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Macrocyclic Scaffold for the Collagen Triple Helix

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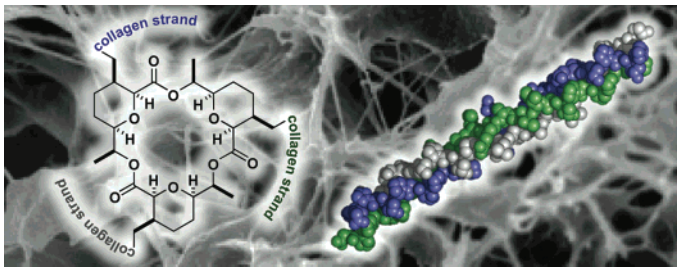
ABSTRACT



Three strands of natural collagen are linked by covalent bonds prior to their folding into a triple helix. We report on a synthetic collagen in which the strands are pendent on a rigid macrocyclic scaffold of C_3 symmetry. The scaffold confers substantial conformational stability upon the collagen triple helix and makes its folding independent of concentration, both desirable attributes for exploring and exploiting synthetic collagens.

Collagen is the most abundant protein in animals. The folding of natural collagen into its canonical triple helix is an intramolecular process involving three strands that are cross-linked by disulfide bonds.[1] In marked contrast to natural collagen, synthetic collagen is typically comprised of three untethered strands.[2]

The tethering of synthetic collagen strands provides a more realistic model of natural collagen. Moreover, the folding of a tethered collagen is independent of its concentration, facilitating kinetic analyses of folding and thermodynamic analyses of conformational stability.[3] Further, proper tethering enhances the conformational stability of triple helices formed from short strands, which are easier to synthesize and more amenable to physicochemical analyses.

A wide variety of scaffolds have been used to tether synthetic collagen strands. Goodman and co-workers have shown that *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid (Kemp's triacid), tris(2-aminoethyl)amine (TREN), and metal chelates can serve as scaffolds for synthetic collagen triple helices.[4] These scaffolds have C_3 symmetry, congruent with that of the collagen triple helix. Other workers have used asymmetric branched peptides to tether collagen strands.[5] Finally, disulfide bonds between strands provide a biomimetic tether.[6]

Here, we report on the first macrocyclic scaffold for the collagen triple helix. This scaffold belongs to a class of 18-membered cyclic hydropyran oligolides with alternating ester

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(1) For reviews, see: (a) Engel, J.; Bächinger, H. P. *Top. Curr. Chem.* **2005**, *247*, 7−34. (b) Koide, T.; Nagata, K. *Top. Curr. Chem.* **2005**, *247*, 85−114.

(2) For reviews, see: (a) Fields, G. B.; Prockop, D. J. *Biopolymers* **1996**, *40*, 345−357. (b) Jenkins, C. L.; Raines, R. T. *Nat. Prod. Rep.* **2002**, *19*, 49−59. (c) Raines, R. T. *Protein Sci.* **2006**, *15*, 1219−1225.

(3) Bachmann, A.; Kiefhaber, T.; Boudko, S.; Engel, J.; Bächinger, H. P. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13897−13902.

(4) (a) Feng, Y.; Melacini, G.; Taulane, J. P.; Goodman, M. *J. Am. Chem. Soc.* **1996**, *118*, 10351−10358. (b) Goodman, M.; Bhumralkar, M.; Jefferson, E. A.; Kwak, J.; Locardi, E. *Biopolymers* **1998**, *47*, 127−142. (c) Kwak, J.; De Capua, A.; Locardi, E.; Goodman, M. *J. Am. Chem. Soc.* **2002**, *124*, 14085−14091. (d) Kinberger, G. A.; Taulane, J. P.; Goodman, M. *Inorg. Chem.* **2006**, *45*, 961−963. (e) Cai, W.; Kwok, S. W.; Taulane, J. P.; Goodman, M. *J. Am. Chem. Soc.* **2004**, *126*, 15030−15031.

