Incorporation of Achiral Peptoid-Based Trimeric Sequences into Collagen Mimetics

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Abstract: This report represents initial studies of collagen mimetics with achiral peptoid-based trimeric sequences. The incorporation of achiral units into collagen-like structures is of considerable interest for the structural simplification of collagen-like biomaterials. The achiral unit Gly-Nleu-Nleu (where Nleu represents *N*-isobutylglycine) was positioned between Gly-Pro-Hyp trimeric repeats in collagen-like structures in order to examine the effect of an achiral block on triple helicity. A series of single chain structures, Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH$_2$ (where $n = 1-3$), and a template-assembled structure, KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$]$_3$ (where KTA represents *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid), were investigated. Biophysical studies were carried out in both H_2O and ethylene glycol (EG)/H_2O (2:1, v/v) solvents, using circular dichroism and optical rotation measurements. Highly cooperative melting curves from optical rotation determinations were obtained for Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH$_2$ ($n = 2, 3$) and KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$]$_3$, revealing that the achiral trimer can participate in triple helical structures. These results were also supported by circular dichroism spectroscopy. For the molecules Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH$_2$ and KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$]$_3$, the presence of collagen-like structures was also supported by ^{1}H NMR spectroscopy in H_2O. For each structure, a distinct set of resonances, obtained at low temperature, disappeared once a thermal denaturation temperature was reached. Furthermore, the analysis of NOE cross-peaks established the close packing of Pro, Hyp, and Nleu. The spatial proximity of Pro and Nleu residues and of Hyp and Nleu residues belonging to different chains was confirmed by molecular modeling of triple helical Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH$_2$.

Introduction

The design of synthetic collagen-like structures is important for the development of novel biomaterials. Such biomaterials have diverse applications which include drug delivery systems, ocular devices, and wound healing materials.[1] Collagen proteins have large triple helical domains made up of three polyproline II-like helices supercoiled in a right-handed manner.[2−7] The primary sequence consists of repeating trimeric units of (Gly-X-Y)$_n$, in which Gly-Pro-Hyp sequences are the most common structural repeats.

Collagen-like structures can have enhanced biostability against enzymatic degradation through the incorporation of unnatural residues, such as peptoid residues.[8] We have discovered that the peptoid residue *N*-isobutylglycine (Nleu) is an excellent proline surrogate by investigating single-chain structures and template assembled structures with (Gly-Pro-Nleu)$_n$[9−11] and (Gly-Nleu-Pro)$_n$[12,13] repeats. These sequences are capable of forming triple helical structures once a certain chain length is reached. We decided to take this work a leap forward and determine if entirely achiral trimeric sequences could participate in triple helical arrays. The incorporation of achiral repeats is of considerable interest in order to simplify the overall synthesis and primary structure of collagen-like biomaterials. This simplification arises from the ease of synthesis of the achiral units which do not involve the protections and racemizations-free activations required to prepare optically pure sequences.

We report here the first studies exploring the incorporation of an achiral sequence into collagen mimetics. The collagen mimetics in this study consist of structures with achiral trimeric units of Gly-Nleu-Nleu (**1**), positioned between Gly-Pro-Hyp

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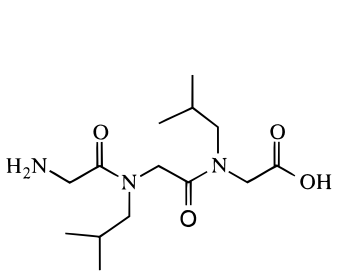
repeats. These compounds include the following acetyl-



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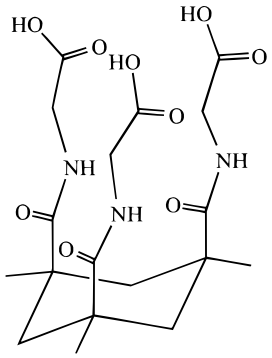


(2)

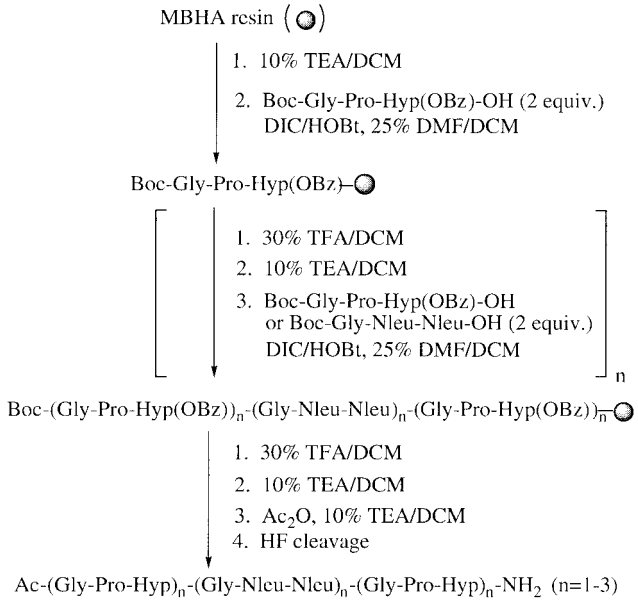
terminated structures as well as a template-assembled stucture: Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH_2 (where $n = 1-3$) and KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$ (where KTA represents *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid, the Kemp triacid[14]). For the single-chain compounds, triple helix formation would occur as an intermolecular process, while for the template-assembled compound, triple helix formation would follow an intramolecular process. From our previous studies, the KTA-(Gly-OH)$_3$ template (**2**) is known to enhance triple helix formation significantly.[9−11,15−17]

The triple helical propensity of the Gly-Nleu-Nleu-containing structures was investigated using circular dichroism (CD) spectroscopy and temperature-dependent optical rotation measurements. The conformational characterization of Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH_2 (where $n = 1, 2$) and KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$were studied using 1D and 2D ^{1}H NMR spectroscopy in H_2O. These analyses provided sequence-specific assignments and NOE connectivities. Molecular modeling analysis of triple helices of Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 assembled with the three chains, staggered by one residue, confirms the interchain proton connectivities found experimentally.

Results and Discussion

Synthesis. The single-chain peptoid-containing structures, Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH_2 ($n = 1-3$), were assembled by solid-phase segment condensation methods, first introduced by Sakakibara and co-workers.[18] The compounds were synthesized on a 4-methylbenzhydrylamine (MBHA) resin using Boc (*tert*-butyloxycarbonyl) synthetic strategies, according to Scheme 1. For each segment condensation, 2 equiv of building block, either Boc-Gly-Nleu-Nleu-OH[19] or Boc-Gly-Pro-Hyp(OBzl)-OH,[17] was used with coupling reagents diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt). Each coupling reaction was monitored to completion (4−8 h) using the Kaiser ninhydrin test.[20] After the desired chain length was synthesized, the N-terminus was

Scheme 1



acetylated with acetic anhydride. The product was removed from the resin using standard HF cleavage techniques.

