Conformation and Morphology of 4-(NH$_2$/OH)-Substituted L/D-Prolyl Polypeptides: Effect of Homo- and Heterochiral Backbones on Formation of $\beta$-Structures and Nanofibers

Bharath Raj Madhanagopal, Shahaji H. More, Nitin D. Bansode, and Krishna N. Ganesh*

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ABSTRACT: The relative stereochemistry of C2 and C4 in 4-substituted prolyl polypeptides plays an important role in defining the derived conformation in solution. cis-(2S,4S)-Amino/hydroxy-L-prolyl polypeptide (t$_C$-Amp$_S$/t$_C$-Hyp$_S$) shows a PPII conformation in phosphate buffer and a $\beta$-structure in a relatively hydrophobic solvent, trifluoroethanol (TFE). It is now demonstrated that the homochiral enantiomeric cis-substituted $\alpha$-prolyl polypeptide (t$_C$-Amp$_S$/t$_C$-Hyp$_S$) exhibits mirror image $\beta$-structures in TFE. In the case of alternating heterochiral prolyl peptides, it is the trans-substituted [S$_C$(2S,4R)-t$_C$(2R,4S)$_{\alpha}$]$_n$ prolyl polypeptide that shows $\beta$-structures in TFE, while the cis-substituted [S$_C$(2S,4S)-t$_C$(2R,4R)$_{\alpha}$]$_n$ prolyl polypeptide is disordered in both phosphate buffer and TFE. The results highlight the important chirality-specific structural requirements for $\beta$-structure formation. The observed conformation in solution (circular dichroism (CD)) is also correlated with the morphology of the self-assemblies (field emission scanning electron microscopy (FESEM)), with the PPII form leading to spherical nanoparticles and $\beta$-structures leading to nanofiber formation. The results shed light on the role of relative stereochemistry at C2 and C4 in defining the polyproline peptide conformation in solution and how different conformations drive self-assemblies of peptides toward specific nanostructures.

INTRODUCTION

Supramolecular assemblies of polypeptides have found utility in many applications including drug delivery and tissue regeneration. The repertoire of chemical functional groups available in the side chains of natural and synthetic amino acid building blocks, combined with well-defined conformation of the peptide backbone, has led to a plethora of self-assembled peptide nanostructures, such as spheres, fibrils, sheets, hollow vesicles, nanotubes, and networks. Despite this expansion, the understanding of the morphogenesis of these assemblies still remains inadequate to predict and control their morphology through tailoring their chemical structures and manipulating the environmental conditions.

The rational design of peptides for self-assembly involves consideration of various factors, such as inter-/intramolecular hydrogen bonds, hydrophobic effects, electrostatic forces, van der Waals’ interactions, and dipole–dipole interactions arising from the chemical structures. While helices emerge from intramolecular H-bonds, $\beta$-structures engage interchain backbone hydrogen bonds between peptide strands and dominate as a self-assembling peptide motif. Variation of charge and hydrophobicity of peptide side chains provide additional repertoire for designing peptides to grow into extended $\beta$-sheets. The PPII helix form that is prevalent in polyproline and collagen peptides can be used as a versatile scaffold to arrange charged hydrophilic and neutral hydrophobic groups at specific positions along the helix. Several design strategies have been implemented for hierarchical self-assembly of collagen mimetic peptides to yield morphologically well-defined nanostructures. These include the use of metal ions to generate cagelike hollow structures by collagenous peptides bearing metal-binding ligands and the use of electrostatic interactions between negatively and positively charged amino acid residues. By employing the latter strategy, a variety of morphologies, such as nanofibers, hydrogels, and nanosheets, have been created by placing different amino acids at strategic positions along the PPII chains in the collagen triple helices to induce favorable intermolecular salt bridges. Polyproline peptides are also amenable to stereospecific modifications, in particular at C4, to provide a platform to spatially organize functional groups at regular intervals along a PPII helix. Such peptides have been shown to assemble into fibers or into an exquisite molecular interface.

We have earlier demonstrated solvent-dependent changes of conformation from a PPII helix in aqueous (aq) phosphate
buffer to a $\beta$-structure in trifluoroethanol (TFE) in polypeptides derived from cis-(2S,4S)-hydroxy/amino-l-proline (Figure 1A).24,25 The two strands of the $\beta$-sheet-like structure observed in TFE were shown to be aligned in an antiparallel orientation.25 The cis-(2S,4S)-4-hydroxy-l-prolyl polypeptide (1c-Hyp)$_2$ forms a $\beta$-structure upon conjugation with the C$_{12}$-C$_{16}$ chain at the N-terminus and does not show any regular morphology in water but assembles into long nanofibers in TFE.25 The R/S stereochemical nature of C4-((OH/NH$_2$) substitution on l-proline is crucial in influencing the conformation adopted by the polypeptides; only the cis-(2S,4S) disposition of C2 and C4 substituents on l-proline (Figure 1A) leads to a $\beta$-sheet-like structure in TFE. The trans-(2S,4R) orientation of substituents (Figure 1B) failed to show the $\beta$-sheet-like structure and formed only the PPII structure in TFE. In this context, it would be interesting to study the generality of conformational features in 4-substituted l/d-prolyl polypeptides corresponding to all four diastereomers (Figure 1). Herein, we examine the comparative conformational features of the 4(NH$_2$/OH) substituents on l/d-prolines in both homochiral (all-l, or all-d-prolyl units) and heterochiral (alternating l- and d-prolyl units) polypeptides and the role of conformation in solution in the associated morphology arising from self-assembly. It was found that the $\beta$-sheet-like structure in TFE resulted from homochiral l- and d-polypeptides containing cis-2,4-disubstituted scaffolds. On the other hand, in heterochiral polypeptides with backbone having alternating l/d-prolyl units, the same $\beta$-sheet-like structure was formed by the trans-2,4-disubstituted scaffolds.

### RESULTS AND DISCUSSION

**Synthesis of 4(R/S)-Amino-l/d-prolyl Polypeptides.**

Fmoc-protected prolines 1 and 2, along with the two sets of four diastereomers each from 4(R/S)-amino-l/d-proline (3–6) and 4(R/S)-hydroxy-l/d-proline (7–10) (Figure 2) were used for the synthesis of homo- and heterochiral polypeptides. The monomers 1, 2, and 7 were obtained from commercial sources, and the monomers 3, 4, 5, 8, and 9 were synthesized following the procedures reported earlier.25 The d-monomers (4R)-(N$^4$Boc)-N1(Fmoc)-d-Amp (6) and (4R)-O$^4$(Bu)-N1(Fmoc)-d-Hyp (10) were synthesized as shown in Scheme 1, starting from the (4R)-OH-N1(Cbz)-d-Pro-methyl ester (11), which was reported previously.27 The methyl ester 11 was treated with methyl p-toluenesulfonate under Mitsunobu reaction conditions to obtain (4S)-OTs derivative 12, followed by the reaction with NaN$_3$ accompanied by reversion at C4 to yield (4R)-N$_3$ compound 13 that showed a characteristic IR stretching frequency for the azide at 2104 cm$^{-1}$. The azide 13 was reduced by the Staudinger reaction to obtain the (4R)-NH$_2$ derivative, which was in situ protected as its Boc derivative to yield (4R)-N$^4$(Boc)-d-Pro methyl ester 14. Hydrolysis of the ester 14 with aq LiOH and deprotection of N1(Cbz) by hydrogenation followed by reprotection to the N$^3$-Fmoc derivative yielded monomer 6. For the preparation of 10, the reaction of 11 with tert-butyl bromide to yield 4R (O$^4$-tert-butyl) ether 15 was followed by the same sequence of hydrolysis and reductive deprotection and Fmoc protection to afford the final compound. All synthesized monomers were characterized by their NMR and mass spectral data, and the chiral purities of 4 and 6 were confirmed by chiral high-performance liquid chromatography (HPLC). While l-monomers were synthesized as Boc derivatives (3 and 5), d-monomers were synthesized as Fmoc derivatives (4 and 6). For determining the chiral purity, the corresponding enantiomeric pairs 4e and 6e were synthesized. The chromatogram revealed that the two d-diastereomeric monomers (4 and 6) clearly resolved from their respective l-enantiomers (4e and 6e), each exhibiting unique retention times, with no contamination from other diastereomers (Figure S1, Supporting Information).

