[a] Values were calculated as described previously[7] and are corrected for zero-point vibrational energy.

Equilibrium sedimentation experiments were also more consistent with a monomeric species than a trimeric species (data not shown). These results suggest that interstrand fluoro−fluoro interactions are averse to triple-helix stability (Figure 3).

Conformational Stability of a (3*S*-Flp−4*R*-Flp−Gly)$_7$ Triple Helix. We sought alternative residues to preorganize the main chain for triple-helix formation. Because fluoro groups at the 3-position of proline should not suffer from steric conflicts with adjacent strands, we decided to examine the conformational preferences of 3-fluoroprolines with density functional theory calculations. Earlier studies on 4-fluoroprolines at the same level of theory were in gratifying agreement with experiment.[7] As expected, the conformation adopted by the ring is dependent on the stereochemistry of the fluoro group (Table 2). (2*S*,3*R*)-3-Fluoroproline (3*R*-Flp) prefers a C$^\gamma$-exo conformation (strictly, $_{C_i}{}^{\gamma}T^{C_i{}^{\delta}}$), and (2*S*,3*S*)-3-fluoroproline (3*S*-Flp) prefers a C$^\gamma$-endo conformation (strictly, $^{C_i{}^{\gamma}}T_{C_i{}^{\beta}}$).[6] Unlike with 4-fluoroproline, φ is nearly identical for the two diastereomers of 3-fluoroproline. We chose to install 3*S*-Flp in the X-position because it has a strong preference for a C$^\gamma$-endo conformation, with φ and ψ dihedral angles close to those of Pro in the X-position of a triple helix (where φ and ψ are $-72.6 \pm 7.6°$ and $163.8 \pm 8.8°$, respectively[24]), and because 3*S*-Flp has the same heteroatom configuration as does (2*S*,3*S*)-3-hydroxyproline (3*S*-Hyp), which is found at low abundance in the X-position of natural collagen.[52]

To test this strategy, we prepared (3*S*-Flp−4*R*-Flp−Gly)$_7$. This peptide forms a triple helix. At 4 °C, its CD spectrum has a large maximum at 225 nm (Figure 2A). Upon heating, a cooperative decrease in ellipticity was observed, indicative of triple-helix denaturation (Figure 2B). Apparently, moving the fluoro group from the 4-position to the 3-position of a proline residue in the X-position eliminates the interstrand fluoro−fluoro steric interactions that prevented (4*S*-Flp−4*R*-Flp−Gly)$_7$ from folding (Figure 3). Nonetheless, the T_m value of a (3*S*-Flp−4*R*-Flp−Gly)$_7$ triple helix is only 34 °C (Figure 2C), which is substantially lower than that of (Pro−4*R*-Flp−Gly)$_7$ (45 °C).[27] With 4*R*-Flp in the Y-position, replacement of Pro in the X-position with 3*S*-Flp is destabilizing, even though 3*S*-Flp should preorganize the φ and ψ dihedral angles properly.

Why does 3*S*-Flp destabilize a collagen triple helix relative to proline? In a triple helix, the main-chain O of each residue in the X-position forms an interstrand hydrogen bond to the main-chain N−H of a glycine residue. These interstrand C═O···H−N hydrogen bonds are known to be critical to triple-helix stability.[53] An inductive effect from the proximal fluoro group in 3*S*-Flp would weaken these hydrogen bonds, as has been noted in triple helices containing 3*S*-Hyp in the X-position.[52] This effect was less problematic with 4*S*-Flp in the X-position[33,34] because the fluoro group is separated from the main-chain O by an extra bond in 4*S*-Flp. Apparently, the weakening of this hydrogen bond outweighs the benefit of preorganization conferred by 3*S*-Flp. This problem would also plague 3*R*-Flp. Although this consideration is irrelevant for the Y-position, as residues there do not participate in interstrand hydrogen bonds, our calculations indicate that neither 3*R*-Flp nor 3*S*-Flp has proper φ and ψ dihedral angles[31] for the Y-position (where φ and ψ are $-59.6 \pm 7.3°$ and $149.8 \pm 8.8°$, respectively[24]).

Formation of a Heterotrimeric Helix. The peptide (4*S*-Flp−4*R*-Flp−Gly)$_7$ does not fold into a triple helix because unfavorable interstrand steric interactions overwhelm the benefits of preorganization provided by the 4-fluoroproline residues. We reasoned that minimizing these steric interactions could enable the realization of these benefits. Replacing one or two of the (4*S*-Flp−4*R*-Flp−Gly)$_7$ strands in a triple helix with (Pro−Pro−Gly)$_7$ would test this hypothesis. We prepared solutions of (Pro−Pro−Gly)$_7$ and (4*S*-Flp−4*R*-Flp−Gly)$_7$ in varying ratios and examined them by CD spectroscopy. Although a homogeneous solution of either peptide did not produce a triple helix, mixtures resulted in triple-helix formation.[54] The ellipticity maxima at 225 nm were much larger for each mixture than for solutions containing only one peptide (Figure 4A). A larger ratio of positive ellipticity near 225 nm to negative ellipticity near 200 nm (Rpn value) is a reliable indicator of a higher concentration of triple helix.[55] The Rpn values of the solutions can be

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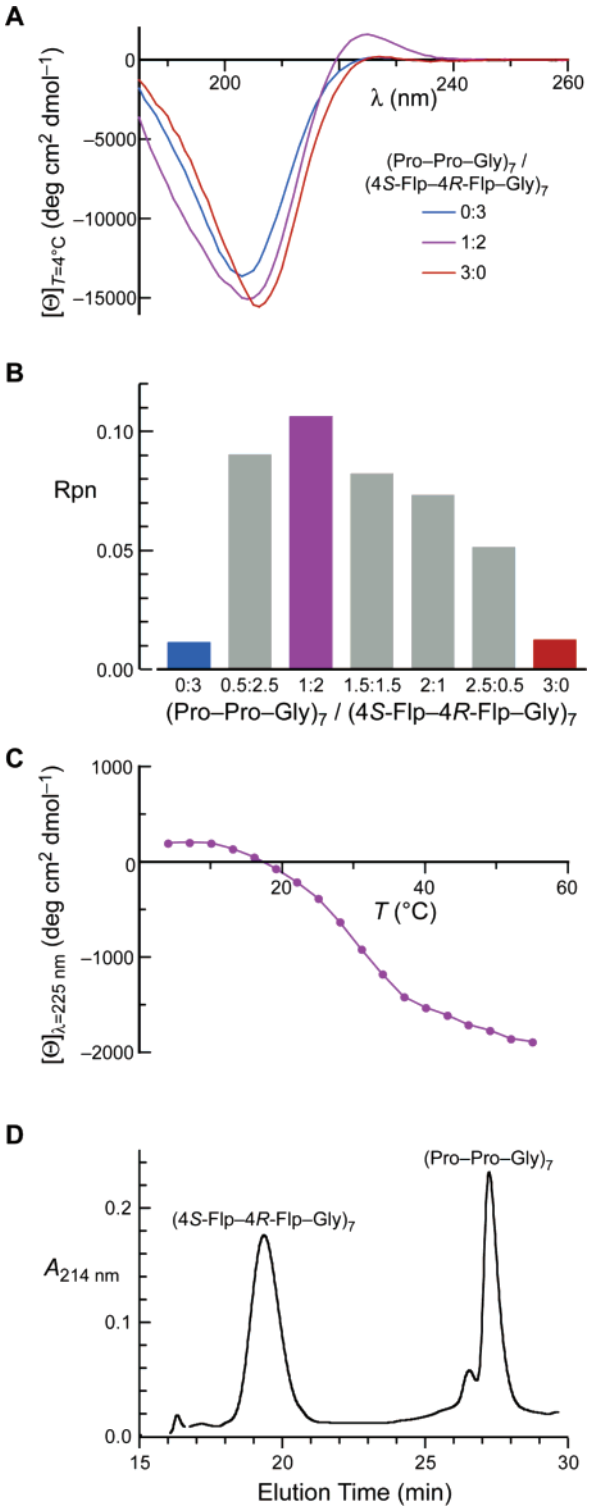

