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

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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^{α}-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_3-CO-Pro_3-Prm_3-Pro_3-NH_2. The leg peptide, the 40-residue thiol Gly-Arg-Gly-Asp-Ser-Pro-Gly-Tyr-Gly-Pro_{30}-Cys-NH_2, 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 ^{13}C NMR spectrometry, the linker peptide was a proline-II helix, as indicated by the presence of only trans Pro-Pro resonances for its β and γ carbons. By circular dichroic spectroscopy, the model peptide CH_3-CO-Pro_9-NH_2, 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_{30} 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.[1] 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 α-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.[2] 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.[3,4] 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.[5] 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_{17}-Ser-Pro_{18} segment in a sulfated surface glycoprotein from volvox,[6] the Pro_{17}-Ser-Pro_{11} segment of nuclear protein EBNA-2 from herpesvirus 4,[7] the Pro_{23} segment in pig acrosin,[8] the Pro_{17} segment of the chicken limb-deformity nuclear protein,[9] the Pro_{15} segment in a fruit-fly steroid hormone receptor,[10] and the Pro_{15} segment of the human homeotic protein Hox B4.[11] Six or more contiguous prolines give a stable proline-II helix,[12] 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.

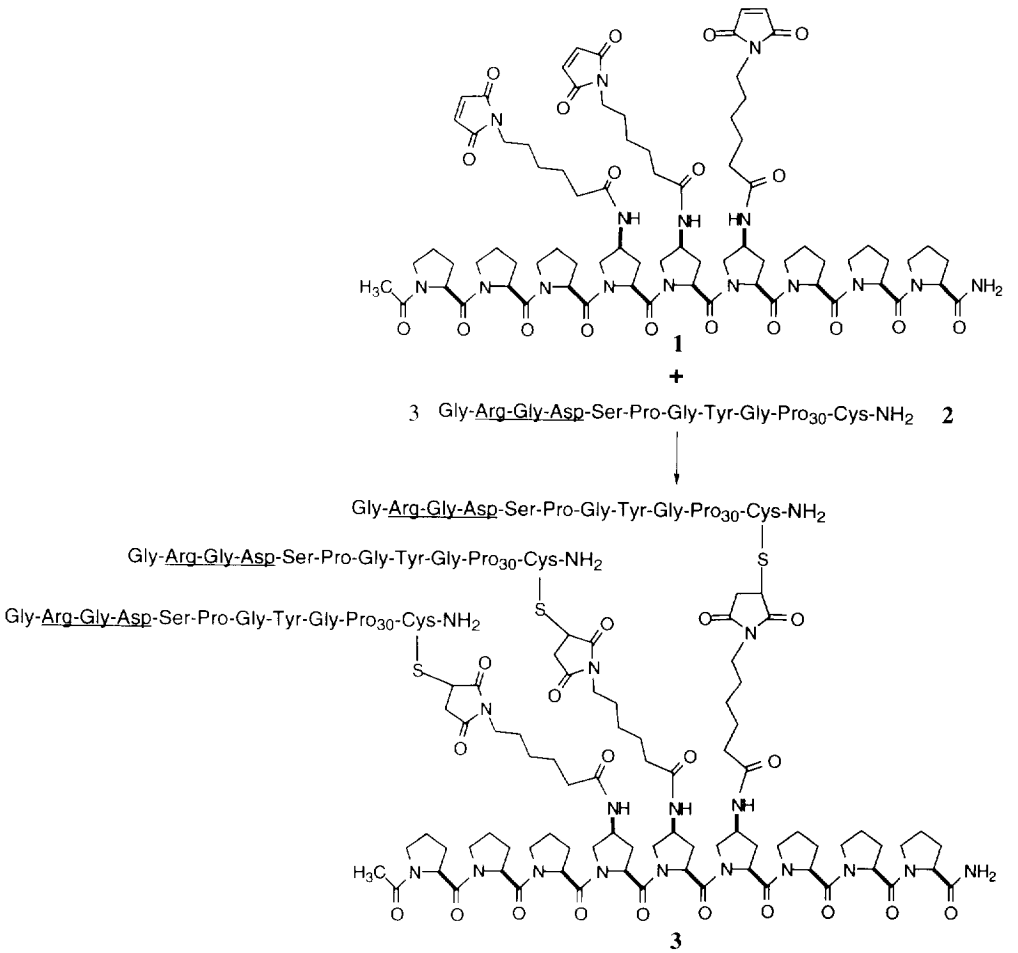
Chemoselective Ligation of Peptide Thiols

