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# Engineering of a 129-Residue Tripod Protein by Chemoselective Ligation of Proline-II Helices

Dewey G. McCafferty, Cheryl A. Slate, Bassam M. Nakhle, Harold D. Graham, Jr.,

Todd L. Austell, Richard W. Vachet, Brian H. Mullis, and Bruce W. Erickson\*

Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3290, U.S.A.

Abstract: A 129-residue tripod protein was designed, synthesized, and biophysically characterized. This receptor-adhesive modular protein contained three 30-residue proline-II helices linked to a 9-residue proline-II helix through thioether bonds. Coupling of 6-maleimidohexanoic acid succinimido ester to  $cis-N^{\alpha}$ -Boc-4-amino-L-proline furnished in 77% yield the maleimido acid cis-N-Boc-4-(6-maleimidohexanamido)-L-proline (Boc-Prm), which was used in the solid-phase synthesis of the linker peptide CH<sub>3</sub>-CO-Pro<sub>3</sub>-Prm<sub>3</sub>-Pro<sub>3</sub>-NH<sub>2</sub>. The leg peptide, the 40-residue thiol Gly-Arg-Gly-Asp-Ser-Pro-Gly-Tyr-Gly-Pro<sub>30</sub>-Cys-NH<sub>2</sub>, was also made by solid-phase synthesis. The tripod protein was prepared by Michael addition of the thiol groups of three leg peptides to the three maleimide groups of the linker peptide. By <sup>13</sup>C NMR spectrometry, the linker peptide was a proline-II helix, as indicated by the presence of only trans Pro-Pro resonances for its  $\beta$  and  $\gamma$  carbons. By circular dichroic spectroscopy, the model peptide CH<sub>3</sub>-CO-Pro<sub>9</sub>-NH<sub>2</sub>, the linker peptide, the leg peptide, and the tripod protein each contained substantial proline-II helix, as indicated by a strong negative band at 205 nm and a weak positive band at 226 nm. Since the Pro<sub>30</sub> proline-II helix of each leg is about 93 Å long, two Arg-Gly-Asp sites on different legs of the tripod protein could be as much as ~250 Å apart.

A receptor-adhesive modular protein (RAMP) is a nongenetic protein designed to contain two or more ligands spatially separated by a fixed distance.<sup>1</sup> A RAMP might use two or more ligands to bind to two or more cell-surface receptors at the same time and thus might bind more tightly to a cell than a single ligand binding to a single receptor. A RAMP contains four types of modules: linker, rod, spacer, and ligand. We have designed and synthesized dimeric RAMPs containing a disulfide bond between two cysteine residues as the linker, a parallel  $\alpha$ -helical coiled coil as the rigid rod, the segment Gly-Tyr-Gly as the flexible spacer, and the segment Gly-Arg-Gly-Asp-Ser-Pro as the receptor ligand.<sup>2</sup> The two ligands were at opposite ends of the coiled coil and were separated by a distance of ~60 Å. This RAMP competed with extracellular matrix proteins for integrin receptor binding but was too short to span the distance of ~120 Å between two integrin receptors.<sup>3,4</sup> We report

here a general method for the design and synthesis of a new class of RAMPs based on the proline-II helix, which can span much longer distances.

## **RESULTS AND DISCUSSION**

#### The Proline-II Helix

Proline oligomers can adopt two helical conformations in solution.<sup>5</sup> The right-handed proline-I helix has cis peptide bonds and 3.3 residues per turn. The more stable left-handed proline-II helix has trans peptide bonds, 3.0 residues per turn, and a length of 3.1 Å per residue. A segment of 15 or more proline residues in a row is present in several natural proteins. Among these are the Pro<sub>17</sub>-Ser-Pro<sub>18</sub> segment in a sulfated surface glycoprotein from volvox,<sup>6</sup> the Pro<sub>17</sub>-Ser-Pro<sub>11</sub> segment of nuclear protein EBNA-2 from herpesvirus 4,<sup>7</sup> the Pro<sub>23</sub> segment in pig acrosin,<sup>8</sup> the Pro<sub>17</sub> segment of the chicken limb-deformity nuclear protein,<sup>9</sup> the Pro<sub>15</sub> segment in a fruit-fly steroid hormone receptor,<sup>10</sup> and the Pro<sub>15</sub> segment of the human homeotic protein Hox B4.<sup>11</sup> Six or more contiguous prolines give a stable proline-II helix,<sup>12</sup> so these oligoproline segments probably form proline-II helices that span distances of about 45-70 Å. Inspired by these natural precedents, we have used the proline-II helix as the rod module and in the linker module of a new class of RAMPs.

#### **Chemoselective Ligation of Peptide Thiols**

An attractive strategy for synthesizing a large protein is by covalently linking smaller unprotected peptides together by chemoselective ligation. Sulfur nucleophiles typically react faster than nitrogen or oxygen nucleophiles toward polarizable electrophiles such as a benzyl bromide, a bromoacetyl, or a maleimide moiety. For example, two molecules of an organic thiol can be selectively cross-linked by alkylation with 3,5-bis(bromomethyl)benzoic acid.<sup>13</sup> Similarly, a peptide bearing a C-terminal thiocarboxylic acid can be selectively alkylated by a peptide bearing an N-terminal bromoacetyl group to form a thioester bond.<sup>14</sup> Furthermore, a peptide thiol can selectively add to a maleimide group to form a stable thioether bond.<sup>15,16</sup> We have used this last approach to link three molecules of a large peptide thiol containing a Pro<sub>30</sub> segment to three maleimide groups attached to a Pro<sub>9</sub> derivative.