Figure 4. Circular dichroism spectroscopy and HPLC analysis of (Pro−Pro−Gly)$_7$, (4*S*-Flp−4*R*-Flp−Gly)$_7$, and mixtures. All peptides were at a total concentration of 0.2 mM in 50 mM potassium phosphate buffer at pH 2.9. (A) Spectra at 4 °C. (B) Ratio of positive maximum to negative minimum (Rpn) in the spectra of mixtures. (C). Effect of temperature on the spectrum of the 1:2 mixture at 225 nm (T_m = 28 °C). (D) HPLC trace of the heterotrimeric helix that was purified from a 2:1 (Pro−Pro−Gly)$_7$/(4*S*-Flp−4*R*-Flp−Gly)$_7$ mixture at 4 °C and then subjected to HPLC at room temperature. Peak integration yielded 14:21 (Pro−Pro−Gly)$_7$/(4*S*-Flp−4*R*-Flp−Gly)$_7$.

distributed into two groups, the lower values (0.011 and 0.012) of the homogeneous solutions and the higher values (0.090, 0.106, 0.082, 0.073, and 0.051) of the mixtures (Figure 4B).

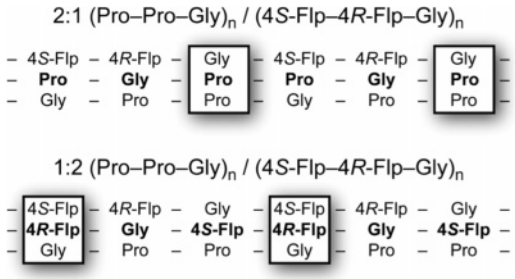

Figure 5. Likely alignment of strands in triple helices consisting of two (Pro−Pro−Gly)$_n$ and one (4*S*-Flp−4*R*-Flp−Gly)$_n$ strand (top), and one (Pro−Pro−Gly)$_n$ and two (4*S*-Flp−4*R*-Flp−Gly)$_n$ strands (bottom). Cross sections that differ in the two triple helices are boxed.

Finally, the mixtures exhibit a cooperative transition characteristic of triple-helix denaturations. The T_m value of the heterotrimer in the 1:2 mixture is 28 °C (Figure 4C), which is greater than that of a (Pro−Pro−Gly)$_7$ homotrimer but less than that of a (4*S*-Flp−Pro−Gly)$_7$ or (Pro−4*R*-Flp−Gly)$_7$ homotrimer (Table 1).

A 1:2 mixture of (Pro−Pro−Gly)$_7$ and (4*S*-Flp−4*R*-Flp−Gly)$_7$ produces a solution with the largest concentration of triple helix (Figure 4B). We interpret this ratio as the stoichiometry of the triple helix that forms in a mixture of these two peptides. This interpretation is supported not only by the 1:2 mixture having the highest Rpn value, but also by a distribution of Rpn values in the various mixtures that is close to that expected for a 1:2 stoichiometry. Purification of the triple helix from a 2:1 mixture and its analysis revealed that the helix was indeed a heterotrimer containing (Pro−Pro−Gly)$_7$ and (4*S*-Flp−4*R*-Flp−Gly)$_7$ in a ratio that is closer to 1:2 than 2:1, despite the 2:1 ratio of the initial mixture (Figure 4D).

At first glance, the 1:2 stoichiometry is surprising. Like a homotrimeric helix of (4*S*-Flp−4*R*-Flp−Gly)$_7$ strands, a heterotrimeric helix formed from one (Pro−Pro−Gly)$_7$ strand and two (4*S*-Flp−4*R*-Flp−Gly)$_7$ strands should still experience interstrand fluoro−fluoro interactions that would not be present if two strands of (Pro−Pro−Gly)$_7$ folded with one strand of (4*S*-Flp−4*R*-Flp−Gly)$_7$. Why then is the stoichiometry 1:2 rather than 2:1?

To explain the stoichiometry of the heterotrimeric helix formed in mixtures of (Pro−Pro−Gly)$_7$ and (4*S*-Flp−4*R*-Flp−Gly)$_7$, we considered cross sections of the relevant triple helices. Each cross section of a collagen triple helix, like each triplet, contains an X, Y, and glycine residue. In the 2:1 and 1:2 stoichiometries, there are 4*R*-Flp:Gly:Pro and 4*S*-Flp:Pro:Gly cross sections (Figure 5). The remaining cross section differs. The triple helix with two strands of (4*S*-Flp−4*R*-Flp−Gly)$_7$ has a 4*S*-Flp:4*R*-Flp:Gly cross section, whereas the other triple helix has a Pro:Pro:Gly cross section. Hence, the observed stoichiometry and the analysis of cross sections suggests that a 4*S*-Flp:4*R*-Flp:Gly cross section confers more stability than does a Pro:Pro:Gly cross section.

