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Orchestration of Structural, Stereoelectronic, and Hydrogen-Bonding Effects in Stabilizing Triplexes from Engineered Chimeric Collagen Peptides (Pro^{X}-Pro^{Y}-Gly)$_6$ Incorporating 4(*R*/*S*)-Aminoproline

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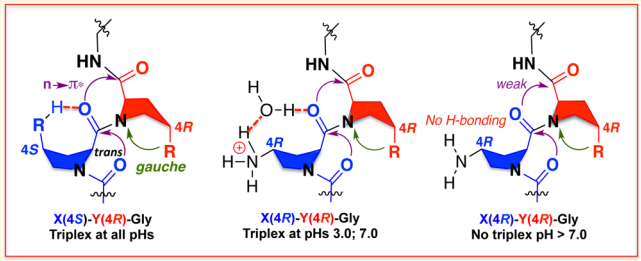
Supporting Information

ABSTRACT: Collagens are an important family of structural proteins found in the extracellular matrix with triple helix as the characteristic structural motif. The collagen triplex is made of three left-handed polyproline II (PPII) helices with each PPII strand consisting of repetitive units of the tripeptide motif X-Y-Gly, where the amino acids X and Y are most commonly proline (Pro) and 4*R*-hydroxyproline (Hyp), respectively. A C4-*endo* pucker at X-site and C4-*exo* pucker at Y-site have been proposed to be the key for formation of triplex, and the nature of pucker is dependent on both the electronegativity and stereochemistry of the substituent. The present manuscript describes a new class of collagen analogues—chimeric cationic collagens—wherein both X- and Y-sites in collagen triad are simultaneously substituted by a combination of 4(*R*/*S*)-(OH/NH_2/NH_3^+/NHCHO)-prolyl units and triplex stabilities measured at different pHs and in EG:H_2O. Based on the results a model has been proposed with the premise that any factors which specifically favor the ring puckers of C4-*endo* at X-site and C4-*exo* at Y-site stabilize the PPII conformation and hence the derived triplexes. The pH-dependent triplex stability uniquely observed with ionizable 4-amino substituent on proline enables one to define the critical combination of factors C4-(*exo*/*endo*), intraresidue H-bonding, stereoelectronic (*R*/*S*) and n → π^* interactions in dictating the triplex strength. The ionizable NH_2 substituent at C4 in *R*/*S* configuration is thus a versatile probe for delineating the triplex stabilizing factors and the results have potential for designing of collagen analogues with customized properties for material and biological applications.



INTRODUCTION

Collagens constitute an important family of structural proteins found in the extracellular matrix having triple helix as the characteristic structural motif.[1] The collagen triplex consists of three left-handed polyproline II (PPII) helices supercoiled in a right-handed manner around a common axis.[2] Each left-handed PPII strand consists of repetitive units of the tripeptide motif X-Y-Gly where the amino acids X and Y are most commonly proline (Pro) and 4*R*-hydroxyproline (Hyp), respectively. The puzzling aspect of collagen triplex structure is the obligatory requirement of Hyp in the Y-position of X-Y-Gly triad in a stereospecific 4*R*-configuration (Figure 1A).[3] Any departure from these structural requisites destabilizes the collagen triple helix.[1d] An early suggestion was the role of water-mediated interchain hydrogen bonds between hydroxyl group of 4*R*-Hyp and the carbonyl group of the peptide backbone of adjacent strand.[2] This failed to explain the greater thermal stability of 4*R*-fluoroprolyl (Flp) collagen peptide[5] since F in C−F bond does not form a H-bond like the OH group.[6] An alternative proposal was based on the β-gauche effect originating from the electronegative C4 substitution on the C4−C5−N1 fragment of the proline ring.[7] This preordinates a C4-*exo* pucker for the pyrrolidine ring in Y-site as confirmed from crystal structure[8] favoring the essential *trans* conformation of prolyl−peptide bond, important for stabilizing n → π^* interaction of C=O_i with C=O_{i-1} (Figure 1B).[9] The crystal structure of the peptide (Pro-Pro-Gly)$_{10}$ in triplex form showed that the proline at Y-position adopts a C4-*exo* conformation while preferring a C4-*endo* pucker at the X-site (Figure 1B).[10] Theoretical calculations supported such dissimilar, complementary ring puckers at X- and Y-sites, necessary for a close packing of the three PPII chains in the triple helix.[11] The recent upsurge in literature on biomimetic analogues of collagen for functional applications[12] and our own previous interests[13] compelled us to examine the hierarchical factors that govern collagen triplex stability, through a rational design of chimeric collagen peptides having 4(*R*/*S*)-substituted prolines concomitantly located at X/Y sites.

We have been interested[13] in the role of cationic 4(*R*/*S*)-aminoprolyl residue in stabilizing the derived collagen triplex relative to that of the neutral 4*R*-OH-proline (Hyp) collagen peptide **1** (Figure 2). 4*R*-NH_2 proline (Amp) was compatible for triplex formation either at Y (**2**) or X (**3**) position, while 4*R*-OH proline (Hyp) formed triplex only when located at Y-site

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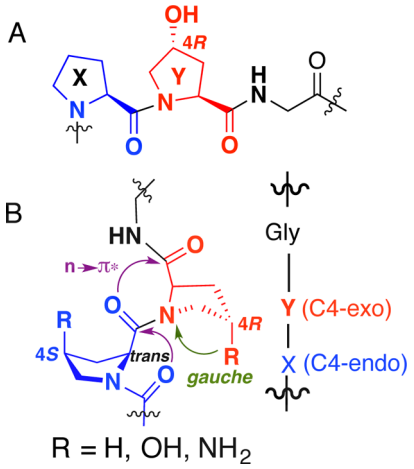


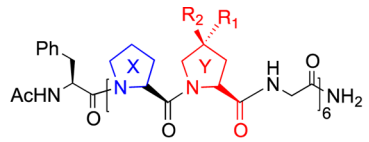
Figure 1. (A) Collagen peptide structure. (B) Preferred ring puckers at X- and Y-sites in collagen peptide.

(**1**). Further, 4*S*-NH_2 proline (amp) was suited for triplex when present at X-site (**4**), but not in Y-site (**19**). The ionizable 4(*R*/*S*)-NH_2 prolines in collagen peptides bestowed a pH dependence on triplex stability.[13] 4*R*-Amp at Y-site (**2**) stabilized the triple helix at both acidic (3.0) and basic (12.0) pHs, while the X-site peptides **3** (4*R*-Amp) and **4** (4*S*-amp) formed triplex only in acidic to neutral pH but not at basic pH 12.0. It is to be expected that cationic 4(*R*/*S*)-NH_2 substitutions on proline ring would induce different stereoelectronic effects in the neutral (NH_2) and protonated (NH_3^+) forms, causing a pH dependence of triplex stability in 4*R*-Amp/4*S*-amp collagen peptides. Another feature modulating the proline ring pucker is the *cis* ↔ *trans* equilibrium of the prolyl tertiary amide bond (rotamers).[14] In this context, 4(*R*/*S*)-aminoproline is a unique ionizable probe to delineate the relative roles of structural and stereoelectronic factors through pH-dependent collagen stability, unlike the nonionizable F, SH, and CH_3 substituents. In order to probe the factors that relatively govern the triplex formation at different pHs, we set out to examine the chimeric collagen peptides **7**−**15**. These are constituted from different combinations of ionizable (NH_2) and neutral (OH/NHCHO), 4(*R*/*S*)-stereo substituents on proline, located concurrently at both X and Y-sites, as compared to analogous monosubstituted peptides **1**−**6** and **16**−**19** (Figure 2).