The template-assembled structure, KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$, was synthesized in a manner similar to that previously described by us for other KTA template assembled analogues.[17] The template KTA-(Gly-OH)$_3$ was allowed to react with the N-terminus of [Gly-Pro-Hyp-(OBzl)$_2$]$_2$-(Gly-Nleu-Nleu)$_2$-[Gly-Pro-Hyp(OBzl)]$_2$-resin. Afterward, the assembly was cleaved from the resin using standard HF cleavage methods.

All compounds were purified to >97% homogeneity by preparative reversed-phase HPLC prior to biophysical characterization. Molecular weights were confirmed by mass spectrometry and were uniformly in agreement with expected values.

Denaturation Studies. Triple helical structures can be distinguished from nonsupercoiled polyproline II-like structures based on thermal denaturation behavior. Triple helices melt in a highly cooperative fashion since their structures stabilized by both intra- and interstrand hydrogen-bonding water networks.[21−23]

Melting curves for the Gly-Nleu-Nleu-containing structures were constructed by monitoring the optical rotation at 365 nm, at various temperatures. Melting curves were obtained in both H_2O and ethylene glycol (EG)/H_2O (2:1, v/v) solutions, at concentrations of 0.2 mg/mL. The solvent system EG/H_2O (2:1, v/v) was chosen since ethylene glycol is known to stabilize triple helices and therefore can be useful for detecting weak triple helices.[24,25] The thermal melting curves are displayed in Figures 1 and 2, and the thermal melting temperatures (T_m) are tabulated in Table 1, along with T_m values for analogous peptides containing only Gly-Pro-Hyp repeats.[17]

The analogue Ac-(Gly-Pro-Hyp)-(Gly-Nleu-Nleu)-(Gly-Pro-Hyp)-NH_2 does not exhibit a cooperative melting curve in neither H_2O nor EG/H_2O (2:1, v/v) solution since its chain length

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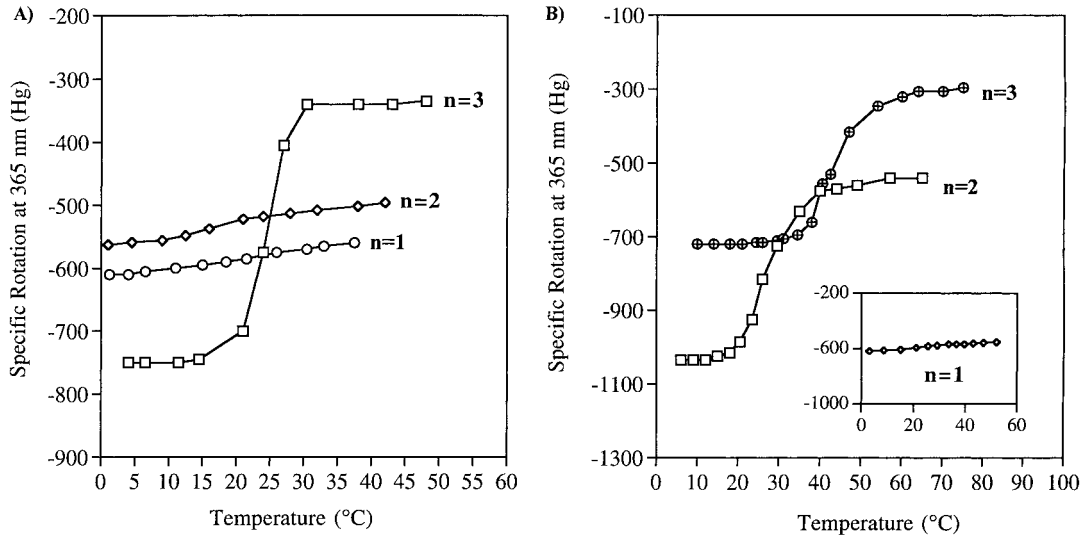

Figure 1. (A) Thermal melting curves for Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH$_2$ ($n = 1-3$) in H$_2$O, at 0.2 mg/mL. (B) Thermal melting curves for Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH$_2$ ($n = 1-3$) in EG/H$_2$O (2:1, v/v), at 0.2 mg/mL.

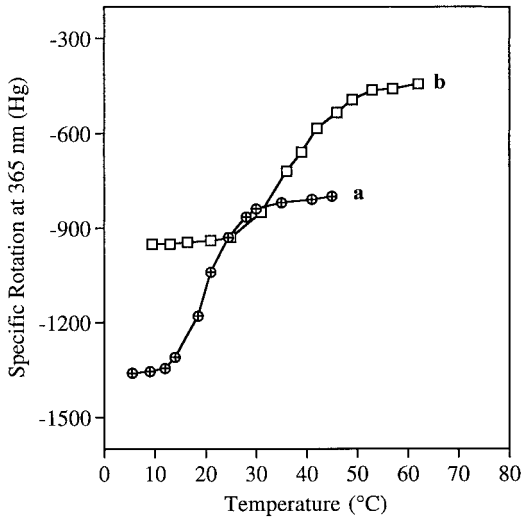

Figure 2. Thermal melting curves for KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$]$_3$ in H$_2$O (a) and EG/H$_2$O (2:1, v/v) (b) solutions.

is too short to support a triple helix. This is consistent with our previous work where we found that Ac-(Gly-Pro-Hyp)$_3$-NH$_2$ is not triple helical.[17] For Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$, a cooperative melting curve was observed in EG/H$_2$O solutions (T_m = 25 °C) but not in H$_2$O solution. For Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH$_2$, a cooperative melting curve was observed in both H$_2$O (T_m = 25 °C) and EG/H$_2$O (2:1, v/v) (T_m = 43 °C) solutions.

The template-assembled compound exhibited cooperative melting curves in both H$_2$O (T_m = 20 °C) and EG/H$_2$O (2:1, v/v) (T_m = 43 °C) solutions (Figure 2). The thermal melting temperature of KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$]$_3$ is 17 °C higher than that obtained for Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$, in EG/H$_2$O (2:1, v/v). This result highlights the stablizing effect that the KTA template has on triple helicity.

The effect of Gly-Nleu-Nleu incorporation on triple helical structures is apparent by comparing T_m values of the Gly-Nleu-Nleu-containing compounds to the T_m values of similar chain length Gly-Pro-Hyp-containing compounds[17] (Table 1). Replacement of three central Gly-Pro-Hyp repeats of Ac-(Gly-Pro-Hyp)$_9$-NH$_2$ by three Gly-Nleu-Nleu repeats causes a decrease in the thermal melting temperature from 67 to 25 °C, in H$_2$O. Similarily, T_m values determined in EG/H$_2$O (2:1, v/v) solution decreased from >95 °C for Ac-(Gly-Pro-Hyp)$_9$-NH$_2$ to 43 °C for Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH$_2$. A T_m value of 80 °C was obtained for KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH$_2$]$_3$ while a T_m value of 20 °C was obtained for KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$]$_3$. It is clear from these comparisons that substitution of Gly-Nleu-Nleu into triple helical structures containing Gly-Pro-Hyp repeats significantly destabilizes the triple helix. However, the co-oligomeric structures are still able to form triple helices. It is probable that the origin of the destabilization effect can arise from the increase in flexibility of the achiral central sequences which in turn weakens the interchain hydrogen bonding.