#### Design of a 129-Residue Tripod Protein Containing Four Proline-II Helices

We have designed a RAMP tripod having three long proline-II helices linked to a short proline-II helix through thioether bonds. The 9-residue linker peptide (CH<sub>3</sub>-CO-Pro<sub>3</sub>-Prm<sub>3</sub>-Pro<sub>3</sub>-NH<sub>2</sub>; **1** in Scheme 1) contains only proline-based residues to make sure that it forms a proline-II helix. Each of the three central residues is *cis*-4-(6-maleimidohexanamido)-L-proline (Prm), which contains a maleimide ring attached to the proline ring by a flexible 7-atom tether. The 40-residue leg peptide (**2**) contains the 6-residue ligand module (Gly-<u>Arg-Gly-Asp</u>-Ser-Pro) and the 3-residue spacer module (Gly-Tyr-Gly) used previously.<sup>1,2</sup> It also contains a Pro<sub>30</sub> rod module, which should form a proline-II helix. Leg peptide **2** ends with a Cys-NH<sub>2</sub> residue, which contains a thiol group for addition to the maleimide group of a Prm residue of linker peptide **1**.

The 129-residue tripod protein (3) was designed to be assembled by Michael addition of the thiol groups of three copies of leg peptide 2 to the three maleimide groups of linker peptide 1 (Scheme 1). As a result, the maleimide group of each Prm residue would be converted into the succinimido group of a residue of

129-Residue tripod protein



Scheme 1. Assembly of the Tripod Protein 3 by Chemoselective Ligation of Three Molecules of the Leg Peptide 2 to the Linker Peptide 1.

*cis*-4-(6-succinimidohexanamido)-L-proline (Prs). Each leg of the tripod protein **3** is attached to the 9-residue linker chain by a thioether bond between the  $\beta$ -sulfur of Cys and 3'-carbon of the succinimido ring of Prs. Thus tripod **3** contains a total of 96 Pro residues and three Prs residues in four proline-II helical domains.

Since each proline residue of an oliogoproline segment folded into a proline-II helix contributes 3.1 Å to its length,<sup>17</sup> the length of the linker chain should be ~28 Å, the length of the  $Pro_{30}$  rod in each leg should be ~93 Å, and the distance between the N terminus of the fully extended 40-residue leg and the axis of the linker chain should be ~140 Å (Figure 1). Thus the distance between two Arg-Gly-Asp ligand modules could be as great as ~250 Å.



**Figure 1.** A Schematic Diagram of the Tripod Protein **3** That Shows the Four Types of Modules and Some of Its Dimensions.

#### Chemistry of the 9-Residue Linker Peptide

Synthesis of Boc-Pra-OH. In order to provide a site for attachment of a maleimide group, a derivative of *cis*-4-amino-L-proline was prepared (Scheme 2). *cis*- $N\alpha$ -Boc-4-azido-L-proline methyl ester (4) was synthesized<sup>18,19</sup> in four steps on a 100-mmol scale from commercially available *trans*-4-hydroxy-L-proline in 53% over-all yield. Catalytic hydrogenation of azide 4 in the presence of 2% (v/v) HCl/ethanol provided *cis*-N-Boc-4-amino-L-proline methyl ester (Boc-Pra-OCH<sub>3</sub>, 5) in 89% yield as the hydrochloride salt. Selective hydrolysis of ester 5 with 2.1 equiv of lithium hydroxide in 2:1 (v/v) methanol/water furnished the protected amino acid *cis*- $N\alpha$ -Boc-4-amino-L-proline (Boc-Pra-OCH, 6) in quantitative yield. Hydrogenation of azide 4 followed by saponification gave amine 6 in 88% over-all yield, which was higher than the 62% over-all yield of amine 6 previously reported<sup>18</sup> for saponification of the ethyl ester analogue of 4 followed by hydrogenation. This improved six-step route provided Boc-Pra-OH in 46% over-all yield from *trans*-4-hydroxy-L-proline.

Synthesis of Boc-Prm-OH. Coupling of 6-maleimidohexanoic acid succinimido ester<sup>15,20</sup> to the 4-amino group of Boc-Pra-OH in the presence of *N*.*N*-diisopropylethylamine (DIEA) provided the N<sup> $\alpha$ </sup>-protected amino acid *cis-N<sup>\alpha</sup>*-Boc-4-(6-maleimidohexanamido)-L-proline (Boc-Prm-OH, 7) in 77% yield. Maleimide 7 was insoluble in water, partially soluble in dimethyl sulfoxide, CH<sub>2</sub>Cl<sub>2</sub>, or CHCl<sub>3</sub>, but fully soluble in dimethylformamide (DMF). Its <sup>1</sup>H nuclear magnetic resonance (NMR) spectrum displayed two maleimide olefinic protons at 6.7 ppm and the CYH proton at 4.5 ppm, which was ~0.35 ppm downfield from the corresponding proton of amino ester 5.

**Synthesis of Linker Peptide 1.** The 9-residue acetylated peptide amide 1 was assembled on 4-methylbenzhydrylamine-polystyrene beads (MBHA resin) by manual solid-phase synthesis using the Boc/benzyl strategy.<sup>21</sup> The loss of Pro-Pro diketopiperazine after deprotection of Boc-Pro-Pro-resin was avoided by forming Boc-Pro-Pro-resin through the coupling of Boc-Pro-Pro to Pro-resin.<sup>22</sup> Michael addition of 1-hydroxybenzotriazole to the Prm maleimide group was avoided by coupling Boc-Prm-OH and



**Scheme 2**. Reagents and conditions: (a) H<sub>2</sub>, 10% Pd/C in 2% HCl/ethanol; (b) LiOH, 2:1 CH<sub>3</sub>OH/H<sub>2</sub>O; (c) 6-maleimidohexanoic acid succinimido ester, DIEA, 1:1 CH<sub>3</sub>CN/H<sub>2</sub>O.