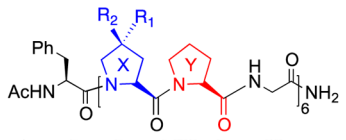
RESULTS AND DISCUSSION

Design and Synthesis of Chimeric Peptides. We had earlier observed that 4*S*-amp and 4*R*-Amp were compatible in collagen tripeptide motif when present at X and Y positions respectively,[13] similar to that seen in 4(*R*/*S*)-fluoroprolyl collagen peptides.[15] We now sought to study the triplex stability of chimeric peptides **7**-**15** having 4*R*-Amp/4*S*-amp/4*R*-Hyp/4*S*-hyp/4*R*-fAmp residues at X and Y-sites in various combinations for comparison with that of the control monosite-substituted peptides **1**−**6**, **16**−**19**. To separate the contributions of charge and H-bonding effects of the ionizable 4(*R*/*S*)-aminoprolines (NH_2/NH_3^+) on triplex stabilities, the chimeric peptides **7**−**13** bearing nonionizable amino derivatives (4*R*/*S*)-NHCHO (fAmp/famp) at X or Y-sites (**16**−**18**) were synthesized. The appropriate monomers required for solid-phase assembly of all 4(*R*/*S*)-(OH/NH_2) peptides were synthesized by methods reported earlier.[13] The 4(*R*/*S*)-NH-formylprolyl monomers **23** and **27** were synthesized as per Scheme 1, starting from 4*R*-hydroxyproline **19** through the known 4*S*-*O*-tosylate **20** and 4*R*-*O*-mesylate **24** derivatives.[13] These were subsequently transformed (Scheme 1) to the corresponding azides (**21**, **25**) that were reductively formylated (**22**, **26**) followed by switching the protecting group at N1 from Boc to Fmoc to get the desired protected monomers 4*R*-fAmp **23** and 4*S*-famp **27**. All the intermediates and final compounds were characterized by relevant spectral and analytical data.

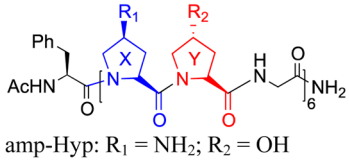
The peptides **7**−**19** were synthesized by solid-phase assembly on Rink amide resin using the appropriately protected 4(*R*/*S*)-OH/NH_2-prolyl monomers as reported earlier[13] and the 4(*R*/*S*)-NHCHO prolyl (**23** and **27**) monomers. After



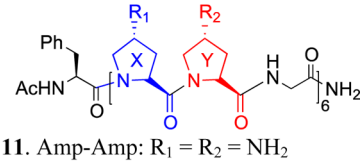
1. Pro-Hyp: R_1 = OH; R_2 = H
2. Pro-Amp: R_1 = NH_2; R_2 = H
16. Pro-fAmp: R_1 = NHCHO; R_2 = OH
19. Pro-amp: R_1 = H; R_2 = NH_2



3. Amp-Pro: R_1 = NH_2; R_2 = H
4. amp-Pro: R_1 = H; R_2 = NH_2
5. hyp-Pro: R_1= H; R_2 = OH
6. Hyp-Pro: R_1=OH, R_2= H
17. famp-Pro: R_1 = H; R_2=NHCHO
18. fAmp-Pro: R_1 = NHCHO; R_2 = H



7. amp-Hyp: R_1 = NH_2; R_2 = OH
8. amp-Amp: R_1 = R_2 = NH_2
9. famp-Amp: R_1 = NHCHO; R_2= NH_2
10. hyp-Amp: R_1 = OH; R_2 = NH_2
15. hyp-Hyp: R_1 = R_2 = OH



11. Amp-Amp: R_1 = R_2 = NH_2
12. Hyp-Amp: R_1 = OH; R_2 = NH_2
13. Amp-Hyp: R_1 = NH_2; R_2 = OH
14. Hyp-Hyp: R_1 = R_2 = OH

Figure 2. Structure of 18-mer monosubstituted (X/Y, **1**−**6**, **16**−**19**) and chimeric (X and Y, **7**−**15**) collagen peptides. (Abbreviations used are as, Amp = 4*R*-aminoproline, amp = 4*S*-aminoproline, Hyp = 4*R*-hydroxyproline, hyp = 4*S*-hydroxyproline, famp = 4*S*-formylproline, fAmp = 4*R*-formylproline.)

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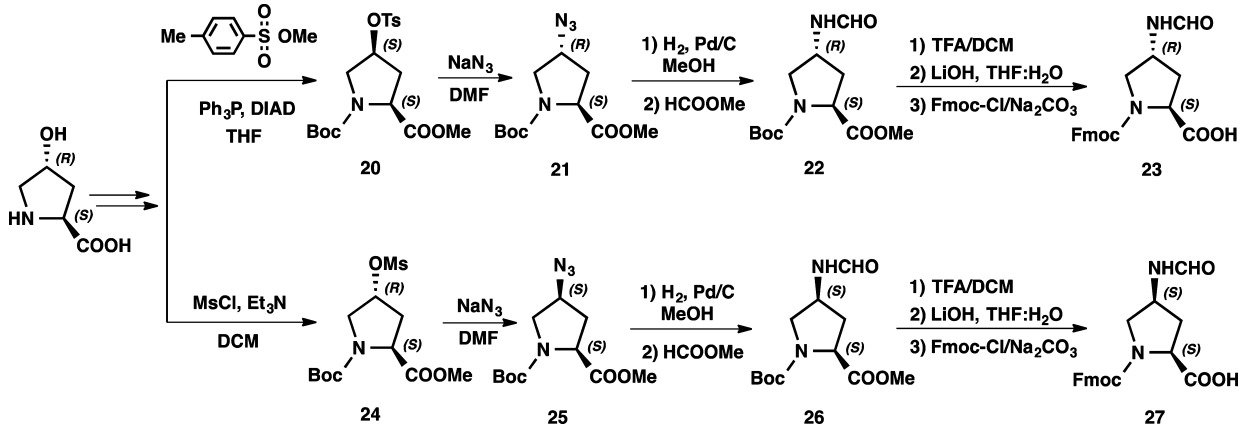
Scheme 1. Synthesis of 4*R*/*S*-NHCHO-prolyl Monomers 23 and 27[a]



[a]Synthesis of **20** and **24** from **19** was done by following literature methods.[13]

synthesis, all the peptides were capped at N-terminus by acetylation and then cleaved from the resin to yield peptides in C-amidated form. Such N,C-capping eliminates the destabilizing effects arising from charge−charge repulsive interactions in parallel oriented, terminally charged (NH_3^+ and COO^-) groups in peptide strands of collagen triplex. Phenylalanine was incorporated at the N-terminus of all peptides to enable accurate measure of peptide concentrations by UV absorbance at 259 nm (ε = 200 M^{-1} cm^{-1}). The crude peptides were purified by semipreparative HPLC using C18 reverse-phase (RP) column, and the final purity confirmed by analytical RP HPLC. The structural identity of all peptides was confirmed by MALDI-TOF mass spectral data.