Circular Dichroism Studies. Natural collagen possesses a unique CD spectrum with a small positive peak about 220 nm, a crossover near 213 nm, and a large negative peak about 200 nm.[24,25] Triple helical Ac-(Gly-Pro-Hyp)$_9$-NH$_2$, a synthetic collagen-like structure, has a CD spectrum in H$_2$O with a positive peak at 224 nm, a crossover at 218 nm, and a negative peak at 198 nm.[17] The CD spectra of the structures containing the Gly-Nleu-Nleu repeat are displayed in Figures 3 and 4, and the CD data is tabulated in Table 2. Table 2 also contains the CD data of analogous collagen-like structures containing only Gly-Pro-Hyp repeats.

In our previous studies, we defined Rpn values as the ratio of positive peak intensity, at ca. 220 nm, over negative peak intensity, at ca. 200 nm.[17] These values have been useful for establishing the presence of triple helical conformations in solution.[17]

For the single-chain compounds, in H$_2$O solvent, a higher Rpn value is obtained for Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH$_2$ (Rpn = 0.18) compared to both Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH$_2$ (Rpn = 0.04) and Ac-(Gly-Pro-Hyp)-(Gly-Nleu-Nleu)-(Gly-Pro-Hyp)-NH$_2$ (Rpn = 0.03). This comparison of Rpn values suggests that Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH$_2$ contains triple helical conformations, while the shorter chain analogues do not. This conclusion is consistent with a previous result where triple helical Ac-(Gly-Pro-Hyp)$_9$-NH$_2$ was found to have an Rpn value of 0.14.[17]

Table 1. Thermal Melting Temperatures of Collagen-like Structures[a]

compound	T_m values	
	H_2O	EG/H_2O (2:1, v/v)
Ac-(Gly-Pro-Hyp)-(Gly-Pro-Hyp)-(Gly-Pro-Hyp)-NH_2[b]	no transition	no transition
Ac-(Gly-Pro-Hyp)$_3$-NH_2[c]	no transition	no transition
Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2[b]	no transition	25 °C
Ac-(Gly-Pro-Hyp)$_6$-NH_2[c]	36 °C	59 °C
Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2[b]	25 °C	43 °C
Ac-(Gly-Pro-Hyp)$_9$-NH_2[c]	67 °C	>95 °C
KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$[b]	20 °C	43 °C
KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH_2]$_3$[c]	81 °C	>95 °C

[a] Concentration = 0.2 mg/mL. [b] This work. [c] Reference 17.

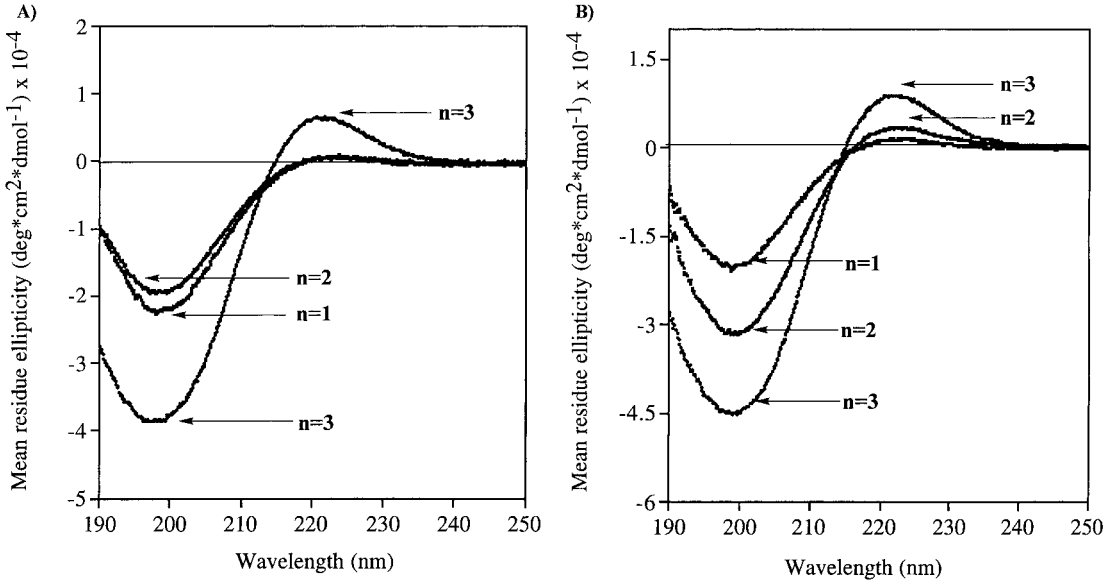

Figure 3. (A) CD spectra of Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH_2 (n = 1−3) in H_2O, at 0.2 mg/mL. (B) CD spectra of Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH_2 (n = 1−3) in EG/H_2O (2:1, v/v), at 0.2 mg/mL.

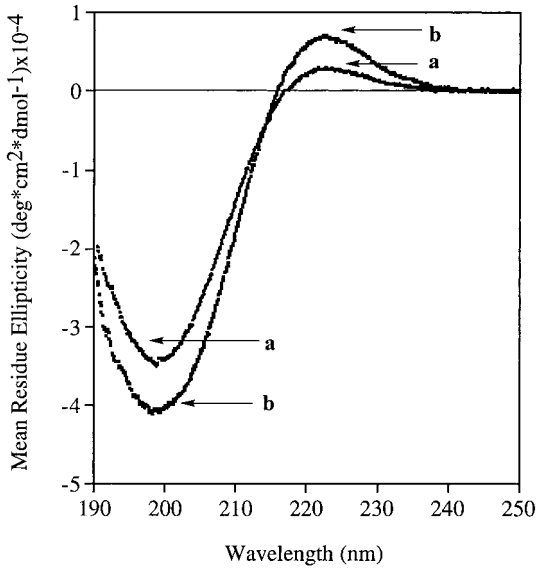

Figure 4. CD spectra of KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$ in H_2O (a) and EG/H_2O (2:1, v/v) (b) solutions.

In EG/H_2O (2:1, v/v) solvent, Ac-(Gly-Pro-Hyp)-(Gly-Nleu-Nleu)-(Gly-Pro-Hyp)-NH_2 exhibits an Rpn value of 0.07 while the longer chain structures, Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH_2 (n = 2, 3) have Rpn values of 0.11 (n = 2) and 0.20 (n = 3). Analogous triple helical structures, Ac-(Gly-Pro-Hyp)$_n$-NH_2 (n = 6, 9) have Rpn values of 0.16 (n = 6) and 0.17 (n = 9), in EG/H_2O (2/1) solvent. This comparison suggests that the structures Ac-(Gly-Pro-Hyp)$_n$-(Gly-Nleu-Nleu)$_n$-(Gly-Pro-Hyp)$_n$-NH_2 (n = 2, 3) have triple helical character.

The template-assembled structure, KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$, has Rpn values of 0.09 and 0.17 in H_2O and EG/H_2O (2:1, v/v) solutions, respectively. As a comparison, triple helical KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH_2]$_3$ has Rpn values of 0.12 and 0.17 in H_2O and EG/H_2O solutions, respectively. This comparison suggests that KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$ also contains triple helical conformations in both solvents.

