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Published on Web 11/05/2002

TREN (Tris(2-aminoethyl)amine): An Effective Scaffold for the Assembly of Triple Helical Collagen Mimetic Structures

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Received July 15, 2002

Abstract: A new scaffold, TREN-(suc-OH)$_3$ where TREN is tris(2-aminoethyl)amine and suc is the succinic acid spacers, was incorporated to assemble triple helices composed of Gly-Nleu-Pro sequences (Nleu denotes *N*-isobutylglycine). Extensive biophysical studies which include denaturation studies, CD and NMR spectroscopy, and molecular modeling demonstrated that TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ ($n = 5$ and 6) form stable triple helical structures in solution. A comparative analysis of TREN-assembled and KTA-assembled collagen mimetics (KTA denotes Kemp triacid, 1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid) indicates that the flexibility of the TREN scaffold is superior to the KTA scaffold in inducing triple helicity. This effect most likely arises from the flexibility of the TREN scaffold which allows the three peptide chains to adjust their register for a tighter triple helical packing.

Introduction

Many substances of biological origin have been employed as biomaterials. For example, macromolecules derived from connective tissue have been isolated, chemically modified, and used in medical applications such as drug-delivery systems and as tissue repair agents.[1,2] Connective tissue of vertebrates consists of cells and extracellular fibers embedded in a matrix composed of collagen, elastin, fibronectin, laminin, and proteoglycans.[1] Collagen is the major component of connective tissue and makes up 30% of all proteins in the body, primarily in tissues for mechanical strength, bone, cartilage, skin, and tendon. It is responsible for the structural framework of blood vessels and organs.[3−6]

Collagen is defined by a triple helix motif, where three left-handed polyproline II-like chains supercoil together to form a right-handed super helix.[7−9] In this supercoiled structure, the three chains are arrayed together by a register shift of one residue. This unique packing of the three polypeptide chains requires a specific amino acid sequence. Collagen is composed primarily of Gly-Xaa-Yaa repeating trimeric units, where Xaa and Yaa residues are predominantly Pro and Hyp, respectively.[10−12]

In synthetic collagen structures, the primary sequence of peptides can significantly affect folding and conformational stability as studied by Brodsky, Raines and Goodman.[13−20] However, the incorporation of a template or scaffold into the design of peptidomimetics can reinforce intramolecular folding of peptides. Mutter and collaborators introduced the TASP (template-assembled synthetic proteins) concept in their synthesis of 4α-helical bundles.[21,22] In their studies, template assembly of peptides maintained the integrity of the folded structure with high conformational stability. DeGrado and co-workers expanded on the concept of templates and synthesized a multiheme binding protein that assembles into a four-helix bundle dimer accommodating two bis-histidyl groups.[23,24] Ghadiri and co-workers demonstrated that 15 to 20-residue amphiphilic polypeptides, with N-terminal pyridyl or bis-pyridyl functionality can undergo intermolecular self-assembly upon Ru(II) complexation to form stable three- or four-helix bundle metallo-

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(1) Jorge-Herrero, E. *Anal. Quim. Int. Ed.* **1997**, *93*, S32−S37.
(2) Lee, C. H.; Singla, A.; Lee, Y. *Int. J Pharm.* **2001**, *221*, 1−22.
(3) Pachence, J. M. *J. Biomed. Mater. Res. (Appl. Biomat.)* **1996**, *33*, 35−40.
(4) Nimni, M. E.; Chueng, D.; Strates, S. B.; Kodama, M.; Skiekh, K. *J. Biomed. Mater. Res.* **1987**, *21*, 741−771.
(5) Nimni, M. E.; Harkness, R. D. In *Collagen*; Nimni, M. E., Ed.; Molecular Structure and Functions of Collagen; Vol. 1; Boca Raton: CRC Press: 1988; Chapter 1.
(6) Eyre, E. R. *Science* **1980**, *207*, 1316.
(7) Rich, A.; Crick, F. H. C. *J. Mol. Biol.* **1961**, *3*, 483−506.
(8) Van Der Rest, M.; Garrone, R. *FASEB* **1991**, *5*, 2814−2823.
(9) Linsenmayer, T.; Hay, E. D. *Cell Biology of Extracellular Matrix*; Plenum Press: New York, 1991; pp 7−44.
(10) Ramachandran, G. N. *Treatise on Collagen*; Ramachandran, G. N., Ed.; New York: Academic Press: 1967; Vol. 1, pp 103−183.
(11) Fietzek, P. P.; Kuhu, K. *Mol. Cell Biochem.* **1975**, *8*, 141−157.
(12) Fraser, R. D. B.; McRae, T. P. *Conformations in Fibrous Proteins*; Academic Press: New York, 1973.
(13) Long, C. G.; Braswell, E.; Zhu, D.; Apigo, J.; Baum, J.; Brodsky, B. *Biochemistry* **1993**, *32*, 11688−11695.
(14) Shah, N. K.; Ramshaw, J. A. M.; Kirkpatrick, A.; Shah, C.; Brodsky, B. *Biochemistry* **1996**, *35*, 10262−10268.
(15) Holmgren, S. K.; Taylor, K. M.; Bretscher, L. E.; Raines, R. T. *Nature* **1998**, *392*, 666−667.
(16) Holmgren, S. K.; Bretscher, L. E.; Taylor, K. M.; Raines, R. T. *Chem. Biol.* **1999**, *6*, 63−70.
(17) Panasik, N. Jr.; Eberhardt, E. S.; Edison, A. S.; Powell, D. R.; Raines, R. T. *Int. J. Pept. Protein Res.* **1994**, *44*, 262−269.
(18) Jefferson, E. A.; Locardi, E.; Goodman, M. *J. Am. Chem. Soc.* **1998**, *120*, 7420−7428.
(19) Kwak, J.; Jefferson, E. A.; Bhumralkar, M.; Goodman, M. *Bioorg. Med. Chem.* **1999**, *7*, 153−160.
(20) Goodman, M.; Melacini, G.; Feng, Y. *J. Am. Chem. Soc.* **1996**, *44*, 10928−10929.
(21) Tuchscherer, G.; Domer, B.; Sila, U.; Kamber, B.; Mutter, M. *Tetrahedron* **1993**, *17*, 3559−3575.
(22) Grove, A.; Mutter, M.; Rivier, J. E.; Montal, M. *J. Am. Chem. Soc.* **1993**, *115*, 5919−5924.
(23) Handel, T. M.; DeGrado, W. F. *J. Am. Chem. Soc.* **1990**, *112*, 6710−6711.
(24) Handel, T. M.; Williams, S. A.; DeGrado, W. F. *Science* **1993**, *261*, 879−885.