The stability conferred by a cross section can be inferred from the stability of its analogous homotrimeric triple helix. As (Pro−Pro−Gly)$_7$ and (4*S*-Flp−4*R*-Flp−Gly)$_7$ did not form stable homotrimeric helices in normal solution conditions (Figures 2 and 4), we attempted to form triple helices of these peptides in the presence of trimethylamine oxide (TMAO), which is a natural osmylate that enhances the conformational stability of collagen[56,53] and other proteins.[57] At higher concentrations of

Table 3. Values of T_m (°C) for Triple Helices of (Pro−Pro−Gly)$_7$ and (4*S*-Flp−4*R*-Flp−Gly)$_7$ (0.2 mM) in 50 mM Acetic Acid Containing Trimethylamine Oxide

[TMAO] (M)	(Pro–Pro–Gly)$_7$	(4*S*-Flp–4*R*-Flp–Gly)$_7$
3.0	18	32
2.5	14	28
2.0		24
0[a]	−6	8

[a] Values at 0 M TMAO were estimated by linear regression analysis.[53]

TMAO, both (Pro−Pro−Gly)$_7$ and (4*S*-Flp−4*R*-Flp−Gly)$_7$ folded into triple helices (Table 3). At all concentrations of TMAO, the T_m value of (4*S*-Flp−4*R*-Flp−Gly)$_7$ was greater than that of (Pro−Pro−Gly)$_7$. Extrapolation indicates that (4*S*-Flp−4*R*-Flp−Gly)$_7$ (T_m = 8 °C) forms a more stable triple helix in the absence of TMAO than does (Pro−Pro−Gly)$_7$ (T_m = −6 °C). Because a 4*S*-Flp−4*R*-Flp−Gly triplet contributes more to triple-helix stability than does a Pro−Pro−Gly triplet, (Pro−Pro−Gly)$_7$[(4*S*-Flp−4*R*-Flp−Gly)$_7$]$_2$ should form a more stable triple helix than does [(Pro−Pro−Gly)$_7$]$_2$(4*S*-Flp−4*R*-Flp−Gly)$_7$, as was observed (Figure 4).

The analysis of cross sections explains the preference of two (4*S*-Flp−4*R*-Flp−Gly)$_7$ strands to fold with one (Pro−Pro−Gly)$_7$ strand rather than a third (4*S*-Flp−4*R*-Flp−Gly)$_7$ strand, even though a 4*S*-Flp−4*R*-Flp−Gly triplet contributes more to stability than does a Pro−Pro−Gly triplet. The presence of one (Pro−Pro−Gly)$_7$ strand generates the three cross sections shown in Figure 5, whereas a (4*S*-Flp−4*R*-Flp−Gly)$_7$ homotrimer has only 4*S*-Flp:4*R*-Flp:Gly cross sections. Because both Pro:4*R*-Flp:Gly and 4*S*-Flp:Pro:Gly cross sections provide much more stability than does a 4*S*-Flp:4*R*-Flp:Gly cross section, the presence of a single strand of (Pro−Pro−Gly)$_7$ greatly increases triple-helix stability.

A Code for Strand Association. The self-assembly of heterotrimeric helices has an additional implication—the possibility of developing a "code" for collagen strand association. This code would not be based on hydrogen-bonding patterns, as in the Watson−Crick paradigm.[58] Rather, the code would rely on stereoelectronic and steric effects like those encountered herein. In analogy to the A:T and G:C base pairs of the DNA double helix, collagen strand association would be inferable a priori by the analysis of cross sections (Figure 5). We believe that the judicious imposition of stereoelectronic and steric effects on proline derivatives could lead to the rational self-assembly of triple helices with defined 1:2 and even 1:1:1 strand stoichiometries.

Conclusions

The conformational stability of the collagen triple helix is enhanced by proper preorganization of its individual polypeptide strands. We find that modifications that use stereoelectronic effects to preorganize proline residues can also introduce adverse steric and inductive effects, thus complicating the design of more stable triple helices. A balance can be attained between the benefits of preorganization and the detriments of these adverse effects. The spontaneous formation of a heterotrimeric helix from (4*S*-Flp−4*R*-Flp−Gly)$_7$ and (Pro−Pro−Gly)$_7$ strands is the manifestation of such a balance, and represents a first step in the development of a code for the self-assembly of complex triple-helical structures.

Experimental Procedures

General Experimental. Commercial chemicals were of reagent grade or better, and were used without further purification. Anhydrous THF, DMF, and CH_2Cl_2 were obtained from a CYCLE-TAINER solvent delivery system (Baker). Other anhydrous solvents were obtained in septum-sealed bottles. In all reactions involving anhydrous solvents, glassware was either oven- or flame-dried. Flash chromatography was performed with columns of silica gel 60, 230−400 mesh (Silicycle), or with a Flashmaster Solo instrument (Argonaut).

NMR spectra were acquired with a Bruker AC+ 300 spectrometer (^{1}H, 300 MHz; ^{13}C, 75.4 MHz) at the Magnetic Resonance Facility in the Department of Chemistry or (as indicated) a Bruker DMX-400 Avance spectrometer (^{1}H, 400 MHz; ^{13}C, 100.6 MHz) at the NMR Facility at Madison (NMRFAM). NMR spectra were obtained on samples dissolved in $CDCl_3$ unless indicated otherwise. Compounds with a carbamate protecting group (e.g., Boc or Fmoc) exist as mixtures of *Z* and *E* isomers that do not interconvert on the NMR time scale. Accordingly, these compounds exhibit two sets of NMR signals. In ^{13}C data, signals that clearly arise from a minor *Z* or *E* isomer are listed within parentheses.

Mass spectrometry was performed with either a Micromass LCT (electrospray ionization, ESI) or a Micromass AutoSpec (electron impact ionization, EI) mass spectrometer in the Mass Spectrometry Facility in the Department of Chemistry or with a Perkin-Elmer Voyager (matrix-assisted laser desorption/ionization, MALDI) mass spectrometer in the Biophysics Instrumentation Facility.

N-9-Fluorenylmethoxycarbonyl-(2*S*,4*S*)-4-fluoroproline (**8**) was synthesized according to the procedure of Kobayashi and co-workers.[42]

***N-tert*-Butoxycarbonyl-(2*S*,4*S*)-4-hydroxyproline Benzyl Ester (3).** A solution of Cs_2CO_3 (3.6 g, 11 mmol) in water (60 mL) at 0 °C was added to a solution of *N-tert*-butoxycarbonyl-(2*S*,4*S*)-4-hydroxyproline (**2**) (5.2 g, 22 mmol) in methanol (90 mL) at 0 °C. The mixture was concentrated by rotary evaporation to a mixture of colorless oil and solid, and DMF (110 mL) was added to the residue. The suspension was cooled in a 0 °C bath before the addition of benzyl bromide (2.6 mL, 22 mmol). The resulting suspension was stirred overnight, and then vacuum-filtered to remove the white precipitate. The filtrate was concentrated by rotary evaporation to a white solid, which was dissolved in ethyl acetate (150 mL). The organic layer was washed with water (3 × 50 mL), dried over $MgSO_4$(s), and concentrated by rotary evaporation to a colorless solid (**3**; 6.7 g, 21 mmol, 95%). ^{1}H NMR δ 1.34 and 1.41 (s, 9H), 2.07−2.15 (m, 1H), 2.34 (dddd, J = 4.7, 9.9, 14.3, 14.3 Hz, 1H), 3.17−3.40 (m, 1H), 3.49−3.73 (m, 2H), 4.29−4.46 (m, 2H), 5.20 (s, 1.1H), 5.14 and 5.32 (d, J = 12.3 Hz, 0.9H), 7.29−7.42 (m, 5H); ^{13}C NMR δ 28.1 (28.3), 38.6 (37.7), 55.3 (55.8), 57.9 (57.8), 67.3, 70.1 (71.1), 80.4 (80.3), 128.1, 128.2, 128.5, 128.6, 135.0 (135.3), 153.6 (154.3), 174.5 (174.6); HRMS-ESI (*m*/*z*) [M + Na]$^+$ calcd for $C_{17}H_{23}NO_5Na$ 344.1474, found 344.1458.