CD Spectroscopic Study. The thermal stabilities of triplexes constituted from the peptides were examined by temperature-dependent CD spectral data. A large negative band around 200−205 nm, a crossover near 215 nm, and a positive band around 220−225 nm in CD spectra are the hallmarks of collagen-like triplex structure.[16] Triple-helix formation involves association of three strands of polypeptide chains, and increasing the concentration of peptides should promote triplex formation. The ratio of positive to negative band intensity ($R_{p/n}$) is a measure of the triple helical content.[16b] The steady increase in $R_{p/n}$ with concentration reaches saturation at the critical triple helical concentration (CTC) above which the triplex association is complete. It was found that the CTC for triplex forming 18-mer collagen peptides was around 0.15 mM, and hence all CD studies were performed at a peptide concentration of 0.2 mM. As judged by $R_{p/n}$ criterion, the monosite **1**−**4** and chimeric peptides **7**−**13** expediently formed triplex, the X-site monosubstituted 4*S*-hyp peptides **5**, **6** and 4*S*-amp in Y-site (peptide **19**) failed to show triplex. As an illustrative example, Figure 3A shows the CD spectra of triplexes formed by the chimeric peptide amp-Amp (**8**) at different pHs. Similar data for other peptides are provided in the Supporting Information. The melting temperature T_ms were obtained by recording the CD spectra as a function of temperature, and the plot of ellipticity versus temperature shows a sigmoidal transition for triplex forming peptides (e.g., Figure 3B). The midpoint of such plots corresponds to the T_m, and the pH dependence of T_m for the triplex forming peptides **7**−**13** obtained at different pHs of 3.0 (acidic), 7.0 (physiological), 9.0 (~pK_a of NH_2) and 12.0 (alkaline) is summarized in Table 1.

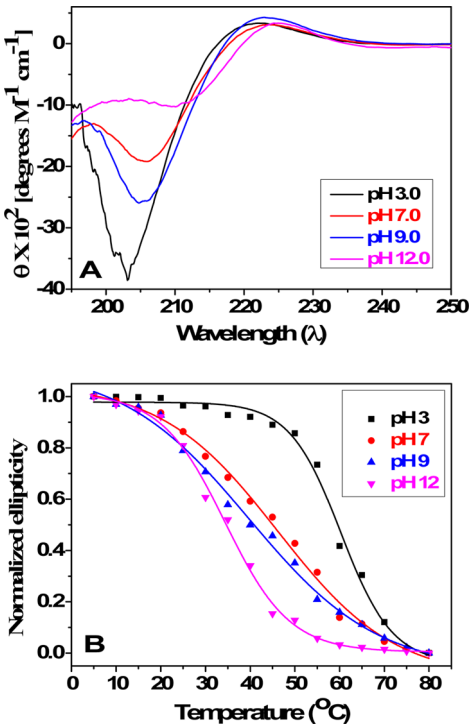


Figure 3. (A) CD spectra of peptide **8** as a function of pH. (B) CD thermal melting curves of peptide **8** at different pHs.

Several significant facts emerge from data in Table 1: (a) the ionizable amp/Amp peptides (**2**−**4**, **7**−**9**, **11**−**13**) form triplexes with most stability at acidic pH, (b) the non-ionizable peptides with 4*R*/*S*-OH/NHCHO substitution on proline at either X/Y-sites (**1**, **16**, **17**) or both (**14**) do not show pH-dependence of triplex T_m, (c) peptides with 4*R*-Amp in Y-site (**2**, **9**, **11**-**12**) have the least triplex stability at pH 9.0 with the T_ms of peptides **2**, **9** and **12** enhanced on either side of pH range (Figure 4), (d) the chimeric peptide amp-Amp **8** exhibited a pH−T_m profile linearly decreasing with increasing pH, (e) peptides having ionizable 4*S*-amp in X-site (**4** and **7**) form triplexes only in the pH range 3.0−9.0, (f) peptides with 4*R*-Amp at X-site (**3**, **11**, **13**) show triplex only at pH 3.0−7.0 but not at alkaline pH 12.0, and (g) peptide with 4*S*-amp at Y-site (**19**) does not form triplex at any pH. Overall the results clearly imply a preference for 4*R*-(OH/NH_2/NHCHO)-proline

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Table 1. pH-Dependent T_m of Chimeric Peptides[a]

peptide	(X-Y)	pH 3.0	pH 7.0	pH 9.0	pH 12.0	EG:H_2O
1	Pro-(4*R*)Hyp[b]	27.0	28.0	27.0	27.0	31.0
2	Pro-(4*R*)Amp[b]	60.0	54.7	26.0	49.0	23.0
3	(4*R*)Amp-Pro[b]	36.0	33.0	–	–	35.0
4	(4*S*)amp-Pro[b]	44.0	37.0	34.0	–	39.0
7	(4*S*)amp-(4*R*)Hyp	49.8	39.3	34.0	–	42.0
8	(4*S*)amp-(4*R*)Amp	61.0	46.6	40.5	34.0	37.0
9	(4*S*)famp-(4*R*)Amp	58.0	56.0	24.0	44.0	47.0
10	(4*S*)hyp-(4*R*)Amp	–	–	–	–	–
11	(4*R*)Amp-(4*R*)Amp	51.5	40.8	25.0	–	34.0
12	(4*R*)Hyp-(4*R*)Amp	55.8	51.5	31.0	53.0	31.2
13	(4*R*)Amp-(4*R*)Hyp	43.1	36.0	29.0	–	30.5
14	(4*R*)Hyp-4(*R*)Hyp	31.8	32.0	30.9	30.0	29.8
15	(4*S*)hyp-(4*R*)Hyp	–	–	–	–	–
16	Pro-(4*R*)fAmp	42.0	41.0	41.0	40.0	40.0
17	(4*S*)famp-Pro	23.2	21.0	21.0	20.0	31.0
18	(4*R*)fAmp-Pro	no triplex formation				

[a]All T_ms were obtained from the first derivative plots and are accurate to ±0.5 °C. Buffers: pH 3.0 (20 mM acetate), pH 7.0 (20 mM phosphate), 9.0 (20 mM phosphate), pH 9 and 12 (20 mM borate), EG (ethylene glycol). All buffers contain 10 μM NaCl. (−) indicates no triplex seen. [b]Taken from ref 13.

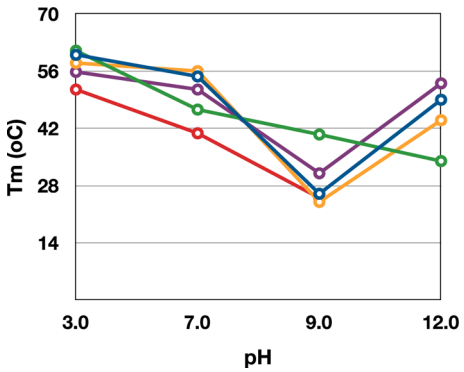

Figure 4. CD-T_m of 4*R*-Amp(Y) collagen peptides as a function of pH. Pro-Amp **2** (blue line), amp-Amp **8** (green line), famp-Amp **9** (yellow line), Amp-Amp **11** (red line) and Hyp-Amp **12** (dark-red line).

at Y-site and 4*S*-(OH/NH_2/NHCHO)-proline at X-site and pH-dependent triplex forming properties of all ionizable 4*R*/*S*-NH_2-prolyl peptides.

Triplex Stabilities in EG:H_2O. It is known that ethylene glycol (EG), a relatively more hydrophobic solvent than H_2O, favors H-bonding-dependent stability of the collagen triplex.[17] The CD-T_m of peptides measured in EG:H_2O (3:1) is shown in the last column of Table 1. Comparison of the ΔT_m relative to the T_ms at neutral pH 7.0 (Figure 5) reveals that peptides bearing charged NH_2 substitution at X-site (**3**, **4**, and **7**) showed increase in triplex stability by 2−3 °C, but peptide **17** having neutral formyl group at X-site exhibited a much higher T_m ($\Delta T_m \approx 10$ °C). In contrast, peptides with ionic 4*R*-Amp at Y-site (**2**, **8**, **9**, **11**, and **12**) exhibited severe destabilization ($\Delta T_m \approx 31.7°$, 9.7°, 9.0°, 6.0°, and 20.2 °C, respectively, ΔT_m are accurate to ±0.5 °C).