Boc-Pro-OH to the peptide-resin using *N*,*N*'-dicyclohexylcarbodiimide (DCC) as the activating agent and *N*-methylmorpholine (NMM) as the base. After each residue was double coupled, it was treated with acetic anhydride and NMM to acetylate any unreacted amino groups. After acetylation of its N terminus, the nonapeptide was cleaved from the resin using anhydrous HF in the absence of scavengers. Loss of the maleimide groups on treatment with 10-30% acetic acid was avoided by washing the peptide from the resin with glacial acetic acid. Reversed-phase HPLC (Table 1) provided 62 mg of pure linker peptide 1, which gave the expected mass of 1558 Da by electrospray-ionization (ESI) mass spectrometry (MS). Its <sup>1</sup>H NMR spectrum displayed six maleimide olefinic protons at 6.8 ppm, so the maleimide group survived the chemistry of solid-phase assembly and HF cleavage.

**Synthesis of CH<sub>3</sub>-CO-Prog-NH<sub>2</sub>.** This nine-residue acetylated peptide amide (8) was assembled on a 0.5-mmol scale on the MBHA resin with an automatic solid-phase synthesizer. Preparative HPLC provided 160 mg of pure peptide 8, which gave the expected mass of 934 Da by fast-atom bombardment (FAB) MS. It served as a model Prog peptide for NMR and circular dichroic (CD) studies of linker peptide 1.

## Chemistry of the 40-Residue Leg Peptide

Synthesis of Leg Peptide 2. The 40-residue peptide amide 2 was assembled on the MBHA resin by automatic solid-phase methods using double coupling and the Boc/benzyl strategy.<sup>21</sup> Analytical HPLC of the cleaved peptide in the presence of dithiothreitol (DTT) showed that the major component was the leg peptide 2. Early HPLC fractions contained several deletion peptides. By ESI-MS their masses were smaller than that of peptide 2 by multiples of 194 Da, which was consistent with partial loss of Pro-Pro diketopiperazine during chain assembly.<sup>22</sup> Later HPLC fractions provided 86 mg of pure leg peptide 2, which gave the expected mass of 3881 Da by ESI-MS.

Synthesis of Leg Peptide Dimer 9. Air oxidation of the leg peptide 2 in 0.05 M phosphate buffer at pH 7.5 afforded the 80-residue disulfide-bridged homodimer (9). Preparative HPLC provided 8.2 mg of pure dimer 9, which gave the expected mass of 7759 Da by ESI-MS. In aqueous solutions, the majority of

	Code	Retention Time $a$ , min		
Peptide		Preparative b	Analytical c	
Linker Peptide	1	55	34	
Leg Peptide	2	40	31	
Leg Peptide Dimer	9	52	34	
Tripod Protein	3	62 d	36	

**Table 1.** HPLC Retention Times for the Reversed-Phase

 Chromatography of Four Proline-Based Synthetic Peptides.

 $^{\it a}$  HPLC column was eluted with the indicated linear gradient of CH\_3CN in 0.06% CF\_3CO\_2H/H\_2O.

<sup>b</sup> Preparative butyl-silica column (Vydac C4, 300-Å pore size, 250 mm x 10 mm) was eluted at 5 mL/min for 10 min at 0% CH<sub>3</sub>CN and for 80 min with 0-75% CH<sub>3</sub>CN.

<sup>c</sup> Analytical butyl-silica column (Vydac C4, 300-Å pore size, 150 mm x 2.1 mm) was eluted at 1 mL/min for 5 min at 0% CH<sub>3</sub>CN and for 60 min with 0-75% CH<sub>3</sub>CN.

<sup>d</sup> Poly(vinyl alcohol)-silica column (S5 120A PVA-SIL (YMC, Inc.), 120-Å pore size, 4.6 x 250 mm) was eluted at 1 mL/min for 5 min with 0% CH<sub>3</sub>CN, for 7 min with 0-12% CH<sub>3</sub>CN, for 16 min at 12% CH<sub>3</sub>CN, and for 45 min with 12-100% CH<sub>3</sub>CN.

peptide thiol **2** underwent air oxidation within 10 min to produce the disulfide-bridged dimer **9**. This oxidation occured rapidly even in argon-bubbled solvents containing EDTA, but it was negligible in an aqueous solution that had been previously degassed by at least three freeze-pump-thaw cycles.

#### Synthesis of the 129-Residue Tripod Protein 3

The four-chain trivalent RAMP **3** was synthesized by Michael addition of three molecules of the peptide thiol **2** to the three maleimide groups of linker peptide **1** (Scheme 1). Specifically, thiol **2** was reacted with an excess of linker peptide **1** under argon using DIEA as the base and freeze-pump-thaw degassed 1:1 (v/v) acetonitrile/water as the solvent. After 24 h >95% of thiol **2** was consumed. Tripod **3** was purified by preparative reversed-phase HPLC in the presence of DTT (Table 1). HPLC purification using the butyl-silica column was not successful because tripod protein **3** co-eluted with linker peptide **1**. But use of a *poly*(vinyl alcohol)-silica column resulted in baseline separation of peptide **1** and protein **3**. Lyophilization afforded 3.1 mg of tripod protein **3**, which was homogeneous by analytical HPLC (Table 1). It gave the expected mass of 13,200 Da for its 1853 atoms by ESI-MS and contained no disulfide dimer **9** because no thiol **2** was detected by analytical HPLC after treatment with DTT.