***N-tert*-Butoxycarbonyl-(2*S*,4*R*)-4-fluoroproline Benzyl Ester (4).** A solution of **3** (6.55 g, 20.4 mmol) in anhydrous THF (100 mL) was cooled in a −78 °C bath under Ar(g). To the stirred, colorless solution was added morph-DAST (7.3 mL, 60 mmol) dropwise by syringe. The solution was allowed to warm to room temperature gradually and stirred for 2 d, during which it became yellow. After cooling of the solution in a 0 °C bath, methanol was added to quench the reaction. Saturated aqueous $NaHCO_3$ (5 mL) was added, which resulted in vigorous bubbling. The mixture was then concentrated by rotary evaporation. The residue was extracted with ethyl acetate (150 mL), and the organic layer was washed with saturated aqueous $NaHCO_3$ (50 mL) and water (50 mL). The organic layer was dried over $MgSO_4$(s) and concentrated by rotary evaporation to a yellow oil. Flash chromatography (2% $CH_3OH/CHCl_3$) did not provide sufficiently pure product as indicated by

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TLC and ^{1}H NMR, and impurities persisted after a second flash chromatographic step (3:1 hexane/EtOAc). The partially purified oil crystallized upon standing and was recrystallized from hexane to afford **4** (2.37 g, 7.33 mmol, 36%) as colorless needles. ^{1}H NMR δ 1.36 and 1.47 (s, 9H), 1.95−2.21 (m, 1H), 2.50−2.68 (m, 1H), 3.51−3.71 (m, 1H), 3.75−3.99 (m, 1H), 4.45 (dd, J = 8, 8 Hz, 0.6 H), 4.54 (dd, J = 8, 8 Hz, 0.4H), 5.08−5.31 (m, 3H), 7.31−7.40 (m, 5H); ^{13}C NMR δ 28.1 (28.3), 37.5 (J_{C-F} = 23.1 Hz) (36.5 (J_{C-F} = 23.3 Hz)), 52.9 (J_{C-F} = 23 Hz) (53.2 (J_{C-F} = 23 Hz)), 66.9, 80.6 (80.5), 91.0 (J_{C-F} = 178.6 Hz) (91.8 (J_{C-F} = 178.8 Hz)), 128.1, 128.2, 128.3, 128.5, 128.6, 135.2 (135.5), 153.5 (154.1), 172.4 (172.1); HRMS-ESI (m/z) [M + Na]$^+$ calcd for $C_{17}H_{22}FNO_4Na$ 346.1431, found 346.1443.

***N*-*tert*-Butoxycarbonyl-(2*S*,4*R*)-4-fluoroproline (5).** Methanol was added carefully to a mixture of **4** (2.29 g, 7.08 mmol) and Pd/C (10% w/w, 0.74 g, 0.70 mmol Pd) under Ar(g). The resulting black suspension was stirred for 18 h under H_2(g) delivered from a balloon. The suspension was filtered through Celite, and the filtrate was concentrated by rotary evaporation to a colorless oil that slowly solidified into a white powder. No further purification was required for **5** (1.65 g, 7.07 mmol, 100%). ^{1}H NMR δ 1.44 and 1.49 (s, 9H), 2.06−2.74 (m, 2H), 3.44−3.77 (m, 1H), 3.83−4.00 (m, 1H), 4.43 (dd, J = 8.5, 8.5 Hz, 0.5H), 4.54 (dd, J = 8.5, 8.5 Hz, 0.5H), 5.10−5.34 (m, 1H), 10.0 (br s, 1H); ^{13}C NMR δ 28.1 (28.3), 37.4 (J_{C-F} = 22.1 Hz) (36.0 J_{C-F} = 22.1 Hz), 52.9 (J_{C-F} = 22.6 Hz) (53.4 (J_{C-F} = 22.8 Hz)), 57.5, 81.2 (81.8), 90.9 (J_{C-F} = 179.6 Hz) (91.4 (J_{C-F} = 179.3 Hz)), 153.6 (155.6), 178.1 (175.5); HRMS-ESI (m/z) [M − H]$^-$ calcd for $C_{10}H_{15}FNO_4$ 232.0985, found 232.0995.

***N*-*tert*-Butoxycarbonyl-(2*S*,4*R*)-4-fluoroprolyl-glycine Benzyl Ester (6).** To a solution of **5** (1.49 g, 6.39 mmol) in anhydrous CH_2Cl_2 (75 mL) at 0 °C under Ar(g) was added the tosyl salt of glycine benzyl ester (2.15 g, 6.38 mmol), *N*,*N*′-diisopropylethylamine (DIEA; 2.3 mL, 13.2 mmol), and PyBOP (3.34 g, 6.42 mmol). The solution was stirred overnight, and then washed with 10% w/v aqueous citric acid (3 × 25 mL), saturated aqueous $NaHCO_3$ (3 × 25 mL), brine (25 mL), and water (25 mL). The organic layer was dried over $MgSO_4$(s) and concentrated by rotary evaporation to a colorless oil. Flash chromatography (1:1 hexane/EtOAc) afforded 1.92 g (5.05 mmol, 79%) of **6** as an oily solid. ^{1}H NMR δ 1.46 (s, 9H), 2.06−2.70 (m, 2H), 3.31−3.63 (m, 1H), 3.81−4.18 (m, 3H), 4.31−4.59 (m, 1H), 5.06−5.30 (m, 3H), 7.25−7.45 (m, 5H); ^{13}C NMR δ 20.2, 34.9 (J_{C-F} = 20 Hz), 41.4, 53.4 (J_{C-F} = 22.7 Hz), 58.1, 67.1, 81.1, 91.8 (J_{C-F} = 175 Hz), 128.3, 128.6, 135.1, 155.7, 169.4, 171.5; HRMS-ESI (m/z) [M + Na]$^+$ calcd for $C_{19}H_{25}FN_2O_5Na$ 403.1645, found 403.1633.

(2*S*,4*R*)-4-Fluoroprolyl-glycine Benzyl Ester-HCl (7). Dipeptide **6** (1.9 g, 5.0 mmol) was dissolved in 38 mL of 4 M HCl in dioxane. After 1 h, the solution was concentrated by rotary evaporation to a white solid. Ether was added to the solid, forming a suspension that was allowed to stand overnight. The suspension was filtered under vacuum, affording **7** (1.6 g, 5.0 mmol, 100%) as a white solid. ^{1}H NMR (CD_3OD) δ 2.24 (dddd, J = 3.3, 10.4, 14.2, 38.8 Hz, 1H), 2.72−2.88 (m, 1H), 3.51−3.76 (m, 2H), 4.05 and 4.13 (d, J = 17.7 Hz, 2H), 4.59 (dd, J = 7.5, 10.5 Hz, 1H), 5.17 and 5.19 (d, J = 12.3 Hz, 2H), 5.35−5.57 (m, 1H), 7.29−7.40 (m, 5H); ^{13}C NMR (CD_3OD) δ 37.9 (J_{C-F} = 21.8 Hz), 42.3, 53.4 (J_{C-F} = 24.4 Hz), 59.7, 68.1, 93.7 (J_{C-F} = 177.3 Hz), 129.4, 129.6, 137.0, 169.4, 170.6; ESI-MS (m/z) [M − Cl]$^+$ 280.1.