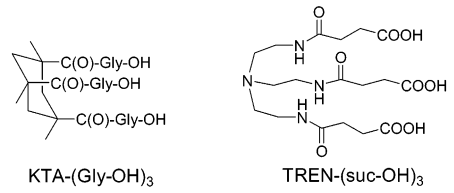


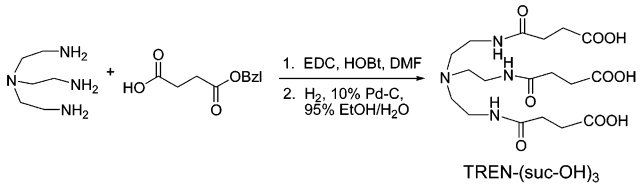
Figure 1. The KTA-(Gly-OH)$_3$ and the TREN-(suc-OH)$_3$ scaffolds. KTA = Kemp triacid; TREN = tris-(2-aminoethyl)amine; suc = succinic acid.

proteins.[25,26] These constructs established the feasibility of binding peptides to cofactors which lead to nativelike folding. In all cases, de novo design by scaffold-mediated folding is an effective technique for the design of peptidomimetics.

Template-assembled collagen arrays have been shown to stabilize triple helicity. The incorporation of a template or scaffold (while we have used the term "template" in the past, we now use "scaffold" to refer to the same design technique for the assembly of triple helical collagen structures) into the synthesis of collagen structures favors intramolecular folding of triple helices and increases stability by changing the entropic effects. The appropriate collagen mimetic scaffold has three functional groups connected to three peptide chains which allow the proper packing with the one amino acid register shift of adjacent chains. Heidemann et al. employed 1,2,3-propane carboxylic acid and lysine dimer scaffolds for the assembly of collagen-like triple helices.[27,28] Fields and co-workers utilized the lysine dimer scaffold for the design and synthesis of bioactive collagen-like structures in which sequences from collagen types I and IV were incorporated between helical Gly-Pro-Hyp structural units.[29−31] Tanaka et al. derivatized the Lys dimer template for both N- and C-terminal attachment to peptide chains to design di-template arrays of triple helices.[32] Moroder and co-workers further developed the scaffold concept by utilizing the disulfide chemistry of Cys residues to anchor the three peptides at the C terminus.[33] Moroder utilized a strategy of orthogonal protection of Cys residues which facilitated the chemoselective syntheses of heterotrimeric collagen structures.

Our laboratories have established a collagen mimetic program to investigate the structural requirements for the development of novel collagen mimetic biomaterials. In previous work, we reported the synthesis and biophysical analysis of scaffold-assembled collagen structures, using the Kemp triacid[34] (KTA, *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid) as the scaffold for triple helical formation.[35] The KTA-(Gly-OH)$_3$ scaffold (Figure 1) is a conformationally constrained molecule in which the carboxyl groups are directed in the axial position. The Gly residues were incorporated as spacers for peptide chain attachment to reduce the steric hindrance of the three peptide chains and to provide flexibility for the adjustment of the one-residue register shift necessary for collagen-like triple helical conformations.

Scheme 1



Triple helicity of collagen structures composed of Gly-Pro-Hyp,[36,37] Gly-Pro-Nleu,[20,38,39] and Gly-Nleu-Pro[40,41] sequences where Nleu denotes *N*-isobutylglycine, were investigated. The KTA-(Gly-OH)$_3$ scaffold was shown to facilitate and enhance the formation of triple helical structures as compared to their single-chain acetylated counterparts. In addition, these studies demonstrated that structures composed of Gly-Nleu-Pro sequences have higher triple helical propensities than structures composed of the isomeric Gly-Pro-Nleu sequences.

In our efforts toward the design of novel collagen mimetics, we expanded our scaffold studies to include a flexible scaffold. Similar to KTA-assembled structures, we synthesized a TREN (tris(2-aminoethyl)amine) scaffold (Figure 1), attached a series of collagen mimetic structures composed of Gly-Nleu-Pro sequences, and assessed their triple helical propensities by thermal denaturation and CD spectroscopy as well as by NMR spectroscopy and molecular modeling. In the present study, we show that the TREN scaffold, though flexible, enhances triple helicity over that of the KTA scaffold. Incidentally, while functionalization for further development of macromolecular arrays cannot be achieved readily for KTA-assembled triple helices, the tertiary amine core of the TREN can be derivatized to create structural assemblies via complexation or quarternization.

The TREN Scaffold. The TREN molecule is a flexible tripodal structure with three aminoethyl groups. Scheme 1 shows the synthesis of TREN-(suc-OH)$_3$, where suc denotes succinic acid. The succinic acid groups extend the flexibility of the scaffold and provide terminal carboxylates for attachment of peptide chains. In addition, the succinic acid spacers relieve any steric hindrance by extending the reactive sites for ease in synthesis. Monobenzylated succinate was prepared by literature procedures.[42] The amino groups of TREN were coupled to monobenzylated succinate using 1-ethyl-3-[3-(dimethylamino)-propyl] carbodiimide (EDC) and *N*-hydroxybenzotriazole (HOBt) as the activating reagents. The benzyl ester groups were removed by hydrogenation to afford the tricarboxylic acid, TREN-(suc-OH)$_3$.