NMR Spectroscopy. For the acetyl-terminated analogue with nine trimeric repeats and the KTA analogue with six trimeric repeats, ^{1}H NMR spectroscopy was used to verify that the Gly-Nleu-Nleu repeats were actually taking part in the triple helical assemblies.

For the interpretation of the NOESY spectra, three polypeptide chains were considered to have identical repetitive sequences symmetrically and closely packed. Because of the overlap of spectral resonances arising from the same type of residue along the sequence or belonging to different peptide chains, it is not possible directly to characterize triple-helices using NOE-based distance constraints. A given NOE cross-peak can result from either interchain or intrachain interactions. However, Brodsky et al.[26] used X-ray fiber diffraction data from triple helical Gly-Pro-Hyp sequences to identify NOEs that are predicted to arise uniquely from interchain resonances in a triple

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Table 2. Circular Dichroism Data for Collagen-Based Structures[a]

	H_2O				EG/H_2O (2:1, v/v)			
compounds	max (nm)	cross (nm)	min (nm)	Rpn[b]	max (nm)	cross (nm)	min (nm)	Rpn
Ac-(Gly-Pro-Hyp)-(Gly-Nleu-Nleu)-(Gly-Pro-Hyp)-NH_2	224 (7.10×10^2)	219	199 (-2.20×10^4)	0.03	223 (1.33×10^3)	217	200 (-2.01×10^4)	0.07
Ac-(Gly-Pro-Hyp)$_3$-NH_2c	223 (1.8×10^3)	218	200 (-2.3×10^4)	0.08	223 (2.2×10^3)	217	199 (-2.4×10^4)	0.09
Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2	223 (7.50×10^2)	218	199 (-1.90×10^4)	0.04	222 (3.49×10^3)	216	199 (-3.12×10^4)	0.11
Ac-(Gly-Pro-Hyp)$_6$-NH_2c	224 (3.4×10^3)	218	198 (-2.9×10^4)	0.12	223 (5.3×10^3)	217	198 (-3.4×10^4)	0.16
Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2	221 (6.75×10^3)	215	199 (-3.84×10^4)	0.18	222 (8.84×10^3)	215	199 (-4.49×10^4)	0.20
Ac-(Gly-Pro-Hyp)$_9$-NH_2c	224 (4.4×10^3)	218	198 (-3.1×10^4)	0.14	223 (6.7×10^3)	217	197 (-4.0×10^4)	0.17
KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$	223 (3.18×10^3)	216	198 (-3.42×10^4)	0.09	223 (6.94×10^3)	216	199 (-4.06×10^4)	0.17
KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH_2]$_3$c	224 (4.1×10^3)	218	198 (-3.4×10^4)	0.12	224 (3.7×10^3)	218	199 (-2.2×10^4)	0.17

[a] CD spectra were obtained at 20 °C, at 0.2 mg/mL. The peak intensities are included in parentheses. [b] Rpn represents the ratio of positive peak intensity over negative peak intensity. [c] Reference 17.

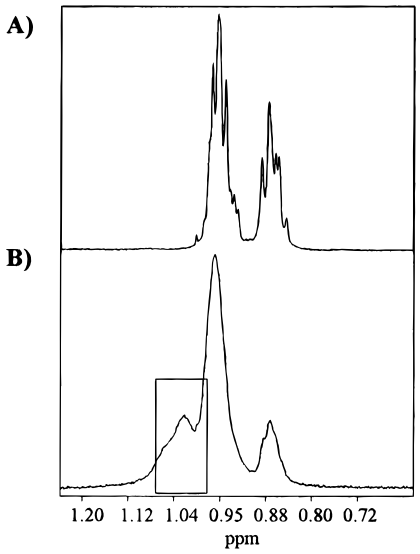

Figure 5. (A) Ac-(Gly-Pro-Hyp)-(Gly-Nleu-Nleu)-(Gly-Pro-Hyp)-NH_2. (B) Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2. ^{1}H NMR spectral regions including the assembled (boxed peaks) and the unassembled Nleu methyl resonances at 1.02 ppm and at 0.87 ppm, respectively. All spectra were acquired in D_2O at 275 K.

helical model. These connectivities provide a critical test for the triple helical tendency of modified sequences and give insight into triple helical packing.

The two acetyl analogues ($n = 1, 3$) were found to be distinguishable by NMR spectroscopy in aqueous solution at low temperature since an additional set of resonances appear for the Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 compound. Resonances arising from the methyl protons of the Nleu residues are displayed in Figure 5. A similar high field set of resonances has been shown to correspond to collagen-like triple helical structures and therefore can be denoted as the assembled set of resonances.[9,13]

The triple helical character of Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Nleu-Nleu)$_3$-NH_2 is supported by three independent experimental observations: (1) the new set of resonances is absent in the derivative with only one repeat for each unit; (2) as the temperature increases, the intensities of the new resonances decrease and disappear at temperatures above the T_m value determined by optical rotation measurements (Figure 6); (3) the new resonances are involved in "interunit" NOEs between Nleu and Pro, and Nleu and Hyp residues which are not observed in the NOESY spectrum acquired for the analogue with the shorter sequence (Figure 7, 8).

The same pattern of interchain NOEs was found for the KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$ as reported in Figure 9, confirming its triple-helical conformation in solution.

A list of the "interunit" NOEs, referring to both the acetyl-terminated and the KTA-assembled analogues, is reported in Table 3. Since these connectivities cannot arise from non-supercoiled polyproline II-like structures (distances > 5 Å), these data represent clear evidence of assembled structures and provide insight into the packing of the polypeptide chains. Specifically, the NOE pattern is consistent with the hypothesis of assembled structures between the Gly-Pro-Hyp and Gly-Nleu-Nleu trimeric sequences. Some connectivities between Pro and Hyp residues anticipated to be interchain from the crystal model of triple helices composed of Gly-Pro-Hyp repeats are determined as well.

The ensemble of interchain NOEs is useful to check modeled collagen-like triple helices of the Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 derivative. The lowest energy structure arising from the molecular modeling simulations is reported in Figure 10. Only the central region of the modeled triple helical structures is highlighted. All experimental interchain NOEs between Nleu and Pro and between Nleu and Hyp residues are observed. Therefore, this modeled structure represents the most reasonable triple helical pattern and can be used to provide an insight into the close packing of the Pro, Hyp, and Nleu residues.