The protected monomers (1–10) were used for the synthesis of polypeptides P1–P12 on a solid support (4-methyl benzhydrylamine (MBHA) or Rink amide resin) using either t-Boc (for P2 and P3) or Fmoc chemistry (for P1 and P4–P12) following standard protocols.28–30 The sequences of various homochiral (all-l, P1–P5, and all-d, P6–P10) and heterochiral (alternating l/d, P11 and P12) polypeptides synthesized are shown in Figure 3. The l- and d-peptides were capped at the N-terminus by l- or d-phenylalanine,
respectively, to enable the measurement of peptide concentrations using their extinction coefficients at 257 nm (195 M⁻¹ cm⁻¹).³¹ Tyrosine or tryptophan has a better extinction coefficient than phenylalanine but were not preferred at the N-terminus to avoid their effects on peptide folding. Tyrosine with phenolic hydroxyl and the indole ring in tryptophan may be active in imparting H-bonding and aromatic stacking at the N-terminus to influence the chirality of peptide chains. All peptides were purified by HPLC, and their identity was established by mass spectral data (Table S1, Supporting Information).

Circular Dichroism (CD) Conformational Studies of Homochiral Enantiomeric LC-Amp₉ and DC-Amp₉ Polypeptides. Figure 4A shows the CD spectra of unsubstituted prolyl enantiomeric peptides L-Pro₉ (P1) and D-Pro₉ (P6) in phosphate buffer and trifluoroethanol (TFE). The CD spectra are exact mirror images as expected for true enantiomers, and no changes in the conformation of peptides were seen in the two solvents. The 4-amino-substituted homochiral enantiomeric peptides LC-Amp₉ (P3) and DC-Amp₉ (P8) showed CD spectra in buffer with bands at 225 and 205–207 nm corresponding to the n → π⁺ and π → π⁺ transitions of the peptide bonds³² (Figure 4B). The CD spectra were mirror images with respect to signs of the bands but not exactly with respect to the intensities: the L-peptide P3 had a slightly higher intensity at the 205–207 nm region than the D-peptide P8.

Scheme 1. Synthesis of (4R)-N⁴(Boc)-N₁(Fmoc)-D-Amp (6) and (4S)-O⁴-tBu-N₁-Fmoc-D-Hyp (10)⁴⁵

⁴⁵(a) PPh₃, diisopropyl azodicarboxylate (DIAD), methyl p-toluenesulfonate, dry tetrahydrofuran (THF) (56%); (b) NaN₃, 60 °C, dimethylformamide (DMF), 3 h (73%); (c) PPh₃, dry THF; (d) (Boc)₂O, dioxane/water (50:50) (93%); (e) 2 N aq LiOH; (f) H₂, Pd/C, methanol; (g) Fmoc-Cl, 10% Na₂CO₃, dioxane/water (50:50) (50%). (h) tBu-Br, Ag₂O (90%); (i) 2 N LiOH; (j) H₂, Pd/C; (k) Fmoc-Cl, 10% Na₂CO₃ (50%).

Figure 3. Structures of prolyl polypeptides. P1−P5: L-(2S)-homopolypeptides; P6−P10: D-(2R)-homopolypeptides; P11: heterochiral, alternating, all-cis [lc−dc]-polypeptide; P12: heterochiral, alternating, all-trans [lc−dc] polypeptide; and P13: L-Phe-[l-Pro-d-Pro]-l-Pro.
The conformational profile of L-amp9 (P3) in buffer is typical of left-handed PPII (similar to P1)\textsuperscript{32} and hence the mirror image CD spectral profile of enantiomeric D-amp9 (P8) (similar to D-pro9 P6) represents a right-handed PPII form. This is consistent with an earlier observation that has reported mirror image CD spectra for D-proline.\textsuperscript{33} The CD spectra of enantiomeric peptides LC-amp9 (P3) and DC-amp9 (P8) in TFE are shown in Figure 4C. LC-amp9 (P3) exhibited a large negative band at 220 nm arising from the n → π\* transition, with the appearance of a large positive band at 197 nm and disappearance of the mild positive band at 225 nm. This profile is characteristic of a \( \beta \)-sheet-like structure with a right-handed twist as established before.\textsuperscript{34} The CD spectra of enantiomeric D-amp9 (P8) showed an inversion in the sign of bands, with a strong positive band at 215 nm and a negative band at 197 nm representing a mirror image \( \beta \)-sheet-like structure with a left-handed twist, which is unknown in the literature. Although mirror images in terms of their sign, the CD spectra of P3 and P8 in TFE are not exactly mirror images in terms of intensities and depict slight asymmetry in their band positions (Figure 4C). In view of the fact that unsubstituted L-pro9 (P1) and D-pro9 (P6) peptides have exact mirror image CD profiles in both aqueous phosphate buffer and TFE, the slight asymmetric effects seen in CD spectra of P3 and P8 point to subtle conformational differences induced by the C4-amino substituent at the microstructural level.

Conversely, the CD spectra of enantiomeric trans peptides L-amp9 (P2) and D-amp9 (P7) were similar in aqueous phosphate buffer and TFE. They exhibited CD bands that were relatively weaker for the n → π\* transition at 220–225 nm and a stronger band for the \( \pi \) → π\* transition at 200–205 nm (Figure S2, Supporting Information). These bands were of opposite sign, and D-amp9 (P7) showed lower-intensity bands than the enantiomeric peptide L-amp9 (P2) in both buffer and TFE. The CD profiles of trans peptides L-amp9 (P2) and D-amp9 (P7) in TFE indicated non-formation of the \( \beta \)-structure by either in TFE.

**CD Conformational Studies of Homochiral Enantiomeric Lc-Hyp9 and Dc-Hyp9 Polypeptides.** As reported earlier,\textsuperscript{25} the Lc-hyp9 (P5) peptide showed a PPII conformation in buffer and \( \beta \)-sheet-like CD spectra in TFE with a negative band at around 212 nm and a positive band at around...
The enantiomeric peptide \( \text{D-L-Hyp}_9 \) (P10) showed almost perfect mirror image CD spectra in aqueous phosphate buffer (Figure 5A), and in TFE, the spectra showed inversion of bands at almost similar wavelength, but the intensity of the positive band at 215 nm was significantly larger. The disparity of CD band intensities in enantiomeric peptides in \( \text{L-L-Hyp}_9 \) (P5) and \( \text{D-L-Hyp}_9 \) (P10) was qualitatively similar to that seen in enantiomeric \( \text{L-Amp}_9 \) (P3) and \( \text{D-Amp}_9 \) (P8) (Figure 4C) and largely amplified in TFE, with \( \beta \)-peptide showing higher intensity than \( \alpha \)-peptide.

The trans-\( \text{L-L-Hyp}_9 \) (P4) and \( \text{D-L-Hyp}_9 \) (P9) polypeptides in aqueous phosphate buffer showed CD spectra with inversion in sign of bands at 225 and 205–207 nm (Figure S2, Supporting Information), with the CD profile of \( \alpha \)-peptide P4 characteristic of the left-handed PPII conformation.45 Thus, the mirror image CD profile of \( \beta \)-peptide P9 corresponds to the right-handed PPII conformation. Although such inversion is established in unsubstituted \( \beta \)-proline polypeptides, it is unknown in 4-substituted \( \alpha \)-prolyl peptides. However, in TFE, both \( \alpha \)- and \( \beta \)-peptide did not exhibit a \( \beta \)-sheet-like CD profile seen with the corresponding cis-\( \text{L-L} \)- and \( \text{D-L-Hyp}_9 \) polypeptides23 and retained their PPII conformation with minor differences in band intensities.