***N*-9-Fluorenylmethoxycarbonyl-(2*S*,4*S*)-4-fluoroprolyl-(2*S*,4*R*)-4-fluoroprolyl−glycine Benzyl Ester (9).** To a solution of Fmoc-4*S*-FlpOH[42] (**8**, 0.22 g, 0.62 mmol) in anhydrous CH_2Cl_2 (1 mL) was added 0.20 g (0.63 mmol) of **7**, which was insoluble. After cooling of the suspension in a 0 °C bath, PyBroP (0.29 g, 0.62 mmol) and DIEA (0.32 mL, 1.8 mmol) were added. The DIEA addition caused the suspension to dissolve. After 5 min, the reaction was removed from the cold bath and stirred for 90 min, at which point TLC (1:1:0.04 $CHCl_3$/EtOAc/AcOH) showed no remaining **8**. The reaction mixture was diluted with CH_2Cl_2 (15 mL) and washed with 10% w/v aqueous citric acid, saturated aqueous $NaHCO_3$, brine, and water (15 mL of each). The organic layer was dried over $MgSO_4$(s) and concentrated by rotary evaporation to a light yellow oil. Flash chromatography (EtOAc) afforded **9** (0.30 g, 0.48 mmol, 79%) as a colorless oil. ^{13}C NMR δ 33.7 (J_{C-F} = 20.8 Hz), 33.9 (J_{C-F} = 21.3 Hz), 35.5 (J_{C-F} = 21.7 Hz), 36.5 (J_{C-F} = 21.2 Hz), 41.3, 47.1, 47.4, 52.8 (J_{C-F} = 23.7 Hz), 53.0 (J_{C-F} = 23.1 Hz), 58.0, 58.4, 60.3, 66.9, 67.1. 67.6, 91.5 (J_{C-F} = 181 Hz), 91.7 (J_{C-F} = 181 Hz), 119.8, 120.0, 124.8, 125.1, 127.1, 127.7, 128.2, 128.3, 128.4, 128.5, 135.3, 141.3, 143.6, 144.0, 144.4, 154.7, 162.5, 169.2, 170.5; HRMS-ESI (m/z) [M + Na]$^+$ calcd for $C_{34}H_{33}F_2N_3O_6Na$ 640.2235, found 640.2247.

***N*-9-Fluorenylmethoxycarbonyl-(2*S*,4*S*)-4-fluoroprolyl-(2*S*,4*R*)-4-fluoroprolyl−glycine (1).** Under an Ar(g) atmosphere, **9** (0.30 g, 0.49 mmol) was dissolved in a mixture of acetic acid (7 mL) and methanol (7 mL). Palladium on carbon (10% w/w, 0.035 g, 0.033 mmol Pd) was added to the solution, and the resulting black suspension was stirred for 2 h under H_2(g) delivered from a balloon. The mixture was then filtered through Celite, and the filtrate was concentrated by rotary evaporation. The oily residue was triturated with methanol, and the resulting solid was recrystallized from methanol, providing **1** (0.10 g, 0.19 mmol, 38%) as colorless needles. ^{13}C NMR (DMSO-d_6) δ 34.9 (J_{C-F} = 20.9 Hz), 35.9 (J_{C-F} = 20.2 Hz), 36.0 (J_{C-F} = 21.8 Hz), 40.8, 46.8, 47.0, 53.3 (J_{C-F} = 23 Hz), 53.8 (J_{C-F} = 25 Hz), 57.2, 57.4, 58.2, 58.3, 67.0, 67.4, 90.9 (J_{C-F} = 176.9 Hz), 91.9 (J_{C-F} = 178.3 Hz), 92.9 (J_{C-F} = 175.9 Hz), 92.9 (J_{C-F} = 175.1 Hz), 120.3, 120.4, 125.4, 125.5, 127.4, 127.9, 140.8, 141.0, 143.8, 143.9, 144.0, 153.9, 154.0, 169.1, 169.4, 171.3, 171.4; HRMS-ESI (m/z) [M + Na]$^+$ calcd for $C_{27}H_{27}F_2N_3O_6Na$ 550.1766, found 550.1763.

***N*-*tert*-Butoxycarbonyl-(2*S*,3*S*)-3-hydroxyproline (12).** To a suspension of **11** (4.92 g, 37.5 mmol) in a mixture of THF (40 mL) and H_2O (20 mL) was added 10% w/v aqueous NaOH (20 mL) and a solution of di-*tert*-butyl dicarbonate (12.3 g, 56.3 mmol) in THF (40 mL) and H_2O (20 mL). The resulting biphasic mixture was stirred overnight. Most of the THF was removed by rotary evaporation, and the concentrate was washed with EtOAc (6 × 75 mL). The aqueous layer was then acidified to pH 2−3 with 10% w/v aqueous $KHSO_4$, saturated with NaCl, and extracted with 2:1 $CHCl_3$/EtOH (3 × 100 mL). The combined organic extracts were dried over $MgSO_4$(s), filtered, and concentrated by rotary evaporation to provide **12** (6.9 g, 30 mmol, 80%) as an oily solid. ^{1}H NMR δ 1.43 and 1.47 (s, 9H), 1.82−1.93 (m, 1H), 1.97−2.11 (m, 1H), 3.46−3.62 (m, 2H), 4.10 and 4.17 (s, 1H), 4.36−4.41 (m, 1H), 4.95 (s, 2H); ^{13}C NMR δ 28.6 (28.7), 32.8 (33.4), 45.3 (45.8), 69.4 (69.1), 75.8 (75.0), 81.5 (81.3), 156.1 (156.4), 174.3 (174.0); HRMS-ESI (m/z) [M − H]$^-$ calcd for $C_{10}H_{16}NO_5$ 230.1028, found 230.1019.