Conclusion

A major goal of this report focuses on co-oligomer collagen mimetic structures. We explored the effect of the achiral trimeric sequence, Gly-Nleu-Nleu, on triple helical structures. We found that the achiral sequences can participate in the formation of an overall chiral, triple helical array where the achiral sequence is sandwiched between Gly-Pro-Hyp repeats. The Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 and the KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$ structures were found to be triple helical in both H_2O and EG/H_2O (2:1, v/v) solvents while the Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2 compound was found to be triple helical only in EG/H_2O (2:1, v/v) solution (concentrations = 0.2 mg/mL). Both optical rotation measurements and CD spectroscopy support these conclusions. NMR spectroscopy also provides support for the triple-helical arrays since the NOE patterns are consistent with the proximity of Nleu to Pro and Nleu to Hyp residues which implies packing among different trimeric sequences of different chains. Furthermore,

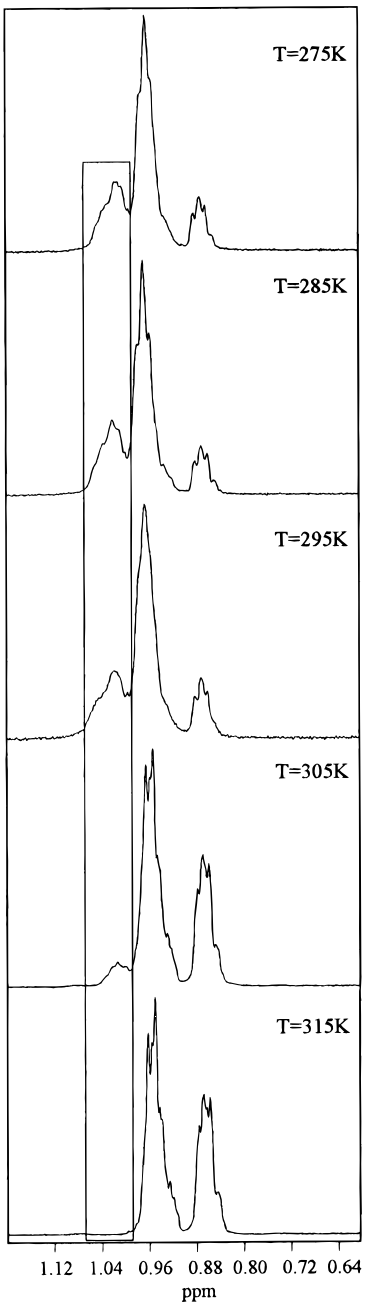

Figure 6. ^{1}H NMR spectral regions for Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH$_2$ including the assembled (boxed peaks) and the unassembled Nleu methyl resonances at 1.02 ppm and at 0.87 ppm, respectively. All the spectra were acquired in D_2O.

some NOE cross-peaks between Pro and Hyp residues predicted to be unique interchain connectivities based on the molecular modeling of the triple helical (Pro-Hyp-Gly)$_{10}$[26] confirm the close packing of these residues and their involvement in a coiled-coil collagen-like structure.

Our results demonstrate that the achiral trimeric sequence, Gly-Nleu-Nleu, can participate in triple helical structures. This is of importance since much effort in the field of biomaterials is directed toward structural simplifications. Other oligomeric achiral sequences are now under investigation in our laboratories.

Experimental Section

Materials. All amino acids are of the L-configuration. Protected amino acids and EDC were purchased from Bachem. Reagent grade and HPLC-grade solvent were purchased from Fisher Scientific. Palladium on activated carbon (10% palladium) was purchased from Acros. HOBt, DIC, TFA (HPLC-grade), and BOP were purchased from Chem-Impex International. THF solvent was dried by refluxing over Na in the presence of benzophenone.

General Procedures. All reactions carried out in solution were monitored by thin-layer chromatography on precoated silica gel 60F-54 plates (Merck). Compounds were visualized by UV or ninhydrin.

Preparative reversed-phase HPLC purification and analysis was carried out on a MILLENNIUM 2010 system consisting of a Waters 715 Ultra WISP sample processor, a Waters TM 996 photodiode array detector, two Waters 510 pumps, and a NEC Power Mate 486/33I computer. Solvents used in HPLC purification were as follows: solvent A, 0.1% trifluoroacetic acid (TFA)/H_2O; solvent B, 0.1% TFA/CH_3CN. The flow rate was 8 mL/min for the preparatory column (Vydac C-18, 25 × 2.2 cm) and 1.0 mL/min for the analytical column (Vydac C-18, 25 × 0.46 cm).

Mass spectra were determined at the Scripps Research Institute using fast atom bombardment, electrospray ionization, and MALDI mass spectrometric techniques.

Circular dichroism (CD) measurements were carried out on a modified Cary-61 spectropolarimeter as described previously.[17] Spectra were obtained using a 0.05 cm path length cell by signal averaging 10 scans from 190 to 300 nm with a scan speed of 1.0 nm/s.

Optical rotations were measured with a Perkin-Elmer 241 polarimeter equipped with a Model 900 isotemp refrigerator circulator (Fisher Scientific), and data were collected at 365 nm (Hg). At each temperature point, the sample was allowed to equilibrate until the optical rotation was time independent. To allow for equilibration of triple helix formation, sample solutions were stored at 4 °C, for at least 1 week prior to the optical rotation determinations.

The NMR samples were prepared in H_2O/D_2O (9:1) and D_2O (purchased from Isotec, Inc.) with a peptide concentration of about 1.5 mg/mL. The two linear mimetics were dissolved in H_2O and kept at 5 °C for at least 2 weeks prior to measurements. The KTA-assembled mimetic was dissolved in H_2O and kept at 5 °C, overnight. All NMR experiments were carried out on an AMX-500 Bruker spectrometer. 1D spectra of 32K data points were acquired at temperatures between 275 and 315 K in order to follow the denaturation of the triple helical structure. The 2D NMR spectra were acquired at 275 K to improve the nuclear Overhauser effect (NOE). Depending on the experimental sensitivity, a variable number of scans in the range between 64 and 144 was acquired for each t_1 increment, and 450−512 t_1 increments were collected of 2K data points each. Acquisition was performed in the phase-sensitive mode using the time proportional phase increment method (TPPI). Free induction decays were multiplied by appropriate windows functions in both time domains and zero filled to 2K×2K real points.

The water signal was suppressed by preirradiation with a selective gated pulse during the relaxation delay. For nuclear Overhauser effect spectroscopy (NOESY), preirradiation was also applied during the mixing time. The carrier frequency was placed at the frequency of water resonance spanning a SW of 6004 Hz. Chemical shift assignments were obtained in a straightforward manner[27] by the combined use of DQF−COSY[28−30] and NOESY[31] experiments. The TOCSY[32] experiments were acquired using the MLEV-17 spin-lock sequence typically at a field strength of 10 kHz for a total mixing time of 75−80 ms. NOESY spectra were carried out with a mixing time of 200 and 300 ms.