**Figure 2.** Illustration of the trans-trans (tt) and cis-trans (ct) conformations for the N-terminal segment CH<sub>3</sub>-CO-Pro<sup>1</sup>-Pro<sup>2</sup>-... of CH<sub>3</sub>-CO-Pro<sup>1</sup>-Pro<sup>2</sup>-Pro<sup>3</sup>-Prm<sup>4</sup>-Prm<sup>5</sup>-Prm<sup>6</sup>-Pro<sup>7</sup>-Pro<sup>8</sup>-Pro<sup>9</sup>-NH<sub>2</sub> (1) and CH<sub>3</sub>-CO-Pro<sup>1</sup>-Pro<sup>2</sup>-Pro<sup>3</sup>-Pro<sup>4</sup>-Pro<sup>5</sup>-Pro<sup>6</sup>-Pro<sup>7</sup>-Pro<sup>8</sup>-Pro<sup>9</sup>-NH<sub>2</sub> (8).

# **Biophysical Studies**

<sup>13</sup>C NMR Spectrometry. The solution conformations of the Pro9 peptide **8** and its maleimide derivative **1** were studied in D<sub>2</sub>O by <sup>13</sup>C NMR spectrometry. Listed in Table 2 are the <sup>13</sup>C chemical shifts and assignments for the carbons of peptides **1** and **8**. As shown in Figure 2, their N-terminal segment CH<sub>3</sub>-CO-Pro<sup>1</sup>-Pro<sup>2</sup>-... can exist in either the trans-trans or the cis-trans conformation. Figure 3 depicts the well-resolved resonances due to the  $\beta$ ,  $\gamma$ , and <u>C</u>H<sub>3</sub>CO carbons of **1** and **8**. The chemical shifts of these carbons are diagnostic of the cis and trans amide conformations of this N-terminal segment.<sup>23,24</sup> The ratios of the peak heights for the C<sup>β</sup>, C<sup>γ</sup>, and <u>C</u>H<sub>3</sub>CO carbons of these conformations indicated that the trans-trans/cis-trans ratio was 4:1 for peptide **8**. Similarly, the ratio of the peak heights for the <u>C</u>H<sub>3</sub>CO carbon revealed that the trans-trans/cis-trans ratio was also 4:1 for linker peptide **1**. In other words, about 20% of the amide bond between the acetyl group and Pro<sup>1</sup> was in the cis conformation but essentially all of the peptide bond between Pro<sup>1</sup> and Pro<sup>2</sup> was in the trans conformation (Figure 2). In addition, the chemical shifts of the  $\beta$  ( $\beta$  29.3 ppm,  $\gamma$  26.0 ppm) are closer to those reported<sup>25</sup> for polyproline-II ( $\beta$  28.6 ppm,  $\gamma$  25.4 ppm) than to those for polyproline-II ( $\beta$  32.2 ppm,  $\gamma$  22.5 ppm). These results indicate that the entire peptide backbones of model Pro9 peptide **8** and linker peptide **1** exist in the proline-II helical conformation with trans-peptide bonds.

**CD Spectroscopy.** The solution conformations of the oligoproline segments of linker peptide **1**, leg peptide **2**, tripod protein **3**, and Pro<sub>9</sub> peptide **8** were characterized by ultraviolet CD spectroscopy. Typically, a right-handed proline-I helix shows a negative band at 199 nm, a stronger positive band at 215 nm, and a weak negative band at 230 nm.<sup>26</sup> In contrast, a left-handed proline-II helix exhibits a strong negative band at ~205 nm and a weak positive band at ~226 nm.<sup>26</sup> Figure 4 shows the CD spectra for **1**, **2**, **3**, and **8** in water, a solvent that favors formation of a proline-II helix.<sup>27</sup> Each CD spectrum shows a strong negative band at 205-206 nm and a weak positive band at 226-228 nm (Table 3), which is consistent with substantial folding as a proline-II helix. When linker peptide **1** was slowly heated in water at pH 7.0 from 5 °C to 95 °C, the amplitude of its negative CD band at 205 nm decreased linearly from -49,700 to -39,300 deg cm<sup>2</sup> dmol<sup>-1</sup>. This modest 20% decrease in the amplitude of  $\{\theta\}_{205}$  with increasing temperature and its linear rather than sigmoidal shape indicates that linker peptide **1** remains substantially folded as a proline-II helix even at 95 °C.