***N*-*tert*-Butoxycarbonyl-(2*S*,3*S*)-3-hydroxyproline Methyl Ester (13).** A solution of diazomethane etherate was prepared in a Mini-Diazald reactor (Aldrich) from 6.1 g (29 mmol) of Diazald. The solution was added carefully behind a blast shield to a suspension of **12** (3.72 g, 16.1 mmol) in ether. Vigorous bubbling occurred. After the solution was left to stand overnight, the solid had dissolved, and a yellow color persisted. A few drops of acetic acid were added to quench and the mixture was concentrated by rotary evaporation to a colorless viscous oil. Residual acetic acid, indicated by ^{1}H NMR spectroscopy, was removed as an azeotrope with both benzene and hexanes. Further concentration of the residue under reduced pressure did not remove all traces of solvent, as benzene and hexanes were still present according to ^{1}H NMR spectroscopy. The mass of the remaining residue was 4.06 g, of which greater than 90% appeared to be **13**. ^{1}H NMR δ 1.41 and 1.47 (s, 9H), 1.85−1.99 (m, 1H), 2.03−2.19 (m, 1H), 2.44 (br s, 1H), 3.54−3.70 (m, 2H), 3.75 (s, 3H), 4.18 (s, 0.6H), 4.30 (s, 0.4H), 4.44 (s, 1H); ^{13}C NMR δ 28.2 (28.4), 31.2 (32.5), 44.2 (44.6), 52.1 (52.3), 68.0 (67.7), 75.1 (74.1), 80.2, 154.0 (154.7); ESI-MS (m/z) [M + Na]$^+$ calcd for $C_{11}H_{19}NO_5Na$ 268.1, found 268.1.

***N*-*tert*-Butoxycarbonyl-(2*S*,3*R*)-3-hydroxyproline Benzyl Ester (14).** To a solution of **13** (7.7 g, 31 mmol) in anhydrous THF (250 mL) at 0 °C were added PPh_3 (8.85 g, 32.9 mmol) and *p*-nitrobenzoic

acid (5.49 g, 32.9 mmol), which caused the solution to develop a light yellow color. A solution of diisopropylazodicarboxylate (DIAD, 6.69 g, 33.1 mmol) in anhydrous THF (25 mL) was added dropwise to the reaction mixture. The orange color of the DIAD solution dissipated rapidly, and the color had faded to a pale yellow minutes after the addition was complete. After stirring of the reaction for 3 h, the addition of a 0 °C solution of 1 M NaOH (100 mL) caused the mixture to turn red. After 1 h, the mixture was concentrated by rotary evaporation until no THF was present. The mixture was then diluted with water (50 mL) and washed with ethyl acetate (3 × 50 mL). The aqueous layer was acidified to pH ∼2 with 10% w/v aqueous $KHSO_4$, which caused the formation of a beige precipitate, which was removed by vacuum filtration and identified as *p*-nitrobenzoic acid. The filtrate was saturated with NaCl and extracted with 2:1 $CHCl_3$/EtOH (6 × 100 mL). The combined organic extracts were dried over $MgSO_4$(s) and concentrated by rotary evaporation to 7.1 g of yellow oil. As indicated by ^{1}H NMR, the oil contained *p*-nitrobenzoic acid in addition to *N*-*tert*-butoxycarbonyl-(2*S*,3*R*)-3-hydroxyproline. The oil was dissolved in methanol (128 mL), and the resulting solution was cooled in a 0 °C bath. A 0 °C solution of Cs_2CO_3 (5.0 g, 15 mmol) in water (87 mL) was added, and the resulting mixture was then concentrated by rotary evaporation to an orange oil. DMF (250 mL) was added to the oil, which caused much solid to precipitate. The mixture was cooled in a 0 °C bath before the addition of benzyl bromide (3.6 mL, 30 mmol). The mixture was allowed to warm to room temperature and stirred overnight. A colorless precipitate had formed and was removed by vacuum filtration. The orange filtrate was concentrated by rotary evaporation, and the resulting residue was dissolved in ethyl acetate (200 mL). The solution was washed with saturated aqueous $NaHCO_3$ (100 mL) and water (2 × 100 mL). After drying over $MgSO_4$(s), the organic layer was concentrated by rotary evaporation, and the resulting oil was purified by flash chromatography (1.5:1 hexane/EtOAc), affording **14** (5.3 g, 16 mmol, 53%) as a light yellow oil. ^{1}H NMR δ 1.34 and 1.46 (s, 9H), 1.94−2.17 (m, 2H), 2.31 (d, J = 5.7 Hz, 1H), 3.40−3.70 (m, 2H), 4.34−4.66 (m, 2H), 5.09−5.35 (m, 2H), 7.28−7.41 (m, 5H); ^{13}C NMR δ 27.8 (28.0), 31.7 (32.3), 43.5 (43.9), 63.6 (63.2), 66.4, 72.0 (71.1), 79.9 (79.8), 127.7, 128.0, 128.2, 135.2 (135.4), 153.5 (154.0), 170.0 (169.8); HRMS-ESI (*m*/*z*) $[M + Na]^+$ calcd for $C_{17}H_{23}NO_5Na$ 344.1474, found 344.1416.

***N*-*tert*-Butoxycarbonyl-(2*S*,3*S*)-3-fluoroproline Benzyl Ester (15).** A solution of **14** (5.2 g, 16 mmol) in anhydrous THF (160 mL) under Ar(g) was cooled in a −78 °C bath, followed by the dropwise addition of morph-DAST (8.0 mL, 66 mmol). The solution was allowed to warm to room temperature and stirred overnight, during which it developed a light yellow color. After cooling in a 0 °C bath, the reaction was quenched with methanol (5 mL), causing vigorous bubbling. The reaction mixture was concentrated by rotary evaporation to an oily solid, which was dissolved in ethyl acetate (150 mL). The organic layer was washed with additional saturated aqueous $NaHCO_3$ (3 × 50 mL) and water (50 mL), dried over $MgSO_4$(s), and concentrated by rotary evaporation. Repeated Flashmaster purification failed to eliminate an impurity, which was an alkene produced by elimination during the fluorination reaction. According to ^{1}H NMR, the ratio of **15** to the alkene was 6.5:1, suggesting that 1.26 g (3.89 mmol, 24%) of **15** was prepared. A small amount was purified further for spectroscopic analysis. ^{1}H NMR δ 1.34 and 1.47 (s, 9H), 1.93−2.28 (m, 2H), 3.48−3.80 (m, 2H), 4.51 (d, J = 22.3 Hz, 0.6H), 4.65 (d, J = 21.9 Hz, 0.4H), 5.03−5.30 (m, 3H), 7.30−7.42 (m, 5H); ^{13}C NMR δ 27.9 (28.1), 30.1 (J_{C-F} = 20.8 Hz) (30.9 (J_{C-F} = 21.3 Hz)), 43.8 (44.1), 66.1 (J_{C-F} = 24.2 Hz) (65.8 (J_{C-F} = 23.9 Hz)), 67.0, 80.1, 94.8 (J_{C-F} = 185.7 Hz) (93.9 (J_{C-F} = 185.5 Hz)), 127.8, 128.2, 128.3, 128.4, 128.5, 134.9 (135.1), 153.3 (154.0), 169.0 (169.2); HRMS-EI (*m*/*z*) M^+ calcd for $C_{17}H_{22}FNO_4$ 323.1533, found 323.1539.