The data on triplex stability in EG:H_2O can be comprehensively rationalized as follows. In X-site peptides **4**, **7**, and **17**, the 4*S*-substitution, which favors the necessary C4-*endo* pucker stabilizes the triplex. The *endo* pucker arises from a

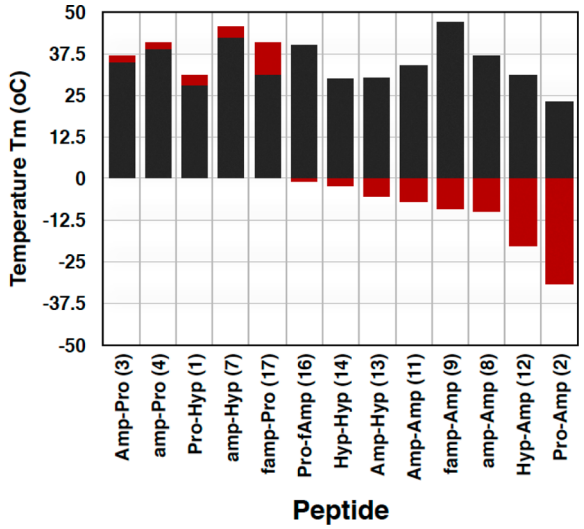

Figure 5. Triplex T_ms (gray bars) of peptides in EG:H_2O and ΔT_ms (red bars, + stabilization, − destabilization) compared to corresponding T_ms at pH 7.0. Peptides on *X*-axis arranged according increasing stabilization and destabilization.

stronger intraresidue H-bonding in EG:H_2O between the axial 4*S*-(NH_2/NHCHO) and C2 amide carbonyl on proline, which are in *cis*-stereochemical disposition (Figure 6). In 4*R*-peptides

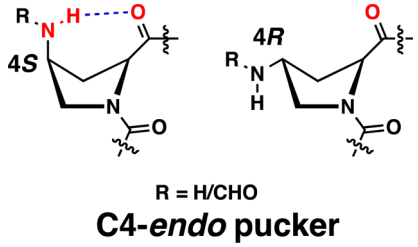

Figure 6. Intraresidue H-bond in 4*S*-amp which absent in 4*R*-Amp.

3 and **11**, the preferred C4-*endo* pucker at X-site would have equatorial amino/formyl group at C4, not amenable to form H-bonding with the C2-carbonyl on proline, leading to a lower triplex stability. It is interesting to note the different behavior of X-site 4*R* peptides **3** and **11**, with the former slightly stabilizing, while the latter destabilizing compared to corresponding neutral pH 7.0. A general observation in EG:H_2O is that 4*R*-Amp peptides either in Y-site (**2**, **8**, **9**, **11**, **12**) or X-site are all destabilized, perhaps due to unfavorable electrostatic charge effects; this also explains the slightly higher destabilization of charged peptide **14** compared to neutral **13**. In comparison, 4*S*-(NH_2/famp) peptides (**4**, **7**, and **17**) are stabilized due to H-bonding effects.

Since all peptides are terminally capped, the observed pH dependence of triplex T_ms of peptides **2**−**4**, **7**−**9**, **11**−**13** arises solely from the ionizable 4-NH_2 group on proline ring. This is supported by the pH invariance of T_ms of peptides **1**, **6**, **14**, **16**, and **17** devoid of ionizable 4-NH_2 group. The peptides with single site 4-NH_2 prolyl residue in the Pro(X)-Pro(Y)-Gly triad (**2**, **9**, **12**, **13**) showed a minima in T_m at pH 9.0, near the pK_a of amino group. In the chimeric peptides with two substitutions of 4-NH_2-proline per triad (**8** and **11**), T_m decreased with increasing pH (Figure 4), but no triplex formed at pH 12.0 for peptide **11**. The relative difference in group electronegativity of the neutral NH_2 and the protonated NH_3^+ functions would

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result in differential H-bonding capacities. The ionization status of 4-NH_2 group depends on pH, which may influence the *endo*/*exo* nature of proline ring pucker. At extremes of pH (3.0 and 12.0), a single pucker dominates due to the presence of either NH_3^+ or NH_2 form. However, at pH around the pK_a (9.0), simultaneous existence of neutral and protonated amino groups results in a mixture of ring puckers causing a conformational heterogeneity in helical strands, leading to lower triplex stability. In case of peptides **8** and **11** that have ionizable 4-NH_2-proline at both X and Y-sites, the mutual interaction of charges on adjacent prolines dictates their protonation and hence their relative ring puckers. Increasing pH enhances the neutral forms at both X/Y-sites, synergistically driving prolines to unfavorable puckered states resulting in a lower triplex stability at higher pH. Such pH-dependent triplex stabilities have been observed in case of collagen peptides having other charged side chains (e.g., lysine, arginine).[16a,18] Theoretical calculations on 4(*R*/*S*)-NH_2-proline at monomer and dipeptide levels have suggested a possible switch of axial/equatorial orientation of the 4-substituent through pucker change upon protonation.[19] Differential solvation of the axial/equatorial substituents may distinctly stabilize the relevant *exo*/*endo* puckered states ordained by the nature of the 4-NH_2/NH_3^+ substituent.[20]

The protonation of NH_2 to NH_3^+ at acidic pH enhances not only its H-bonding potential but also amplifies the consequences of electrostatic charges (electron-withdrawing and gauche effects, puckering, and solvation). In order to soften the effects of charge, neutral 4N-formylproline collagen peptides (**16**−**18**) were studied. The *N*-formyl group should enhance the H-bonding ability due to its amide character without bestowing any charge effects, unlike the 4-NH_3^+ group. It was found that (i) both the 4*R*-fAmp (**16**) in Y-site and 4*S*-famp (**17**) in X-site substitutions on proline were compatible for triplex formation, akin to the corresponding parent 4(*R*/*S*)-NH_2 proline peptides; (ii) the Y-site peptide **16** was more effective than X-site peptide **17** in aqueous buffer; and (iii) stability of X-peptide **17** was enhanced by ∼10 °C in EG:H_2O (compared to that at pH 7.0), while the triplex stability was not affected much for Y-site peptide **16**. This highlights the important role of H-bonding at X-site (iv) the chimeric peptide famp-Amp **9** with neutral 4*S*-famp in X-site and cationic 4*R*-Amp in Y-site exhibited a pH-dependent triplex stability to the same extent as that of Y-site 4*R*-Amp peptides **2** (1 × NH_2) and **11** (2 × NH_2) in aqueous buffer, but the T_m was enhanced enormously in EG:H_2O ($\Delta T_m \approx 13$ °C), over that at neutral pH.

The role of charge effects in stabilizing the triplexes of cationic peptides is reflected in the enhancement of T_ms in the presence of salt (NaCl). The T_m of 30 °C for peptide **11** (Figure 7) without salt at pH 7.0 enhanced in the presence of increasing amounts of salt to 45 °C (50 mM), 51.5 °C (100 mM), and 52 °C (200 mM). Such T_m changes were negligible for the nonionic peptide **14**, whose T_m remained constant at 30 ± 1 °C with increasing salt concentrations. Salt effects arise from the screening of peptide positive charges by its own counterion and breaking of water structure.[21]

INTERDEPENDENCE OF STEREOELECTRONIC, RING PUCKER, AND H-BONDING EFFECTS

The cumulative data on triplex stability of chimeric peptides presented here can be comprehensively rationalized in terms of a complex interplay of stereoelectronic β-gauche effects, ring pucker, and intra residue H-bonding as a function of pH. These are summarized in Table 2.