Assignment for	Chemical Shift (ppm)		Assignment for		
CH <sub>3</sub> -CO-Pro <sub>9</sub> -NH <sub>2</sub> (8)	8	1	CH <sub>3</sub> -CO-Pro <sub>3</sub> -Prm <sub>3</sub> -Pro <sub>3</sub> -NH <sub>2</sub> (1)		
Pro <sup>9</sup> CONH <sub>2</sub>	178.4	178.3	Pro <sup>9</sup> CONH <sub>2</sub>		
		177.4	Maleimide CO		
		174.6	Hexanoyl CO		
$CH_3\underline{C}O(ct)$	173.8	173.8	CH3CO (ct)		
$Pro^{1} CONR_{2} (tt)$	173.7				
CH3CO (tt) &	173.4	173.4-172.2	CH3CO (tt) &		
$Pro^{1} CONR_{2} (ct)$			Pro and Prm CONR <sub>2</sub>		
Pro <sup>2-8</sup> CONR <sub>2</sub>	173.1-172.8				
		135.8	Maleimide HC=CH		
$Pro^{\dagger} C^{\alpha}$	61.6	61.6	$Pro^{\dagger} C^{\alpha}$		
Pro <sup>2-8</sup> C <sup>α</sup>	60.0	60.4-58.8	$Pro^{2,3,7-9} C^{\alpha} \&$		
			Prm $C^{\alpha}$		
$Pro^9 C^{\alpha}$	59.7				
		53.8-54.1	Prm C <sup>γ</sup>		
$Pro^{1} C^{\delta}(tt)$	50.1	50.0-50.4	$Pro^{1} C^{\delta}(tt) \&$		
			Prm C <sup>δ</sup>		
Pro <sup>2-9</sup> C <sup>δ</sup>	49.0	49.1	Pro <sup>2,3,7-9</sup> C <sup>δ</sup>		
$Pro^{1} C^{\delta}(ct)$	48.6				
		38.9	Hexanoyl C <sup>ε</sup>		
		37.0	Hexanoyl C <sup>α</sup>		
		34.0-34.2	Prm C <sup>β</sup>		
$Pro^{1} C^{\beta} (ct)$	31.5				
$Pro^9 C^{\beta}$	30.9	30.9	Pro <sup>9</sup> C <sup>β</sup>		
$Pro^{1}C^{\beta}(tt)$	29.9	30.0	$Pro^{1} C^{\beta} (tt)$		
$Pro^{2-8} C^{\beta}$	29.3-29.5	29.1-29.4	Pro <sup>2,3,7,8</sup> C <sup>β</sup>		
		28.9	Hexanoyl C <sup>8</sup>		
		27.0	Hexanoyl C <sup>β</sup>		
Pro <sup>2-9</sup> C <sup>γ</sup>	26.0-26.2	26.0-26.1	Pro <sup>2,3,7-9</sup> Cγ		
		26.0	Hexanoyl C <sup>Y</sup>		
$Pro^{1} C^{\gamma}(tt)$	25.7	25.7	$Pro^{\dagger} C^{\gamma}(tt)$		
$Pro^{\dagger} C^{\gamma} (ct)$	23.9				
$\underline{C}H_{3}CO(tt)$	22.7	22.7	<u>C</u> H <sub>3</sub> CO (tt)		
$\underline{C}H_{3}CO(ct)$	22.6	22.6	$\underline{C}H_{3}CO(ct)$		

Table 2.  $^{13}$ C NMR Chemical Shifts and Carbon Assignments for Proline-Based Peptides 1 and 8 in D<sub>2</sub>O.



Figure 3. Partial <sup>13</sup>C NMR spectra for CH<sub>3</sub>-CO-Pro<sub>3</sub>-Prm<sub>3</sub>-Pro<sub>3</sub>-NH<sub>2</sub> (1, upper) and CH<sub>3</sub>-CO-Pro<sub>9</sub>-NH<sub>2</sub> (8, lower) in D<sub>2</sub>O at 100 MHz. The resonances are marked for the trans-trans (tt) and cis-trans (ct) conformers of the N-terminal segment CH<sub>3</sub>-CO-Pro<sup>1</sup>-Pro<sup>2</sup>-... (see Figure 2).



Figure 4. CD Spectra of Four Oligoproline-Based Synthetic Peptides in Water at 25 °C and pH 7.0.

## Summary

This paper has described (a) an improved synthesis of  $cis-N^{\alpha}$ -Boc-4-amino-L-proline, a protected form of a conformationally constrained  $\alpha,\gamma$ -diamino acid; (b) the first synthesis of the thiol-reactive amino acid  $cis-N^{\alpha}$ -Boc-4-(6-maleimidohexanamido)-L-proline (Boc-Prm); (c) use of this reagent in the solid-phase synthesis of a 9-residue linker peptide, which contains three adjacent Prm residues and folds as a proline-II helix; (d) synthesis of a 40-residue leg peptide, which contains a Pro<sub>30</sub> proline-II helix; and (e) chemoselective ligation of three leg peptides to this linker peptide to form a tripod protein, a branched four-chain structure having substantial proline-II helical conformation. These results provide experimental support for the idea that an oligoproline segment of a natural protein with nine or more prolines in a row folds into a proline-II helix, which may function as a rigid spacer.

Peptide	Code	$[\theta]$ , deg cm <sup>2</sup> dmol <sup>-1</sup>		$[\theta]_{OP}$ , deg cm <sup>2</sup> dmol <sup>-1</sup>	
		205 nm	228 nm	205 nm	228 nm
Linker Peptide	1	-48,800	7,800	-48,800	7,800
Leg Peptide	2	-33,800	1,300	-45,100	1,700
Tripod Protein	3	-23,400	1,500	-30,100	1,900
CH3-CO-Pro9-NH2	8	-46,400	2,100	-46,400	2,100

**Table 3.** Mean Residue Ellipticities,  $[\theta]$ , and Mean Oligoproline Ellipticities,  $[\theta]_{OP}$ , for Four Synthetic Oligoproline-Based Peptides in Water (25 °C, pH 7.0).