***N*-9-Fluorenylmethoxycarbonyl-(2*S*,3*S*)-3-fluoroproline (16).** To a solution of **15** (∼1.26 g, 3.89 mmol, impure) in methanol (50 mL) was added Pd/C (10% w/w, 0.52 g, 0.49 mmol) carefully. The resulting black suspension was stirred overnight under H_2(g) delivered from a balloon. The suspension was vacuum filtered through Celite, and the filtrate was concentrated to a colorless oil. The oil was dissolved in 4 M HCl in dioxane (30 mL). After 90 min, the solution was concentrated by rotary evaporation to an oily solid. The residue was dissolved in water (15 mL) and diluted with dioxane (30 mL). To this solution were added FmocOSu (1.32 g, 3.91 mmol) and $NaHCO_3$ (0.69 g, 8.2 mmol). The resulting suspension was stirred overnight, and then concentrated by rotary evaporation to a thick oil. The residue was dissolved in 5% w/v aqueous $KHCO_3$ (40 mL) and washed with ethyl acetate (3 × 20 mL). According to TLC, some product entered the organic layer during the wash, so the organic layer was extracted with 5% w/v aqueous $KHCO_3$ (3 × 14 mL). The aqueous layers were acidified to pH ∼2 by the addition of 2 N HCl, causing a milky white precipitate to form. The precipitate was extracted from each aqueous layer with ethyl acetate (3 × 20 mL). The combined organic extracts were dried over $MgSO_4$(s) and concentrated by rotary evaporation to an oily solid. Flashmaster purification (70 g silica; $CHCl_3$ containing AcOH (2% v/v)) afforded **16** (0.82 g, 2.3 mmol, 59%) as a sticky white solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 2.00−2.31 (m, 2H), 3.50−3.62 (m, 1H), 3.67−3.74 (m, 1H), 4.17−4.55 (m, 4H), 5.21−5.26 (m, 0.5H), 5.34−5.39 (m, 0.5H), 7.27−7.42 (m, 4H), 7.57−7.66 (m, 2H), 7.76−7.82 (m, 2H); ^{13}C NMR (CD_3OD, 100.6 MHz) δ 31.9 (J_{C-F} = 21.1 Hz) (31.0 (J_{C-F} = 21.7 Hz)), 45.7 (45.4), 48.3, 67.8 (J_{C-F} = 19.7 Hz) (67.5 (J_{C-F} = 19.7 Hz)), 69.2 (68.9), 96.6 (J_{C-F} = 184.3 Hz) (95.7 (J_{C-F} = 184.1 Hz)), 120.9, 126.0, 126.1, 126.2, 126.3, 128.2, 128.8, 129.2, 129.9, 142.5, 142.6, 145.0, 145.1, 145.2, 156.3 (156.7), 171.9 (171.7); HRMS-ESI (*m*/*z*) $[M - H]^-$ calcd for $C_{20}H_{17}FNO_4$ 354.1142, found 354.1128.

***N*-9-Fluorenylmethoxycarbonyl-(2*S*,3*S*)-3-fluoroprolyl-(2*S*,4*R*)-4-fluoroprolyl−glycine Benzyl Ester (17).** To a solution of **16** (0.77 g, 2.2 mmol) in anhydrous CH_2Cl_2 (30 mL) at 0 °C were added **7** (0.70 g, 2.2 mmol), PyBroP (1.03 g, 2.21 mmol), and DIEA (1.2 mL, 6.9 mmol). DIEA addition caused **7** to dissolve, resulting in a colorless solution. The reaction mixture was allowed to warm to room temperature and stirred for 21 h. The reaction mixture was then diluted with CH_2Cl_2 (70 mL) and washed with 10% w/v aqueous citric acid (50 mL), saturated aqueous $NaHCO_3$ (50 mL), brine (50 mL), and water (50 mL). The combined organic extracts were dried over $MgSO_4$(s) and concentrated by rotary evaporation to a yellow oil. Flashmaster purification (50 g silica; hexane containing EtOAc (0−60% v/v)) afforded **17** (1.05 g, 1.7 mmol, 77%) as a colorless solid. ^{1}H NMR (CD_3OD, 400 MHz) δ 1.99−2.31 (m, 3H), 2.45−2.59 (m, 1H), 3.46−3.58 (m, 1H), 3.64−4.52 (m, 8H), 4.55−4.62 (m, 1H), 4.67 (d, J = 21.4 Hz, 0.5H), 4.79 (d, J = 22.1 Hz, 0.5H), 5.05−5.41 (m, 4H), 7.24−7.42 (m, 9H), 7.51−7.57 (m, 1H), 7.59−7.66 (m, 1H), 7.75−7.82 (m, 2H); ^{13}C NMR (CD_3OD, 100.6 MHz) δ 30.8 (J_{C-F} = 23.6 Hz), 32.0 (J_{C-F} = 21.4 Hz), 36.8 (J_{C-F} = 21.0 Hz), 36.9 (J_{C-F} = 22.4 Hz), 42.1, 45.7, 46.0, 48.5, 54.6 (J_{C-F} = 22.0 Hz), 54.9 (J_{C-F} = 23.2 Hz), 60.2, 66.5 (J_{C-F} = 23.3 Hz), 66.9 (J_{C-F} = 23.6 Hz), 67.8, 68.6, 68.9, 93.2 (J_{C-F} = 179.7 Hz), 95.0 (J_{C-F} = 185.3 Hz), 95.7 (J_{C-F} = 184.1 Hz), 120.9, 125.8, 125.9, 126.0, 126.1, 128.1, 128.3, 128.7, 128.8, 129.3, 129.4, 136.9, 142.3, 142.4, 142.5, 144.7, 144.9, 145.1, 145.2, 151.5, 155.9, 168.9, 169.1, 170.6, 173.0, 173.7; HRMS-ESI (*m*/*z*) $[M + Na]^+$ calcd for $C_{34}H_{33}F_2N_3O_6Na$ 640.2235, found 640.2255.

***N*-9-Fluorenylmethoxycarbonyl-(2*S*,3*S*)-3-fluoroprolyl-(2*S*,4*R*)-4-fluoroprolyl−glycine (10).** To a mixture of **17** (1.00 g, 1.62 mmol) and Pd/C (10% w/w, 0.16 g, 0.15 mmol Pd) under Ar(g) was added methanol (40 mL) carefully. The resulting black suspension was stirred for 1 h under H_2(g) delivered from a balloon. TLC (1:1 hexane/EtOAc) then showed no starting material remaining. The suspension was vacuum-filtered through Celite, and the filtrate was concentrated by rotary evaporation to a colorless solid. Flashmaster purification (50 g silica) afforded **10** (0.54 g, 1.0 mmol, 63%) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 2.06−2.32 (m, 3H), 2.52−2.65 (m, 1H), 3.46−3.59 (m, 1H), 3.65−4.52 (m, 8H), 4.57−4.64 (m, 1H), 4.69 (d, J = 20.7 Hz, 0.5H), 4.81 (d, J = 22.4 Hz), 5.21−5.46 (m, 2H), 7.28−7.42