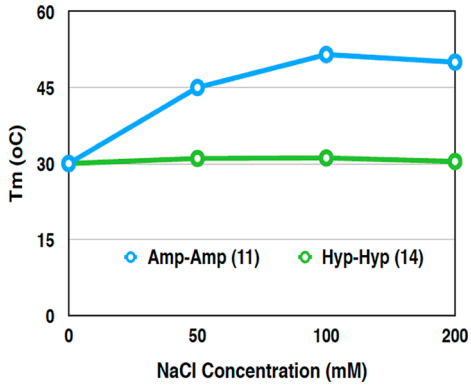

Figure 7. Plot of T_m versus concentration of NaCl for peptide **11** and peptide **14**.

Column A lists the necessary factors that favor PPII single strand conformation and thereby increase the triplex stability of collagen. These comprise of (i) necessary C4-*endo* and C4-*exo* pucker at X and Y sites, respectively; (ii) stabilizing gauche effect through (Z−C4−C5−N1) bonds at Y-site (iii) favored n → π^* interaction from $CO_{i(n)}$ to $CO_{i-1(\pi^*)}$ promoted by a *trans* orientation of N1-tertiary amide bonds at X and Y sites; and (iv) intraresidue H-bonding between C4(*S*) substituent with C2(O) of the amide. The reasoning is built on the basic premise that any factors that specifically favor the ring puckers of C4-endo at X-site and C4-exo at Y-site stabilize the PPII conformation and hence the derived triplexes. In the chimeric peptides having X(4*S*)-Y(4*R*)-substituted prolines (Column B), stabilization of triplexes is based on highly favorable stereoelectronic gauche effect in C4-*exo* pucker with the axial 4*R*-substituent. The ensuing *trans* prolyl amide bond at Y-site synergistically encourages n → π^* interaction with the C2 carbonyl (C═O_i) at both acidic (pH 3.0) and alkaline (pH 12.0) conditions. At X-site, 4*S*-substituent is able to form intraresidue H-bonding with C2 carbonyl favoring the necessary C4-*endo* pucker. Such a H-bond of 4*S*-substituent with C═O_i abetting its *trans* orientation is consistent with earlier reports.[22] It was reported by Wennemers et.al.[22b] that introduction of single 4*S*-amino proline at X site resulted in destabilization of triplex at acidic and basic pH due to competition between intramolecular H-bond versus interstand H-bond. This different observation is perhaps due to the fact that these peptides had only one substitution (unlike six in this work) and the solvent system used for CD was different. Thus, 4-amino-substituted chimeric peptides **7**−**9** (Column B) with X(4*S*)-Y(4*R*) combination form triplexes at all pH conditions. In case of X(4*R*)-Y(4*R*) chimeric peptides **11**-**13** (Column C), while the Y-site is stabilized at all pHs in axial 4*R* substitution, we hypothesize that the protonated equatorial group (NH_3^+) at X-site at acidic pH 3.0, due to its positive charge and greater H-bonding capacity, can engage the C2 carbonyl through water-mediated H-bonding. However, with the 4*R* neutral substituents (OH/NHCHO) or with NH_2 (at pH 12.0), weak or absence of H-bonding at X-site is unable to retain the C2 carbonyl in a *trans* orientation, due to which its n → π^* interaction with the CO at Y-site is lost. This leads to very weak or nonobservance of the triplex from X(4*R*)-Y(4*R*) chimeric peptides **11**−**13** at pHs 9.0 and 12.0. In case of X(4*S*)-Y(4*S*)-substituted prolines (Column D) in the preferred C4-*exo*

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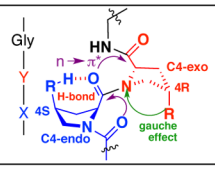
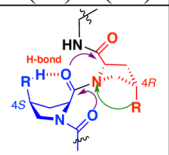
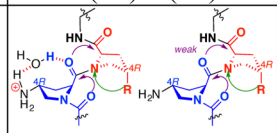
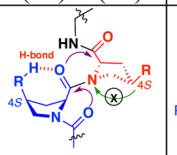
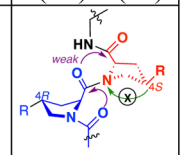
Table 2. Comparative Structural Features at X/Y-Sites[a] at Different pHs and Consequences on Triplex Stability[a]

	A	B	C	D	E
	Features	X(4*S*)-Y(4*R*)	X(4*R*)-Y(4*R*)	X(4*S*)-Y(4*S*)	X(4*R*)-Y(4*S*)
1			 pH = 3.0, 7.0 pH > 9.0		
2	*Proline pucker X(C4-endo)-Y(C4-exo)*	Favorable at both sites	pH 3.0,7.0: favorable at both sites; pH>9.0: not favorable at X-site	Favorable at both sites	Favorable at both sites
3	R_1-C4-C5-N1-gauche effect at Y-site	Favorable	Favorable	Unfavorable	Unfavorable
4	n➔π* interaction in $CO_{i(n)}$-$CO_{i-1(\pi^*)}$	Favorable at both sites	pH 3.0,7.0 – favorable at Y; pH >9.0 – not favorable at X-site	Favorable	Weak Unfavorable
5	Intraresidue H-bonding at X-site	Favorable	Possible only in NH_3^+	Possible	Not possible
6	Peptides	**7,8,9**	**11-13**	No examples from current work with chimeric peptides[b]	
	Remarks	Triplex at all pHs	Triplex only at pH 3.0, 7.0		

[a] R_1 = R_2 = NH_2/OH/NHCHO; At pH 3.0 and 7.0, R_1 = R_2 = NH_2, ⊗ = effect not favored. [b] See refs 14d and 22b for examples of non chimeric (4*S*)hyp(X)-Pro(Y) and Pro(X)-(4*S*)amp)(Y) peptides.

pucker at Y-site, the 4*S*-substituents (R_1) assume an equatorial orientation where the stereo electronic gauche effect is ineffective. The amide carbonyl at the Y-site proline devoid of such a stabilizing factor cannot have productive n → π* interaction with the C2 carbonyl ($C{=}O_{i-1}$) of proline, destabilizing the triplex in both acidic and alkaline pHs. This seems to be the overriding factor in determining the triplex stabilities, although features at X-site are suitable for triplex stabilization. With X(4*R*)-Y(4 *S*) chimeric peptides (Column E), structural factors at both sites are not conducive for triplex formation.

Interestingly, the peptides **10** and **15** carrying 4*S*-hyp at X-site failed to form triplex in water or EG:H_2O when either 4*R*-Amp or 4*R*-Hyp occupies Y-site. Destabilization of triple helix with 4*S*-hyp in X-site and Pro in Y-site for $(4S\text{-hyp-Pro-Gly})_n$ is known earlier in the literature.[14d,22b] It was attributed to originate from a combination of unfavorable enthalpic and entropic effects. Both the favored transannular intrachain H-bonding of 4*S*-OH with amide CO competing with the interstrand H-bonding to form triplex and the accompanying distortion of C^{γ}-*endo* pucker discourage the triplex formation. In this context, the observed triplex formation from X-site 4*S*-amp/4*R*-Amp chimeric peptides is noteworthy, and the differing nature and extent of competing stabilizing/destabilizing factors in case of 4-NH_2 compared to 4-OH need to be analyzed.

Thus, the dependence of relative triplex stabilities in chimeric collagen peptides on the nature of 4(*R*/*S*) substituents and pH are the consequences of combined intraresidue H-bonding, proline ring pucker, n → π* interaction in $CO_{i(n)}{-}CO_{i-1(\pi^*)}$ and stereoelectronic gauche effects. The analytical understanding of such combinatorial factors determining the collagen triplex stability has been possible due to the versatile nature of 4-aminoproline as an ionizable, H-bonding, and stereoelectronic probe of proline pucker. The direct proof for the above model in the future needs detailed NMR investigations and theoretical calculations.