## **EXPERIMENTAL PROCEDURES**

Materials and Methods. NMM was distilled from ninhydrin and stored in the dark. Melting points were obtained on a Thomas-Hoover apparatus and are uncorrected. <sup>1</sup>H NMR spectra were recorded at 200 MHz, 250 MHz, or 400 MHz on a Bruker AC200, WM250, or Varian XL-400 spectrometer. <sup>13</sup>C NMR spectra were recorded at 100.56 MHz on a Varian XL-400 spectrometer with 1% dioxane as the internal reference (67.4 ppm downfield from (CH<sub>3</sub>)<sub>4</sub>Si). Thin-layer chromatography (TLC) was performed on Whatman Diamondback F-254 silica-gel plates. FAB mass spectra were recorded for compounds 5 and 8 with a VG SEQ70 hybrid MS/MS spectrometer (Fission Instruments). Positive-ion ESI mass spectra were obtained with a Vestex 201A quadrupole mass spectrometer (Perseptive Biosystems) for compounds 1, 2, and 9, and with a Finnegan MAT quadrupole ion-trap spectrometer<sup>28</sup> for compound 3. CD spectra were recorded with an AVIV Model 62DS CD spectrophotometer at integral wavelengths from 190-250 nm using a 4-s averaging time and seven-point smoothing.<sup>2</sup> Individual data points were within 2,000 deg  $cm^2$  dmol<sup>-1</sup> of the plotted curve. Mean residue ellipticity,  $[\theta]$ , was based on the total number of residues. Mean oligoproline ellipticity,  $[\theta]_{OP}$ , was based on only the number of Pro plus Prm or Prs residues present in oligoproline segments (e.g., 99 for the 129-residue tripod protein). Peptide and protein concentrations (15-75  $\mu$ M) for CD studies were quantitated by amino acid analysis of stock solutions. Elemental analysis was conducted by Atlantic Microlab (Norcross, GA).

cis-1-(1,1-Dimethylethoxycarbonyl)-4-amino-L-proline Methyl Ester, Boc-Pra-OCH3 (5).  $cis-N^{\alpha}$ -Boc-4-azido-L-proline methyl ester<sup>18,19</sup> (4, 20 g, 74 mmol) was dissolved in 200 mL of 2% (v/v) HCl/ethanol and added under a blanket of argon gas to a glass hydrogenation vessel containing 10% palladium-on-carbon catalyst (5.0 g). The vessel was shaken in a Parr hydrogenator with  $H_2$  (45 psi) for 16 h. The reaction mixture was filtered through a pad of diatomaceous earth and concentrated by rotary evaporation. The residue was triturated with cold diethyl ether (3 x 200 mL), collected by suction filtration, washed with cold diethyl ether (5 x 100 mL), and dried under vacuum overnight to afford amino ester 5 as the hydrochloride salt (18.4 g, 66 mmol) in 89% yield. The product was homogeneous by TLC (Rf 0.19, 85:15 (v/v) CHCl<sub>3</sub>/CH<sub>3</sub>OH) and was a mixture of the trans and cis rotamers about the CO-NR<sub>2</sub> bond in 9:1 (v/v) $CD_3CN/D_20$  (trans/cis = 3:2) and in  $D_2O$  (trans/cis = 5:2) by NMR: mp 178.0-179.0 °C dec; FAB-MS (calcd for C<sub>11</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub> [MH<sup>+</sup>]: 244.3 Da) 245, 189, and 145 Da; <sup>-1</sup>H NMR (9:1 (v/v) CD<sub>3</sub>CN/D<sub>2</sub>O) δ 1.38 and 1.44 (2 s, trans & cis, 9 H, C(CH<sub>3</sub>)<sub>3</sub>), 2.10 and 2.17 (2 t, trans & cis, each J = 5.5 Hz, 1 H, C<sup>β</sup>H), 2.59-2.70 (m, 1 H,  $C^{\beta}$ H), 3.50-3.56 (2 d, trans & cis, each J = 4.4 Hz, 1 H,  $C^{\delta}$ H), 3.67-3.77 (m, 1 H,  $C^{\delta}H$ ), 3.77 (s, 3 H, OCH<sub>3</sub>), 3.87 (pentet J = 1.1 Hz, 1 H, CYH), and 4.24 and 4.29 ppm (2 d, trans & cis, each J = 5.3 Hz, 1 H, C<sup> $\alpha$ </sup>H); <sup>13</sup>C NMR (D<sub>2</sub>O)  $\delta$  176.6 (<u>C</u>OCH<sub>3</sub>, trans), 176.3 (<u>C</u>OCH<sub>3</sub>, cis), 156.6 (CONR<sub>2</sub>, cis), 155.9 (CONR<sub>2</sub>, trans), 84.1 ((CH<sub>3</sub>)<sub>3</sub>C), 60.0 (C<sup>α</sup>, trans), 58.6 (C<sup>α</sup>, cis), 54.5 (OCH<sub>3</sub>), 51.4  $(C^{\gamma}, cis), 50.4 (C^{\gamma}, trans), 50.2 (C^{\delta}, cis), 49.6 (C^{\delta}, trans), 34.5 (C^{\beta}, trans), 33.9 (C^{\beta}, cis), 28.7 ((CH_3)_3C, C^{\beta})$ cis), and 28.5 ppm ((<u>CH3)</u><sub>3</sub>C, trans); anal. (calcd for C<sub>11</sub>H<sub>21</sub>N<sub>2</sub>O<sub>4</sub>Cl: C 47.06, H 7.54, N 9.98) C 47.00, H 7.50, N 9.77.