As seen from the above discussion, the 4-NH\(_2\)/OH-substituted homochiral prolyl polypeptides (P3/P8; P5/P10) show a \( \beta \)-like structure in TFE only when the substituents at C2 and C4 (NH\(_2\)/OH) are cis in relative orientation (2S,4S for \( \text{L-L} \) and 2R,4R for \( \text{D-L} \)-prolyl polypeptides). The trans-substituted L/\( \beta \)-prolyl polypeptides (2S,4R; \( \text{L-L} \)-P2/P7; \( \text{D-L} \)-P4/P9) yielded only the PPII form in both aqueous phosphate buffer, and TFE and no \( \beta \)-sheet-like structure in TFE was seen, unlike that of \( \text{L-L} \)/D-\( \beta \)-peptides. This unambiguously suggested the importance of relative stereochemistry at both C2 and C4 for formation of \( \beta \)-sheet-like structures from all dysteroremers of 4-substituted prolyl peptides.

**CD Conformational Studies of Alternating Hetero-Chiral [L\(_4\)-C\(_2\)-L\(_4\)-C\(_2\)] and [L\(_4\)-D\(_7\)-L\(_4\)-L\(_7\)] Hyp\(_9\) Polypeptides.** In view of the unique properties exhibited by homochiral 4-NH\(_2\)/OH cis-substituted \( \text{L-L} \)- and \( \text{D-D} \)-polypeptides, it was thought to assess heterochiral alternating \( \alpha \)- and \( \beta \)-Hyp\(_9\) polypeptides [L\(_4\)-C\(_2\)-L\(_4\)-C\(_2\)] (P11) and [L\(_4\)-D\(_7\)-L\(_4\)-L\(_7\)] (P12). The CD spectra of alternating [L\(_4\)-C\(_2\)-L\(_4\)-C\(_2\)] (P11) (Figure 6A) showed a strong negative CD band at 195 nm in phosphate buffer, an intense negative CD band at 215 nm in TFE, and absence of any band at higher wavelength. This indicates a random conformation, in contrast to ordered structures of homochiral all-cis L\(_4\)-C\(_2\)-Amp\(_9\) (P3)/L\(_4\)-Hyp\(_9\) (P8) and D\(_4\)-Amp\(_9\) (P5)/D\(_4\)-Hyp\(_9\) (P10) polypeptides that assumed a PPII form in phosphate buffer and a \( \beta \)-sheet-like structure in TFE.

The heterochiral trans peptide [L\(_4\)-D\(_7\)-L\(_4\)-L\(_7\)] (P12) in buffer showed a moderately positive band at 195 nm, a strong negative CD band at 207 nm, and a weak positive CD band at 225 nm, indicative of a left-handed PPII structure (Figure 6B), characteristic of \( \alpha \)-polypeptides (L\(_4\)-Amp\(_9\) (P3)/L\(_4\)-Hyp\(_9\) (P5)). In TFE, P12 showed a negative band at 200 nm and a strong positive band at 218 nm, similar to the characteristic \( \beta \)-sheet-like structure of \( \beta \)-polypeptides P8 and P10. Thus, in the alternating heterochiral trans peptide [L\(_4\)-D\(_7\)-L\(_4\)-L\(_7\)] (P12), the left-handed \( \beta \)-PII structure seen in buffer assumes a right-handed twisted \( \alpha \)-structure in TFE. In heterochiral peptides, the determinant for handedness of PPII in buffer seems to be the \( \beta \)-proline, while in TFE, the handedness of the twisted \( \beta \)-structure seems to be directed by \( \alpha \)-proline. The molecular origin of differential adoption of handedness in the PPII conformation and twisted \( \beta \)-sheet-like structures cannot be deciphered from the present work.53 What is interesting is the fact that homochiral (\( \text{L-L} \) and \( \text{D-D} \)) polypeptides show a \( \beta \)-structure in TFE, while with alternating heterochiral peptides (\( \text{L-L} \)-D\(_7\)), it is the \( \beta \)-peptide that forms the \( \beta \)-structure.

The heterochiral polypeptide [L\(_4\)-D\(_7\)] \( \alpha \)-peptides with alternating \( \beta \)/\( \alpha \) conformations are reported to adopt conformations other than typical PPII and PPII in water, depending on the \( \beta \)/\( \alpha \) nature of N-terminus amino acid.54 When peptide length \( n \) is odd (N-terminus \( \beta \) ), the CD spectra resemble the \( \beta \)-structure, and with number of residues \( n \) even (N-terminus \( \alpha \) ), the peptide has an undefined or a random conformation. This arises from the tendency of the L-Pro–D-Pro peptide amide bonds to adopt alternating trans and cis geometries at \( \beta \)/\( \alpha \)- and D–L steps.56,57 The peptide L-Phe–(L-Pro–D-Pro)–L-Pro (P13) exhibited a CD spectrum typical of the left-handed PPII form similar to that of L-Pro\(_9\) (P1) (Figure 6), which has all amides in trans form. The observed CD profile is thus dictated by the C-terminus L-Pro. In TFE, the peptide P13 adopted a \( \beta \)-like conformation with resemblance to the CD pattern reported for [L-Pro–D-Pro]–L-Pro in water.

Given this solvent-dependent conformational behavior of heterochiral unsubstituted poly(L–D) proline peptides, the interpretation of the conformational properties of alternating 4-R/S-substituted peptides is complex due to superimposition of chiral effects at both the backbone and side chains (C4) on the overall conformation of the peptide in TFE. The CD spectrum of trans-[L\(_4\)-D\(_7\)+L\(_4\)-L\(_7\)] (P12) recorded in TFE (Figure 6) suggests that in 4-Hyp peptides with an alternating L–D backbone, the handedness of \( \beta \)-structure is dominated by the D-Hyp residues rather than L-Hyp. In cis-[L\(_4\)-D\(_7\)] (P11), the conformation adopted by the peptide is random (lack of 215 nm band) in both aqueous phosphate buffer and TFE (Figure 6). These effects perhaps arise from unfavorable combinations of cis/trans-prolyl amide bonds on the backbone (Table 1).
Role of TFE and β-Structure Formation. It is well established that trans LT-Amp9 (P2) and LT-Hyp9 (P4) polypeptides adopt the PPII conformation in aqueous solvents with prolyl rings in the C4-exo conformation (Figure 7A). It is stabilized by secondary factors such as stereoelectronic effects, N1-C4 gauche effects, and n−π* interactions from N1-tertiary amide carbonyl to C2 carbonyl.29,36 The cis-LC-Amp9 (P3) and LC-Hyp9 (P5) peptides show the PPII conformation with prolyl ring assuming C4-endo pucker (Figure 7B,D).39 In this conformation, the (4S)-(OH/NH2) substituent is

Table 1. CD Spectral Features of Heterochiral Polyprolyl Peptides in Buffer and TFE

| peptide                          | water/PBS | TFE remarks                          |
|----------------------------------|-----------|--------------------------------------|
| [1c0c]Lc-Lc (P11)a                | 190 nm (slightly +ve) | 190 nm (+ve) unfavorable cis–trans-amide geometry |
|                                  | 205–210 nm (−ve) | 215 nm (−ve) |
|                                  | random/undefined | random/undefined |
| [1a0l]Lc-La (P12)a                | 190–195 nm (+ve) | 195 nm (+ve) all-trans-amide bonds in both phosphate buffer and TFE |
|                                  | 205–210 nm (−ve) | 200 nm (−ve) |
|                                  | 225 nm (+ve) | 220 nm (+ve) |
|                                  | left-handed, lβ-like | lβ-like |
| [l-Pro-D-Pro]Lc-L-Pro (P13)a      | 190–195 nm (+ve) | 195 nm (+ve) alternating cis–trans-amide bonds |
|                                  | 205–210 nm (−ve) | 212 nm (−ve) |
|                                  | 225 nm (+ve) | 220 nm (+ve) |
|                                  | left-handed, lβ-like | lβ-like |
| Boc[l-Pro-D-Pro]l-Pro (n = 1–3)b  | 195 nm (+ve) | alternating cis–trans-amide bonds |
|                                  | 215–220 nm (−ve) | |
|                                  | lβ-like | |
| Boc[l-D-Pro-l-Pro]l-Pro (n = 1–3)b| 190 nm (nearly zero) | alternating cis–trans-amide bonds |
|                                  | 210 nm (−ve) | |
|                                  | random/undefined | |

“Present work. bData from ref 36; PBS: phosphate-buffered saline, 10 mM, pH 7.0; TFE: trifluoroethanol.

