Articles

Convergent Synthesis of Repeating Peptides (Val-X-Leu-Pro-Pro-Pro)$_8$ Adopting a Polyproline II Conformation†

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The *N*-terminal domain of maize γ-zein has a repetitive structure (Val-His-Leu-Pro-Pro-Pro)$_8$ that has recently been shown to adopt an amphipathic polyproline II type conformation in aqueous solution. We report here the synthesis and conformational analysis of three model peptides (Val-X-Leu-Pro-Pro-Pro)$_8$ (X = Ala (**1**), Glu (**2**), Lys (**3**)). The three compounds have been synthesized in a very efficient way using a convergent solid-phase strategy. Circular dichroism shows unequivocally that the three model peptides adopt polyproline II (PPII) type conformations under a variety of experimental conditions and that neither the presence of histidine nor amphipathicity of the peptide is an absolute requirement for adopting the native conformation. These results open the door for the *de novo* design of compounds with PPII conformations and must be taken into account in the structure prediction of protein structures from sequence data banks.

Polyproline II (PPII) is a conformation characteristic of homopolymeric proline in water. The amide bond adopts a *trans* conformation ($\omega = +180°$) and the angles ϕ and ψ values of $-78°$ and $+146°$, respectively.[1] The PPII conformation is a left-handed helix with a periodicity of 3.0 residues per turn.[2] The importance of the PPII structure in biology is becoming more and more apparent, and it is clear that several proline-rich domains adopt this type of structure in their native states.[3] Furthermore, analysis of data banks containing known three-dimensional structures of proteins demonstrates that this type of structure occurs with a certain frequency, even in sequences where proline is relatively scarce.[4] Recently it has been established that the adoption of a PPII conformation is a prerequisite for the recognition of a peptide or protein fragment by SH3 domains, which are important in signal transduction processes.[5] The PPII structure has also been found in peptides bound to class II histocompatibility proteins.[6] Finally, Silligardi has proposed that the conformation adopted preferentially by many small peptides in solution, and whose NMR and CD data are normally interpreted as indicative of the random coil, are in fact PPII conformations.[4b]

Proline-rich protein domains are especially abundant in plant proteins.[7] The *N*-terminal domain of the γ-zein protein of maize is composed of the sequence (Val-His-Leu-Pro-Pro-Pro)$_8$. Geli et al. have elegantly demonstrated that this repetitive sequence is responsible for the targeting of γ-zein to the endoplasmic reticulum.[8] CD studies clearly show that, in contrast to proline-rich domains in other maize proteins,[9] that of γ-zein adopts a PPII type structure in aqueous solution.[2] These results suggest that the adoption of PPII type structure may be the structural feature responsible for the targetting of the protein.

An unusual characteristic of the *N*-terminal domain of γ-zein is that it acquires a marked amphipathic character on adopting a PPII conformation. Effectively, on adopting a helical structure with 3.0 residues per turn, the side chains of the hydrophobic residues valine and leucine are aligned on the same side of the helix whereas the imidazole groups, which are polar and may be charged depending upon pH, are aligned on the opposite side. This observation prompts a series of questions:

1. Is amphipathicity necessary for the adoption of PPII type structures in repetitive structures such as the *N*-terminal domain of γ-zein?

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† Abbreviations: Boc, *tert*-butoxycarbonyl; CSPPS, convergent solid-phase peptide synthesis; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; ESMS, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HMPB, 4-(4-(hydroxymethyl)-3-methoxyphenoxy)butyric acid; HOAc, acetic acid; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; IRAA, internal reference amino acid; MeCN, acetonitrile; MPLC, medium pressure liquid chromatography; OtBu, *tert*-butyl ester, RPHPLC, reversed-phase high-performance liquid chromatography; RPMPLC, reversed-phase medium pressure liquid chromatography; TFA, trifluoracetic acid; Trt, trityl.

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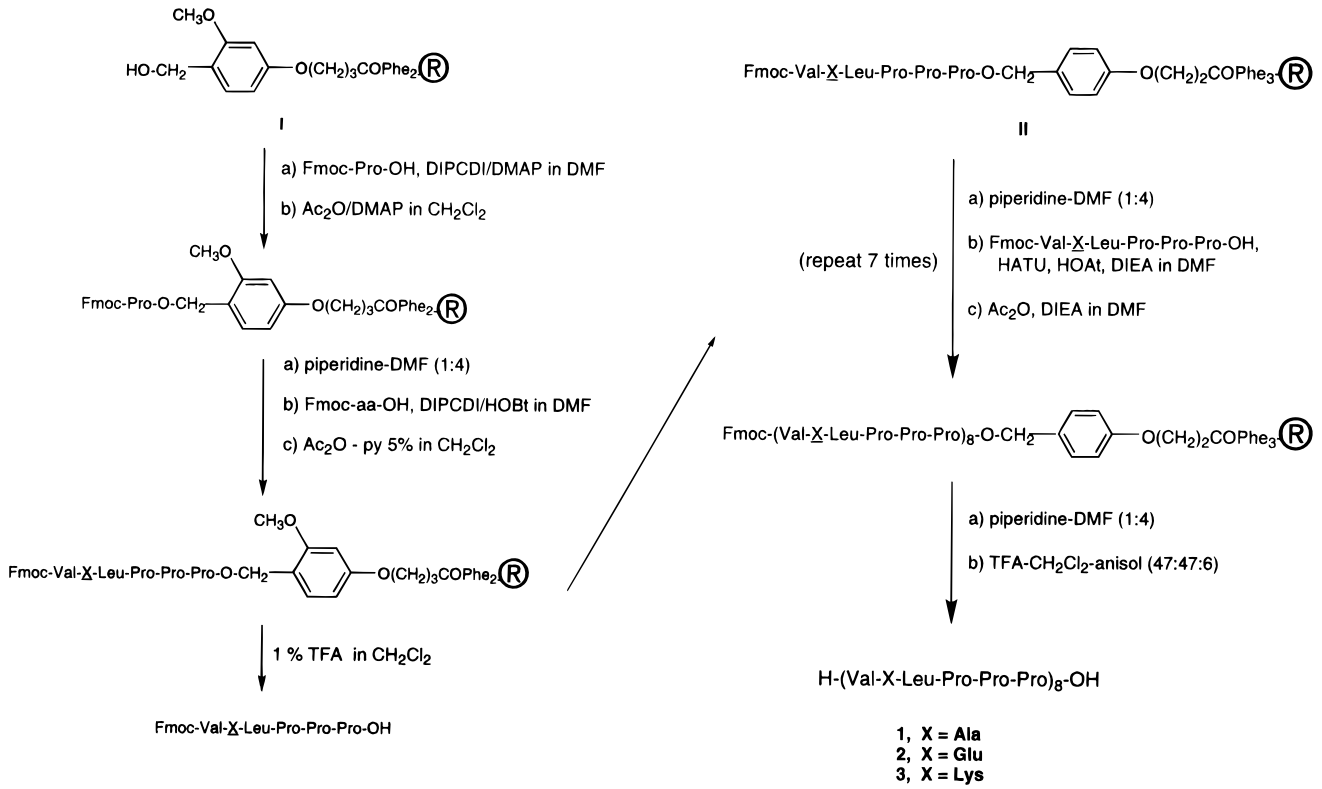
Scheme 1. Scheme of the H-(Val-X-Leu-Pro-Pro-Pro)$_8$-OH Syntheses[a]



[a] **X**, protected amino acid; **X**: deprotected amino acid.