Scaffold assembly of collagen mimetic structures was prepared on a solid support (Scheme 2). The synthesis of the tripeptide building block and the peptide chains composed of

(25) Ghadiri, M. R.; Soares, C.; Choi, C. *J. Am. Chem. Soc.* **1992**, *114*, 4000−4002.
(26) Ghadiri, M. R.; Case, M. A. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1594−1597.
(27) Greiche, Y.; Heidemann, E. *Biopolymers* **1979**, *18*, 2359−2361.
(28) Roth, W.; Heidemann, E. *Biopolymers* **1980**, *19*, 1909−1917.
(29) Fields, C. G.; Grab, B.; Lauer, J. L.; Fields, G. B. *Anal. Biochem.* **1995**, *231*, 57−64.
(30) Grab, B.; Miles, A. J.; Furch, L. T.; Fields, G. B. *J. Biol. Chem.* **1996**, *271*, 12234−12240.
(31) Miles, A.; Skubitz, A. P. N.; Furch L. T.; Fields, G. B. *J. Biol. Chem.* **1994**, *269*, 30939−30945.
(32) Tanaka, Y.; Suzuki, K.; Tanaka, T. *J. Pept. Res.* **1998**, *51*, 413−419.
(33) Ottl, J.; Moroder, L. *J. Am. Chem. Soc.* **1999**, *121*, 653−661.
(34) Kemp, D. S.; Petrakis, K. S. *J. Org. Chem.* **1981**, *46*, 5140−5143.
(35) Goodman, M.; Feng, Y.; Melacini, G.; Taulane, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 5156−5157.
(36) Feng, Y.; Melacini, G.; Taulane, J. P.; Goodman, M. *J. Am. Chem. Soc.* **1996**, *118*, 10351−10358.
(37) Melacini, G.; Feng, Y.; Goodman, M. *J. Am. Chem. Soc.* **1996**, *118*, 10359−10364.
(38) Feng, Y.; Melacini, G.; Taulane, J. P.; Goodman, M. *Biopolymers* **1996**, *39*, 859−872.
(39) Melacini, G.; Feng, Y.; Goodman, M. *J. Am. Chem. Soc.* **1996**, *118*, 10725−10732.
(40) Feng, Y.; Melacini, G.; Goodman, M. *Biochemistry* **1997**, *36*, 8716−8724.
(41) Melacini, G.; Feng, Y.; Goodman, M. *Biochemistry* **1997**, *36*, 8725−8732.
(42) Lay, L.; Nicotra, F.; Pangrazio, C.; Panza, L.; Russon, G. *J. Chem. Soc., Perkin Trans. 1* **1994**, 333−338.

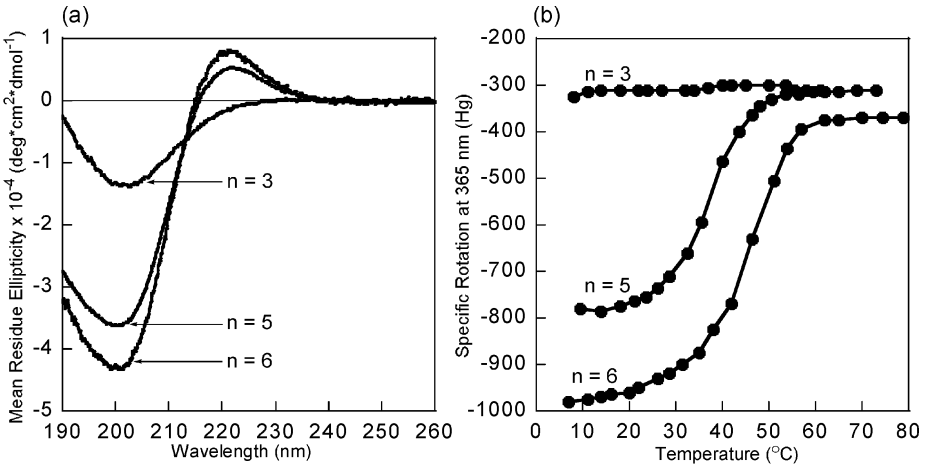
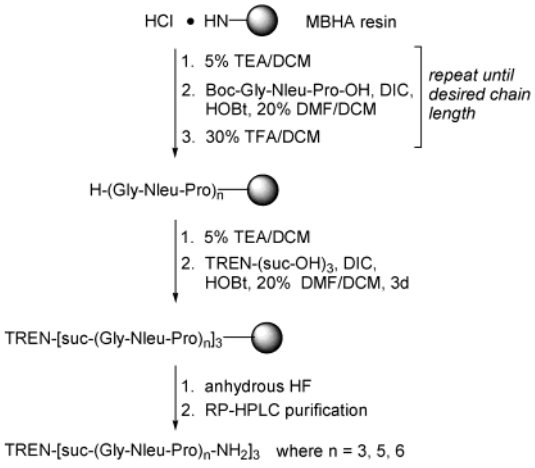


Figure 2. (a) CD spectra of TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ where n = 3, 5, 6 in solution at room temperature. (b) Melting transition curves of TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 3, 5, 6), monitored by temperature-dependent optical rotation measurements. Measurements were carried out at 0.2 mg/mL in H_2O.

Scheme 2



Gly-Nleu-Pro sequences were achieved in a manner similar to that previously reported.[40] To obtain the optimum yield of the desired scaffold-assembled peptide, TREN-(suc-OH)$_3$ was used as the limiting reagent. Treatment with anhydrous HF and purification by RP-HPLC afforded the target scaffold-assembled compounds (details in Supporting Information). Mass spectrometry and analytical RP-HPLC were employed to characterize the desired peptide structures.

Results and Discussion

CD Spectroscopy. Natural collagen has a unique CD spectrum in which a small positive peak appears at about 220 nm, a crossover near 213 nm and a large trough at approximately 197 nm.[43−45] These features have been used as a reference to determine the presence of synthetic collagen-like triple helices in solution. The CD profiles of our synthetic collagen mimetics were measured in both H_2O and ethylene glycol:H_2O (EG:H_2O, 2:1, v/v). The EG:H_2O mixture is known to stabilize helical structures and is useful to amplify and detect small amounts of weak triple helical collagen-like structures.[44,45] The CD spectra for the TREN-assembled collagen mimetic structures in H_2O are shown in Figure 2a (see Supporting Information for spectra in EG:H_2O).