CONCLUSIONS

In collagen model peptides, any 4(*R*/*S*)-substitutions on proline that inherently promote a C4-*endo*-pucker at X-site and C4-*exo* pucker at Y-site strengthen the triplex formation. Unsubstituted proline is degenerate with respect to C4-*exo*/*endo* puckers but prefers C4-*exo* at Y and C4-*endo* at X sites for effective steric packing to form a triplex.[10] In case of chimeric collagen peptides composed of 4(*R*/*S*)-substituted prolines at both X and Y-sites, the triple helical stability effectively depends on the stereochemical nature of the residue at X site, since Y-site stabilizes triplex only in C4-*exo* pucker. The different factors contributing to the triplex formation by chimeric peptides $[Pro(X)\text{-}Pro(Y)\text{-}Gly]_6$ with 4*R*/*S*-NH_2/OH/H/NHCHO prolines incorporated at X/Y-sites in various combinations have been delineated through pH-dependent triplex stabilities. The pattern of relative strengths of triplexes can be explained through a composite model (Figure 5) amalgamating the preferred proline pucker in C4-*endo*/*exo* form at X/Y sites with *trans* prolyl amide conformation for effective n → π* interaction, the stereoelectronic gauche effect, and intraresidue H-bonding. In case of 4-NH_2-proline peptides, each of the above factors is a function of stereochemistry (*R*/*S*), X/Y-site location of the substituted proline, and pH. In chimeric peptides that have dual site modifications, the intraresidue H-bonding of the 4*S*-substituents with the X-site C2-carbonyl with C4-*endo* proline pucker is the determinant for stabilization of triplexes. Deployment of the ionizable 4(*R*/*S*)-aminoproline in collagen peptide has uniquely allowed pH as an additional parameter to examine the structural factors determining the triplex stability, which is not feasible with other nonionizable substituents (OH/F/SH/CH_3). The amino group being a chemically reactive functionality, the results would be

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useful in design of chimeric hyperstable collagen peptides with potential to hybridize with natural collagens and cross-link further to form fibrils. Such supramolecular composites may have implications for creating new functional materials for biomedical applications, drug/gene delivery, and tissue engineering.[23]

EXPERIMENTAL SECTION

Materials and reagents were of the highest commercially available grade and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 F254 plates. Compounds in TLC were visualized by UV and stain by ninhydrin. Gravity column chromatography was performed using silica gel 60−120 and 100−200 particle size. ^{1}H and ^{13}C NMR spectra were recorded on 200/400/600 MHz spectrometers. Chemical shifts are reported in ppm using TMS and $CDCl_3$ as a reference. High-resolution mass spectra (HRMS) were recorded in electrospray ionization-time-of-flight (ESI-TOF) mode. MALDI-TOF mass spectra of all peptides were obtained in positive reflectron mode using α-cyano-4-hydroxy cinnamic acid (CHCA) or 2,5-dihydroxy benzoic acid (DHB) as a matrix. N^1-Fmoc-N^4-Boc-4*R*/*S*-aminoproline was synthesized from 4*R*-hydroxyproline by sequential protection/deprotection strategies as described previously.[13]

Synthesis of Compounds 21−27. *(2S-4R)N-(t-Butyloxycarbonyl)-4-azido-prolinemethylester* ***21***. A solution of compound **20**, 8.5 g, (25 mmol), and NaN_3 13 g, (200 mmol) in dry DMF (80 mL) was stirred at 60 °C for 8 h. DMF was removed, and the residue was dissolved in water and extracted with ethyl acetate. After workup, the organic layer dried over anhydrous Na_2SO_4 and concentrated to obtain crude **21** that was purified by silica gel chromatography (40% ethyl acetate/hexane elute); (Yield 6.5 g, 96%); ^{1}H NMR (600 MHz, $CDCl_3$) δ 1−42−1.47 (d, *J* = 30 Hz 9H), 2.16−2.20 (m, 1H), 2.29−2.37 (m, 1H) 3.46−3.61 (ddd, *J* = 3.67, 3.48, 1.65 Hz, 1H), 3.70−3.73 (q, 1H), 3.75−3.76 (d, *J* = 6.24, 3H), 4.18−4.21 (m, 1H), 4.32−4.43 (m, 1H); ^{13}C NMR (600 MHz, $CDCl_3$) δ 28.2, 28.3, 35.3, 36.3, 51.2, 51.4, 57.4, 57.8, 58.7, 59.2, 80.7, 80.8, 153.4, 154.0, 172.8, 173.0; HRMS (ESI-TOF) (*m*/*z*) $[M + Na]^+$ calculated for $C_{11}H_{18}N_4O_4Na$ 293.1225, found 293.1238.

(2S,4R)N^1-(t-Butyloxycarbonyl)-4-N-formyl-aminoproline methylester ***22***. To a solution of azide **21** (6 g, 22.2 mmol) in methanol (6 mL) was added Raney Nickel catalyst (2 mL), and the reaction mixture was hydrogenated in a Parr shaker for 4 h at room temperature under H_2 pressure of 45−50 psi. The catalyst was filtered, and solvent was evaporated under vacuum to yield free amine compound, which was immediately stirred with methylformate for 4 h. After completion of reaction the solvent was removed, and the crude product was purified by silica gel column chromatography (EtOAc/Petroleum ether [7:3]) to get a pale yellow liquid of *N*-formyl compound **22** (Yield 5.5 g, 90.9%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.37−1.41 (d, *J* = 16.94, 9H), 2.18−2.29 (m, 2H), 3.28−3.41 (ddd, *J* = 4.58, 4.12, 3.66, 1H), 3.70 (s, 3H), 3.72−3.81 (m, 2H), 4.25−4.37 (m, 1H), 4.49−4.59 (m, 1H), 6.74−6.68 (dd, *J* = 6.87, 6.41, 1H), 8.04−8.09 (d, *J* = 12.82, 1H); ^{13}C NMR, (400 MHz, $CDCl_3$) δ 28.3, 28.4, 35.5, 36.7, 46.8, 47.3, 51.2, 52.0, 52.3, 52.5, 57.5, 57.8, 80.7, 80.8, 153.8, 154.4, 161.3, 161.4, 172.7, 173.0; HRMS (ESI-TOF) (*m*/*z*) $[M + Na]^+$ calculated for $C_{12}H_{20}N_2O_5Na$ 295.1269, found 295.1282.