2. If it is necessary, is it dependent upon the nature of the charge (positive or negative) on the polar charged side chains?

3. If positive charges are required, can these be provided by other protonated basic amino acid side chains?

Recently we have described a convergent solid-phase synthetic method that allows the straightforward preparation of proline-rich protein domains.[10] The objective of the present work is to try to answer the questions posed above, applying this method to the synthesis of other peptides with repeating structures. We describe the synthesis of the compounds **1**, **2**, and **3** and their conformational analysis by CD. In compound **1** the side chain of alanine is neutral and apolar at any pH so that the putative PPII helix cannot have amphipathic character. In compound **2**, at pH 7, the side chains of glutamic acid will be negatively charged so that the PPII helix would have a charge opposite in sign to that in native γ-zein. Finally, at pH 7, compound **3** in a PPII conformation would have the same charge sign as that of native γ-zein on the polar side of the helix, but these would now be due to the presence of alkylammonium side chains rather than the imidazole side chains of the native protein.

$$(\text{Val-Ala-Leu-Pro-Pro-Pro})_8 \quad (1)$$

$$(\text{Val-Glu-Leu-Pro-Pro-Pro})_8 \quad (2)$$

$$(\text{Val-Lys-Leu-Pro-Pro-Pro})_8 \quad (3)$$

Results

Peptide Synthesis. In order to synthesize peptides **1**, **2**, and **3** by a convergent solid-phase methodology, the protected segments Fmoc-Val-Ala-Leu-Pro-Pro-Pro-OH, Fmoc-Val-Glu(OtBu)-Leu-Pro-Pro-Pro-OH, and Fmoc-Val-Lys(Boc)-Leu-Pro-Pro-Pro-OH were prepared (Scheme 1). The syntheses of these protected peptides were carried out by following a scheme parallel to that previously described for the synthesis of the native γ-zein protected monomer Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH.[10] 4-Methylbenzhydrylamine resin was used as solid support, with two residues of phenylalanine as internal reference amino acid (IRAA)[11] and a highly acid-labile 4-(4-(hydroxymethyl)-3-methoxyphenoxy)butyric acid (HMPB) handle.[12] The Fmoc group was used for the temporary α-amino protection, Boc and OtBu groups for the protection of the side chain of lysine and glutamic acid, respectively. The HMPB handle was coupled with DIPCDI/HOBt in DMF. In each peptide-resin, the first amino acid (Fmoc-Pro-OH) was incorporated with DIPCDI/DMAP (double coupling for 1 h each) and the completeness of the coupling was monitored by amino acid analysis. An acetylation step was carried out after this incorporation to cap possible remaining hydroxyl groups. The second and third proline residues were incorporated as Fmoc-Pro-Pro-OH to avoid diketopiperazine (DKP) formation.[13] This coupling, as were all the remaining amino acid couplings, was carried out using DIPCDI/HOBt in DMF. All couplings were followed by an

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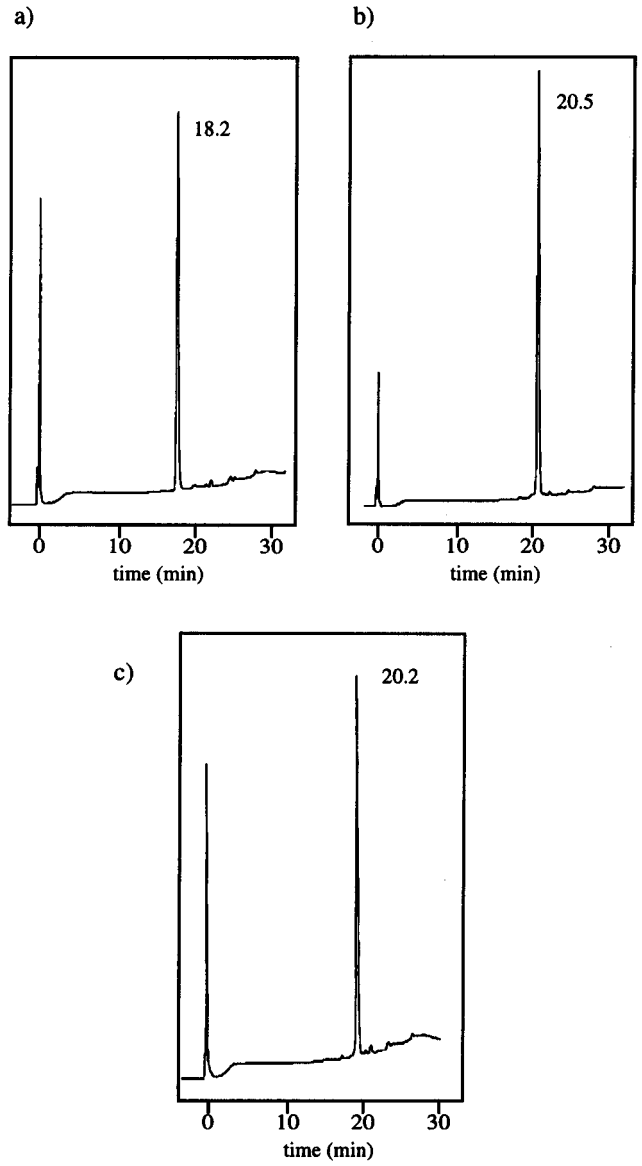

Figure 1. Analytical HPLC on reversed-phase C_{18} of the crude peptides (a) Fmoc-Val-Ala-Leu-Pro-Pro-Pro-OH, (b) Fmoc-Val-Glu(OtBu)-Leu-Pro-Pro-Pro-OH, and (c) Fmoc-Val-Lys(Boc)-Leu-Pro-Pro-Pro-OH. Elution conditions: A, H_2O−0.045% TFA; B, CH_3CN−0.036% TFA; linear gradient from 10% to 100% B in 25 min; flow rate, 1 mL/min; $\lambda = 220$ nm.