Table 1. CD Parameters of Synthetic TREN-Assembled Collagen Mimetic Structures Composed of Gly-Nleu-Pro Sequences

peptide	max (nm)	crossover (nm)	min (nm)	Rpn
TREN-[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$[a]	- - -	- - -	203 (-1.3×10^4)	- - -
TREN-[suc-(Gly-Nleu-Pro)$_5$-NH$_2$]$_3$[a]	222 (0.5×10^4)	216	200 (-3.6×10^4)	0.14
TREN-[suc-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$[a]	221 (0.8×10^4)	215	200 (-4.3×10^4)	0.19
natural collagen[b]	198 (0.7×10^4)	213	198 (-5.4×10^4)	0.13
(Gly-Pro-Hyp)$_{10}$-NH$_2$[c]	225 (0.4×10^4)	218	198 (-3.4×10^4)	0.13

[a] Measurements were carried out at 0.2 mg/mL in H_2O. The CD spectra were taken at room temperature. [b] References 44 and 45. [c] Reference 47.

The CD spectrum for TREN-[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ exhibits a spectrum typical of a disordered array as seen by the shallow trough and lack of the positive peak. However, the CD spectra for the longer analogues TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 5 and 6) are indicative of triple helical structures, where the crossover is near 215 nm, the peak at 222 nm, and the trough around 202 nm. These spectral absorbances and Rpn values,[36,40] listed in Table 1, are comparable to known collagen mimetic triple helical conformations exhibiting natural collagen and (Gly-Pro-Hyp)$_{10}$-NH$_2$.[44,45,47]

Denaturation Studies. The melting transitions are consistent with our observations from CD spectroscopy. Temperature-dependent optical rotation measurements were used to monitor the transition from triple helical to nontriple helical structures. Figure 2b shows the observed melting transitions for the TREN peptides in H_2O. While TREN-[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ exhibits no transition, the T_m (melting temperature) values for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 5, 6) are 38 and 46 °C, respectively. As the peptide chain length increases, the thermal stability of the TREN-assembled structures increases which is in agreement with the increase in Rpn values from the

(43) Sakakibara, S.; Kishida, Y.; Okuyama, K.; Tanaka, N.; Ashida, T.; Kakudo, M. *J. Mol. Biol.* **1972**, *65*, 371−373.
(44) Brown, F. R., III; Carver, J. P.; Blout, E. R. *J. Mol. Biol.* **1969**, *39*, 307−313.
(45) Brown, F. R., III; DiCorato, A.; Lorenzi, G. P.; Blout, E. R. *J. Mol. Biol.* **1972**, *63*, 85−99.
(46) Brodsky-Doyle, B.; Leonard, K. R.; Reid, K. B. M. *Biochem. J.* **1976**, *159*, 279−286.
(47) Venugopal, M. G.; Ramshaw, J. A. M.; Braswell, E.; Zhu, D.; Brodsky, B. *Biochemistry* **1994**, *33*, 7948−7956.

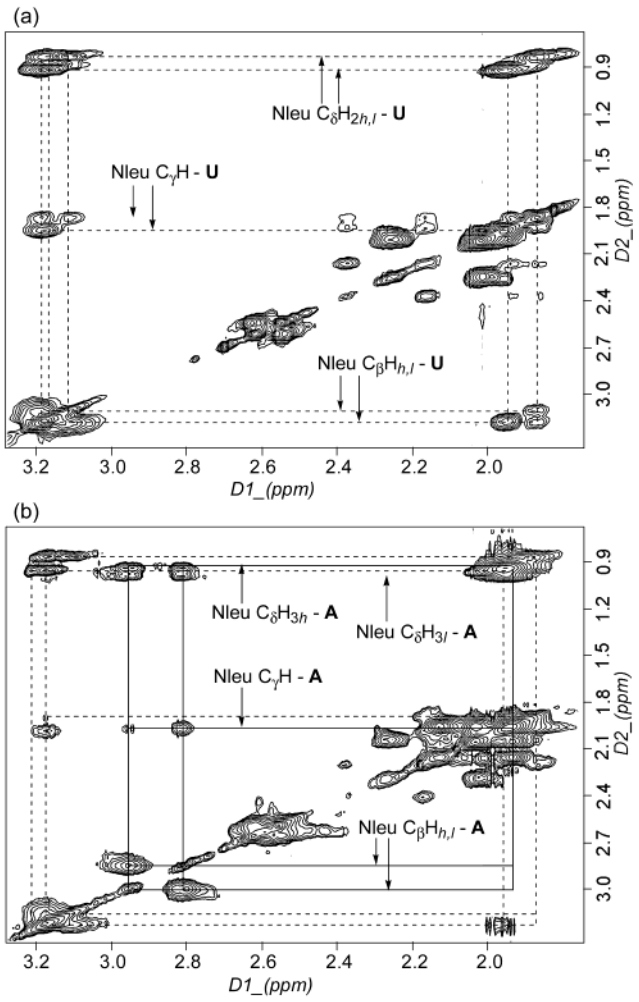


Figure 3. Comparison of TOCSY spectra for TREN-[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ (a) and TREN-[suc-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ (b). The dashed lines indicate Nleu resonances for nonhelical or unassembled (U) structures and the solid lines indicate the Nleu resonances for triple helices or assembled (A) structures. Measurements were carried out in D_2O at 27 °C.