Figure 7. Schematic diagrams of proline puckering in cis-(2S,4S) peptides P3 (X=NH) and P8 (X=O) in water (A, B) and in TFE (C). Hydrogen bonding schemes in l-Hyp9 (P8) (D) PPPII form in water with C4-endo proline pucker by intrarresidue H-bonding and (E) β-structure in TFE with C4-exo proline pucker by interchain H-bonding.
favorably placed for intramolecular H-bonding with C2 amide carbonyl, retaining the stabilizing effects of n−π* interactions. The cis disposition of C4 and C2 substituents allows intramolecular H-bonding between them, unlike that in the trans L-I-substituted prolyl peptide (Figure 7A) where the C2 and C4 substituents are oriented in opposite directions. Both 4S- and 4R-substituted I-prolyl polypeptides adopt the PPII conformation in aqueous phosphate buffer. TFE as a solvent is generally known to stabilize the a-helix in peptides. The bulk dielectric constant for pure TFE is about one-third that of water, and it is a much weaker hydrogen-bond acceptor (less negative charge on O due to vicinal electron-withdrawing CF3) but relatively a better H-bond donor than water. The polar hydrophobicity of TFE (due to CF3) changes the water structure, leading to hydrophobic dehydration (Figure 7C). The higher H-bonding donor ability of the OH group of TFE results in its complexation with the 2 carbonyl group, hampering its intrasidue H-bonding with the (4S)-(NH2/OH) group (Figure 7C). The loss of H-bonding and interfering steric effects of the CF3 group flips the C4-endopuck of proline to C4-exo pucker, pushing the (4S)-NH2/OH group into a pseudo-equatorial position, where it is now available for possible interchain H-bonding to generate a b-structure (Figure 7E). Such proline ring puckering to accommodate solvent effects is reasonable as ring pucker interconversion at room temperature has a low activation barrier of 2−5 kcal/mol with time scales of picoseconds, while trans-cis amide bond conversion has a higher activation barrier ~20 kcal/mol with time scales of seconds to minutes at room temperature.

As already observed, the enantiomeric cis polypeptide DC-Amp9 (P8) exhibits CD spectra corresponding to the right-handed PPII form in aqueous phosphate buffer and the β-sheet-like structure (left-handed) in TFE. The CD spectra of cis peptide L-I-Amp9(P3) recorded in other hydrophobic solvents such as methanol and acetonitrile show formation of β-structures (Figure S3, Supporting Information). These results imply the generality of the β-structure of cis-4-(NH2/OH)-substituted homochiral I-/I-prolyl polypeptides in solvents that are more hydrophobic than water. The formation of β-structure in these peptides (P3 and P8) is facilitated by a flip at C4 in proline puckering to place the 4-substituent in a more accessible pseudo-equatorial position (Figure 7C). Although there is no direct proof for this structure at this time, it has been reported earlier that poly-isoleucine, which shows α-helix in aqueous solvents, switches to a β-conformation in TFE caused by low dielectric constant and steric effects of the solvent.

The slight asymmetry in the mirror images of CD spectra observed for enantiomeric 4-substituted proline derivatives (but not in unsubstituted L- and D-proline) for β-structures in TFE is surprising, but it has some literature precedence in enantiomeric linear peptides. The conformational preferences in polypeptide structures are governed by an intricate balance among the nature of 4-substituent, stereoelectronic factors, gauche effects (N1-C4H), and n−π* interactions. A stereoinversion at C2 (L to D) might differentially affect such nonbonded secondary interactions, which may not necessarily translate into mirror image effects, particularly in five-membered puckered systems. Only one report exists on linking n−π* interactions to chirality at C2 in prolines from studies of five diastereomeric sets of 4R-substituted proline monomers from available crystal structure data. The results suggest that n−π* interactions can induce pyramidalization of C2 carbonyl, creating a stereogenic center at the carbonyl through electronic delocalization. Being adjacent to an existing chiral center at C2 (L/D), such induced chiral effects from n−π* interactions in monomers additively build up in proline oligomers and further reinforced by chirality of C4 substituents. This enantiomer-specific effect may contribute to imparting some nonmirror image profiles in CD spectra of enantiomeric pairs. It is also known that the major contributions to CD spectra of peptides are from the backbone secondary structure with minor contributions from side chains. The fact that unsubstituted L- and D-prolyl polypeptides show exact mirror images but not the 4-substituted L/ I-prolyl peptides suggests that the observed distortion from ideal mirror image CD profiles may originate from nonbonding interactions of 4(R/S)-substituents.

It may be recalled that way back in 1953, Linus Pauling had proposed that polypeptide chains possessing alternate L- and D-amino acids can give rise to rippled sheet structures. It was later found that helices from peptides with alternating L- and D-amino acids have torsional angles in relevant β-regions of the Ramachandran plot and form either single- or double-stranded helices. In this background, the β-structure observed for the alternating heterochiral peptide L-DT-P12 in TFE resembling that of d-cis peptide DC- Hyp9 (P8) is in conformity with the predictions of Pauling and substantiates the projections from the Ramachandran plot.

**Morphology of Self-Assembled 4(R/S)-Amino-L-/I-prolyl Polypeptides.** It was previously observed that the conformation adopted in solution manifested in the self-assembly-induced morphology as seen by field emission scanning electron microscopy (FESEM) images. The PPII conformation of Hyp polypeptides in buffer led to spherical nanoparticles, while the β-like structure resulted in long twisted nanofibers. Figure 8 shows FESEM and atomic force microscopy (AFM) images of the enantiomeric pairs L-I-Amp9 (P3) and DC-Amp9 (P8). Both peptides in water showed irregular aggregates of random shapes (Figure 8A,D), while in TFE, they self-assembled into nice long twisted fibrous structures (Figure 8B,E) with a helical pitch of around 80 nm and a width of 15−20 nm. The individual fibrils intertwined around each other to form longer and thicker fibers up to 60 nm in width and several micrometers in length (Figure S4, Supporting Information), similar to protofilaments of amyloid fibers. The left-handed helical nature of the
individual fibrils was retained in fibers and strongly pronounced during the hierarchical assembly. The AFM images (Figure 8C,F) revealed heights of nanofibrils to be 2–3 nm (Figure S5, Supporting Information), suggesting stacks of the polypeptides in fibrils with some particles still bound to fibers.