acetylation step to ensure the absence of deletion peptides. The Fmoc groups were cleaved by piperidine:DMF (1:4) prior to each coupling. The syntheses of these protected segments were monitored by the ninhydrin[14] or chloranil[15] tests. Amino acid analyses of the peptide-resins after synthesis gave the correct amino acid composition. The protected peptides were obtained on acidolysis of the peptide-resins using 1% TFA in CH_2Cl_2. These cleavage conditions permit all the protecting groups used for the peptides side chains to be maintained. The yields of these cleavages were >95% as judged by amino acid analyses of the peptide-resins after acidolysis. The crude peptides showed high HPLC purities (Figure 1). The molecular weights of the peptides were confirmed by FAB mass spectrometry.

For the convergent solid-phase synthesis of **1**, **2**, and **3** by segment condensation (Scheme 1), the crude peptides Fmoc-Val-Ala-Leu-Pro-Pro-Pro-OH, Fmoc-Val-Glu-(OtBu)-Leu-Pro-Pro-Pro-OH, and Fmoc-Val-Lys(Boc)-Leu-Pro-Pro-Pro-OH were used without further purification. As described for the native γ-zein *N*-terminal domain,[10] these convergent syntheses involved the preparation of other monomer-resins to couple the protected segments. To assemble these monomer-resins, the solid support used was aminomethylated polystyrene resin with three residues of phenylalanine as internal standard. The handle 3-(4-(hydroxymethyl)phenoxy)propionic acid was incorporated to the support as its 2,4,5-trichlorophenyl ester[16] with HOBt in DMF. The amino acid incorporation proceeded as described above for the synthesis of the protected segments. The amino acid analyses of the peptide-resins after synthesis gave the correct values. The segment condensations were carried out with the protected segments corresponding to each Fmoc-Val-**X**-Leu-Pro-Pro-Pro-resin (with **X**: Ala, Glu, or Lys), HATU, HOAt, and DIEA in DMF (4 equiv of the protected peptide and the coupling reagents; 1 h each coupling at 25 °C). Fmoc groups were removed with piperidine:DMF (1:4) prior to each coupling. After coupling an acetylation (Ac_2O and DIEA in DMF) step was carried out in order to cap any unreacted amino groups. The monitoring of these syntheses was done in the Fmoc-(Val-**X**-Leu-Pro-Pro-Pro)$_3$-resins by amino acid analysis. ESMS of crude Fmoc-(Val-**X**-Leu-Pro-Pro-Pro)$_3$-OH after cleavage from the resins confirmed completion of the couplings and the total deprotection of the α-amino terminal group. Couplings were then repeated until the different Fmoc-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-resins were obtained. Cleavages were carried out with TFA:CH_2Cl_2:anisole (47:47:6) for 1.5 h. The crude peptides H-(Val-Ala-Leu-Pro-Pro-Pro)$_8$-OH (**1**), H-(Val-Glu-Leu-Pro-Pro-Pro)$_8$-OH (**2**), and H-(Val-Lys-Leu-Pro-Pro-Pro)$_8$-OH (**3**) were purified by reversed-phase HPLC. The presence of H-(Val-**X**-Leu-Pro-Pro-Pro)$_n$-OH or Ac-(Val-**X**-Leu-Pro-Pro-Pro)$_n$-OH where $n < 7$, possible deletion sequences in these syntheses, was not observed by ESMS. The molecular weights were in accordance with the expected values.[17]

CD Spectroscopy. The circular dichroism (CD) of peptides is very sensitive to secondary structure. The CD spectra of the peptides H-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-OH at pH 7 and 5 °C are shown in Figure 2. The CD patterns were very similar to those observed in poly-L-proline and the *N*-terminal domain of γ-zein H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH,[9] with a λ_{min} at 202 nm and a λ_{max} at 228 nm. This indicates that the PPII conformation is substantially populated in all three compounds **1**, **2**, and **3**.

Peptides **1**, **2**, and **3** give a CD spectra showing a minimum at 202 nm and a small maximum at 228 nm. This pattern, although not too different from that found in random-coil peptides, can be assigned to a polyproline II conformation on the basis of the presence of the 228 nm maximum and the characteristic changes with temperature and addition of $CaCl_2$. It is known that the CD spectrum of PPII changes in the presence of 4−6 M $CaCl_2$

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(17) A high affinity of these peptides, when **X** is alanine or glutamic acid, to bind cations as sodium and potassium, was observed on ESMS analysis.

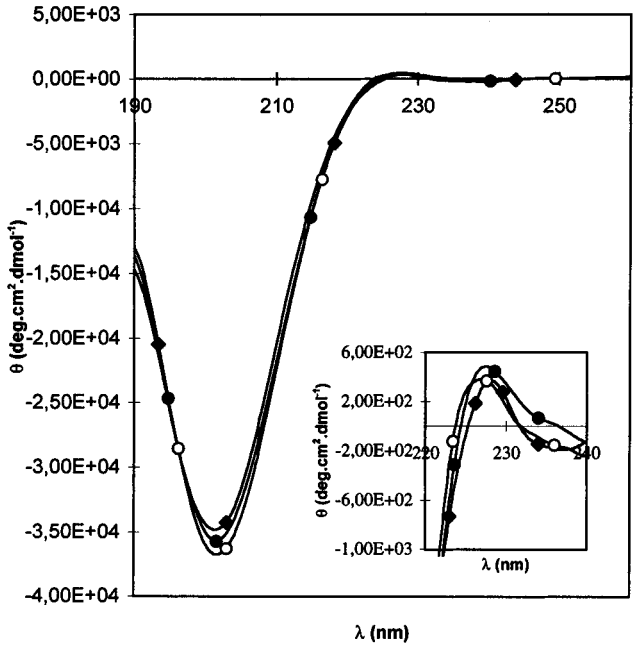

Figure 2. CD spectra of H-(Val-Ala-Leu-Pro-Pro-Pro)$_8$-OH (**1**) (◆), H-(Val-Glu-Leu-Pro-Pro-Pro)$_8$-OH (**2**) (○), and H-(Val-Lys-Leu-Pro-Pro-Pro)$_8$-OH (**3**) (●) in 1 mM sodium citrate/1 mM sodium borate/1 mM sodium phosphate buffer, 15 mM NaCl at pH 7 and 5 °C.