CD analysis of respective analogues. In addition, melting transitions for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$, (n = 3, 5, 6) were assessed in EG:H_2O (2:1, v/v) solutions. Transitions for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 5, 6) were observed at 55 and 67 °C, respectively (see Supporting Information). As expected, the T_m values in EG:H_2O are higher than those measured in H_2O since it is known that EG:H_2O mixtures enhance the stability of helical conformations.[44,45]

NMR Spectroscopy. The formation of triple helical conformations results in unique NMR spin systems.[48,49] Studies by NMR spectroscopy were carried out for the TREN-assembled structures according to a systematic procedure described by Melacini et al.[37,39,41] Analysis by DQF-COSY and TOCSY experiments demonstrated the presence of an additional set of resonances for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 5, 6). These resonances were not observed in the spectra for TREN-[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ under the same conditions. Figure 3 shows the difference in the TOCSY spectra between TREN-

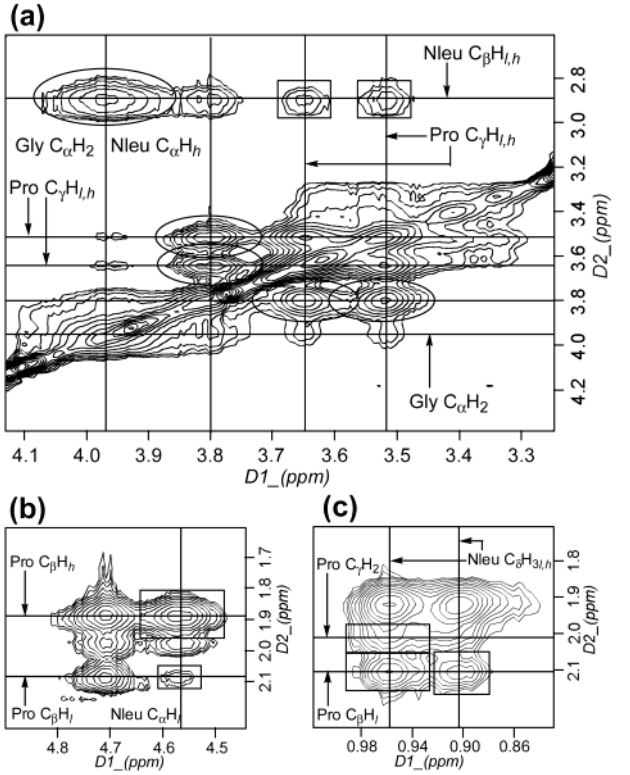


Figure 4. Portions of the NOESY spectra for TREN-[suc-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$. The circled resonances in (a) indicate sequential intrachain NOEs between Gly, Nleu, and Pro, whereas the boxed resonances represent the ensemble interchain NOEs between Pro and Nleu residues. Additional ensemble interchain NOEs between Pro and Nleu side chains are shown in (b) and (c).

[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ and TREN-[suc-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ in D_2O at 27 °C.

This new set of resonances was established as the assembled set for triple helical structures TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 5, 6) by a series of experimental observations similar to that described previously.[37,39,41] The relative intensity of the new resonances decreased with increasing temperature, a low hydrogen-exchange rate of the Gly NH was measured at low temperatures, and the new resonances originated from conformations which exchange slowly on the NMR time scale.[48,50] In addition, the NOESY spectra (Figure 4) exhibits sequential (intrachain) connectivities within a distinct tripeptide unit from Gly to Nleu, Nleu to Pro, and Pro to Gly, which are consistent with the new set of resonances for assembled structures in the TOCSY spectrum (Figure 3b). The NOESY spectrum also contains information for interchain NOEs between new resonances which are discussed below under Molecular Modeling.

Table 2 contains a listing of the chemical shifts of the assembled set of resonances. From this information, Figure 5 shows a comparison of the 1D spectra in the region 3.10−2.83 ppm of the three TREN compounds. This region contains the resonances for the assembled Nleu $C_\beta H_{l,h}$ as seen in the longer analogues (boxed in Figure 5b,c). Conversely, the unassembled Nleu $C_\beta H_{l,h}$ resonances are in the region from 3.14 to 3.22 ppm which is evident for TREN-[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ (Figure 5a). The assembled and unassembled resonances for Nleu $C_\beta H_{l,h}$

(48) Li, M.-H.; Fan, P.; Brodsky, B.; Baum, J. *Biochemistry* **1993**, *32*, 7377−7387.
(49) Brodsky, B.; Li, M.; Long, C. G.; Apigo, J.; Baum, J. *Biopolymers* **1992**, *32*, 447−451.
(50) Miller, M. H.; Nemethy, G.; Scheraga, H. A. *Macromolecules* **1980**, *13*, 910−913.

Table 2. Chemical Shift Assignments of Proton Resonances for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ Where n = 5, 6

		chemical shift (ppm)[a]					
resonance set	residue	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	other
assembled[b]	Gly	7.61	3.96				
	Nleu		4.61, 3.79	2.98, 2.83	2.90	0.97, 0.91	
	Pro		4.57	2.12,1.92	2.01	3.62, 3.50	
	TREN						3.50, 3.27
	suc						2.60, 2.50

[a] The ^{1}H chemical shifts are reported relative to tetramethylsilane (TMS). [b] Refer to ref 40 for the assignments of unassembled resonances.

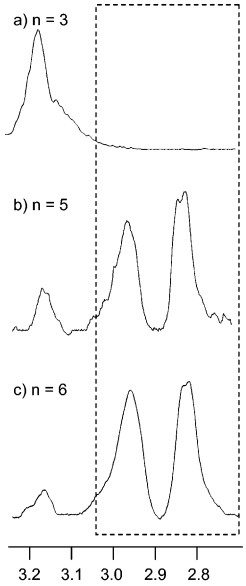


Figure 5. Comparison of the 1D ^{1}H NMR spectral regions of the Nleu $C_\beta H_2$ for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ where n = 3, 5, 6. The boxed area shows the appearance of new resonances for the assembled structures (n = 5 and 6). Measurements were carried out at in D_2O at 27 °C.

are well-resolved with no significant overlap and can therefore be used to identify and quantify triple helical conformations.