Contrary to expectations, the nanofibers from enantiomeric homochiral peptides \( \text{L-} \text{Amp}_9 \) (P3) and \( \text{D-} \text{Amp}_9 \) (P8) exhibited similar left-handed helical twists in the FESEM images (Figure 8B,E). Thus, although in TFE solution the enantiomeric polypeptides exhibited opposite helicity (Figure 4C), it was not translated into the self-assembled morphological forms. Both polypeptides displayed an inherent preference for left-handedness in the formation of supramolecular twisted fibers. The peptides \( \text{L-} \text{Amp}_9 \) (P2) and \( \text{D-} \text{Amp}_9 \) (P7) that failed to show \( \beta \)-structure in TFE solution formed nanoparticles of random shapes in both water and TFE (Figure 9). Similarly, nanofibrous morphology was found to be inaccessible to unsubstituted \( \text{L-Pro}_9 \) (P1) and \( \text{D-Pro}_9 \) (P6) peptides (Figure S6, Supporting Information). Although anisotropic straw- or spindle-like structures were observed, the morphologies of \( \text{L-Pro}_9 \) (P1) and \( \text{D-Pro}_9 \) (P6) were distinctly different from those of well-defined nanofibers formed by \( \text{L-} \text{Amp}_9 \) (P3) and \( \text{D-} \text{Amp}_9 \) (P8). Thus, the discrete nanoparticles seen in FESEM images corroborated with the PPII conformation seen in solution and nanofibers with a \( \beta \)-like structure in solution, underlining the role of peptide secondary structures in dictating the morphology of the supramolecular assemblies.

To test if the supramolecular associations arise from the natural assembly process in solution or consequences of aggregation during solvent evaporation on a surface, dynamic light scattering (DLS) data in buffer was recorded for \( \text{L-} \text{Amp}_9 \) (P3) in solution (Figure S7, Supporting Information). The data indicated a size distribution of 100–500 nm particles in TFE, which was absent in water. The peptide \( \text{L-} \text{Amp}_9 \) (P2) that did not form \( \beta \)-structures in TFE, showed the absence of large size particles in both water and TFE. This suggested self-assembly of \( \text{cis} \) peptide \( \text{L-} \text{Amp}_9 \) (P3) only in TFE, and in the case of \( \text{L-} \text{Amp}_9 \) (P2), no such assemblies were noticed in both water and TFE. Thus, the conformation seen from CD in solution very well correlates with images seen in FESEM.

**Figure 9.** FESEM micrographs of \( \text{trans} \) peptides. \( \text{L-} \text{Amp}_9 \) (P2) in (A) water and (B) TFE. \( \text{D-} \text{Amp}_9 \) (P7) in (C) water and (D) TFE.

**Effect of Concentration on Morphology of \( \text{L-} \text{Amp}_9 \) (P3) in TFE.** Since self-assembly is promoted at higher concentrations, FESEM images of \( \text{cis} \) peptide \( \text{L-} \text{Amp}_9 \) (P3) in TFE were recorded at increasing concentrations (2 \( \mu \)M, 50 \( \mu \)M, 100 \( \mu \)M, 150 \( \mu \)M, 200 \( \mu \)M, and 2 mM) after incubation for 12 h. At a low concentration of 2 \( \mu \)M, P3 formed spherical and elongated structures of <100 nm in width (Figure 10A), and at 50 \( \mu \)M concentration, fibers emerged along with spherical nanoparticles (Figure 10B). In the concentration range 100–200 \( \mu \)M, nanofibers became more prominent (Figure 10C–E), and at 2 mM, well-defined fibrils of lengths in the range 1–3 \( \mu \)m formed with good dispersion. These results illustrate that while the assembly of the peptide strands is stunted at low concentrations (2–50 \( \mu \)M) to spherical or irregular nanoparticles, they grow into helical nanofibers at higher concentrations (50 \( \mu \)M to 2 mM).

**Time-Dependent Changes in the Morphology of \( \text{L-} \text{Amp}_9 \) (P3).** To examine if the initially formed smaller nanoparticle under kinetic control evolves into thermodynamically more stable nanofibers, the self-assembly of \( \text{L-} \text{Amp}_9 \) (P3) at 200 \( \mu \)M concentration was recorded by FESEM as a function of time. After incubation for 1 h, the peptide showed uniform spherical particles of 20–100 nm (Figure 11A), aligned along the thin fibrous axis. After 6 h, the nanofibers emerged along the axis through fusion and superposition of initially formed nanoparticles by mass transfer (Figure 11B). After 12 h, the spherical particles completely vanished with conversion into fully grown nanofibers (Figure 11C). The observed time-dependent changes in FESEM images pointed out that the early emerging spherical particles are a product of the self-assembly process under initial kinetic control, which over a period of 12 h slowly transformed into thermodynamically more stable helical nanofibrous structures, similar to those reported in the literature. The nanofibers even at <6 h were already helical, indicating that the development of helical twists of the fibers was synchronous with fibrils spinning out of the spherical particles rather than development at a later stage after fiber formation.

**Self-Assembly of the Enantiomeric Mixture of \( \text{L-} \text{Amp}_9 \) (P3) and \( \text{D-} \text{Amp}_9 \) (P8).** The enantiomeric peptides \( \text{L-} \text{Amp}_9 \) and \( \text{D-} \text{Amp}_9 \) formed nanoparticles of random shapes in both water and TFE (Figure 10A), and at 50 \( \mu \)M concentration, fibers emerged along with spherical nanoparticles (Figure 10B). In the concentration range 100–200 \( \mu \)M, nanofibers became more prominent (Figure 10C–E), and at 2 mM, well-defined fibrils of lengths in the range 1–3 \( \mu \)m formed with good dispersion. These results illustrate that while the assembly of the peptide strands is stunted at low concentrations (2–50 \( \mu \)M) to spherical or irregular nanoparticles, they grow into helical nanofibers at higher concentrations (50 \( \mu \)M to 2 mM).

**Figure 10.** FESEM images of morphologies formed by peptide \( \text{L-} \text{Amp}_9 \) (P3) in TFE at different concentrations: (A) 2 \( \mu \)M, (B) 50 \( \mu \)M, (C) 100 \( \mu \)M, (D) 150 \( \mu \)M, (E) 200 \( \mu \)M, and (F) 2 mM. Images were recorded after an incubation period of 12 h.
(P3) and DC-Amp9 (P8) show mirror image CD profiles in aqueous phosphate buffer and TFE, but their supramolecular assembly into nanofibers displayed same handedness and helical twist. To probe their co-assembly, the two enantiomeric peptides LC-Amp9 (P3) and DC-Amp9 (P8) were mixed in 1:1 molar ratio and FESEM images of the mixture are shown in Figure 12. Interestingly, the enantiomeric blend exhibited nanofibers with larger thickness (∼40 nm width) compared to that of individual fibers (20 nm width) accompanied by tighter winding of the helix. The helical twist remained left-handed as in the constituent peptides (Figure 8). This is perhaps a consequence of the same handedness of enantiomeric peptides, facilitating further winding of nanofibers around each other and reinforcing the formation of a thicker fiber with a tighter twist.

Figure 11. FESEM images of growth of nanofibers of peptide LC-Amp9 (P3) in TFE at different incubation times: (A) 1 h, (B) 6 h, and (C) 12 h. The concentration of the peptide was 200 μM.

Figure 12. Morphology of nanofibers of the enantiomeric mixture of LC-Amp9 (P3) and DC-Amp9 (P8) in TFE. (A) L-Peptide LC-Amp9 (P3). (B) 1:1 mixture of the peptide enantiomers, LC-Amp9 (P3) and DC-Amp9 (P8). (C) D-Peptide DC-Amp9 (P8).

Figure 13. FESEM images of alternating peptide [L-D-T]4L (P12) conjugated with fatty acid (C14) (A) in water and (C) in TFE. AFM images of P12 (B) in water and (D) in TFE. Insets in (B) and (D) show the height profiles of the nanostructures.