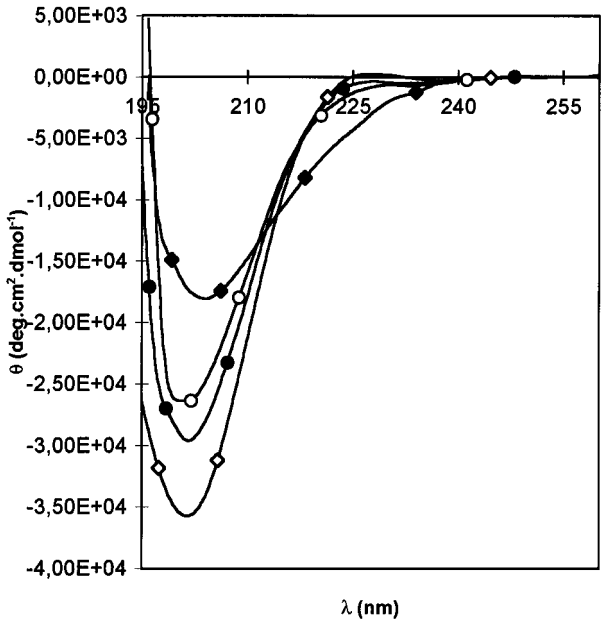

Figure 3. CD spectra of H-(Val-Ala-Leu-Pro-Pro-Pro)$_8$-OH in 1 mM sodium citrate/1 mM sodium borate/1 mM sodium phosphate buffer, 15 mM NaCl at pH 7 and 5 °C: (◇) 0 M $CaCl_2$; (●) 1.5 M $CaCl_2$; (○) 3.0 M $CaCl_2$; (◆) 6.0 M $CaCl_2$.

due to disruption of the extended helix.[18,19] In the CD spectrum of polyproline II, the absolute molar ellipticities in the λ_{min} and λ_{max} decrease until the positive band disappears. CD spectra of the peptides **1**, **2**, and **3** show the same changes on $CaCl_2$ addition, confirming the presence of a PPII conformation. Figure 3 illustrates the CD spectrum of H-(Val-Ala-Leu-Pro-Pro-Pro)$_8$-OH (**1**) in presence of $CaCl_2$. The same behavior is observed for each peptide H-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-OH on $CaCl_2$ addition (data in supporting information).

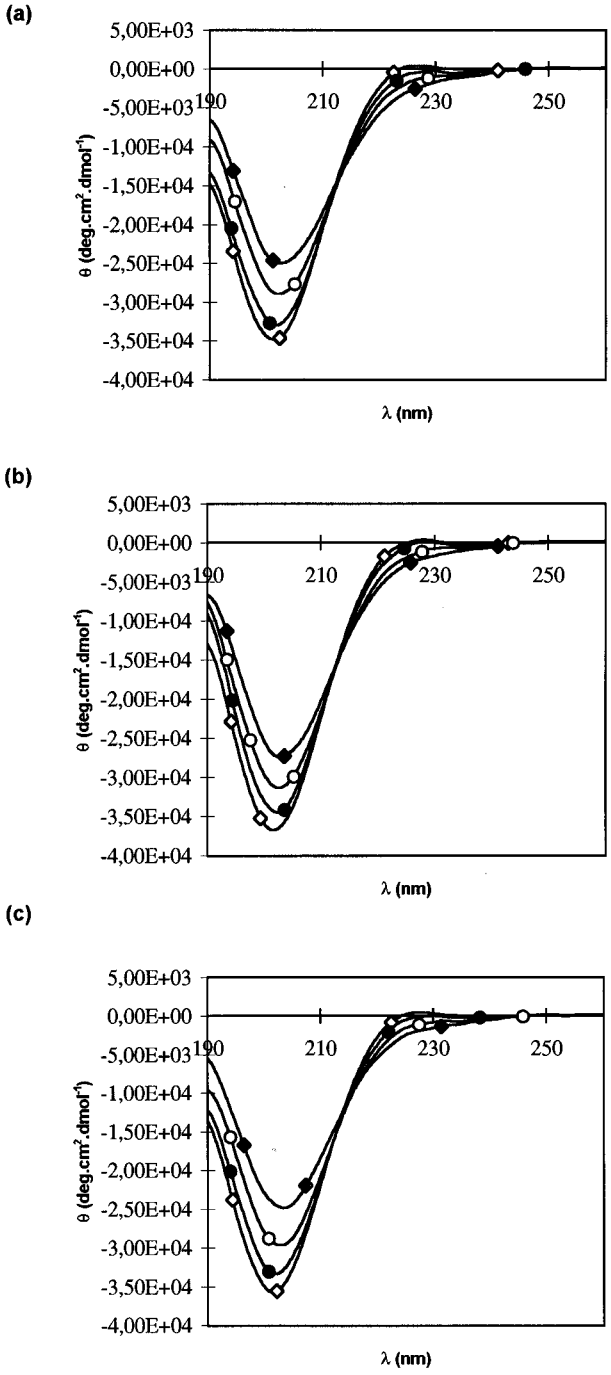

Figure 4. CD spectra of H-(Val-Ala-Leu-Pro-Pro-Pro)$_8$-OH (a), H-(Val-Glu-Leu-Pro-Pro-Pro)$_8$-OH (b), and H-(Val-Lys-Leu-Pro-Pro-Pro)$_8$-OH (c) in 1 mM sodium citrate/1 mM sodium borate/1 mM sodium phosphate buffer, 15 mM NaCl at pH 7: (◇) 5 °C, (●) 25 °C; (○) 50 °C; (◆) 80 °C.