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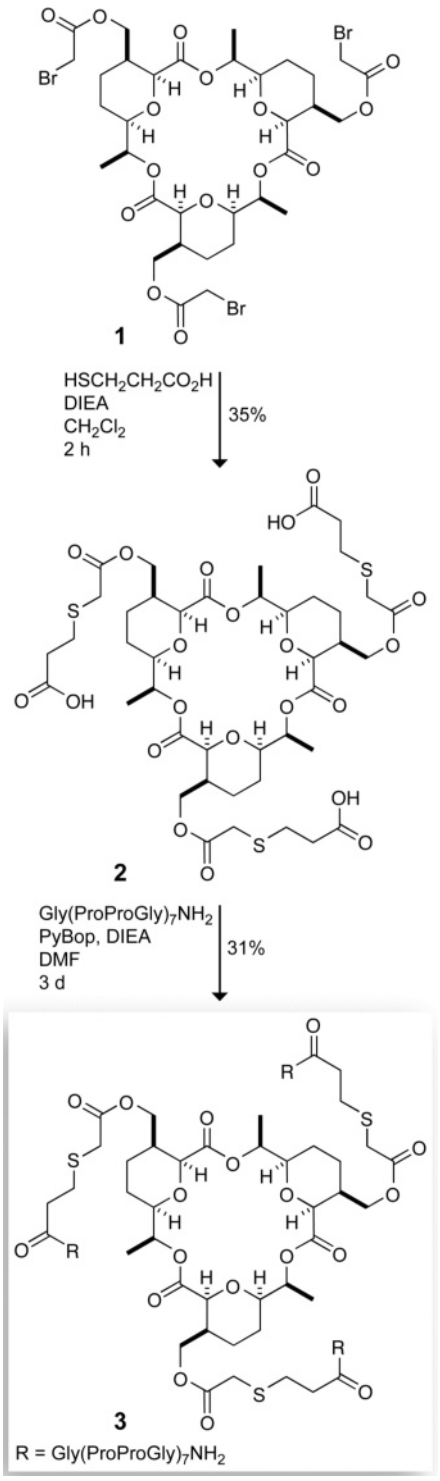
Scheme 1



and ether linkages.[7,8] In this scaffold, three foci for ligand attachment form an equilateral triangle on one face. Previously, this macrocycle was used to present saccharide ligands to a lectin in a defined manner.[9] We recognized that these loci were poised to serve as tethers for the three strands of a collagen triple helix. We now demonstrate the utility of the scaffold for that purpose.

A tethered collagen was synthesized by the route shown in Scheme 1. Briefly, the reaction of macrocycle **1**[10] with 3-mercaptopropionic acid produced macrocycle **2**. Gly-(ProProGly)$_7$NH$_2$ was chosen as a collagen strand because a similar peptide, (ProProGly)$_7$, does not self-associate to form a stable triple helix at room temperature.[11] The N-terminus of Gly(ProProGly)$_7$NH$_2$ was attached to macrocycle **2** by PyBOP-mediated coupling to form macrocycle **3**. The identity of tethered [Gly(ProProGly)$_7$NH$_2$]$_3$ (**3**) was confirmed by mass spectrometry.[12]

Circular dichroism (CD) spectroscopy provides a reliable means to detect a collagen triple helix.[13] To determine whether our macrocyclic scaffold enables tethered [Gly-(ProProGly)$_7$NH$_2$]$_3$ to form a triple helix, solutions of macrocycle **3** and untethered Gly(ProProGly)$_7$NH$_2$ were incubated at 4 °C for ≥24 h and then analyzed by CD