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.[13] 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.[14] Furthermore, a peptide thiol can selectively add to a maleimide group to form a stable thioether bond.[15,16] We have used this last approach to link three molecules of a large peptide thiol containing a Pro_{30} segment to three maleimide groups attached to a Pro_9 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_3-CO-Pro_3-Prm_3-Pro_3-NH_2; **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-Arg-Gly-Asp-Ser-Pro) and the 3-residue spacer module (Gly-Tyr-Gly) used previously.[1,2] It also contains a Pro_{30} rod module, which should form a proline-II helix. Leg peptide **2** ends with a Cys-NH_2 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



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 β-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 oligoproline segment folded into a proline-II helix contributes 3.1 Å to its length,[17] 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.

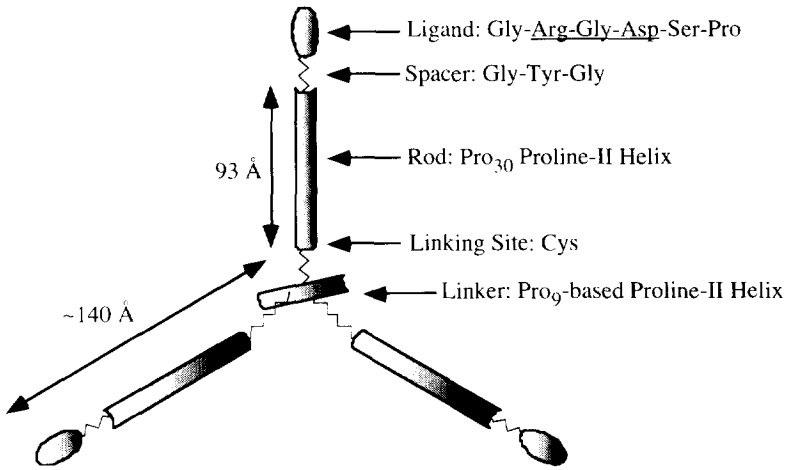


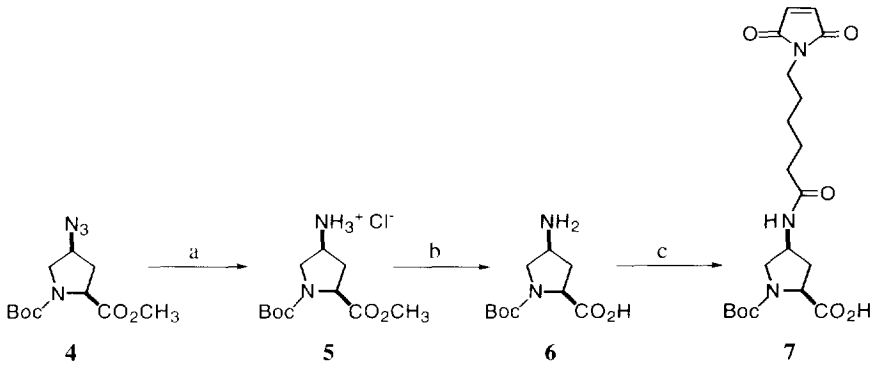
Figure 1. A Schematic Diagram of the Tripod Protein **3** That Shows the Four Types of Modules and Some of Its Dimensions.

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^{α}-Boc-4-azido-L-proline methyl ester (**4**) was synthesized[18,19] 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_3, **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^{α}-Boc-4-amino-L-proline (Boc-Pra-OH, **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[18] 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[15,20] to the 4-amino group of Boc-Pra-OH in the presence of *N,N*-diisopropylethylamine (DIEA) provided the N^{α}-protected amino acid *cis*-N^{α}-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_2Cl_2, or $CHCl_3$, but fully soluble in dimethylformamide (DMF). Its ^1H nuclear magnetic resonance (NMR) spectrum displayed two maleimide olefinic protons at 6.7 ppm and the $C^{\gamma}H$ 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.[21] The loss of Pro-Pro diketopiperazine after deprotection of Boc-Pro-Pro-resin was avoided by forming Boc-Pro-Pro-Pro-resin through the coupling of Boc-Pro-Pro to Pro-resin.[22] 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_2, 10% Pd/C in 2% HCl/ethanol; (b) LiOH, 2:1 CH_3OH/H_2O; (c) 6-maleimidohexanoic acid succinimido ester, DIEA, 1:1 CH_3CN/H_2O.