Figure 4 shows the CD spectra of H-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-OH as a function of the temperature over the range of 5−80 °C. The decrease in the molar ellipticities in the λ_{min} and the λ_{max} points to a decrease of PPII character as the temperature is raised.[19,20] An isosbestic point is also observed, indicating that the conformational behavior of the system can be described as an equilibrium between two conformational states. The width of the thermally induced melting curve is larger than that

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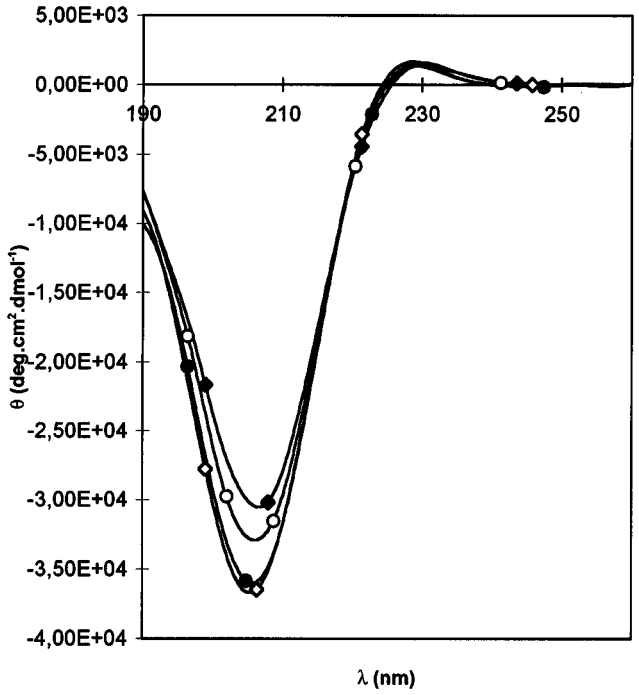

Figure 5. CD. spectra of oligomeric H-(Pro)$_n$-OH (mean size n = 42) in 1 mM sodium citrate/1 mM sodium borate/1 mM sodium phosphate buffer, 15 mM NaCl at pH 7: (◇) 5 °C, (●) 25 °C; (○) 50 °C; (◆) 80 °C.

normally found for a cooperative process such as thermal α-helix denaturation. Again this is characteristic of PPII, a conformation not stabilized by hydrogen bonds between the helix turns.

Discussion

The shape of the CD spectra of **1**, **2**, and **3** (Figure 2) is similar to that observed in polyproline in aqueous solution and to the native γ-zein *N*-terminal domain, indicating that **1**, **2**, and **3** also adopt PPII conformations. The CD spectra as a function of $CaCl_2$ addition and temperature also support a PPII type structure. These results indicate that the PPII conformation adopted by the peptides H-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-OH is not influenced by the nature of the amino acid **X** and, thus, the presence of histidine, or a charged amino acid, is not an absolute requirement for the native γ-zein *N*-terminal domain adopting a PPII type conformation.

An estimation of the amount of polyproline II conformation in (V**X**LPPP)$_8$ sequences by comparison of $[\theta]_M$ with that measured for polyproline is hindered by the possible effect of size. For this reason an oligomeric PP II of comparable size was prepared. This molecule was synthesized *via* stepwise coupling (48 synthetic cycles) of Boc-Pro-OH on a 4-methylbenzhydrylamine resin. The molecular weight distribution of the synthetic material after a single-step purification by gel filtration showed that the mean size of the H-(Pro)$_n$-OH oligomers was n = 42. The CD spectra of this oligomeric PPII at different temperatures are shown in Figure 5. By comparision with these data, the amount of PPII conformation in compounds **1**, **2**, and **3** at 5 °C can be estimated to be 66%, 66% and 67%, respectively.

The similarity of $[\theta]_M$ magnitudes in the CD spectra of **1**, **2**, and **3** (Figure 2) led us to conclude that the changes of the amino acid **X** (**X**: Ala, Glu, or Lys) had no

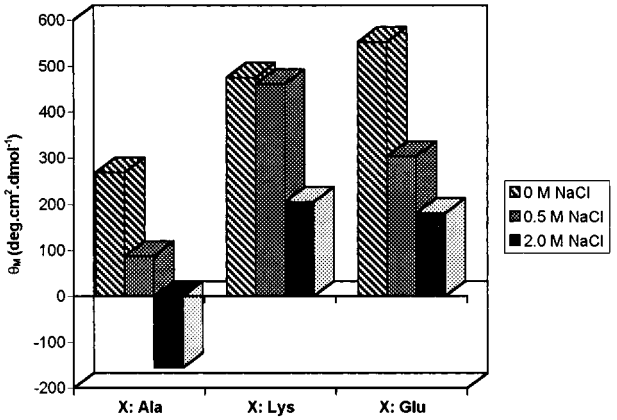

Figure 6. Molar ellipticity per residue at 228 nm as function of NaCl concentration.

effect on the conformation of H-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-OH. This means that the PPII conformation is adopted independently of the sign of charge on the charged amino acid (**X** = Glu, Lys) and of residue **X** being charged or uncharged (**X** = Ala). In this way, the results obtained with **X** = Ala indicate that the amphipathicity is not an essential requirement for H-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-OH adopting a PPII conformation.

It is well-known that addition of alkaline salts destabilizes the PPII conformation.[21] This is the behavior observed on addition of NaCl on a solution of **1** (Figure 6). PPII helices from compounds **2** and **3** are amphipathic at pH 7 due to the ionic character of the Glu and Lys side chains, respectively, at this pH. One could expect, therefore, that for these two compounds the ionic strength increase due to the addition of NaCl would promote intermolecular association *via* interaction between the hydrophobic faces of the helices. The results for **2** and **3** shown in Figure 6 are in agreement with this expectation—the addition of NaCl still destabilizes the PPII conformation but to a lesser extent than that found in the case of **1**.

The effect of the pH on the conformation of **1**, **2**, and **3** has also been examined (Figure 7). At pH 3 the CD spectra of the peptides with **X** = alanine and **X** = lysine show an increase of intensities in λ_{max} with respect to pH 7 (Figures 7a and 7c). This tendency to increase the PPII content at acid pH is in accordance with previously reported data.[19,22,23] This effect may reflect the fact that solvation of proline carbonyl groups is optimal in a PPII conformation, and this solvation requirement is stronger at acidic pH, when carbonyl groups can start to become protonated.[19,22−25] When **X** is glutamic acid (Figure 7b), the magnitude of the λ_{min} and λ_{max} values decrease as the pH is lowered to pH 3. This anomalous behavior of H-(Val-Glu-Leu-Pro-Pro-Pro)$_8$-OH is probably due to protonation of the carboxyl groups in the side-chain of glutamic acid residues at pH 3, and side chain−side chain and/or side chain−backbone interactions, *via* hydrogen bonding, may then become more important and affect the conformational equilibrium.