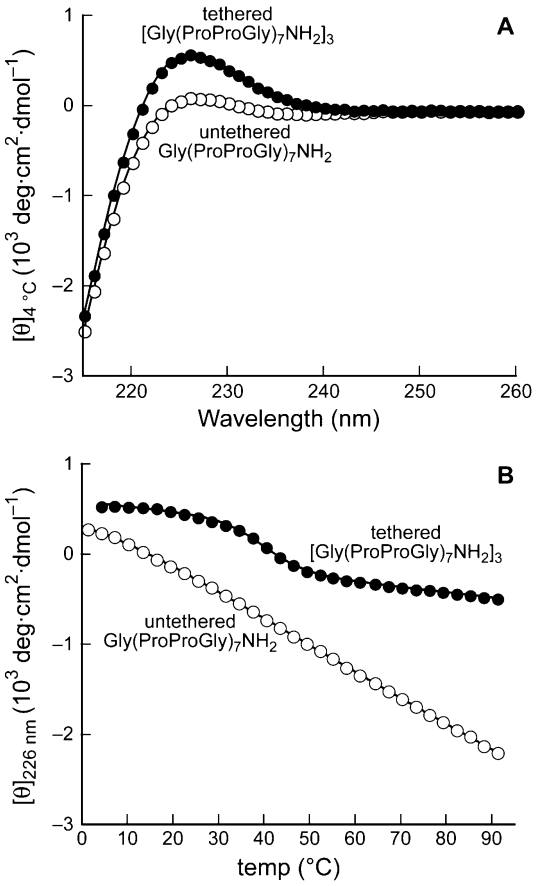


Figure 1. (A) CD spectra of tethered [Gly(ProProGly)$_7$NH$_2$]$_3$ (**3**) and untethered Gly(ProProGly)$_7$NH$_2$ at 4 °C. (B) Thermal denaturation curves of tethered [Gly(ProProGly)$_7$NH$_2$]$_3$ (**3**; T_m = 39.9 ± 0.4 °C) and untethered Gly(ProProGly)$_7$NH$_2$ (T_m < 0 °C) at 226 nm. All data were obtained with solutions of analyte (1.2 mg/mL) in 50 mM HOAc.

(5) (a) Fields, C. G.; Lovdahl, C. M.; Miles, A. J.; Matthias Hagen, V. L.; Fields, G. B. *Biopolymers* **1993**, *33*, 1695−1707. (b) Tanaka, Y.; Suzuki, K.; Tanaka, T. *J. Pept. Res.* **1998**, *51*, 413−419. (c) Thakur, S.; Vadolas, D.; Germann, H. P.; Heidemann, E. *Biopolymers* **1986**, *25*, 1081−1086.

(6) (a) Ottl, J.; Moroder, L. *J. Am. Chem. Soc.* **1999**, *121*, 653−661. (b) Kotch, F. W.; Raines, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3028−3033.

spectroscopy. In the CD spectra, macrocycle **3** had much greater molar ellipticity at 226 nm, as shown in Figure 1A. This attribute is a defining characteristic of a collagen triple helix.[13]

The response of the CD spectrum to increasing temperature reveals the stability of a triple helix. Thermal denaturation experiments monitored by CD spectroscopy revealed a cooperative transition for macrocycle **3** but only a linear decrease in ellipticity for untethered $Gly(ProProGly)_7NH_2$, as shown in Figure 1B. The cooperative unfolding transition is diagnostic of the loss of a triple-helical conformation[13] and indicates that tethered $[Gly(ProProGly)_7NH_2]_3$ but not untethered $Gly(ProProGly)_7NH_2$ forms a stable triple helix. Using a simple two-state model to fit the denaturation curve, the value of T_m[14] for macrocycle **3** was found to be (39.9 ± 0.4) °C. This value is much greater than that of untethered $Gly(ProProGly)_7NH_2$, which had $T_m < 0$ °C.[15]

As a termolecular process, the folding of three collagen strands into a triple helix and the conformational stability of that triple helix are highly dependent on concentration.[1] In contrast, thermal denaturation curves of macrocycle **3** at different concentrations overlap, and the resulting T_m values were indistinguishable, as shown in Figure 2. Thus, the conformational stability of tethered $[Gly(ProProGly)_7NH_2]_3$ was independent of its concentration.

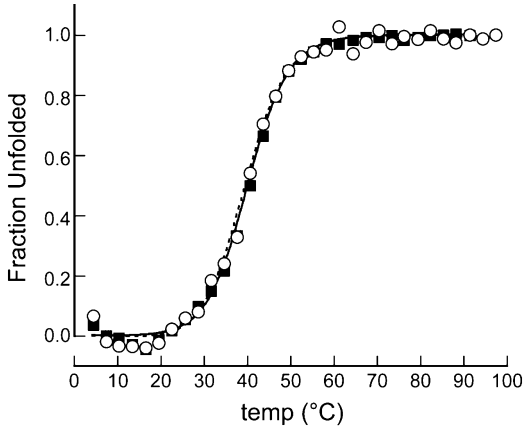


Figure 2. Thermal denaturation curves at two concentrations of tethered $[Gly(ProProGly)_7NH_2]_3$ (**3**) in 50 mM HOAc: 0.4 mg/mL (○;- - -) and 1.2 mg/mL (■; —).

The macrocyclic scaffold is suited for preorganizing collagen strands for triple-helix formation. The arrangement of its axial foci defines an equilateral triangle in which the vertexes are separated by approximately 9.2 Å (Figure 3).