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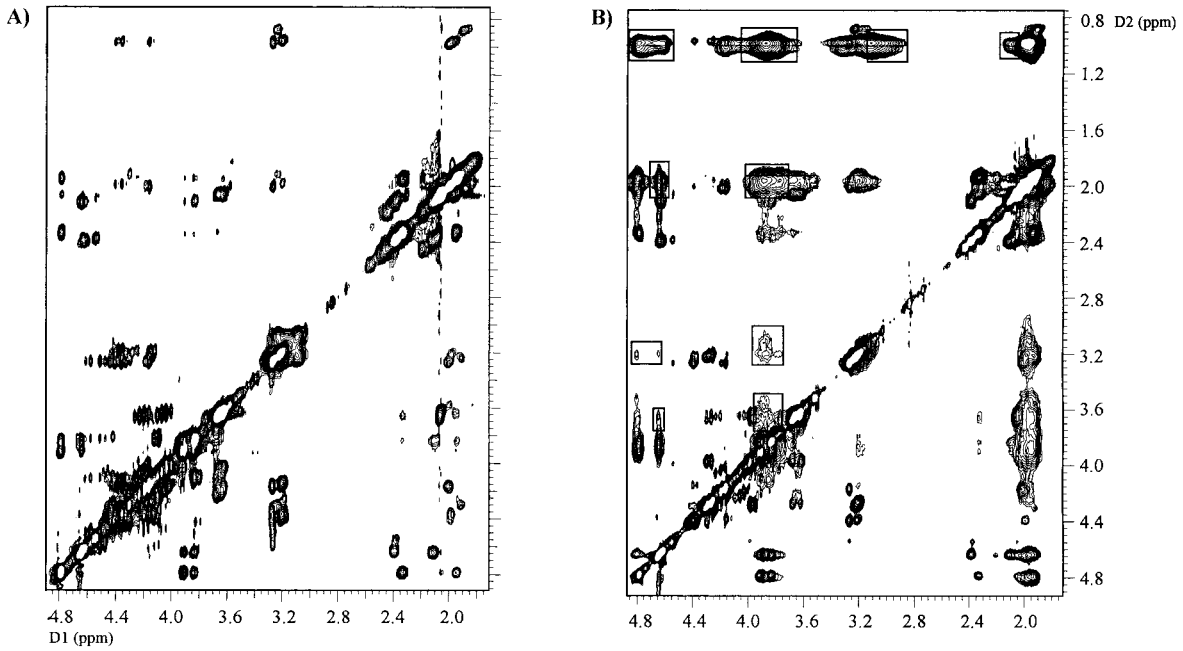

Figure 7. Aliphatic regions of the NOESY spectra of Ac-(Gly-Pro-Hyp)-(Gly-Nleu-Nleu)-(Gly-Pro-Hyp)-NH_2 (A) and Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 (B) in D_2O at 275 K. The ensemble interchain NOE cross-peaks are indicated by rectangles.

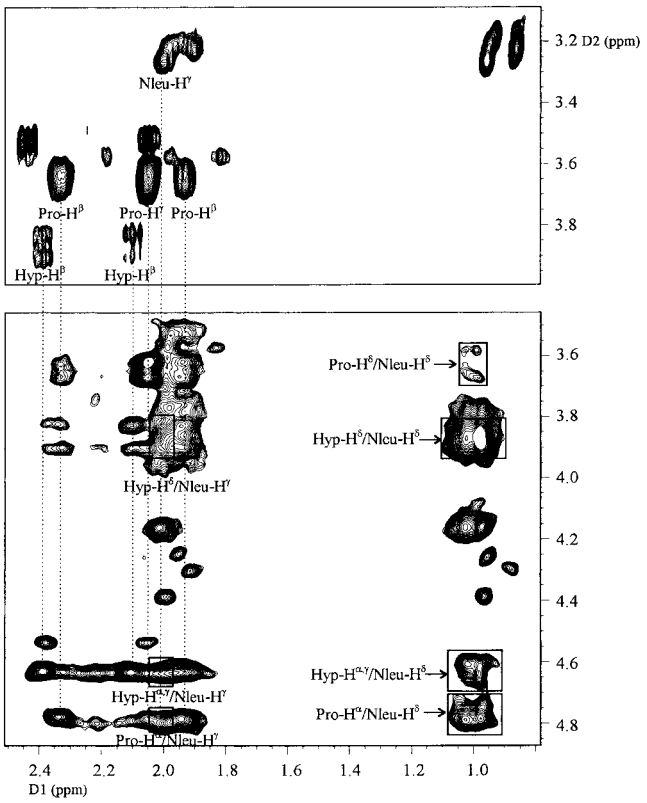

Figure 8. Expanded regions of a TOCSY (top) and NOESY (bottom) spectra of Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 in D_2O at 275 K. In the latter spectrum, interchain connectivities between Nleu and Pro/Hyp residues are indicated.

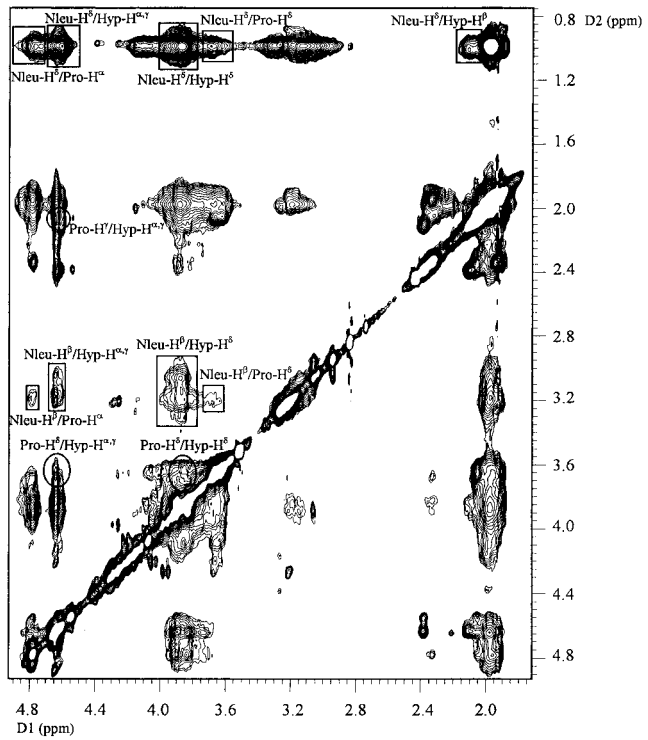

Figure 9. Aliphatic region of the NOESY spectrum of KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$ in D_2O solution at 275 K. The expected unique interchain NOEs based on the triple helical model of (Pro-Hyp-Gly)$_{10}$[26] are indicated by circles and "interunit" NOEs between Nleu and Pro, Hyp residues are indicated by rectangles.

Molecular modeled structures were built using Insight II.[33] Energy minimizations were carried out using the Discover program[34] and the force field CFF91 which provides high-quality parameters for all of the Nleu internal coordinates. A distant dependent dielectric constant was used to approximate the solvent effect. For the molecular modeling of triple helices which include the Gly-Nleu-Nleu trimeric sequence, we focused on the linear analogue Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2. The three chains staggered by one residue were constrained to have the same backbone torsions and interchain hydrogen-bond pattern as the structure proposed by Miller, Nemethy, and Sheraga[35] for (Gly-Pro-Hyp)$_n$. This structure was shown not to be sensitive toward substitutions of other amino acids in place of Pro