Self-Assembly of L-Hyp9 and D-Hyp9 Polypeptides.
The homochiral enantiomeric LC-Hyp9 (P5) and DC-Hyp9 (P10) polypeptides in water self-assembled into rice grain-like elongated nanoparticles with size ∼100 nm. However, in TFE, while the enantiomeric LC-Hyp9 (P5) assembled into nanorods of 1 μm length as reported earlier,25 DC-Hyp9 (P10) showed aggregates of disordered particles of 200 nm size (Figure S8, Supporting Information). Thus, unlike Amp9 peptides, free Hyp9 polypeptides in TFE do not form fibers and show nanofiber formation only when they are conjugated with fatty acid chains.25 The heterochiral alternating [L-D-C]4L peptide (P11) aggregated in water to form disordered particles of 200 nm size, but in TFE, it formed rods or spindle-shaped aggregates of length 200 nm and width 20 nm (Figure S9, Supporting Information) similar to those seen with homochiral LC-Hyp9.
The heterochiral trans peptide \([l_T D_T]_{A-T}\) (P12), which showed a left-handed PPII helix in aqueous phosphate buffer, self-assembled to form nanospheres of \(\sim 200\) nm diameter. In TFE, it exhibited short nanorods of length \(\sim 200\) nm and width \(20\)–\(30\) nm (Figure S9, Supporting Information). Since chemical conjugation to fatty acid at the N-terminus of the peptide has been shown to promote \(\beta\)-structures exhibiting homochiral \(\omega\)-Hyp to assemble into nanofibers in TFE,\(^{25}\) the peptide \([l_T D_T]_{A-T}\) (P12) was conjugated with C14 fatty acid and, its self-assembly in TFE was examined. The peptide \([l_T D_T]_{A-T}\) (P12) displayed nanofibrous morphology (Figure 13C), with nanofibers around 2–3 \(\mu\)m in length and 20 nm in height with a left-handed twist generating grooves with depth around 4–6 nm (Figure 13D).

**Conformation vs Morphology.** The observed \(\beta\)-structure in TFE for the cis-4-substituted L/D-prolyl polypeptides correlates well with the self-assembly of these peptides into nanofibers\(^{24,25,30}\) as seen from the FESEM images. The polypeptides with the PPII form assemble into irregular or spherical nanostructures (Figures 8A,D and 9A,C) in buffer. When the peptides are dissolved in TFE, hydrophobic desolvation of the peptide side chain and backbone leads to favorable formation of an interdigitated interchain H-bonding network (side chain to backbone) (Figure 14),\(^{54}\) resulting in \(\beta\)-structures with a chiral twist to form nanofibrils that associate into macroscopic fiber bundles.

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**Figure 14.** Possible mechanism of self-assembly of \(\omega\)-Amp\(_{P}\) (P3).

The morphological changes observed in the time-dependent FESEM studies suggest an interplay between kinetic and thermodynamic factors involved in the supramolecular assembly of Lc-Amp\(_{P}\) (P3).\(^{53}\) The intermolecular H-bonding interactions between peptide chains to form \(\beta\)-structures possibly trigger a kinetically favored nucleation process, resulting in the rapid formation of irregular achiral spherical aggregates within first 1 h. These spherical aggregates transform into thermodynamically stable supramolecular helical fibrils, which further combine laterally and extend along the axis to form larger fibers and subsequently into bundles over a period of 12 h (Figure 14). Only the cis peptides \(\omega\)-Amp\(_{P}\) (P3) and \(\delta\)-Amp\(_{P}\) (P8) exhibiting \(\beta\)-structures in TFE form nanofibers. The unsubstituted polypeptide peptides \(\omega\)-Pro\(_{A}\) (P1) and \(\delta\)-Pro\(_{A}\) (P6) and the trans peptides \(\omega\)-Amp\(_{P}\) (P2) and \(\delta\)-Amp\(_{P}\) (P7)] with no potential for \(\beta\)-structures do not self-assemble into nanofibers. On a similar line, we have recently reported that speiglermic collagen peptides constituting \(\alpha\)trans-(4R)-amino-\(\omega\)-proline and \(\alpha\)trans-(4S)-amino-\(\delta\)-proline that assume left- and right-handed PPII helical forms, respectively, self-assemble into achiral spherical nanostructures\(^{57}\) but not into nanofibers. This observation emphasizes the importance of stereospecific cis disposition of H-bonding C2 and C4 substituents on proline to favor \(\beta\)-structures in TFE and enable helical self-assembly in homochiral peptides.

The twist in the backbone of \(\beta\)-strands plays a critical role in determining the chirality of supramolecular fibrous helical assemblies.\(^{25}\) Peptides with an intrinsic right-handed twist assemble into left-handed helical nanofibers.\(^{34,51,52,55}\) Generally, the naturally occurring \(\omega\)-peptides exhibit a right-handed backbone twist, and several amyloid proteins and peptides are known to form left-handed helical assemblies.\(^{56}\) Peptides composed of \(\alpha\)-amino acids show helical assemblies with right-handed helicity, opposite of their \(\omega\)-counterpart.\(^{57}\) Nature employs proline-rich regions to prevent fibrillation of proteins.\(^{58}\) Introduction of a stretch of polypeptides in proteins was found to reduce the tendency of the protein to form \(\beta\) sheets, thereby reducing the chances of their aggregation into amyloidogenic fibers.\(^{58}\) Interestingly, while unsubstituted polypeptides adopt the PPII form, which prevents fibrillation, stereospecific C4(S)-NH\(_2\)/OH substitution favors fibrillation in TFE through formation of a \(\beta\)-structure-like conformation.

**CONCLUSIONS**

The unique conformational and self-assembling features of 4(NH\(_2\)/OH)-substituted cis-(2S,4S)-\(\omega\)-prolyl (P3) and cis-(2R,4R)-\(\omega\)-prolyl (P8) polypeptides reveal that mirror image PPII and \(\beta\)-structures were adopted by the enantiomeric peptides as seen by CD spectra. However, these were not perfect mirror images unlike those of unsubstituted \(\omega\)-prolyl enantiomeric pairs P1/P6. The observed slight asymmetry in band positions and intensities in CD spectra of enantiomeric peptides reveal that the substitution at C4 with an amino group leads to subtle changes at microstructural levels among the enantiopairs, perhaps induced by nonbonded interactions. The PPII form in aqueous buffer self-assembles to spherical nanoparticles as visualized by FESEM, while the \(\beta\)-structure, adopted in TFE, results into nanofibers in TFE. However, the opposite helical handedness of peptide PPII helices in aqueous phosphate buffer or \(\beta\)-structure in TFE does not translate into self-assembled nanofibers that were similar for both enantiopairs. The peptide \(\omega\)-Amp\(_{P}\) (P3) initially assemblies into kinetically favored aggregates from which thermodynamically stable helical nanofibers are spun out over a period of 12 h, followed by further hierarchical assembly to form larger bundles. The present study highlights the significance of C4-substituents on proline in determining the strength of intermolecular interactions, which directs the self-assembly of this class of peptides. The results have implications for understanding how different conformations of peptides in solution drive self-assemblies toward specific nanostructures.

The future potential applications of the work may involve generation of nanofibers of the peptides from TFE to explore their application as water-sensitive organogels.\(^{59}\) Given the remarkable way the supramolecular chirality is delinked from the chirality of the peptide building block, it will be interesting to study chirality induction properties of homo and heterochiral polypeptide polypeptides on immobilized metal nanoparticles. The conformational transition (PPII to \(\beta\)) of these peptides can be integrated into dynamic nanosystems that reconfigure in response to the hydration levels of the environment. Further, as cationic peptides, Amp\(_{P}\) peptides are
analogues of poly-

i-lysine that are used to functionalize surfaces for promoting cell adhesion. The cross-linked polyaminopropyl peptides could thus be useful in surface functionalization for material and tissue engineering applications. The cell penetrating and membrane disrupting properties of these peptides are being explored for potential use as antimicrobial peptides.