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 ^1H 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_3-CO-Pro$_9$-NH_2. 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 Pro_9 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.[21] 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.[22] 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

Table 1. HPLC Retention Times for the Reversed-Phase Chromatography of Four Proline-Based Synthetic Peptides.

Peptide	Code	Retention Time [a], min	
		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

[a] HPLC column was eluted with the indicated linear gradient of CH_3CN in 0.06% CF_3CO_2H/H_2O.

[b] 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_3CN and for 80 min with 0-75% CH_3CN.

[c] 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_3CN and for 60 min with 0-75% CH_3CN.

[d] *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_3CN, for 7 min with 0-12% CH_3CN, for 16 min at 12% CH_3CN, and for 45 min with 12-100% CH_3CN.

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.

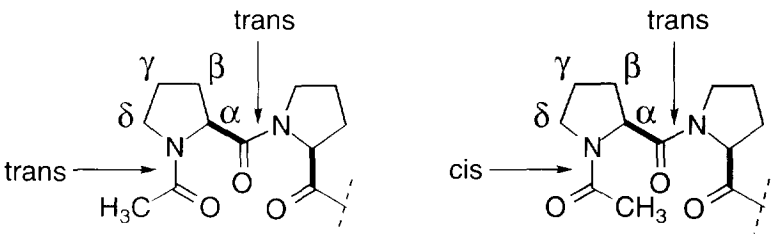


Figure 2. Illustration of the trans-trans (tt) and cis-trans (ct) conformations for the N-terminal segment CH_3-CO-Pro^1-Pro^2-... of CH_3-CO-Pro^1-Pro^2-Pro^3-Prm^4-Prm^5-Prm^6-Pro^7-Pro^8-Pro^9-NH_2 (**1**) and CH_3-CO-Pro^1-Pro^2-Pro^3-Pro^4-Pro^5-Pro^6-Pro^7-Pro^8-Pro^9-NH_2 (**8**).

Biophysical Studies

^{13}C NMR Spectrometry. The solution conformations of the Pro_9 peptide **8** and its maleimide derivative **1** were studied in D_2O by ^{13}C NMR spectrometry. Listed in Table 2 are the ^{13}C chemical shifts and assignments for the carbons of peptides **1** and **8**. As shown in Figure 2, their N-terminal segment CH_3-CO-Pro^1-Pro^2-... can exist in either the trans-trans or the cis-trans conformation. Figure 3 depicts the well-resolved resonances due to the β, γ, and $\underline{C}H_3CO$ 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.[23,24] The ratios of the peak heights for the C^β, C^γ, and $\underline{C}H_3CO$ 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 $\underline{C}H_3CO$ 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^1 was in the cis conformation but essentially all of the peptide bond between Pro^1 and Pro^2 was in the trans conformation (Figure 2). In addition, the chemical shifts of the β and γ carbons of the non-terminal Pro residues of peptide **1** (β 29.4 ppm, γ 26.1 ppm) and peptide **8** (β 29.3 ppm, γ 26.0 ppm) are closer to those reported[25] for polyproline-II (β 28.6 ppm, γ 25.4 ppm) than to those for polyproline-I (β 32.2 ppm, γ 22.5 ppm). These results indicate that the entire peptide backbones of model Pro_9 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_9 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.[26] In contrast, a left-handed proline-II helix exhibits a strong negative band at ~205 nm and a weak positive band at ~226 nm.[26] Figure 4 shows the CD spectra for **1**, **2**, **3**, and **8** in water, a solvent that favors formation of a proline-II helix.[27] 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^2 $dmol^{-1}$. 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.

Table 2. ^{13}C NMR Chemical Shifts and Carbon Assignments for Proline-Based Peptides **1** and **8** in D_2O.