(33) Biosym Technologies, Inc., San Diego, CA.
(34) Discover 95.0/3.00 User Guide, Biosym/MSI, San Diego, CA, 1995.
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Table 3. Expected Unique Interchain NOEs (distances smaller than 4.5 Å) Based on the Model of Triple Helical (Pro-Hyp-Gly)$_{10}$ and Observed "Interunit" NOEs[a]

expected NOEs	exptl NOEs	interunit NOEs	
Hyp $C_\delta H_2$-Pro $C_\delta H_1$	w	Hyp $C_{\alpha,\gamma}$H-Nleu C_βH	w
Hyp $C_{\alpha,\gamma}$H-Pro $C_\delta H_1$	m	Hyp C_δH-Nleu C_βH	w
Pro $C_\gamma H_1$-Hyp $C_{\alpha,\gamma}$H	m	Hyp C_δH-Nleu C_γH	n.r.
Pro $C_\delta H_1$-Hyp $C_\beta H_1$	n.d.	Hyp $C_{\alpha,\gamma}$H-Nleu C_γH	n.r.
Pro $C_\delta H_1$-Hyp $C_\beta H_2$	n.d.	Hyp C_δH-Nleu C_δH	s
Pro $C_\delta H_2$-Hyp $C_\beta H_2$	n.d.	Hyp $C_{\alpha,\gamma}$H-Nleu C_δH	s
		Hyp C_βH-Nleu C_δH	m
		Pro C_αH-Nleu C_βH	w
		Pro C_δH-Nleu C_βH	w (only for the KTA-analog)
		Pro C_αH-Nleu C_γH	n.r.
		Pro C_αH-Nleu C_δH	s
		Pro C_δH-Nleu C_δH	w

[a] Connectivities which cannot be identified because of spectral overlap are not included in the table. Observed NOEs are indicated with w (weak), m (medium), and s (strong) based upon their intensities. The hydrogens are named according to ref 33. NOE cross-peaks refer to the Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 and the KTA-[(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$ unless otherwise indicated in parentheses. The n.d. stands for not determined NOE. The n.r. stands for not well resolved for integration.

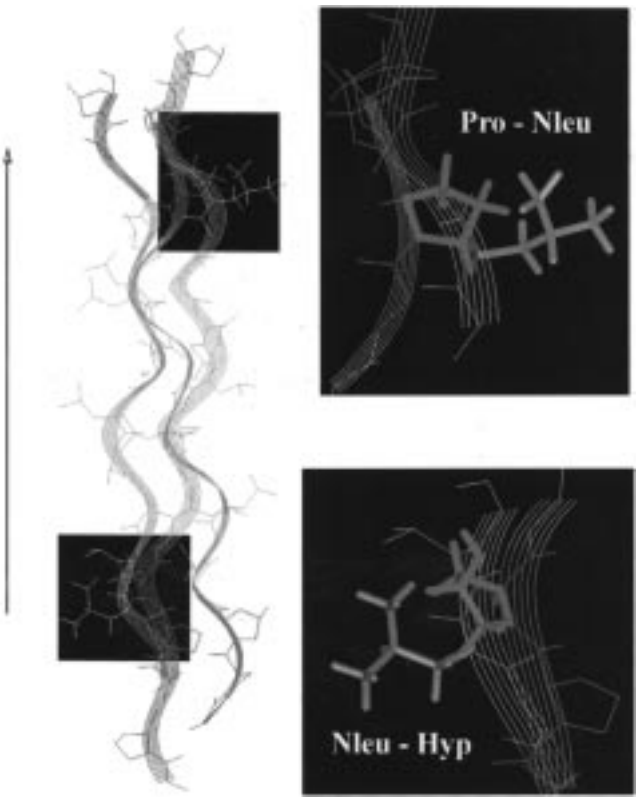

Figure 10. Central region of modeled triple helical structures of Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2. The two trimeric sequences (Gly-Pro-Hyp) and (Gly-Nleu-Nleu) are highlighted in red and green, respectively. On the left side the backbone ribbon diagram is shown for each of the three chains which are staggered by one residue. On the right side enlarged views of the N-terminus (bottom) and the C-terminus (top) of the (Gly-Nleu-Nleu)$_3$ sequence are reported showing the interchain proximities between Nleu and Hyp residues and between Pro and Nleu residues.

and Hyp residues[36] and therefore represents a good starting point as backbone conformation for molecular modeling. Energy minimizations were achieved fixing the backbone torsions (ϕ, ψ, and ω) at their original values and searching energy minima in the χ^1 ($C_\alpha-N-C_\beta-C_\gamma$), χ^2 ($N-C_\beta-C_\gamma-C_\delta$) torsional space of the Nleu residues as it has been done in our laboratories for (Gly-Pro-Nleu)$_n$[9] and (Gly-Nleu-Pro)$_n$[13] sequences. The ensemble interchain NOEs provide a critical test for the modeled structures.

Syntheses of Trimeric Building Blocks. Boc-Gly-Pro-Hyp(OBzl)-OH was synthesized by coupling Boc-Gly-Pro-OH[37] with Hyp(OBzl)-OBzl[38] using EDC and HOBt as coupling reagents. Hydrolysis of Boc-Gly-Pro-Hyp(OBzl)-OBzl with KOH, in THF/H_2O (1:1, v/v) solution afforded Boc-Gly-Pro-Hyp(OBzl)-OH.[39]

Boc-Gly-Nleu-Nleu-OH[19] was synthesized by coupling the peptoid containing dimer Boc-Gly-Nleu-OH[12] with Nleu-OEt[40] using BOP as the activating reagent to afford Boc-Gly-Nleu-Nleu-OEt. The trimer was then saponified with KOH in H_2O/THF (1:1, v/v) to give Boc-Gly-Nleu-Nleu-OH. The detailed procedures for the synthesis of Boc-Gly-Nleu-Nleu-OEt and Boc-Gly-Nleu-Nleu-OH are outlined below.

(A) Boc-Gly-Nleu-Nleu-OEt. A solution of Boc-Gly-Nleu-OH (26 g, 92 mmol) and HCl·Nleu-OEt (18 g, 92 mmol) was cooled to 0 °C. The coupling reagent, BOP (41 g, 92 mmol), was then added portionwise to the solution, followed by the addition of triethylamine (26 mL, 183 mmol). The ice bath was removed, and the solution was left stirring overnight. The DMF was removed by rotary evaporation under reduced pressure, and H_2O (800 mL) and EtOAc (800 mL) were added. The EtOAc layer was washed with H_2O (800 mL), $NaHCO_3$ (2 × 800 mL), brine (800 mL), 2 N $NaHSO_4$ (2 × 800 mL), and brine (3 × 800 mL). The organic layer was dried over anhydrous Na_2SO_4, the volatiles were removed in vacuo, and the residue was purified by flash chromatography (SiO2, 1/1, hexanes/EtOAc) to give 28 g (72% yield) of Boc-Gly-Nleu-Nleu-OEt: $R_f = 0.2$ (1/1 hexanes/EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.49 (br s, 1H), 4.22−4.01 (m, 8 H), 3.19−3.06 (m, 4 H), 1.93−1.82 (m, 2 H), 1.40 (s, 9 H), 1.24 (t, 3 H) 0.85−0.98 (m, 12 H).

FAB-MS: $m/z = 430$ (M + H) calcd for $C_{21}H_{39}N_3O_6$ + H 430, obsd 430.