The integration for the assembled Nleu $C_\beta H_2$ resonances (2.98−2.83 ppm) with increasing temperatures was used to monitor the melting of triple helical conformations. The measurements were normalized to reflect helical content of the structures under investigation. As described earlier, the shorter analogue TREN-[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ does not exhibit a cooperative melting curve in H_2O, indicating that the peptide chain length is too short to support a triple helix. Figure 6 shows that the transition from helical to nonhelical for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 5, 6) is cooperative with melting temperatures in D_2O at 38 and 48 °C, respectively. These results are consistent with the observed transitions from temperature-dependent optical rotation measurements.

Further insight into the relationship between the TREN scaffold and triple helix conformations can be obtained from the differences in the methylene chemical shifts of the core TREN molecule (see NMR spectra in the Supporting Information). At high temperature (T = 50 °C), only two distinguishable resonances at 3.50 and 3.27 ppm were observed for TREN-[suc-(Gly-Nleu-Pro)$_5$-NH$_2$]$_3$, indicating spectral overlap of the six methylene groups from the three chains. This overlap results from the rotational ternary symmetry of the TREN structure. At T = 4 °C, the triple helix for the TREN-[suc-(Gly-Nleu-Pro)$_5$-NH$_2$]$_3$ is favored, and a signal splitting of these resonances

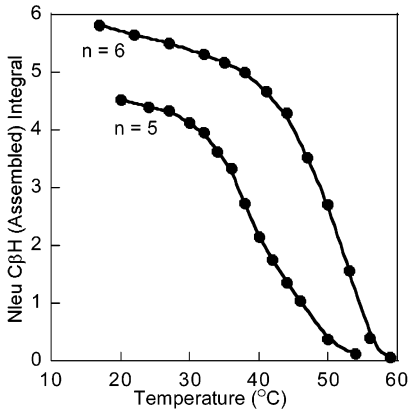


Figure 6. Melting transitions for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ where n = 5 and 6 in D_2O. Denaturation was monitored by temperature-dependent integration of assembled Nleu $C_\beta H$ resonances.

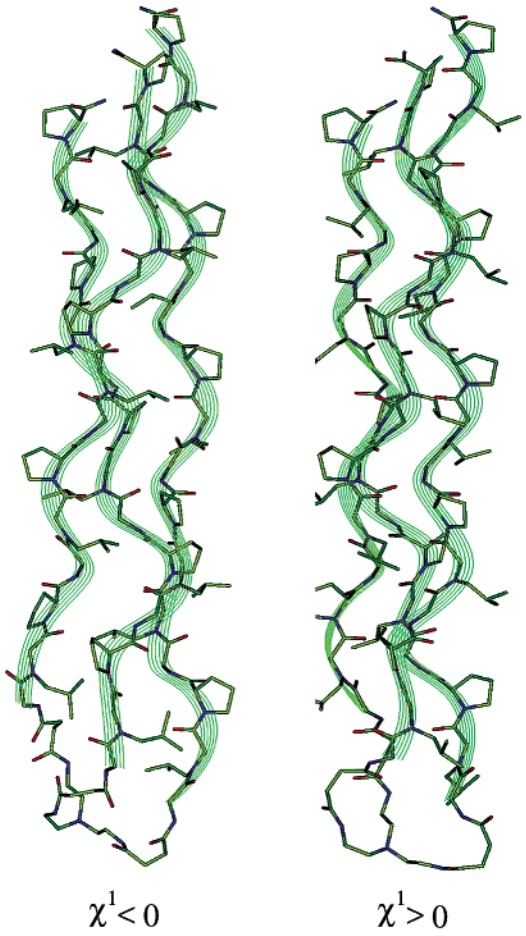


Figure 7. Lowest-energy structures of TREN-[suc-(Gly-Nleu-Pro)$_5$-NH$_2$]$_3$ from each of the two family of clusters generated from molecular modeling. The two families are based on the orientation of the isobutyl groups of the Nleu side chains: $\chi^1 < 0$ (pointing in) and $\chi > 0$ (pointing out).

was observed. Hence, the rotational three-fold symmetry of the TREN template is disrupted by the triple helical register (screw symmetry). At low temperature, the six methylene groups in the succinic acid linkers exhibit the same signal splitting as noted above for the TREN structure as a consequence of triple helix formation. In the absence of the triple helix, the signal splitting for the methylene groups in the succinic acid linkers disappears, and only two resonances are observed (2.60 and 2.54 ppm). In

Table 3. Predicted and Observed Interchain NOEs for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 5, 6)

predicted NOEs	observed NOEs	Nleu side chain orientation	predicted NOEs	observed NOEs	Nleu side chain orientation
Gly NH−Nleu C_γH	X[a]	in	Nleu C_γH − Pro $C_\beta H_r$	?	in
Nleu $C_\alpha H_s$ - Pro C_αH	X	out	Nleu C_γH − Pro $C_\beta H_s$	?	in
Nleu $C_\alpha H_s$ - Pro $C_\beta H_s$	X	out	Nleu C_γH − Pro $C_\beta H_r$	?	in
Nleu $C_\beta H_s$ - Pro C_αH	X	out	Nleu C_γH − Pro $C_\gamma H_2$	?	in
Nleu $C_\beta H_r$ - Pro C_αH	X	out	Nleu C_γH − Pro $C_\delta H_r$	?	in
Nleu $C_\beta H_s$ - Pro $C_\beta H_r$	X	out	Nleu $C_\delta H_{3,r}$ - Pro C_αH	X	in
Nleu $C_\beta H_s$ - Pro $C_\beta H_r$	X	out	Nleu $C_\delta H_{3,s}$ - Pro C_αH	X	in
Nleu $C_\beta H_s$ - Pro $C_\beta H_s$	X	out	Nleu $C_\delta H_{3,r}$ - Pro $C_\beta H_r$	X	in
Nleu $C_\beta H_r$ - Pro $C_\beta H_s$	X	out	Nleu $C_\delta H_{3,r}$ - Pro $C_\beta H_s$	X	in
Nleu $C_\beta H_s$ - Pro $C_\gamma H_2$	X	out	Nleu $C_\delta H_{3,s}$ - Pro $C_\beta H_s$	X	in
Nleu $C_\beta H_r$ - Pro $C_\gamma H_2$	X	out	Nleu $C_\delta H_{3,r}$ - Pro $C_\gamma H_2$	X	in
Nleu $C_\beta H_s$ - Pro $C_\delta H_r$	X	out	Nleu $C_\delta H_{3,s}$ - Pro $C_\gamma H_2$	X	in
Nleu $C_\beta H_r$ - Pro $C_\beta H_r$	X	out	Pro $C_\gamma H_2$ - Gly $C_\alpha H_2$	X	
Nleu C_γH − Pro C_αH	?[b]	in	Pro $C_\delta H_r$ - Gly $C_\alpha H_2$	X	

[a] The symbol "X" indicates observed NOEs. [b] The symbol "?" indicates that an NOE cross-peak was obscured by spectral overlap.