## EXPERIMENTAL METHODS

### General Procedures

All starting materials and reagents used were of the highest grade available commercially and were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 F254 plates. Compounds on TLC were visualized by UV and by ninhydrin spray. All column chromatography purifications were performed using 100–200 mesh silica gel as the stationary phase. 1H and 13C NMR spectra were recorded on Bruker 400 MHz in CDCl3 and chemical shifts in δ parts per million (ppm) were referenced to tetramethylsilane (TMS). High-resolution mass spectrometry (HRMS) was used in electrospray ionization time of flight (ESI-TOF) mode to measure the exact mass of the compounds. The molecular weights of all of the peptides were characterized by matrix-assisted laser desorption and ionization time of flight (MALDI-TOF) spectrometry operated in positive reflection mode using either α-cyano-4-hydroxy cinnamic acid (CHCA) or 2,5-dihydroxy benzoic acid (DHB) as matrix.

### Synthesis of Compounds 6, 10, 12–15

(4S)-Azido-N1-(t-Boc)-D-aminoproline Methyl Ester 13. The compound 12 (0.8 g, 4.3 mmol) and NaN3 (1.4 g, 22 mmol) were dissolved in dryDMF (20 mL) and stirred for 8 h at 60 °C under a nitrogen atmosphere. After completion of reaction, DMF was removed under reduced pressure and the residue was dissolved in water and extracted with ethyl acetate.

The combined organic layers were washed with brine, dried over anhydrous Na2SO4 and concentrated on a rotary evaporator to yield colorless thick oil. The crude product obtained was purified by column chromatography.

Yield: 0.4 g, 71%; (SiO2, 20% ethyl acetate/hexane); specific rotation: [α]25 +17 (c, 2%, MeOH); 1H NMR (400 MHz, CDCl3): 7.38–7.27 (m, 5H), 5.13 (dd, J = 33.8, 16.9, 7.7 Hz, 2H), 4.46 (dd, J = 26.2, 8.9, 3.6 Hz, 1H), 4.18 (ddq, J = 7.4, 3.7, 1.7 Hz, 1H), 3.89–3.59 (m, 1H), 3.61–3.47 (m, 4H), 2.53–2.35 (m, 1H), 2.22 (dt, J = 13.5, 3.5 Hz, 1H); 13C{1H} NMR (100 MHz, CDCl3): δ: 171.0, 154.6, 154.2, 136.0, 128.6, 128.2, 127.6, 59.5, 58.3, 57.8, 52.5, 51.2, 35.5, 35.0; IR (neat, cm⁻¹): 2954, 2104, 1747, 1701, 1413, 1347, 1267, 1203, 1169, 1112, 1056; HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C12H23N2O4Na 327.1069; found 327.1072.

(4R)-Azido-N1-(t-Boc)-D-aminoproline Methyl Ester 14. The azido compound 13 (4 g, 12.5 mmol) was reacted with PPh3 (5 g, 19.2 mmol) in dry THF (40 mL). The resulting solution was stirred overnight; when complete consumption of azide was observed, then water (4 mL) was added followed by stirring for another 2 h and THF was evaporated under reduced pressure to yield the crude amine product. The residue was dissolved in dioxane–water (1:1) containing NaHCO3 followed by slow addition of (Boc)2O (3.45 g, 16 mmol) and stirred for another 4 h. After completion of reaction, the reaction mixture was concentrated and extracted with ethyl acetate and purified by column chromatography to obtain 14. Yield: 2.0 g, 41%; (SiO2, 15% ethyl acetate/hexane); specific rotation: [α]25 +9 (c, 2%, MeOH); 1H NMR (400 MHz, CDCl3): δ: 7.36–7.26 (m, 5H), 5.09 (dd, J = 21.6, 12.3 Hz, 2H), 4.36 (dd, J = 20.9, 9.6, 3.0 Hz, 2H), 3.85–3.55 (m, 4H), 3.51 (dd, J = 22.8, 10.4 Hz, 1H), 2.46 (ddd, J = 13.5, 9.7, 6.3 Hz, 1H), 2.08–1.89 (m, 1H), 1.41 (s, 9H); 13C{1H} NMR (100 MHz, CDCl3): δ: 174.1, 155.2, 154.8, 154.1, 136.3, 128.6, 128.5, 128.1, 127.9, 67.5, 58.0, 57.7, 52.7, 52.5, 37.0, 35.9, 28.1; HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C21H25N3O4Na 401.1688 found 401.1689.

(4R)-N4-(t-Boc)-N1-(Cbz)-D-proline Methyl Ester 15. The compound 14 (1 g, 3.3 mmol) was hydrolyzed in THF/water (1:1, 20 mL) using LiOH 2 N, for 1 h. THF was removed under vacuum, and the aqueous layer was washed with ethyl acetate, acidified with aq KH2SO4 and extracted with ethyl acetate to obtain the crude product (acid). The crude product obtained was dissolved in dry methanol (15 mL) and hydrogenated in the presence of 10% Pd/C (0.5 g) for 6 h. The reaction mixture was filtered through celite. The filtrate was concentrated under reduced pressure. The product obtained as a white solid powder was dissolved in water/dioxane, 1:1 (60 mL). This was treated with 10% Na2CO3 at 0 °C to bring to pH 9.0 and stirred for 30 min. Fmoc-Cl (1.4 g, 5.6 mmol) was added portionwise over a period of 45 min maintaining the temperature at 0 °C for 4 h and then allowing to come to room temperature and stirred for 18 h. After completion of the reaction, the mixture was acidified with aq KH2SO4 to pH 4.0 and followed by work up to obtain the crude product, which was then purified by column chromatography. Yield: 1.05 g, 53%; (SiO2, 1.5% methanol/dichloromethane (DCM)); specific rotation: [α]25 +3 (c, 2%, MeOH); 1H NMR (400 MHz, CDCl3): δ: 7.76–7.28 (9H, m), 5.19–4.96 (3H, m), 4.52–4.40 (4H, m), 3.72–3.67 (3H, m), 3.60–3.50 (3H, m), 2.44–2.31 (4H, m); 13C{1H} NMR (100 MHz, CDCl3): δ: 171.6, 154.4, 136.2, 130.2, 130.0, 128.9, 128.2, 128.1, 127.8, 67.5, 67.1, 57.6, 56.6, 52.5, 51.7, 37.2, 36.1, 29.7, 21.7; IR (neat, cm⁻¹): 3022, 2957, 1747, 1709, 1599, 1419, 1357, 1213, 1177, 1114, 1053, 1013; HRMS (ESI-TOF) m/z: [M + Na]+ calcd for C14H25N3O3SNa 456.1092; found 456.1087.

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2.33 (m, 1H), 2.21

[2R,4R]-N’-((Benzoyl)carbonyl)-4-(tert-butoxy)-hydroxyproline Ester 15. The mixture of compound 11 (5.0 g, 179 mmol), Ag₂O (8.5 g 358 mmol), and tert-butyl bromide (6 mL, 537 mmol) in cyclohexane (50 mL) was stirred for 24 h at room temperature. The resulting suspension was filtered out, the filtrate was concentrated under reduced pressure, and the crude product was purified by silica gel chromatography eluting with petroleum ether/ethyl acetate (80:20) to give a desired product 15. Yield: 5.11 g, 85%; 1H NMR (400 MHz, CDCl₃): δ: 7.35–7.24 (m, 5H), 5.18–5.01 (m, 2H), 4.37–4.32 (m, 1H), 4.15–4.12 (m, 1H), 3.74–3.56 (m, 4H), 3.34–3.30 (m, 1H), 2.34–2.28 (m, 1H), 2.06–2.02 (m, 1H), 1.13 (s, 9H); 13C{1H} NMR (100 MHz, CDCl₃): δ: 172.7, 172.5, 154.9, 154.4, 136.7, 136.5, 128.5, 128.1, 128.0, 127.9, 74.0, 69.4, 68.6, 67.2, 67.1, 57.8, 57.5, 53.6, 53.4, 38.8, 37.9, 28.3; HRMS (ESI-MS) m/z: [M + Na⁺] calcd for C₂₅H₂₈N₂NaO₆Na 475.1845; found 475.1850.