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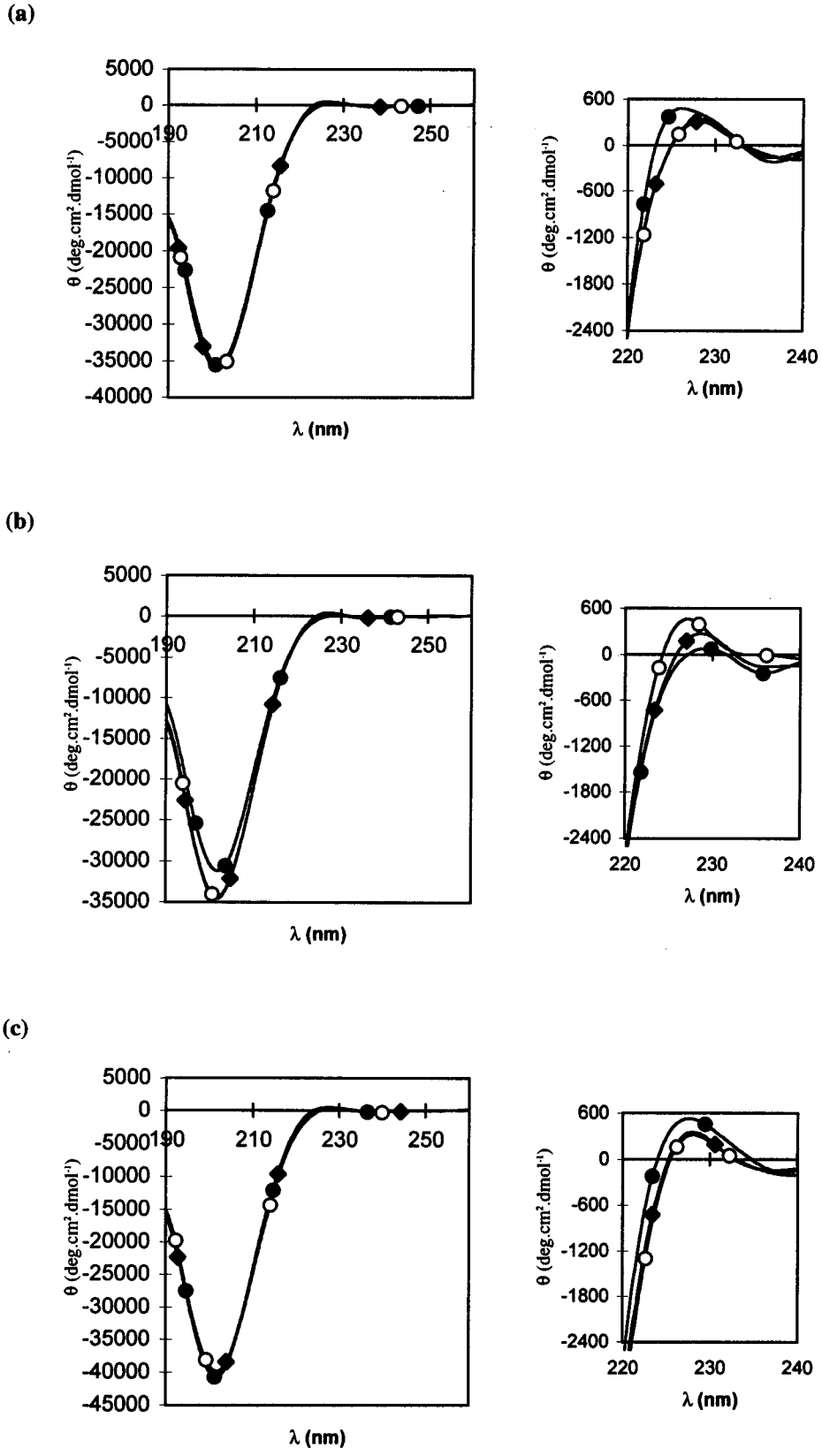

Figure 7. CD spectra of H-(Val-Ala-Leu-Pro-Pro-Pro)$_8$-OH (a), H-(Val-Glu-Leu-Pro-Pro-Pro)$_8$-OH (b), and H-(Val-Lys-Leu-Pro-Pro-Pro)$_8$-OH (c) in 1 mM sodium citrate/1 mM sodium borate/1 mM sodium phosphate buffer, 15 mM NaCl at 5 °C: (●) pH 3; (◆) pH 7, and (○) pH 10.5. On the right, details of the 228 nm regions.

In summary, the results obtained by CD spectroscopy provide clear evidence that the peptides H-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-OH with **X** = Ala, Glu, or Lys adopt PPII type conformations. This is supported by $CaCl_2$ addition and temperature dependency experiments. The shape of the CD spectra is the same as that of the native γ-zein *N*-terminal domain H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH, indicating that histidine is not an essential requirement for the γ-zein to adopt the PPII conformation.

The similarity of the CD patterns and $[\theta]_M$ magnitudes

for H-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-OH with X = Ala, Glu, or Lys led us to conclude that the nature of the amino acid **X** does not affect the PPII character of these peptide sequences. The PPII content of the peptides is increased in acidic medium, except when **X** is glutamic acid. In H-(Val-Glu-Leu-Pro-Pro-Pro)$_8$-OH, the PPII content is lower when the carboxyl group of the glutamic acid side chain is in the carboxylic acid form. This is probably due to side chain−side chain and/or side chain−backbone interactions that destabilize the PPII helix.

Finally, the sequence V**X**LPPP appears to have a great PPII conformational inducing effect. This observation is very important both for the *de novo* design of compounds with PPII conformations and in the analyses of naturally occuring protein sequences.

Experimental Section

Protected amino acids were from NovaBiochem AG (Läufelfingen, Switzerland) and Advanced ChemTech (Maidenhead, England). Fmoc-Pro-Pro-OH·HCl was obtained according to the method of Carpino and Han.[26] DIPCDI and HOBt were from Fluka Chemika (Buchs, Switzerland) and HATU and HOAt reagents were from PerSeptive Biosystems (Massachusetts). The handles 4-(4-(hydroxymethyl)-3-methoxyphenoxy)butyric acid and 3-(4-(hydroxymethyl)phenoxy)-propionic acid were from NovaBiochem AG. DCM, TFA, and DMF were synthesis grade. DMF was bubbled with nitrogen and kept stored over activated 4 Å molecular sieves. 4-Methylbenzhydrylamine resin and aminomethylated polystyrene resin were from NovaBiochem AG. These supports were thoroughly washed as follows: 5 × 30 s CH_2Cl_2; 1 × 1 min TFA−CH_2Cl_2 (4:6); 1 × 10 min TFA−CH_2Cl_2 (4:6), 5 × 30 s CH_2Cl_2; 3 × 2 min DIEA−CH_2Cl_2 (1:19); 5 × 30 s CH_2Cl_2. Peptide syntheses were performed manually in a polypropylene syringe fitted with a polyethylene disc.