Assignment for	Chemical Shift (ppm)		Assignment for
CH_3-CO-Pro_9-NH_2 (**8**)	**8**	**1**	CH_3-CO-Pro_3-Prm_3-Pro_3-NH_2 (**1**)
Pro^9 $CONH_2$	178.4	178.3	Pro^9 $CONH_2$
		177.4	Maleimide CO
		174.6	Hexanoyl CO
$CH_3\underline{C}O$ (ct)	173.8	173.8	$CH_3\underline{C}O$ (ct)
Pro^1 $CONR_2$ (tt)	173.7		
$CH_3\underline{C}O$ (tt) &	173.4	173.4-172.2	$CH_3\underline{C}O$ (tt) &
Pro^1 $CONR_2$ (ct)			Pro and Prm $CONR_2$
Pro^{2-8} $CONR_2$	173.1-172.8		
		135.8	Maleimide HC=CH
Pro^1 C^α	61.6	61.6	Pro^1 C^α
Pro^{2-8} C^α	60.0	60.4-58.8	$Pro^{2,3,7-9}$ C^α &
			Prm C^α
Pro^9 C^α	59.7		
		53.8-54.1	Prm C^γ
Pro^1 C^δ (tt)	50.1	50.0-50.4	Pro^1 C^δ (tt) &
			Prm C^δ
Pro^{2-9} C^δ	49.0	49.1	$Pro^{2,3,7-9}$ C^δ
Pro^1 C^δ (ct)	48.6		
		38.9	Hexanoyl C^ε
		37.0	Hexanoyl C^α
		34.0-34.2	Prm C^β
Pro^1 C^β (ct)	31.5		
Pro^9 C^β	30.9	30.9	Pro^9 C^β
Pro^1 C^β (tt)	29.9	30.0	Pro^1 C^β (tt)
Pro^{2-8} C^β	29.3-29.5	29.1-29.4	$Pro^{2,3,7,8}$ C^β
		28.9	Hexanoyl C^δ
		27.0	Hexanoyl C^β
Pro^{2-9} C^γ	26.0-26.2	26.0-26.1	$Pro^{2,3,7-9}$ C^γ
		26.0	Hexanoyl C^γ
Pro^1 C^γ (tt)	25.7	25.7	Pro^1 C^γ (tt)
Pro^1 C^γ (ct)	23.9		
$\underline{C}H_3CO$ (tt)	22.7	22.7	$\underline{C}H_3CO$ (tt)
$\underline{C}H_3CO$ (ct)	22.6	22.6	$\underline{C}H_3CO$ (ct)