4R-N-Formyl-N^1-(Fmoc)aminoproline ***23***. The compound **22** (5 g 18.35 mmol) was stirred for 2 h in 1:1 mixture of CH_2Cl_2 and TFA (8 mL). The solvent was removed under reduced pressure, the resulting slurry was dissolved in diethyl ether, and removal of ether yielded white compound of trifluoroacetate salt. The above obtained salt was dissolved in a mixture of methanol (25 mL) and 0.5 M LiOH solution (20 mL). The reaction mixture was stirred for 30 min. The reaction mixture was then neutralized to pH 7, and methanol was removed under reduced pressure. The aqueous layer was cooled in an ice−water bath, and dioxane (50 mL) and 2N sodium carbonate (50 mL) were added to it. The solution of Fmoc-Cl (9.1 g 35.2 mmol) in 10 mL dioxane was added slowly over a period of 75 min, the reaction mixture was stirred for 6 h at room temperature, and the pH of the reaction was maintained at about 8−9 during the reaction. The dioxane was removed under reduced pressure, and the aqueous layer was cooled and extracted with diethyl ether (2 × 25 mL). Ether layer was discarded, and aqueous layer was covered with ethyl acetate layer and acidified with KHSO4 solution to pH 2−3. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate. The combined organic layer was washed with water followed by saturated brine solution and dried over anhydrous Na_2SO_4. The ethyl acetate was removed under reduced pressure, and the crude product was purified by column chromatography (EtOAc/Petroleum ether [9:1]) to offer a white amorphous solid of monomer **23** (Yield 5g, 60%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.92- 2.05 (m, 1H), 2.44−2.62 (m, 1H), 3.27−3.66 (m, 2H) 3.75−3.82 (m, 1H), 4.11−4.47 (m, 5H), 7.25−7.28 (m, 2H), 7.32−7.37 (m, 2H), 7.55−7.60 (m, 2H), 7.72−7.75 (m, 2H), 7.85−8.00 (m, 1H), 8.04−8.12 (m, 1H); ^{13}C NMR, (400 MHz, $CDCl_3$) δ 35.1, 36.2, 39.7, 45.9, 46.8, 46.9, 47.5, 51.3, 51.7, 58, 58.3, 67.2, 67.5, 106.4, 119.8, 120, 121.2, 125.2, 127.2, 127.4, 128.8, 137.6, 139.7, 140.8, 143.7, 152.2, 145.3, 161.3, 174.1, 174.4; HRMS (ESI-TOF) (*m*/*z*) $[M + Na]^+$ calculated for $C_{21}H_{20}N_2ONa$ 403.1269, found 403.1277.

(2S,4S)N-(t-Butyloxycarbonyl)-4-azido-prolinemethylester ***25***. A solution of mesyl compound **24** (15.0 g, 46 mmol) and NaN_3 24.0 g, (371 mmol) in dry DMF (120 mL) was stirred at 70 °C for 8 h under argon. DMF was removed under vacuum, and the residue was dissolved in water and extracted with ethyl acetate. The combined organic layer after workup was dried over anhydrous Na_2SO_4 and concentrated. The crude material was purified by silica gel chromatography (40% ethyl acetate/hexane) to afford the azide **25** (Yield 12.0 g; 95.6%) as a colorless oil; ^{1}H NMR, (400 MHz, $CDCl_3$) δ 1.40−1.45 (d, *J* = 21.98 Hz, 9H), 2.12−2.17 (m, 1H), 2.39−2.51 (m, 1H), 3.42−3.50 (m, 1H), 3.65−3.72 (m, 1H), 3.74 (s, 3H), 4.09−4.18 (m, 1H), 4.29−4.43 (ddd, *J* = 4.58, 3.66 Hz, 1H); ^{13}C NMR, (400 MHz, $CDCl_3$) δ 28.3, 28.4, 35.1, 36.1, 50.9, 51.3, 57.4, 57.8, 58.3, 59.3, 80.6, 80.7, 153.5, 154.0, 172.0, 172.3; HRMS (ESI-TOF) (*m*/*z*) $[M + Na]^+$ calculated for $C_{11}H_{18}N_4O_4Na$ 293.1225, found 293.1229.

(2S,4S)N^1-(t-Butyloxycarbonyl)-4-N-formyl-aminoproline methylester ***26***. To a solution of azide **25** (12 g, 44.2 mmol) in methanol (8 mL) was added Raney Nickel catalyst (2 mol %). The reaction mixture was hydrogenated in a Parr shaker for 4 h at room temperature under a H_2 pressure of 45−50 psi. The catalyst was filtered, and solvent was removed to yield a residue of amine that was immediately stirred with methylformate for 4 h. Removal of solvent and purification by silica gel chromatography (EtOAc/Petroleum ether [7:3]) gave pale yellow liquid of 4*S*-*N*-formyl compound **26** (Yield 11.8 g, 78.%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.42−1.46 (d, *J* = 16.94 Hz, 9H), 1.93−1.99 (t, 1H), 2.48−2.58 (m, 1H), 3.45−3.55 (dd, *J* = 10.53, 11.45 Hz 1H), 3.65−3.69 (m, 1H), 3.77−3.78 (d, *J* = 4.58 Hz, 3H), 4.27−4.37 (ddd, *J* = 2.75 Hz, 1H), 4.70−4.71 (m, 1H), 7.05−7.11 (q, 1H), 8.10 (s, 1H); ^{13}C NMR, (400 MHz, $CDCl_3$) δ 28.1, 28.3, 35.3, 36.3, 46.1, 47.0, 52.1, 52.3, 52.5, 52.8, 57.5, 57.7, 80.5, 80.7, 153.3, 154.1, 160.9, 163.4, 174.3, 174.4; HRMS (ESI-TOF) (*m*/*z*) $[M + Na]^+$ calculated for $C_{12}H_{20}N_2O_5Na$ 295.1269, found 295.1277.

(2S,4S)N1-(Fmoc)-4S-N4-Formyl-aminoproline ***27***. By following the same procedure as for compound **23** from **22**, compound **27** was obtained from **26** (Yield 4.5 g, 64%) over three steps; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.02−2.18 (m, 1H), 2.35−2.48 (m, 1H), 3.51−3.57 (m, 1H), 3.60−3.75 (m, 1H), 4.12−4.20 (m, 1H), 4.36−4.47 (m, 2H), 4.64−4.66 (t, 1H), 7.10−7.18 (m, 1H), 7.26−7.29 (t, 2H), 7.32−7.39 (m, 2H), 7.50−7.55 (dd, *J* = 7.58, 6.54 Hz, 2H), 7.69−7.74 (m, 2H), 8.02−8.05 (d, *J* = 14.12 Hz, 1H); ^{13}C NMR, (400 MHz, $CDCl_3$) δ 34.8, 46.8, 52.7, 58.5, 67.9, 119.9, 124.0, 127.0, 127.7, 141.1, 143.5, 143.8, 155.2, 161.8, 175.9; HRMS (ESI-TOF) (*m*/*z*) $[M + Na]^+$ calculated for $C_{21}H_{20}N_2O_5Na$ 403.1269, found 403.1270.

Peptide Synthesis and Purification. Peptides were synthesized manually by solid-phase method on Rink amide resin (0.7 mm/g). Stepwise synthesis of full-length peptides was achieved by coupling Fmoc-potected amino acids to the growing peptide chain using standard HOBt coupling reagent. After completion of synthesis, the peptides were cleaved from the resin by treating with a mixture of TFA:TIPS:DCM, (95:2.5:2.5%) for 2 h. Evaporation of solvent gave

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Table 3. MALDI TOF Data for Peptides 2−19