Peptides and peptide-resins were hydrolyzed with 6 N HCl and 12 N HCl/propionic acid (1:1) at 110 °C for 24 and 48 h, respectively. Amino acid analyses were carried out on a ion-exchange autoanalyzer. Analytical HPLC was performed on a system comprised of two solvent delivery pumps, an automatic injector, and a variable wavelength detector, using a reversed-phase C_{18} column Nucleosil (25 × 0.4 cm, 10 μm). Mass spectra were recorded on a VG Quattro quadrupole instrument. 1,4-Dithiothreitol−1,4-dithioerythreitol (3:1) was used as matrix for FAB spectra. ESMS spectra were recorded in ion-spray mode using water−acetonitrile (1:1) with 1% formic acid as solvent and nitrogen as nebulization gas.

H-(Pro)$_n$-OH (with mean size of $n = 42$) was synthesized on an Applied Biosystems 430A automated peptide synthetizer using the acid-labile Boc group for the temporary protection of the α-amino function. 4-Methylbenzhydrylamine resin (0.1 mmol) and DCC for the couplings (10 equiv each amino acid and DCC, 48 synthetic cycles) were used. The cleavage of the peptide-resin was carried out with anhydrous hydrogen fluoride (HF) for 1 h at 0 °C in the presence of 10% anisole, using a Teflon-Kel F cleavage apparatus. After HF was removed, the resulting peptide/resin mixture was washed with diethyl ether, and the synthetic material was solubilized in AcOH 10% and lyophilized. The crude was purified by chromatography on Sephadex G-25 column using 0.1 N AcOH as eluent. The peptide fractions were combined, and the solvent was removed by lyophilization. The molecular weight distribution of the oligomeric poliproline was determined by ES mass spectrometry (see supporting information).

Peptide Synthesis. (a) Synthesis and Cleavage of Protected Segments Fmoc-Val-X-Leu-Pro-Pro-Pro-OH. For each peptide-resin, two residues of Phe were incorporated on a 4-methylbenzhydrylamine resin (1.0 g, 0.57 mmol/g) using the following synthetic program: (program I) (1) CH_2Cl_2, 4 × 1 min; (2) TFA−CH_2Cl_2 (4:6), 1 × 1 min + 1 × 20 min; (3) CH_2Cl_2, 4 × 1 min; (4) DIEA−CH_2Cl_2 (1:19), 3 × 2 min; (5) CH_2Cl_2; (6) Boc-Phe-OH (0.75 g, 2.8 mmol, 5 equiv) in CH_2Cl_2, after 1 min add equivalent amount of DIPCDI, shake 45 min; (7) CH_2Cl_2, 5 × 1 min; (8) DMF, 4 × 1 min.

After deprotection of Boc-Phe$_2$-resin and neutralization (steps 1−5 of synthetic program I), the Phe$_2$-resin was allowed to stand overnight with the HMPB handle (0.21 g, 0.87 mmol), HOBt (0.12 g, 0.87 mmol), and DIPCDI (0.13 mL, 0.87 mmol) in DMF. After the washings with DMF and CH_2Cl_2, the ninhydrin test was negative. The first Pro residue was coupled as Fmoc-Pro-OH (0.8 g, 2.3 mmol, 4 equiv) using DIPCDI (0.35 mL, 2.3 mmol) and DMAP (28 mg, 0.23 mmol) in DMF (double coupling of 1 h each). The amino acid analyses gave a >99% of incorporation for all resins. At the end of the coupling, an acetylation was carried out in order to cap possible remaining hydroxyl groups as follow: acetic anhydride (0.54 mL, 5.7 mmol) and DMAP (69 mg, 0.57 mmol) in CH_2Cl_2 (2 × 10 min). The rest of amino acids were incorporated according the synthetic following program: (program II) (1) CH_2Cl_2 (4 × 1 min); (2) DMF (4 × 1 min); (3) piperidine−DMF (1:4) (1 × 1 min + 3 × 3 min); (4) DMF (4 × 1 min); (5) CH_2Cl_2 (4 × 1 min); (6) Fmoc-amino acid (2.3 mmol, 4 equiv) in DMF, after 1 min add equivalent amount of HOBt and DIPCDI in DMF and the resin was allowed to stand 60 min at rt with occasional stirring; (7) DMF (4 × 1 min); (8) CH_2Cl_2 (4 × 1 min). This program was used for all amino acids, except for the second and third amino acids that were incorporated in the form a dipeptide as follows: (1) CH_2Cl_2 (4 × 1 min); (2) DMF (4 × 1 min); (3) Fmoc-Pro-Pro-OH (0.5 g, 1.2 mmol, 2 equiv), HOBt (80 mg, 0.6 mmol, 1 equiv) and DIPCDI (0.17 mL, 1.2 mmol, 2 equiv) in DMF (1 × 2 h); (4) DMF (4 × 1 min); (5) CH_2Cl_2 (4 × 1 min). The amino acids **X** used in these synthesis were incorporated as Fmoc-Ala-OH, Fmoc-Glu(OtBu)-OH, and Fmoc-Lys(Boc)-OH. The ninhydrin or chloranil tests were used to monitoring the couplings. At the end of each coupling, an acetylation was carried out with acetic anhydride (10 equiv) in pyridine−CH_2Cl_2 (1:19) (1 × 15 min). The amino acid analyses of the peptide-resins after *N*-terminal deprotection and hydrolysis gave the correct amino acid composition. At the end of the synthesis a portion of each Fmoc-Val-**X**-Leu-Pro-Pro-Pro-resin (130 mg) was washed with CH_2Cl_2 (5 × 1 min) and the cleavage of the peptide from the resin was carried out by alternating washes of 1% TFA in CH_2Cl_2 and CH_2Cl_2 for 20 s (2 mL of solution each time, total volume of 30 mL). This operation was repeated three times. The washes were collected in 30 mL of water (each fraction). The organic solvent of the fractions were evaporated in vacuum, and the protected peptide, precipitaded in the water, was filtered and dried in vacuum over P_2O_5. The cleavage yields were >96% as judged by amino acid analysis. The peptides were about 98% pure by analytical HPLC (Figure 1). **Peptide Characterization: (a) Fmoc-Val-Ala-Leu-Pro-Pro-Pro-OH:** amino acid analysis of the peptide-resin after *N*-terminal deprotection and hydrolysis, $Phe_{2.0}Val_{0.95}Ala_{0.92}Leu_{0.95}Pro_{2.72}$. HPLC: t_R 18.2 min (Nucleosil C_{18} column, HPLC profile is shown in Figure 1a). FABMS [M + Na]: calcd, 838; found, 838. **(b) Fmoc-Val-Glu(OtBu)-Leu-Pro-Pro-Pro-OH:** amino acid analysis of the peptide-resin after *N*-terminal deprotection and hydrolysis, $Phe_{2.0}Val_{1.06}Glu_{1.07}Leu_{1.07}Pro_{3.06}$. HPLC: t_R 20.5 min (Nucleosil C_{18} column, HPLC profile is shown in Figure 1b). FABMS [M + Na] calcd, 952; found, 952. **(c) Fmoc-Val-Lys(Boc)-Leu-Pro-Pro-Pro-OH:** amino acid analysis of the peptide-resin after *N*-terminal deprotection and hydrolysis, $Phe_{2.0}Val_{0.94}Lys_{0.8}Leu_{0.94}Pro_{2.70}$. HPLC: t_R 20.2 min (Nucleosil C_{18} column, HPLC profile is shown in Figure 1c). FABMS [M + Na]: calcd 995; found, 995.