15° C for 3.56 (m, 4H), 3.34 (m, 2H), 2.41 (m, 2H), 2.02 (m, 1H), 1.13 (s, 9H); 13C{1H} NMR (100 MHz, CDCl₃): δ: 7.73–7.24 (m, 8H), 4.50–4.43 (m, 5H), 3.62–3.37 (m, 2H), 2.41–2.33 (m, 1H), 2.21–2.13 (m, 1H), 1.21 (s, 9H); 13C{1H} NMR (100 MHz, CDCl₃): δ: 173.7, 143.8, 143.7, 141.4, 127.8, 127.2, 125.4, 125.1, 120.0, 69.6, 69.2, 68.2, 67.9, 58.6, 54.8, 53.9, 47.2, 47.1, 38.2, 37.0, 28.1, 28.0; HRMS (ESI-MS) m/z: [M + Na⁺] calcd for C₁₉H₂₃NO₅, 342.1787; found 342.1786.

Chiral Purity of Monomers by HPLC. The chiral purity of the d-amino acid monomers 4 and 6 was assessed by comparing their retention times in chiral high-performance liquid chromatography (HPLC) with those of their enantiomers. For this study, an Agilent Technologies 1260 Infinity system equipped with a photodiode array detector (PDA) and a semipreparative RP-18 column (250 × 10 mm², 10 μm). For nonlipidated peptides, the following solutions were used as the mobile phase gradients. Solvent A: 5% CH₃CN in water; solvent B: 50% CH₃CN in water. Both the solvents contained 0.1% TFA. A linear gradient of 0–100% B was achieved in 20 min. In the case of lipidated peptides, solvent A was 5% CH₃CN (with 0.1% TFA) and solvent B was 95% CH₃CN (with 0.1% TFA). The flow rate was maintained at 3 mL/min (P2, P3) or 2 mL/min (P1, P4–P13). The elution of the peptides was monitored at 220 nm. The structural integrity of the purified peptides was confirmed by MALDI-TOF mass spectrometry using CHCA or DHB as a matrix. The retention time data in HPLC and MALDI-TOF for peptides are shown in Table S2, Supporting Information.

Field Emission Scanning Electron Microscopy Studies. Field emission scanning electron microscopy (SEM) imaging was performed using a Zeiss ULTRA Plus scanning electron microscope operating at 3 kV. To avoid the morphology of the self-assembled peptides from being obscured by the salts from the buffer, we used Milli-Q water for scanning electron microscopy (SEM) imaging. Calculated amounts of peptides were dissolved in the desired solvent (water or TFE) and allowed to stand for at least 12 h at room temperature (ca. 25 °C). An aliquot of 2 μL of the peptide solution was then drop-cast on a clean silicon wafer. Samples were allowed to dry at room temperature in vacuum with a Teflon stopcock. While the 4-methyl benzhydrolamine (MBHA) resin (100–200 mesh, Novabiochem), available commercially as a hydrochloride salt, was neutralized prior to coupling of the first amino acid desired in the C-terminus of the peptide for the t-Boc protocol, the Fmoc group on the Rink amide resin (100–200 mesh, Novabiochem) was removed to prepare it for peptide synthesis using the Fmoc protocol. Coupling reactions were carried out using the in situ active ester method, by HBTU as a coupling reagent, HOBt as a racemization suppressor, and N,N-disopropylethylamine (DIPEA) as a catalyst. For t-Boc strategy, 50% trifluoroacetic acid (TFA) in DCM was used as a deprotection solution, while 20% piperidine in DMF was used to deprotect the terminal amine groups at the growing end of the peptide in the Fmoc protocol. The N-terminus of the peptides was capped by reaction with acetic anhydride in dry pyridine. The peptides were cleaved from the MBHA resin using trifluoromethanesulfonic acid (TFMSA) in the presence of thioanisole as scavengers and from the Rink amide resin using 95% TFA in DCM in the presence of trisopropyl silane (TIPS) as a scavenger.

Peptide Purification and Characterization. The crude peptides P1–P13 were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on an Agilent Technologies 1260 Infinity system equipped with a photodiode array detector (PDA) and a semipreparative RP-C18 column (250 × 10 mm², 10 μm). For nonlipidated peptides, the following solutions were used as the mobile phase gradients. Solvent A: 5% CH₃CN in water; solvent B: 50% CH₃CN in water. Both the solvents contained 0.1% TFA. A linear gradient of 0–100% B was achieved in 20 min. In the case of lipidated peptides, solvent A was 5% CH₃CN (with 0.1% TFA) and solvent B was 95% CH₃CN (with 0.1% TFA). The flow rate was maintained at 3 mL/min (P2, P3) or 2 mL/min (P1, P4–P13). The elution of the peptides was monitored at 220 nm. The structural integrity of the purified peptides was confirmed by MALDI-TOF mass spectrometry using CHCA or DHB as a matrix. The retention time data in HPLC and MALDI-TOF for peptides are shown in Table S2, Supporting Information.
desiccators for at least 12 h, and the dried film was sputter-coated with gold prior to imaging.

**Atomic Force Microscopy Studies.** Atomic force microscopy imaging was carried out on a Keysight 5500 atomic force microscope in tapping mode. The cantilever was auto-tuned at 190 kHz frequency prior to measurements. The desired peptide sample (5 μL) was spotted on a freshly cleaved mica surface and allowed to dry at room temperature (ca. 25 °C) under vacuum for about 12 h before imaging in air.

**Dynamic Light Scattering Studies.** The particle size distributions were studied by dynamic light scattering (DLS) with a 633 nm red laser (at 90° angle). Samples were prepared in the appropriate solvent and left to stand for 1 h prior to measurements. Three measurements were carried out for each sample. All of the measurements were recorded at 25 °C. Each measurement consisted of eight runs with data collected for 30 s in each run. An average of three measurements was used for particle size distribution analysis.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c02826. Characterization data (NMR and MS of all new compounds, chiral HPLC of monomers, RP-HPLC and MALDI-TOF of all peptides) and DLS, CD, FESEM, and AFM images of peptides (PDF).

## AUTHOR INFORMATION

### Corresponding Author

Krishna N. Ganesh — Indian Institute of Science Education and Research (IISER), Tirupati, Tirupati S17507, Andhra Pradesh, India; Indian Institute of Science Education and Research (IISER), Pune, 411008, Maharashtra, India; [orcid.org/0000-0003-2292-643X; Email: kn.ganesh@iisertirupati.ac.in](mailto:kn.ganesh@iisertirupati.ac.in)

### Authors

Bharath Raj Madhanagopal — Indian Institute of Science Education and Research (IISER), Tirupati, Tirupati S17507, Andhra Pradesh, India; [orcid.org/0000-0003-1043-8754](orcid.org/0000-0003-1043-8754)

Shahaji H. More — Indian Institute of Science Education and Research (IISER), Tirupati, Tirupati S17507, Andhra Pradesh, India

Nitin D. Bansode — LCPO, ENSCBP, UMR 5629, University of Bordeaux, Pessac 33600, France; [orcid.org/0000-0001-7173-0260](orcid.org/0000-0001-7173-0260)

Complete contact information is available at: [https://pubs.acs.org/doi/10.1021/acsomega.0c02826](https://pubs.acs.org/doi/10.1021/acsomega.0c02826)

### Author Contributions

*B.R.M. and S.H.M. contributed equally to this work.*

### Notes

The authors declare no competing financial interest.

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