cis-1-(1,1-Dimethylethoxycarbonyl)-4-amino-L-proline, Boc-Pra-OH (6). Boc-Pra-OCH<sub>3</sub> hydrochloride (7.04 g, 25.1 mmol) was dissolved in 2:1 (v/v) methanol/water (25 mL) and cooled to 0 °C with an ice bath. Solid lithium hydroxide monohydrate (2.20 g, 52.5 mmol) was added and the peach-colored solution was magnetically stirred for 6 h. Methanol was removed by rotary evaporation and the resulting

aqueous solution was adjusted to pH 7.0 with 12 N HCl. Water was removed by rotary evaporation and the off-white residue was dried overnight in a vacuum dessicator. The crude solid (6.7 g) was used for the next reaction without further purification. An analytical sample of pure Boc-Pra-OH was prepared by reversed-phase chromatography on a short octadecyl-silica column (Waters  $C_{18}$  Sep-Pak cartridge) eluted with 1:1 (v/v) CH<sub>3</sub>CN/water. The melting point and <sup>1</sup>H spectrum of the white solid were identical to the literature values.<sup>18</sup>

cis-1-(1,1-Dimethylethoxycarbonyl)-4-(6-(2,5-dihydro-2,5-dioxo-1H-pyrrolo-1)hexanamido)-L-proline, Boc-Prm-OH (7). 6-Maleimidohexanoic acid succinimido ester<sup>15,20</sup> (2.9 g, 9.4 mmol) and Boc-Pra-OH (6, 2.4 g, 10 mmol) were dissolved in DMF (22 mL). NMM (2.3 mL, 21 mmol) was added, the mixture was stirred at 40 °C overnight, and the DMF was removed by rotary evaporation under high vacuum (0.25 torr). The resulting orange solid was partitioned between  $0.25 \text{ M} \text{ H}_2\text{SO}_4$  (150 mL) and ethyl acetate (200 mL). The aqueous phase was extracted with ethyl acetate (5 x 100 mL) and the combined organic phases were washed with water (2 x 100 mL) and brine (2 x 100 mL), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and rotary evaporated. The residual orange-brown oil was chromatographed twice on silica gel eluted with 85:15 (v/v) CHCl<sub>3</sub>/CH<sub>3</sub>OH (R<sub>f</sub> 0.24) to provide a clear brown oil, which solidified upon trituration with hexanes. The tan solid was collected by suction filtration and dried overnight under vacuum (0.2 torr) to provide in 77% yield the pure acid 7 (3.27 g) as a mixture of urethane rotamers: mp 290 °C dec; <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>) δ 1.21-1.33 (m, 2 H, hexanoyl CYH<sub>2</sub>), 1.40 and 1.44 (2 s, trans & cis, 9 H, C(CH<sub>3</sub>)<sub>3</sub>), 1.60-1.71 (m, 4 H, hexanoyl C<sup> $\beta$ </sup>H<sub>2</sub> & C<sup> $\delta$ </sup>H<sub>2</sub>), 2.08-2.15 (m, 3 H, hexanoyl C<sup>e</sup>H<sub>2</sub> & proline C<sup>β</sup>H), 2.25-2.38 (m, 1 H, proline C<sup>β</sup>H), 3.43-3.60 (m, 4 H, hexanoyl C<sup>α</sup>H<sub>2</sub> & proline  $C^{\delta}H_2$ , 4.26 and 4.42 (2 d, trans & cis, each J = 9.7 Hz, 1 H, proline  $C^{\alpha}H$ ), 4.42-4.61 (m, 1 H, proline CYH), and 6.67 and 6.68 ppm (2 s, 2 H, CH=CH); <sup>13</sup>C NMR (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 175.7 (COOH), 171.5 and 171.1 (hexanoyl C=O & maleimide C=O), 153.4 (Boc C=O), 134.5 (maleimide HC=CH), 78.3 ((CH<sub>3</sub>)<sub>3</sub>C), 58.4-59.8 (C<sup> $\alpha$ </sup>), 51.7-52.4 (C<sup> $\gamma$ </sup>), 46.9-47.7 (C<sup> $\delta$ </sup>), 37.0 (hexanoyl C<sup> $\epsilon$ </sup>), 35.0-35.9  $(C^{\beta})$ , 35.3 (hexanoyl  $C^{\alpha}$ ), 28.1 ((CH<sub>3</sub>)<sub>3</sub>C), 27.8 (hexanoyl  $C^{\delta}$ ), 25.8 (hexanoyl  $C^{\beta}$ ), and 24.7 ppm (hexanoyl CY).

Synthesis of Linker Peptide 1. The 9-residue peptide  $CH_3-CO-Pro_3-Prm_3-Pro_3-NH_2$  was assembled on the MBHA resin (0.62 mmol/g, Applied Biosystems) by manual solid-phase methods. The second and third proline residues were coupled to Pro-resin (1.0 equiv) as the dipeptide Boc-Pro-Pro-OH (2.0 equiv, Fluka) for 2 h using (1-benzotriazoleoxy)tris(dimethylamino)phosphonium hexafluorophosphate (2.2 equiv) as the coupling agent, NMM (4.2 equiv) as the base, and 1-hydroxybenzotriazole (2.2 equiv).<sup>29</sup> In subsequent cycles Boc-Prm or Boc-Pro (4.0 equiv) was coupled using DCC (2.2 equiv, 1.0 M in CH<sub>2</sub>Cl<sub>2</sub>, Applied Biosystems) as the coupling agent and NMM (4.2 equiv) as the base. The coupling time was 2 h for Boc-Pro and 16 h for Boc-Prm. After the N terminus of the Boc-nonapeptide-resin was deprotected with CF<sub>3</sub>CO<sub>2</sub>H, it was acetylated with 3:1 (v/v) acetic anhydride/CH<sub>2</sub>Cl<sub>2</sub> containing NMM (10 equiv). The peptide was cleaved from the resin for 1 h at 4 °C by treatment with anhydrous HF (10 mL) in the absence of scavengers. The resin and peptide were washed with ice-cold diethyl ether (5 x 25 mL), lyophilized, redissolved in HPLC-grade water, lyophilized, and purified by preparative HPLC on butyl-silica (Table 1) to furnish linker peptide 1 (62 mg) as an off-white powder that was pure by analytical HPLC (Table 1): ESI-MS (calcd for  $C_{77}H_{104}N_{16}O_{19}$ : 1557.8 Da) 1558.1 Da; <sup>-1</sup>H NMR (250 MHz, D<sub>2</sub>O)  $\delta$  1.17-1.31 (m, 6 H,