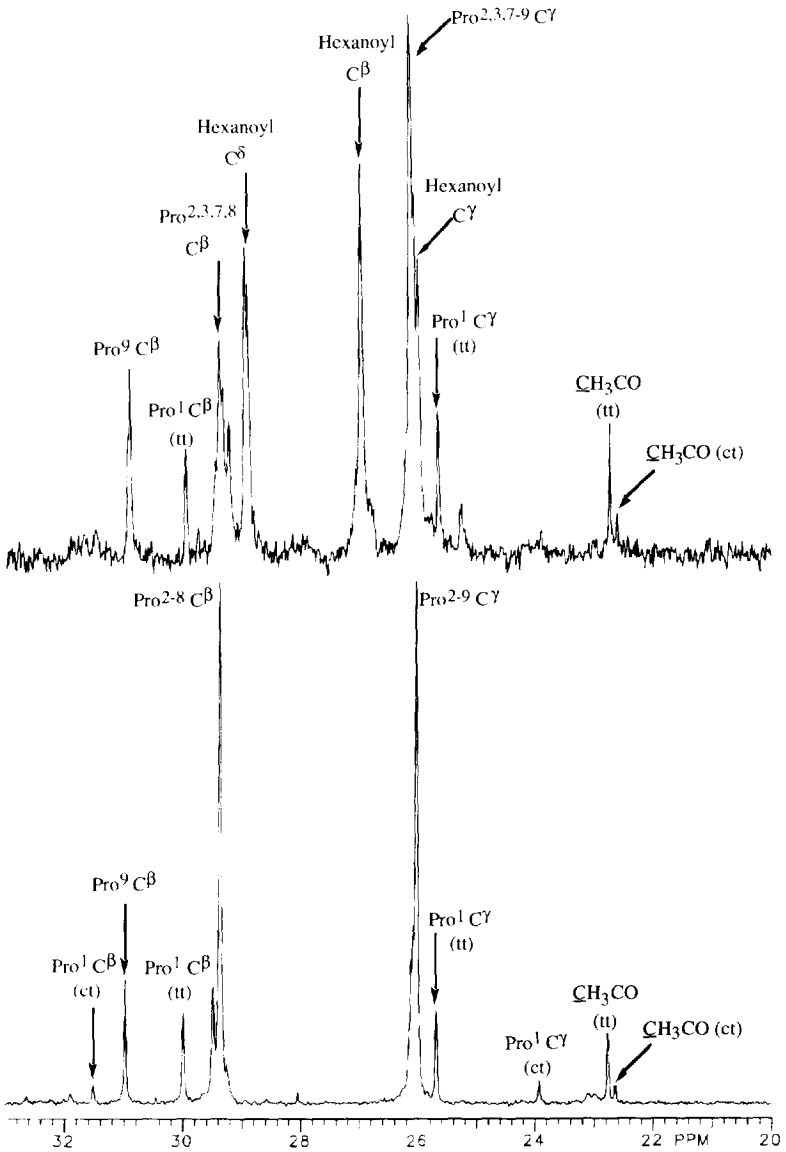


Figure 3. Partial ^{13}C NMR spectra for CH_3-CO-Pro$_3$-Prm$_3$-Pro$_3$-NH_2 (**1**, upper) and CH_3-CO-Pro$_9$-NH_2 (**8**, lower) in D_2O at 100 MHz. The resonances are marked for the trans-trans (tt) and cis-trans (ct) conformers of the N-terminal segment CH_3-CO-Pro^1-Pro^2-... (see Figure 2).

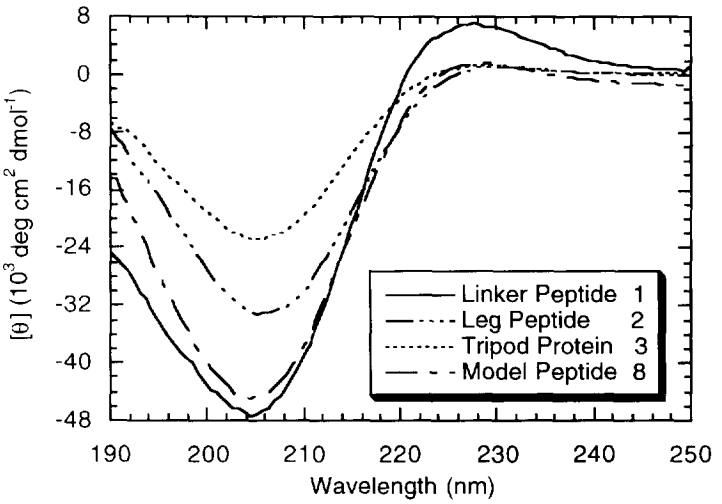


Figure 4. CD Spectra of Four Oligoproline-Based Synthetic Peptides in Water at 25 °C and pH 7.0.

Summary

This paper has described (a) an improved synthesis of *cis*-N^{α}-Boc-4-amino-L-proline, a protected form of a conformationally constrained α,γ-diamino acid; (b) the first synthesis of the thiol-reactive amino acid *cis*-N^{α}-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_{30} 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.

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

		[θ], deg cm² dmol⁻¹		$[\theta]_{OP}$, deg cm² dmol⁻¹	
Peptide	Code	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
CH_3-CO-Pro_9-NH_2	**8**	-46,400	2,100	-46,400	2,100

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. ^{1}H NMR spectra were recorded at 200 MHz, 250 MHz, or 400 MHz on a Bruker AC200, WM250, or Varian XL-400 spectrometer. ^{13}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 $(CH_3)_4Si$). 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[28] 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.[2] Individual data points were within 2,000 deg cm^2 dmol^{-1} 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 μM) 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-OCH$_3$ (5).** *cis*-N^{α}-Boc-4-azido-L-proline methyl ester[18,19] (**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 (R_f 0.19, 85:15 (v/v) $CHCl_3/CH_3OH$) and was a mixture of the trans and cis rotamers about the CO-NR_2 bond in 9:1 (v/v) CD_3CN/D_2O (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 $C_{11}H_{20}N_2O_4$ [MH$^+$]: 244.3 Da) 245, 189, and 145 Da; ^{1}H NMR (9:1 (v/v) CD_3CN/D_2O) δ 1.38 and 1.44 (2 s, trans & cis, 9 H, $C(CH_3)_3$), 2.10 and 2.17 (2 t, trans & cis, each J = 5.5 Hz, 1 H, $C^{\beta}H$), 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_3), 3.87 (pentet J = 1.1 Hz, 1 H, $C^{\gamma}H$), and 4.24 and 4.29 ppm (2 d, trans & cis, each J = 5.3 Hz, 1 H, $C^{\alpha}H$); ^{13}C NMR (D_2O) δ 176.6 ($\underline{C}OCH_3$, trans), 176.3 ($\underline{C}OCH_3$, cis), 156.6 ($CONR_2$, cis), 155.9 ($CONR_2$, trans), 84.1 ($(CH_3)_3\underline{C}$), 60.0 (C^{α}, trans), 58.6 (C^{α}, cis), 54.5 ($O\underline{C}H_3$), 51.4 (C^{γ}, cis), 50.4 (C^{γ}, trans), 50.2 (C^{δ}, cis), 49.6 (C^{δ}, trans), 34.5 (C^{β}, trans), 33.9 (C^{β}, cis), 28.7 (($\underline{C}H_3)_3C$, cis), and 28.5 ppm (($\underline{C}H_3)_3C$, trans); anal. (calcd for $C_{11}H_{21}N_2O_4Cl$: 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_3 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_3CN/water. The melting point and ^1H spectrum of the white solid were identical to the literature values.[18]