(b) General Procedure for the Preparation of H-(Val-X-Leu-Pro-Pro-Pro)$_8$-OH. Each Fmoc-Val-**X**-Leu-Pro-Pro-Pro-resin **II** used for the segments coupling was prepared on a aminomethylated polystyrene resin (0.3 g, 0.88 mmol/g) as follows. Three residues of Phe were incorporated on the resin as internal reference, using synthetic program I. After deprotection of the Boc group, Phe$_3$-resin was allowed to stand overnight with 2,4,5-trichlorophenyl 3′-(4″-(hydroxymethyl)-phenoxy)propionate (0.15 g, 0.4 mmol, 1.5 equiv) and HOBt (54 mg, 0.4 mmol, 1.5 equiv) in DMF and then washed with

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DMF and CH_2Cl_2. The ninhydrin tests for all resins after this incorporation were negative. Fmoc amino acids were incorporated as described above in the synthesis of the protected segments. After the amino acid couplings, all ninhydrin or chloranyl tests were negative. Amino acid analyses of the Fmoc-Val-**X**-Leu-Pro-Pro-Pro-resins gave the correct values. The segment couplings were carried out as follows: Each Fmoc-Val-**X**-Leu-Pro-Pro-Pro-resin II (8 mg, 0.37 mmol/g) was preswollen with CH_2Cl_2 (3 × 10 min) and DMF (3 × 10 min). Then, the Fmoc group was removed by following synthetic program II (steps 1−5). Solutions of the respective protected segment (12 μmol) in DMF, HATU (24 μL of a 0.53 M solution in DMF), HOAt (23 μL of a 0.96 M solution in DMF), and DIEA (11 μL of DIEA−DMF (1:4, v/v)) were added on the peptide-resin, and the mixture was allowed to stand for 1 h at room temperature with occasional agitation. After this time, the resin was washed with DMF and CH_2Cl_2, and an acetylation (20 equiv of acetic anhydride and 20 equiv of DIEA in DMF, 1 × 10 min) was carried out to cap unreacted amino groups. This synthetic procedure was repeated until the desired Fmoc-(Val-**X**-Leu-Pro-Pro-Pro)$_8$-resins were obtained. Prior to cleavage of the peptide-resins, the Fmoc group was eliminated (steps 1−5, synthetic program II). Then, a portion of each peptide-resin (8 mg) was suspended in TFA−CH_2Cl_2−anisole (47:47:6) (2 mL). After 1.5 h at 25 °C each peptide-resin was filtered and washed with TFA−CH_2Cl_2 (1:1) (2 × 1 min) and CH_2Cl_2 (4 × 1 min). The combined filtrates and washings were evaporated to dryness, chased with ether (3 × 30 mL), then dissolved in water, and lyophilized. The cleavage yields were all >97% by amino acid analysis. **Peptide Characterization. (a) H-(Val-Ala-Leu-Pro-Pro-Pro)$_8$-OH.** HPLC: t_R 17.9 min (Nucleosil C_{18} column, elution conditions, A, H_2O−0.045% TFA; B, CH_3CN−0.036% TFA, linear gradient from 32% to 42% B in 20 min; flow rate, 1 mL/min; λ = 220 nm). ESMS: [M] calcd 4616, found 4616; [M + Na − H] calcd 4638, found 4639; [M + 2Na − 2H] calcd 4660; found 4661; [M + 3Na − 3H] calcd 4682, found 4683. **(b) H-(Val-Glu-Leu-Pro-Pro-Pro)$_8$-OH.** HPLC: t_R 14.5 min (Nucleosil C_{18} column, elution conditions, A, H_2O−0.045% TFA; B, CH_3CN−0.036% TFA, linear gradient from 32% to 42% B in 20 min; flow rate, 1 mL/min; λ = 220 nm). ESMS: [M + 3Na − 3H] calcd 5146, found 5146; [M + 4Na − 4H] calcd 5168, found 5169; [M + 5Na − 5H] calcd, 5190; found 5191; [M + 6Na − 6H] calcd 5212, found 5211. **(c) H-(Val-Lys-Leu-Pro-Pro-Pro)$_8$-OH.** HPLC: t_R 14.1 min (Nucleosil C_{18} column, elution conditions, A, H_2O−0.045% TFA; B, CH_3CN−0.036% TFA, linear gradient from 26% to 35% B in 20 min; flow rate, 1 mL/min; λ = 220 nm). ESMS: [M] calcd 5073; found 5073. **Circular Dichroism Spectroscopy.** CD measurements were carried out on a Jasco J700 spectropolarimeter at 0.2 nm intervals over the wavelength range from 190 to 260 nm, a spectral bandwidth of 1 nm and a time constant of 4 s (scan speed 10 nm/min). The CD spectra represent an average of three accumulations and were corrected by subtracting the buffer base line. CD spectra were taken routinely at peptide concentration of 68 μM (3.26 mM per residue) using a 0.02 cm pathlength quartz cuvette at 5 °C. The samples were prepared by dissolving lyophilized peptide previously quantified by amino acid analysis with a 1 mM sodium citrate/1 mM sodium borate/1 mM sodium phosphate buffer,[27] 15 mM NaCl at pH 7. For the peptide pH dependence studies, the pH of the buffer was adjusted by the addition of HCl and NaOH. Data are presented in molar ellipticities per residue ($[\theta]_M$, deg·cm^2·dmol^{-1}).

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Supporting Information Available: FABMS of the peptides Fmoc-Val-X-Leu-Pro-Pro-Pro-OH, ESMS of **1**, **2**, **3**, and the oligomer H-(Pro)$_n$-OH (mean size n = 42), CD spectra of **2** and **3** in the presence of $CaCl_2$, and CD spectra of **1**, **2**, and **3** in the presence of NaCl (12 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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