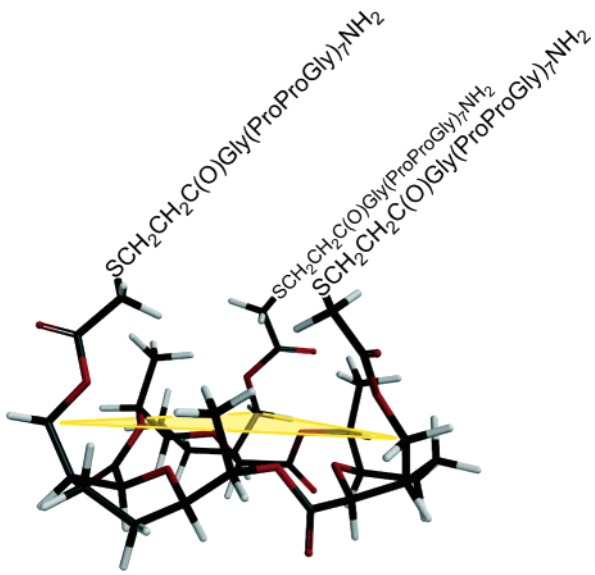


Figure 3. Structure of tethered $[Gly(ProProGly)_7NH_2]_3$ (**3**). The minimum-energy conformation of the macrocycle was calculated with the program Spartan v1.0.3 (Wavefunction, Irvine, CA) and is depicted edge-on. The three loci (gold triangle) are separated by 9.2 Å.

This large distance facilitates the coupling of pendant molecules by diminishing steric interactions. The $(Xaa\text{-}Yaa\text{-}Gly)_n$ strands of collagen are offset in a triple helix such that each cross section contains one Xaa, one Yaa, and one Gly residue. The relevant distances for a scaffold of a triple helix with three identical collagen strands, such as tethered $[Gly(ProProGly)_7NH_2]_3$, are the Xaa···Xaa, Yaa···Yaa, and Gly···Gly distances, which are 6.9, 5.7, and 4.0 Å, respectively, from C^α to C^α.[16] The data depicted in Figures 1 and 2 indicate that these distances can be accommodated by the macrocyclic scaffold.

An attribute of the scaffold of macrocycle **3** suggested a means to modulate the stability of its pendant triple helix. Cyclic hydropyran oligolides, like crown ethers,[17] have

(7) (a) Burke, S. D.; O'Donnell, C. J.; Porter, W. J.; Song, Y. *J. Am. Chem. Soc.* **1995**, *117*, 12649−12650. (b) Burke, S. D.; Heap, C. R.; Porter, W. J.; Song, Y. T. *Tetrahedron Lett.* **1996**, *37*, 343−346. (c) Burke, S. D.; O'Donnell, C. J.; Hans, J. J.; Moon, C. W.; Ng, R. A.; Adkins, T. W.; Packard, G. K. *Tetrahedron Lett.* **1997**, *38*, 2593−2596. (d) Burke, S. D.; McDermott, T. S.; O'Donnell, C. J. *J. Org. Chem.* **1998**, *63*, 2715−2718. (e) Burke, S. D.; Zhao, Q. *J. Org. Chem.* **2000**, *65*, 1489−1500.

(8) For an analogous macrocycle with alternating amide and ether linkages, see: Campbell, J. E.; Englund, E. E.; Burke, S. D. *Org. Lett.* **2002**, *4*, 2273−2275.

(9) Burke, S. D.; Zhao, Q.; Schuster, M. C.; Kiessling, L. L. *J. Am. Chem. Soc.* **2000**, *122*, 4518−4519.

(10) The synthesis of macrocycle **1** is described in ref 7e.

(11) (a) Shaw, B. R.; Schutt, J. M. *Biopolymers* **1975**, *14*, 1951−1985. (b) Hodges, J. A.; Raines, R. T. *J. Am. Chem. Soc.* **2005**, *127*, 15923−15932.

(12) MALDI-TOF *m/z* calcd for $C_{300}H_{429}N_{69}O_{84}S_3$ (M) 6442.22, obsd 6449.05 ($M + H^+$).

(13) Piez, K. A.; Sherman, M. R. *Biochemistry* **1970**, *31*, 119−128.

(14) The value of T_m is the temperature at the midpoint of the thermal transition between the folded and unfolded states.

(15) Acetylation of the N-terminus (as in macrocycle **3**) diminishes deleterious Coulombic interactions and increases the triple-helical stability of untethered collagen-related peptides, but only by 4 °C at pH 3.0 (Babu, I. R.; Ganesh, K. N. *J. Am. Chem. Soc.* **2001**, *123*, 2079−2080).