(m, 4H), 7.52−7.57 (m, 1H), 7.61−7.67 (m, 1H), 7.76−7.82 (m, 2H); ^{13}C NMR (CD_3OD, 100.6 MHz) δ 30.9 (J_{C-F} = 20.5 Hz), 32.0 (J_{C-F} = 21.0 Hz), 36.8 (J_{C-F} = 21.0 Hz), 37.0 (J_{C-F} = 21.6 Hz), 41.8, 45.7, 46.1, 48.2, 48.6, 54.7 (J_{C-F} = 23.9 Hz), 54.9 (J_{C-F} = 22.8 Hz), 60.4, 66.6 (J_{C-F} = 23.4 Hz), 67.0 (J_{C-F} = 23.1 Hz), 68.6, 68.9, 93.4 (J_{C-F} = 179.1 Hz), 95.9 (J_{C-F} = 186.7 Hz), 95.2 (J_{C-F} = 181.5 Hz), 120.9, 125.9, 126.1, 126.1, 126.2, 128.1, 128.3, 128.8, 129.3, 142.4, 142.5, 144.9, 145.0, 145.0, 145.2, 145.3, 155.0, 155.6, 169.0, 169.1, 169.2, 169.2, 172.4, 173.5, 173.6; HRMS-ESI (*m*/*z*) $[M - H]^-$ calcd for $C_{27}H_{26}F_2N_3O_6$ 526.1790, found 526.1775.

Attachment of Fmoc-4*S*-Flp−4*R*-Flp−Gly (1) to 2-Chlorotrityl Resin. Under Ar(g), 32 mg (0.051 mmol) of 2-chlorotrityl resin (loading: 1.6 mmol/g) was swelled in dry CH_2Cl_2 (1 mL). A solution of **1** (18 mg, 0.034 mmol) and DIEA (0.025 mL, 0.14 mmol) in dry CH_2Cl_2 (1 mL) was added by syringe. Additional dry CH_2Cl_2 (0.7 mL) was used to ensure complete transfer. After 2 h, anhydrous CH_3OH (0.2 mL) was added to the mixture to cap any remaining active sites on the resin. The resin-bound peptide was isolated by gravity filtration, washed with several portions of dry CH_2Cl_2 (∼30 mL), and dried at reduced pressure over KOH. The mass of the resin-bound peptide was 38 mg. Loading was measured by ultraviolet spectroscopy[59] to be 0.64 mmol/g.

Attachment of Fmoc-3*S*-Flp−4*R*-Flp−Gly (10) to 2-Chlorotrityl Resin. Under Ar(g), 100 mg (0.16 mmol) of 2-chlorotrityl resin (loading: 1.6 mmol/g) was swelled in dry CH_2Cl_2 (2 mL). A solution of **10** (61 mg, 0.12 mmol) and DIEA (0.070 mL, 0.40 mmol) in dry CH_2Cl_2 (1 mL) was added by syringe. Addition of dry CH_2Cl_2 (1 mL) was used to ensure complete transfer. After 90 min, anhydrous CH_3OH (1 mL) was added to the mixture to cap any remaining active sites on the resin. The resin-bound peptide was isolated by gravity filtration, washed with several portions of dry CH_2Cl_2 (∼30 mL), and dried at reduced pressure over KOH. The mass of the resin-bound peptide was 141 mg. Loading was measured by ultraviolet spectroscopy[59] to be 0.59 mmol/g.

Synthesis of (4*S*-Flp−4*R*-Flp−Gly)$_7$ and (3*S*-Flp−4*R*-Flp−Gly)$_7$. The two 21-mer peptides were synthesized by segment condensation of their corresponding Fmoc-tripeptides (**1** and **10**) on solid phase using an Applied Biosystems Synergy 432A Peptide Synthesizer at the University of Wisconsin-Madison Biotechnology Center. The first trimer was loaded onto the resin as described above to provide resin-bound (4*S*-Flp−4*R*-Flp−Gly) (29 mg resin, 19 μmol) and (3*S*-Flp−4*R*-Flp−Gly) (42 mg resin, 25 μmol trimer). Fmoc deprotection was accomplished by treatment with DMF containing piperidine (20% v/v). Trimers (75 μmol/coupling) were converted to active esters by treatment with HBTU, DIEA, and HOBt. All couplings were allowed to occur for 3 h at room temperature. Peptides were cleaved from the resin in 95:3:2 TFA/triisopropylsilane/H_2O (1 mL), precipitated in ether at 0 °C, and isolated by centrifugation. Peptides were purified by preparative HPLC using a Varian Dynamax C-18 reversed-phase column and a linear gradient (10−50% solvent B) of solvent A (H_2O containing TFA (0.1% v/v)) and solvent B (CH_3CN containing TFA (0.1% v/v)), and analyzed by analytical HPLC using a Vydac C-18 reversed-phase column and the same solvent gradient. Both peptides were ≥90% pure according to both analytical HPLC and MALDI-TOF mass spectrometry (*m*/*z*) $[M + H]^+$ calcd for $C_{84}H_{108}F_{14}N_{21}O_{22}$ 2029, found 2029 for (4*S*-Flp-4*R*-Flp-Gly)$_7$ and 2030 for (3*S*-Flp-4*R*-Flp-Gly)$_7$.

Circular Dichroism Spectroscopy of (4*S*-Flp−4*R*-Flp−Gly)$_7$ and (3*S*-Flp−4*R*-Flp−Gly)$_7$. Peptides were dried under vacuum for at least 48 h before being weighed and dissolved to 0.2 mM in either 50 mM acetic acid (pH 3.0) or 50 mM potassium phosphate buffer (pH 2.9). The solutions were allowed to incubate at ≤4 °C for ≥24 h before their CD spectra were acquired using an Aviv 202SF spectrometer at the Biophysics Instrumentation Facility. Spectra were measured with a band-pass of 1 nm. The signal was averaged for 3 s during wavelength scans and 20 s during denaturation experiments. During the denaturation experiments, CD spectra were acquired at intervals of 3 °C. At each temperature, solutions were allowed to equilibrate for a minimum of 10 min before data acquisition. Values of T_m were determined by fitting the molar ellipticity at 225 or 226 nm to a two-state model.[60]

Purification and Analysis of Heterotrimeric Helix by HPLC. Analytical HPLC was performed on the sample analyzed by CD spectroscopy that contained a 2:1 molar ratio of (Pro−Pro−Gly)$_7$/(4*S*-Flp−4*R*-Flp−Gly)$_7$. The sample, which had been cooled at ≤4 °C for >24 h, was transferred to an autosampler that had been precooled at 4 °C. The sample was analyzed by injection onto a C18 reversed-phase column (4.6 × 250 mm) and elution with a linear gradient (10−35% solvent B). Most of the sample eluted at the same time as (Pro−Pro−Gly)$_7$. A peak corresponding to a triple helix eluted at a longer time. That material was collected, concentrated, and subjected again to HPLC under the same conditions but at room temperature.

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Supporting Information Available: Additional CD spectroscopic data related to Figure 4 (Figure S1) and Table 3 (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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