hexanoyl C<sup>Y</sup>H<sub>2</sub>), 1.51-1.63 (m, 12 H, hexanoyl C<sup>β</sup>H<sub>2</sub> & C<sup>δ</sup>H<sub>2</sub>), 1.88-2.42 (m, 36 H, 3 hexanoyl C<sup>ε</sup>H<sub>2</sub>, 6 Pro C<sup>β</sup>H<sub>2</sub>, 6 Pro C<sup>Y</sup>H<sub>2</sub>, 3 Prm C<sup>β</sup>H & CH<sub>3</sub>CO), 2.68-2.70 (m, 3 H, 3 Prm C<sup>β</sup>H), 3.46-3.78 (m, 18 H, 6 Pro C<sup>δ</sup>H<sub>2</sub> & 3 Prm C<sup>δ</sup>H<sub>2</sub>), 4.19-4.30 (m, 3 H, Prm C<sup>α</sup>H), 4.36-4.80 (m, 9 H, 3 Prm C<sup>Y</sup>H & 6 Pro C<sup>α</sup>H), and 6.84 ppm (s, 6 H, CH=CH); <sup>13</sup>C NMR, see Table 2.

Synthesis of CH<sub>3</sub>-CO-Pro<sub>9</sub>-NH<sub>2</sub> (8). This acetylated nonapeptide amide was assembled on a 0.5-mmol scale from Boc-Pro-OH and MBHA resin (0.62 mmol/g) by using an automatic solid-phase peptide synthesizer (Applied Biosystems Model 430A). After cleavage from the resin with 10:1 (v/v) HF/anisole for 1 h at 4 °C, the peptide was purified by reversed-phase HPLC on butyl-silica (Table 1) to afforded model peptide 8 (160 mg) as an off-white powder: FAB-MS (calcd for C<sub>47</sub>H<sub>68</sub>N<sub>10</sub>O<sub>10</sub> [MH<sup>+</sup>]: 933.1 Da) 934 Da; <sup>13</sup>C NMR, see Table 2.

Synthesis of Leg Peptide 2. The 40-residue peptide amide 2 was assembled on MBHA resin (0.5 mmol, 0.62 mmol/g) using Boc-amino acids and the automatic peptide synthesizer. After the N terminus was deprotected with CF<sub>3</sub>CO<sub>2</sub>H, the peptide was cleaved from the resin by treatment with HF (15 mL), thioanisole (1.8 mL), and dimethylsulfide (1.5 mL) for 1 h at 0 °C. The resin and peptide were washed with ice-cold diethyl ether (4 x 50 mL) into a fine-porosity fritted-glass filter, air-dried for 15 min, and washed with 30% acetic acid (3 x 30 mL). The filtrate was diluted with water (180 mL) and lyophilized. The residual peptide was dissolved in 4 M guanidinium chloride/0.25 M Tris buffer (pH 8) containing DTT (5 mg/mL) and purified by preparative HPLC on butyl-silica (Table 1) to provide pure leg peptide 2 (86 mg) as a white powder: ESI-MS (calcd for  $C_{188}H_{268}N_{44}O_{44}S_1$ : 3880.5 Da) 3881 Da.

Synthesis of Leg Peptide Dimer 9. The 80-residue disulfide-bridged dimer 9 was prepared by stirring a solution of leg peptide 2 (10 mg) in 0.1 M phosphate buffer (pH 7.5, 2.0 mL) in air for 24 h. Preparative HPLC (Table 1) yielded pure homodimer 9 (8.2 mg) as a white powder: ESI-MS (calcd for  $C_{376}H_{534}N_{88}O_{88}S_2$ : 7759.0 Da) 7759.2 Da.

Synthesis of Tripod Protein 3. Solid leg peptide 2 (13 mg) and solid linker peptide 1 (3 mg) were placed in a 1.0-mL conical glass vial sealed with a rubber septum. The vial was flushed with argon and freeze-pump-thaw degassed 1:1 (v/v) acetonitrile/water (0.5 mL) was added by syringe. Addition of DIEA (50  $\mu$ L) instantly turned the solution pink. The solution was swirled at room temperature for 5 min, allowed to stand for 24 h, and quenched by the addition of water (0.25 mL) and solid DTT (1 mg). Preparative HPLC on *poly*(vinyl alcohol)-silica (Table 1) yielded tripod protein 3 (3.1 mg) as a white powder: ESI-MS (calcd for C<sub>641</sub>H<sub>908</sub>N<sub>148</sub>O<sub>153</sub>S<sub>3</sub>: 13,199.3 Da) 13,200 Da. It was homogeneous by analytical HPLC on butyl-silica (Table 1). Since no thiol 2 was detected by analytical HPLC after treatment with DTT, it contained no disulfide dimer 9.

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