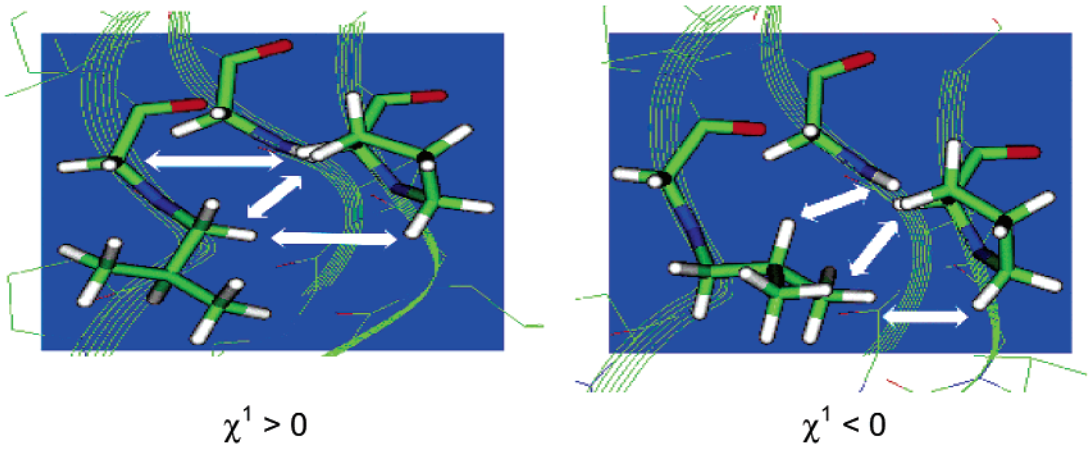


Figure 8. Enlarged portions of the lowest-energy conformations of TREN-[suc-(Gly-Nleu-Pro)$_5$-NH$_2$]$_3$. The white arrows indicate the observed ensemble interchain NOE interactions when $\chi^1 < 0$ (Nleu side chain pointing in) and $\chi^1 > 0$ (Nleu side chain pointing out).

addition, we observed the same two resonances for the nonhelical TREN-[suc-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ at high and low temperatures. Similar results for temperature-dependent scaffold studies were reported for the KTA-assembled structures.[36]

Molecular Modeling. The assignment of the assembled set of resonances to triple helical structures is also supported by the NOESY spectra which were analyzed according to a procedure previously used for NMR studies of other collagen-like structures.[37,39,41,48] This approach relies on the distinction between intra- and interchain NOEs on the basis of triple helical modeled structures. Similar to studies based on KTA-assembled peptides, structures are clustered into two families on the basis of the Nleu χ^1 torsions (details in Supporting Information).[39,41] When χ^1 is negative, the isobutyl group of the Nleu side chain points toward the Pro ring ("*in*" orientation) and when χ^1 is positive, the isobutyl group of the Nleu side chain points away from the Pro ring ("*out*" orientation). The lowest-energy structures from each of the two clusters showing the "*in*" and "*out*" orientation for the Nleu side chain are shown in Figure 7. All experimental interchain NOEs between Gly, Nleu, and Pro residues are observed in the models as distances <4 Å. Therefore, these modeled triple helices represent accessible triple helical conformations and can be used to provide insight into the close packing of the Gly, Nleu, and Pro residues. Furthermore, molecular modeling studies demonstrated that triple helical structures where $\chi^1 > 0$ have lower energies than the corresponding conformations where $\chi^1 < 0$.

As shown in Table 3 and Figure 4, the expected ensemble interchain NOEs are consistent with the observed resonances in the NOESY spectra. The NOE connectivities between the Nleu and Gly residues and between the Nleu and Pro residues indicate an equilibrium between conformations of "*in*" and "*out*" orientations of the Nleu side chain in aqueous solution. These distances were also verified in the molecular modeling as shown by the solid arrows in Figure 8. These results provide a critical test for assembled structures in which $\chi^1 > 0$ for the Nleu side chain and are unambiguously described by four NOEs: Nleu $C_\beta H_{s,r}$ − Pro C_αH and Nleu $C_\beta H_{s,r}$ − Pro $C_\delta H_r$. These NOEs can only arise from ordered triple helical conformations.

The TREN versus KTA Scaffolds. The TREN scaffold was selected for the present study because it contains an additional reactive site at the tertiary amine center which will be employed in future studies and development of biomaterials. Our present study provided evidence that the TREN scaffold induces triple helical formation more effectively than the KTA scaffold for longer peptide chain ensembles.