(B) Boc-Gly-Nleu-Nleu-OH. A solution of Boc-Gly-Nleu-Nleu-OEt (4.7 g, 11 mmol), H_2O (20 mL), and THF (20 mL) was cooled to 0 °C. KOH was dissolved in H_2O (6 mL) and was then added slowly to the solution. The ice bath was removed, and the solution was left stirring at room temperature for 30 min. The THF was then removed in vacuo, and the water was then cooled to 0 °C in an ice bath. Concentrated HCl was added dropwise until the solution had a pH = 2. A white solid precipitated which was extracted with EtOAc. The EtOAc extracts were washed with brine (3 × 30 mL) and then dried over Na_2SO_4. After filtration, the solvent was removed in vacuo and the residual solid was recrystallized from CH_3CN to afford Boc-Gly-Nleu-Nleu-OH (3.4 g, 78% yield): ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.05−0.98 (m, 12 H), 1.42 (s, 9 H), 1.80−2.00 (m, 2 H), 3.10−3.19 (m, 4 H), 4.04−4.21 (m, 6 H), 5.73 (br s, 1 H).

MS-MALDI: $m/z = 402$ calcd for $C_{19}H_{35}N_3O_6$ + H 402, obsd 402; $m/z = 424$ calcd for $C_{19}H_{35}N_3O_6$ + Na 424, obsd 424.

General Solid-Phase Synthetic Methods. Solid-phase segment condensations were carried out using Boc synthetic strategies. The MBHA resin·HCl (200−400 mesh, 1% DVB, 0.45 mmol/g substitution, 0.2 mmol scale) was swollen in DCM for 1 h and washed with 10% TEA in DCM (10 mL, 2×, 2 min) followed by DCM (10 mL, 4×). A solution of 25% DMF in DCM was used as the solvent for the couplings. DIC (2 equiv) and HOBt (2 equiv) were used as the coupling reagents with either Boc-Gly-Pro-Hyp(OBzl)-OH (1.2−1.5 equiv) or Boc-Gly-Nleu-Nleu-OH (1.2−1.5 equiv). The reactions were monitored by the Kaiser ninhydrin test.[20] After completion of each coupling step, the solution was filtered and the resin was washed with MeOH (10 mL, 5 min) and DCM (10 mL, 1 min, 4×). The N-terminal Boc

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protection was removed with 30% TFA in DCM (10 mL, 30 min). After the deprotection, the solution was filtered and the resin washed with MeOH (10 mL, 5 min) and DCM (10 mL, 1 min, 6×). The resin was then neutralized with 10% TEA/DCM (10 mL, 2 min, 2×) followed by washing with DCM (10 mL, 1 min, 6×). The desired peptide chain was assembled by consecutive coupling reactions. To prepare the acetyl-terminated compounds, the N-terminal amines were treated with acetic anhydride (0.24 mL) and TEA (0.35 mL) in a solution of DMF (8 mL). The resin was then washed with MeOH (10 mL, 2×) and DCM (10 mL, 2×) and then dried under vacuum overnight. The peptide−peptoid conjugates were cleaved from the resin by standard HF cleavage methods. The HF cleavage reactions were allowed to proceed for 1 h at −5 to 0 °C. The resin was then dried under vacuum for 2 h in a desiccator containing KOH. The resin and product was washed with Et_2O (anhydrous) several times. The product was then separated from the resin by extracting with a mixture of H_2O and CH_3CN. The crude product was lyophillized.

Ac-(Gly-Pro-Hyp)-(Gly-Nleu-Nleu)-(Gly-Pro-Hyp)-NH_2. Ac-(Gly-Pro-Hyp)-(Gly-Nleu-Nleu)-(Gly-Pro-Hyp)-NH_2 was prepared following the procedures described above in the General Solid-Phase Synthetic Methods section. Preparatory RP-HPLC was used to purify Ac-Gly-Pro-Hyp-Gly-Nleu-Nleu-Gly-Pro-Hyp-NH_2 as a white powder (30% yield based on resin substitution). The analytical HPLC gave a single peak, t_R = 10.5 min (15−40% CH_3CN in 0.1% TFA over 20 min at 1 mL/min).

FAB-MS: m/z = 1009 calcd for $C_{40}H_{64}N_{10}O_{12}$ + Cs 1009, obsd 1009.

Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2. Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2 was prepared following the procedures described above in the General Solid-Phase Synthetic Methods section. Preparatory RP-HPLC was used to purify Ac-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2 as a white powder (20% yield based on resin substitution). The analytical HPLC gave a single peak, t_R = 20.1 min (15−40% CH_3CN in 0.1% TFA over 20 min at 1 mL/min).

FAB-MS: m/z = 1717 calcd for $C_{78}H_{123}N_{19}O_{23}$ + Na 1717, obsd 1717.

Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2. Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 was prepared following the procedures described above in the General Solid-Phase Synthetic Methods section. Preparatory RP-HPLC was used to purify Ac-(Gly-Pro-Hyp)$_3$-(Gly-Nleu-Nleu)$_3$-(Gly-Pro-Hyp)$_3$-NH_2 as a white powder (11% yield based on resin substitution). The analytical HPLC gave a single peak, t_R = 15.3 min (25−55% CH_3CN in 0.1% TFA over 20 min at 1 mL/min).

MALDI-MS: m/z = 2550 calcd for $C_{116}H_{182}N_{28}O_{34}$ + K 2550, obsd 2550.

KTA-[Gly-(Gly-Pro-Hyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$. Boc-[Gly-[(Gly-Pro-Hyp(OBzl)]$_2$-(Gly-Nleu-Nleu)$_2$-[Gly-Pro-Hyp(OBzl)]$_2$-MBHA (0.27 mmol based on resin substitution) was prepared following the procedures described above in the General Solid-Phase Synthetic Methods section. The Boc group was removed with 30% TFA/DCM (15 mL, 1.0 mL of anisol was added as scavenger). The resin was washed with MeOH (1 × 10 mL, 1 min) and DCM (4 × 10 mL, 1 min), followed by 10% TEA in DCM (2 × 15 mL, 2 min) and DCM (4 × 10 mL, 1 min). KTA-(Gly-OH)$_3$ (35 mg, 0.08 mmol) and HOBt (50 mg) were added to the reaction vessel, and a solution of 25% DMF/DCM (15 mL) was added. After 3 d, the resin was washed with MeOH (2 × 15 mL) and DCM (2 × 15 mL), and the resin was then dried overnight under vacuum. HF cleavage methods were used to cleave the material from the resin. The resin was then washed with Et_2O, and the peptide−petoid conjugate was extracted with H_2O. Preparatory RP-HPLC was carried out to obtain the product KTA-[Gly-(Gly-ProHyp)$_2$-(Gly-Nleu-Nleu)$_2$-(Gly-Pro-Hyp)$_2$-NH_2]$_3$ as a white powder (4% yield based on resin substitution). The analytical HPLC gave a single peak, t_R = 14.1 min (27−55% CH_3CN in 0.1% TFA over 20 min at 1 mL/min).

MALDI-MS: m/z = 5357 calcd for $C_{246}H_{384}N_{60}O_{72}$ + Na 5357, obsd 5357.

Acknowledgment. This research was funded by a grant from Ciba Vision Inc.

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