(16) These are average values derived from Protein Data Bank entry 1V7H (Okuyama, K.; Hongo, C.; Fukushima, R.; Wu, G.; Narita, H.; Noguchi, K.; Tanaka, Y.; Nishino, H. *Biopolymers* **2004**, *76*, 367−377). The $C^\alpha\cdots C^\alpha$ distance of the single Gly, Xaa, and Yaa residue in each cross section of a collagen triple helix is 4.9 Å.

(17) (a) Pedersen, C. J. *J. Am. Chem. Soc.* **1967**, *89*, 7017−7036. (b) Izatt, R. M.; Pawlak, K.; Bradshaw, J. S. *Chem. Rev.* **1991**, *91*, 1721−2085.

notable affinity for alkali metal ions.[7] In water, 18-crown-6 ether binds to Na^+, K^+, Cs^+, and H_3O^+ with $\Delta G°$ values of −3.37, −11.70, −5.29, and +2.4 kJ/mol, respectively.[18,19] The conformation of 18-crown-6 ether is altered upon binding to cations.[20] We reasoned that similar changes to the conformation of the scaffold in macrocycle **3** could be transmitted to its pendant triple helix.

The affinity of Na^+ for 18-crown-6 ether is insubstantial. The presence of 0.10 M Na^+ had no measurable effect on the T_m value of triple-helical **3**, consistent with a low affinity for Na^+. This result indicated that the presence of 0.10 M salt alone had no effect on conformational stability (Table 1).

Table 1. Effect of Cations on the Conformational Stability of the Triple-Helical Form of Tethered $[Gly(ProProGly)_7NH_2]_3$[a]

cation (0.10 M)	T_m (°C)
none	39.6 ± 0.7
Na^+	39.8 ± 0.9
K^+	36.9 ± 2.1
Cs^+	43.4 ± 0.7

[a] Values of T_m (±SE) were determined in duplicate or triplicate by monitoring thermal denaturation of solutions of **3** (0.4−1.2 mg/mL in 50 mM HOAc) using CD spectroscopy.

In contrast to Na^+, K^+ has high affinity for 18-crown-6 ether, binding tightly in the plane of the six oxygen atoms.[17,18b] Similarly, a K^+ ion binds 0.6365 Å away from the average plane of the six coordinating oxygens in a macrocyclic scaffold such as that in **3**.[7e] This binding mode could constrain the scaffold of macrocycle **3** to be nearly flat and thereby disperse the $Gly(ProProGly)_7NH_2$ strands. The T_m value of triple-helical **3** decreased by 3 °C in the presence of K^+ (Table 1), providing support for this hypothesis.

Unlike K^+, the larger Cs^+ ion binds out of the plane of the six oxygen atoms of 18-crown-6 ether, instilling a pucker that brings the carbon atoms into closer proximity.[20] Likewise, the binding of Cs^+ outside the plane of the scaffold on the undecorated face of macrocycle **3** could instill a pucker that collocates the $Gly(ProProGly)_7NH_2$ strands. The T_m value of triple-helical **3** increased by 4 °C in the presence of Cs^+ (Table 1), consistent with this notion.[21]

In summary, we have demonstrated that a macrocycle can be an effective scaffold for the collagen triple helix. The scaffold provides a means to endow collagen strands with desirable properties, such as enhanced triple-helical stability and concentration-independent folding. In addition, the binding of alkali metal ions to the scaffold provides a means to modulate the stability of its pendant triple helix. We envision that tethered collagen triple helices such as **3** could aid in the development of synthetic collagens as surrogates for natural collagen and templates for nanotechnological applications.

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Supporting Information Available: Procedures for the synthesis of peptide $Gly(ProProGly)_7NH_2$ and macrocycles **2** and **3**, related analytical data, and denaturation curves that gave rise to the data listed in Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) (a) Gaikwad, A. G.; Noguchi, H.; Yoshio, M. *Anal. Sci.* **1987**, *3*, 217−220. (b) Ozutsumi, K.; Ishiguro, S. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 1173−1175.

(19) The lesser basicity of three of the six coordinating oxygens in the macrocyclic scaffold of **3** could diminish its affinity for cations relative to that of 18-crown-6 ether (ref 7e).

(20) Oxutsumi, K.; Natsuhara, M.; Ohtaki, H. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 2807−2818.

(21) An alternative explanation is that Cs^+ stabilizes the triple helix by also binding to nonbridging oxygens of the cysteic acid moieties on the triple-helical face of macrocycle **3**.