***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[15,20] (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 M H_2SO_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_2SO_4, and rotary evaporated. The residual orange-brown oil was chromatographed twice on silica gel eluted with 85:15 (v/v) $CHCl_3/CH_3OH$ (R_f 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; ^1H NMR (200 MHz, $CDCl_3$) δ 1.21-1.33 (m, 2 H, hexanoyl $C^{\gamma}H_2$), 1.40 and 1.44 (2 s, trans & cis, 9 H, $C(CH_3)_3$), 1.60-1.71 (m, 4 H, hexanoyl $C^{\beta}H_2$ & $C^{\delta}H_2$), 2.08-2.15 (m, 3 H, hexanoyl $C^{\varepsilon}H_2$ & proline $C^{\beta}H$), 2.25-2.38 (m, 1 H, proline $C^{\beta}H$), 3.43-3.60 (m, 4 H, hexanoyl $C^{\alpha}H_2$ & 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 $C^{\gamma}H$), and 6.67 and 6.68 ppm (2 s, 2 H, CH=CH); ^{13}C NMR (400 MHz, $(CD_3)_2SO$) 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 ($(CH_3)_3\underline{C}$), 58.4-59.8 (C^{α}), 51.7-52.4 (C^{γ}), 46.9-47.7 (C^{δ}), 37.0 (hexanoyl C^{ε}), 35.0-35.9 (C^{β}), 35.3 (hexanoyl C^{α}), 28.1 ($(\underline{C}H_3)_3C$), 27.8 (hexanoyl C^{δ}), 25.8 (hexanoyl C^{β}), and 24.7 ppm (hexanoyl C^{γ}).

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).[29] In subsequent cycles Boc-Prm or Boc-Pro (4.0 equiv) was coupled using DCC (2.2 equiv, 1.0 M in CH_2Cl_2, 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_3CO_2H, it was acetylated with 3:1 (v/v) acetic anhydride/CH_2Cl_2 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) and collected on a fine-porosity fritted-glass filter. The peptide was dissolved in glacial acetic acid (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; ^1H NMR (250 MHz, D_2O) δ 1.17-1.31 (m, 6 H,

hexanoyl $C^{\gamma}H_2$), 1.51-1.63 (m, 12 H, hexanoyl $C^{\beta}H_2$ & $C^{\delta}H_2$), 1.88-2.42 (m, 36 H, 3 hexanoyl $C^{\varepsilon}H_2$, 6 Pro $C^{\beta}H_2$, 6 Pro $C^{\gamma}H_2$, 3 Prm $C^{\beta}H$ & CH_3CO), 2.68-2.70 (m, 3 H, 3 Prm $C^{\beta}H$), 3.46-3.78 (m, 18 H, 6 Pro $C^{\delta}H_2$ & 3 Prm $C^{\delta}H_2$), 4.19-4.30 (m, 3 H, Prm $C^{\alpha}H$), 4.36-4.80 (m, 9 H, 3 Prm $C^{\gamma}H$ & 6 Pro $C^{\alpha}H$), and 6.84 ppm (s, 6 H, CH=CH); ^{13}C NMR, see Table 2.

Synthesis of CH_3-CO-Pro$_9$-NH_2 (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 $C_{47}H_{68}N_{10}O_{10}$ [MH^+]: 933.1 Da) 934 Da; ^{13}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_3CO_2H, 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 μL) 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 $C_{641}H_{908}N_{148}O_{153}S_3$: 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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