Peptide	Mol. Formula	Mass. Calc.	Mass Found
Ac-Phe(Pro-Amp-Gly)$_6$-NH$_2$ **2**	$C_{83}H_{122}N_{26}O_{20}$	1804.05	1804.97 $[M]^+$
Ac-Phe(Amp-Pro-Gly)$_6$-NH$_2$ **3**	$C_{83}H_{122}N_{26}O_{20}$	1804.05	1805.0 $[M+H]^+$
Ac-Phe(amp-Pro-Gly)$_6$-NH$_2$ **4**	$C_{83}H_{122}N_{26}O_{20}$	1804.05	1806.0 $[M+2H]^+$
Ac-Phe(hyp-Pro-Gly)$_6$-NH$_2$ **5**[a]	$C_{83}H_{116}N_{20}O_{26}$	1809.97	1831.10 $[M+Na]^+$
Ac-Phe(Hyp-Pro-Gly)$_6$-NH$_2$ **6**[a]	$C_{83}H_{116}N_{20}O_{26}$	1809.97	1831.58 $[M+Na]^+$
Ac-Phe(amp-Hyp-Gly)$_6$-NH$_2$ **7**	$C_{83}H_{122}N_{26}O_{26}$	1898.90	1921.73 $[M+Na]^+$
Ac-Phe(amp-Amp-Gly)$_6$-NH$_2$ **8**	$C_{83}H_{128}N_{32}O_{20}$	1892.99	1914.26 $[M+Na]^+$
Ac-Phe(famp-Amp-Gly)$_6$-NH$_2$ **9**	$C_{89}H_{128}N_{32}O_{26}$	2062.20	2062.41 $[M]^+$
Ac-Phe(hyp-Amp-Gly)$_6$-NH$_2$ **10**	$C_{83}H_{122}N_{26}O_{26}$	1898.90	1920.67 $[M+Na]^+$
Ac-Phe(Amp-Amp-Gly)$_6$-NH$_2$ **11**	$C_{83}H_{128}N_{32}O_{20}$	1892.99	1916.58 $[M+Na]^+$
Ac-Phe(Hyp-Amp-Gly)$_6$-NH$_2$ **12**	$C_{83}H_{122}N_{26}O_{26}$	1898.90	1921.73 $[M+Na]^+$
Ac-Phe(Amp-Hyp-Gly)$_6$-NH$_2$ **13**	$C_{83}H_{122}N_{26}O_{26}$	1900.04	1923.03 $[M+Na]^+$
Ac-Phe(Hyp-Hyp-Gly)$_6$-NH$_2$ **14**	$C_{83}H_{116}N_{20}O_{32}$	1905.95	1931.15 $[M+2H+Na]^+$
Ac-Phe(hyp-Hyp-Gly)$_6$-NH$_2$ **15**	$C_{83}H_{116}N_{20}O_{32}$	1905.95	1931.15 $[M+2H+Na]^+$
Ac-Phe(Pro-fAmp-Gly)$_6$-NH$_2$ **16**	$C_{89}H_{122}N_{26}O_{26}$	1970.90	1993.57 $[M+Na]^+$
Ac-Phe(famp-Pro-Gly)$_6$-NH$_2$ **17**	$C_{89}H_{122}N_{26}O_{26}$	1970.90	1993.26 $[M+Na]^+$
Ac-Phe(fAmp-Pro-Gly)$_6$-NH$_2$ **18**	$C_{89}H_{122}N_{26}O_{26}$	1970.90	1993.15 $[M+Na]^+$
Ac-Phe(Pro-amp-Gly)$_6$-NH$_2$ **19**	$C_{83}H_{124}N_{26}O_{20}$	1804.24	1804.09 $[M]^+$

[a]Taken from ref 24.

the crude peptide, which was dissolved in minimum volume of MeOH and reprecipitated by addition of cold diethyl ether. The peptides were purified by preparative RP-HPLC on RP-4 (15 μM) column. Elution buffers: A, 0.05% TFA in water; B, 0.05% TFA in CH_3CN:H_2O (70:30); gradient 3−70% B over 20 min; flow rate 3 mL/min and monitored at 220 nm. A RP-18e (5 μM) column by using a gradient of 0−100% B in 30 min at a flow rate of 1 mL/min. The absorbance of the eluting was monitored at 220 nm, and structural integrity of purified peptides was confirmed by MALDI-TOF mass spectroscopy by using CHCA matrix. MALDI TOF data for peptides are shown in Table 3.

Solid-Phase Peptide Synthesis. All peptides were synthesized manually in a sintered vessel equipped with a stopcock. Rink amide resin with loading value 0.7−1.0 mmol/g was used, and standard Fmoc chemistry was employed. Resin bound Fmoc group was first deprotected with 20% piperidine in DMF, and the coupling reaction was carried out with in situ active ester method, using HBTU as a coupling reagent and HOBt as a recemization suppresser and DIPEA as a catalyst. All the materials used were of peptide synthesis grade and were used without further purification.

Resin Functionalization. 100 mg resin was taken in sintered and rinsed with 5 mL of dry DCM and filtered, the process was repeated 3 times, and the resulting resin was kept overnight in 5 mL DCM for swelling. DCM was filtered and rinsed 3 times with dry DMF and kept 5 h in 3 mL dry DMF for swelling before putting first for coupling.

General Protocol for Fmoc Deprotection and Amino Acid Coupling. The resin was preswollen overnight, and the following steps were performed for each cycle: (i) Wash with DMF 4 × 5 mL; (ii) deprotection of Fmoc group with 20% piperidine in DMF 3 × 5 mL (20 min for each); (iii) wash resin with DMF (3 × 5 mL), methanol (3 × 5 mL), DCM (3 × 5 mL), and DMF (3 × 5 mL); (iv) test for complete deprotection (ninhydrine or chloronil test); (v) coupling reaction with 3 equiv each amino acid, DIPEA, HOBt, and HBTU in minimum volume of DMF; and (vi) test for completion of coupling reaction (ninhydrin or chloronil test).

This cycle was repeated for each coupling. The completion of coupling and deprotection reactions was monitored by a combination of Kaiser's (ninhydrin) test and chloronil test. In case of positive test after coupling, the second coupling was performed before deprotection of Fmoc group, followed by capping reaction with 1:1:1 mixture of acetic anhydride, DMAP, and DMF.

Peptide Cleavage from the Resin. The dry peptide-resin (20 mg) was placed in a round-bottomed flask, and to this a 5% solution of 95:5 TFA:TIPS in DCM (10 mL) was added. The resulting mixture was stirred gently using magnetic stirrer for 1 h and was filtered through a sintered funnel, and the resin was further washed with 3 × 5 mL of above solution. The filtrate was collected in a round-bottomed flak and evaporated under reduced pressure. The resin was again washed with methanol 3 × 5 mL, and washings were collected in the same round-bottom flask and evaporated to dryness. The crude peptide obtained still may contain a N^4-tBoc group which was deprotected by stirring the peptide solution with 10 mL of 95:5 mixture of TFA:TIPS in DCM for 4 h. The TFA:DCM mixture was removed under reduced pressure, and the residue obtained was dissolved in 0.4 mL of anhydrous methanol, followed by addition of 20 mL anhydrous diethyl ether, and afforded the off-white crude peptide precipitate.

Determination of pK_a of 4-Amino Group of 4*R*/*S*-Aminoproline Monomers and Peptides. The pK_a of 4-*R*/*S* amino group in monomers and selected peptides was determined by titrating an aqueous solution of the corresponding α-N-protected monomers and peptides with 0.01N aq. NaOH. The pH changes during the titration were monitored by a pH meter and plotted against the volume of added NaOH. This plot shows two sharp pH transitions, the lower one corresponding to pK_a of α-carboxylic acid group and higher pK_a corresponding to 4-amino group (Supporting Information). The pK_a values of 4-amino group for 4*R*-Amp and 4*S*-amp monomers are 10.2 and 9.3.

Circular Dichroism (CD) studies. CD measurements were performed on CD spectropolarimeter, using a quartz cylindrical cuvette of 1 cm path length with water circulation jet. The CD spectra were obtained by continuous wavelength scan (average of 5 scans) from 260 to 190 nm at a scan speed of 100 nm/min. All samples were dissolved in corresponding buffer and stored at 4 °C for at least 24 h prior to allow equilibration of strand association to triple helix. The thermal CD spectral studies were conducted using water circulator. The CD spectra of samples were recorded in the range 4−80/90 °C at increments of 5°. The value of T_m was determined by plotting the elipticity at 225 nm against temperature and from the first derivative plots of the sigmoidal transitions.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b01032.

NMR spectra (^{1}H, ^{13}C, DEPT) and HRMS of compounds **21**−**27**, HPLC, MALDI-TOF spectra, and CD data of peptides **7**−**19** (PDF)

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Notes

The authors declare no competing financial interest.

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