The degree of helicity can be quantified by CD measurements and NMR spectroscopy. Rpn values denote the ratio of positive peak over the negative peak in the CD spectra and are useful parameters to measure triple helical conformations in solution. Table 4 contains a listing of Rpn values for TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ (n = 5 and 6), KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$, collagen, and (Gly-Pro-Hyp)$_{10}$-NH$_2$ (the benchmark collagen mimetic structure). The comparison of Rpn values

Table 4. Scaffold Assembled Compounds Studied and Their Degree of Triple Helicity

scaffold-assembled collagen mimetic	Rpn values[a]	average number of assembled Nleu residues per chain[b]
KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$[c]	0.08[c]	1.4[d]
TREN-[suc-(Gly-Nleu-Pro)$_5$-NH$_2$]$_3$	0.14	4.32
TREN-[suc-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$	0.19	5.51
natural collagen[e]	0.13	–
(Gly-Pro-Hyp)$_{10}$-NH$_2$[f]	0.13	–

[a] The ratio of the positive to negative peaks in the CD spectra which were carried out at 0.2 mg/mL in H_2O at room temperature. [b] Calculations were based on the 1D ^{1}H NMR integration of assembled Nleu C$_\beta$H resonances measured in D_2O at 27 °C. [c] Data taken from ref 37. [d] Data taken from ref 38. [e] References 44 and 45. [f] Reference 47.

Table 5. Comparison of T_m Values by Temperature-Dependent Optical Rotation Measurements of KTA-Assembled and TREN-Assembled Collagen Mimetics

	peptide chain (Gly-Nleu-Pro)$_n$					
	$n = 3$		$n = 5$		$n = 6$	
scaffold	H_2O[c]	EG:H_2O[d]	H_2O	EG:H_2O	H_2O	EG:H_2O
none[a]	NT[e]	NT	–	–	26 °C	43 °C
KTA[b]	NT	22 °C	–	–	36 °C	57 °C
TREN	NT	NT	38 °C	55 °C	46 °C	67 °C

[a] Single chain and N-terminal acetylated collagen mimetic structures. [b] Data taken in part from ref 40. [c] Measurements were carried out at 0.2 mg/mL in H_2O. [d] Measurements were carried out at 0.2 mg/mL in EG:H_2O (2:1, v/v). [e] NT denotes no transition observed.

indicates that the TREN analogues in H_2O possess higher triple helicity than equivalent KTA analogues and are comparable to natural collagen and (Gly-Pro-Hyp)$_{10}$-NH$_2$ (Table 4).

The average number of assembled Nleu residues which can be calculated from the 1D ^{1}H NMR integrals of the assembled Nleu C$_\beta$H resonances measured in D_2O, represents another measurement to compare degree of helicity. As seen in Table 4, the average number is greater for the TREN-assembled peptides than for the KTA-assembled structure, indicating the enhancement from the scaffold to form triple helices. This comparison of Rpn values and average number of assembled Nleu residues per chain supports our finding that the flexible TREN scaffold induces a more favorable helical array than the rigid KTA scaffold for longer peptide chain ensembles.

The difference in stabilizing effects from the two scaffolds is also supported by measurements of melting temperatures shown in Table 5. As expected, the single chain Ac-(Gly-Nleu-Pro)$_6$-NH$_2$ is weakly triple helical in H_2O as compared to the scaffold-assembled peptides.[40] However, TREN-[suc-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ is significantly more stable than KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ by more than 10 °C. This difference is also consistent with the thermal melting studies carried out by NMR spectroscopy. Even the TREN analogue TREN-[suc-(Gly-Nleu-Pro)$_5$-NH$_2$]$_3$ forms a more stable triple helix in H_2O than that of KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$.

The KTA scaffold is much more rigid than the TREN scaffold. This rigidity maintains the orientation of the carboxyl attachment sites which facilitate triple helix nucleation (i.e., the initiation for folding). The KTA scaffold induces the folding of KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ (T_m = 22 °C in EG:H_2O) into an "incipient" triple helix (Table 5). The KTA scaffold is better able to stabilize shorter triple helices than the TREN scaffold as a result of the energetics of folding. The optimal folding is a balance between minimizing entropy and maximizing enthalpy. The KTA scaffold is better at minimizing entropy since it is rigid, while the TREN scaffold is better at maximizing enthalpy because of its flexibility. The inherent flexibility of the TREN scaffold allows for a better accommodation of the three longer peptide chains, which in turn contributes to triple helix propagation (i.e., the folding process). Thus, a tighter and more stable packing array can be deduced from the higher T_m values of the structures TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ where $n = 5$ and 6 as compared to KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$. This hypothesis could be analyzed by calorimetric experiments, but such experiments are beyond the scope of this report.

The absence of significant structural distortion of the scaffold in the TREN-assembled triple helices suggests that the TREN scaffold promotes a better-fit triple helix. This proposition can be seen in the molecular modeling where the TREN scaffold is sufficiently flexible to adjust to the two triple helical families for the projection of the Nleu isobutyl groups ($\chi^1 > 0$ and $\chi^1 < 0$). Figure 7 shows the TREN scaffold distended or compressed, bending freely to accommodate and maintain the one-residue register shift for the triple helical folding. On the other hand, previous modeling studies have shown that the KTA cyclohexane ring remains rigid and skewed with respect to the triple helical axes, which does not facilitate triple helicity beyond holding the three peptide chains in intramolecular proximity with respect to each other.[36]

Conclusions

The TREN-(suc-OH)$_3$ scaffold was synthesized and incorporated into collagen mimetic structures composed of Gly-Nleu-Pro sequences. It was shown by biophysical studies that TREN-[suc-(Gly-Nleu-Pro)$_n$-NH$_2$]$_3$ where $n = 5$ and 6 exhibits triple helicity, while the shorter analogue at $n = 3$ is disordered in solution. However, the thermal stabilities and triple helical-inducing properties of the TREN-assembled structures are greater than those of equivalent KTA-assembled structures. The comparison of the two scaffolds by CD and NMR spectroscopy and molecular modeling indicate that the flexibility of the TREN molecule enhances triple helicity over that of the more rigid KTA molecule.

Acknowledgment. We thank Joseph Taulane for his input and advice on the CD spectroscopic techniques and Giuseppe Melacini for his insight and discussion on NMR and molecular modeling studies. We also acknowledge the National Science Foundation Division of Materials Research (DMR-0111617) for financial support.

Supporting Information Available: Detailed procedures for the synthesis of collagen mimetics and intermediates, experimental techniques, and sample preparation are described; CD and NMR studies which supplement results shown in the body of the paper (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0209621