Convergent Solid Phase Peptide Synthesis: An Efficient Approach to the Synthesis of Highly Repetitive Protein Domains[†,‖]

Ionara Dalcol,[‡] Francesc Rabanal,[‡] Maria-Dolors Ludevid,[§] Fernando Albericio,[‡] and Ernest Giralt*[,‡]

Departament de Química Orgànica, Universitat de Barcelona, Martí i Franquès 1, 08028-Barcelona, Spain and Departament de Genètica Molecular, CID-CSIC, Jordi Girona 18, 08034-Barcelona, Spain

Received July 5, 1995[⊗]

Convergent solid phase peptide synthesis is a straightforward approach to the synthesis of protein domains with repetitive structures. The peptide H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH corresponding to the *N*-terminal domain of γ-zein has been synthesized using the protected segment Fmoc-(Val-His(Trt)-Leu-Pro-Pro-Pro)-OH as synthetic precursor. Solid phase repetitive coupling of the segment was achieved effectively using a synthetic protocol centered on the use of the new 7-aza-1-hydroxybenzotriazole-based reagents HATU and HOAt. The protected peptide segment Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH was synthesized on a solid-phase using a combination of the base-labile Fmoc group for the α-amino protection, the acid-labile trityl group for the protection of the side-chain of histidine, and the highly acid-labile 4-(4-(hydroxymethyl)-3-methoxyphenoxy)butyric acid (HMPB) as handle, which allows the cleavage of the protected segment from the resin by treatment with 1% TFA in CH_2Cl_2 with complete retention of the amino acid side-chain protecting groups. This approach also allows the same peptide sequence at different levels of protection to be obtained by varying the cleavage program used and demonstrates that the versatile HMPB handle is completely compatible with the use of the trityl group for protection of the side-chain of histidine. The use of a small quantity of pyridine in the purification of this protected peptide by RPMPLC does not affect the Fmoc group and avoids premature imidazole detritylation. Finally, the general synthetic strategy described in this paper avoids the severe problems previously reported on the synthesis of these highly repetitive structures as demonstrated by a careful analysis of the target compounds by cation-exchange HPLC and electrospray MS.

Proteins with repetitive structures are abundant in nature, especially in the plant kingdom. Within this group of proteins those having proline-rich domains are especially important, and several of their structural and functional properties have been described.[1] Several laboratories, including our own, have recently reported on the conformational analysis of some of these molecules, which have a characteristic tendency to form left-handed helical structures with a pitch of three amino acid residues per turn.[2]

A prerequisite for the structural analysis of these molecules is that efficient methods for their synthesis are available and, although stepwise solid phase peptide synthesis is a possible synthetic approach, single amino acid deletion peptides would be difficult to separate from the desired target peptide since the absence of a single residue would have very little effect on the chromatographic or electrophoretic properties of the molecule. For techniques that demand a high degree of purity, such as X-ray diffraction, or high-field NMR analysis, the separation of such impurities poses a difficult separation problem. Convergent solid-phase peptide synthesis (CSPPS), on the other hand, lends itself well to the synthesis of this type of molecule, since it is only necessary to synthesize and purify one of the protected repetitive units which, by successive couplings on a solid support, then allows the different oligomers to be synthesized.[3] The purification of molecules synthesized by the CSPPS approach is facilitated because segment couplings that do not go to completion give rise to peptides that differ from the target molecule by one complete segment and therefore have appreciably different chromatographic and electrophoretic properties.

The *N*-terminal domain of the γ-zein protein, one of the most abundant in maize, is formed by the repetitive proline-rich sequence:[4,5]

Val-His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-Val-His-Leu-Pro-Pro-Pro-

[†] Abbreviations: Bop-Cl, Bis(2-oxo-3-oxazolidinyl)phosphinic chloride; Bpoc, 2-(*p*-biphenylyl)propyl(2)oxycarbonyl; CSPPS, convergent solid-phase peptide synthesis; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DIPCDI, *N,N'*-diisopropylcarbodiimide; EDT, ethanedithiol; ESMS, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-(fluorenylmethyloxycarbonyl); Fmoc-Cl, 9-fluorenylmethyl chloroformate; HAPyU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-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; NMM, *N*-methylmorpholine; RPHPLC, reversed-phase high performance liquid chromatography; RPMPLC, reversed-phase medium pressure liquid chromatography; Trt, trityl.

[‡] Departament de Química Orgànica, Universitat de Barcelona.

[§] Departament de Genètica Molecular, CID-CSIC.

[‖] This paper is dedicated to Prof. Felix Serratosa, *in memoriam*.

[⊗] Abstract published in *Advance ACS Abstracts*, November 1, 1995.

(1) Williamson, M. P. *Biochem. J.* **1994**, *297*, 249.

(2) Rabanal, F.; Ludevid, M. D.; Pons, M.; Giralt, E. *Biopolymers* **1993**, *33*, 1019–1028.

(3) (a) Marshall, G. R.; Merrifield, R. B. *Biochemistry* **1965**, *4*, 2394. (b) Omenn, G. S.; Anfinsen, C. B. *J. Am. Chem. Soc.* **1968**, *90*, 6571–6572. For recent reviews on CSPPS see: (c) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron* **1993**, *49*, 11065–11133. (d) Benz, H. *Synthesis* **1994**, 337.

0022-3263/95/1960-7575$09.00/0

Recently, Geli et al.[6] have shown that this sequence is the key element involved in the γ-zein protein targeting the endoplasmic reticulum. The mechanism of such targeting is unknown but structural, immunocytochemical, and binding studies could be carried out if efficient synthetic methods were available for the preparation of repetitive sequences. For such studies it would be of interest to prepare oligomers of the type H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH for values of $n \leq 8$, in order to try to establish whether or not there is a minimum requirement with regard to molecular size for the some of the observed effects.

In our previous attempts at the synthesis of the *N*-terminal domain of the γ-zein protein by a CSPPS approach, we used a strategy based upon the use of the Fmoc group for temporary N^{α}-amino group protection and upon the coupling of peptide segments in which His was unprotected at the imidazole function.[7] Although this strategy allowed small amounts of some of the desired oligomers to be obtained, laborious purification procedures were required since analysis of the crude products after the synthesis of a given oligomer of the sequence H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH showed the presence of two types of impurity: (1) those corresponding to deletion oligomers of the type $n - 1$, $n - 2$, etc.; (2) those corresponding to addition oligomers of the type $n + 1$, $n + 2$, etc.

Impurities of type 1 suggest that the *N*-terminal domain of the γ-zein protein has characteristics similar to those of a "difficult sequence"[8] but within the context of a convergent strategy and that conventional coupling methods such as DCC/HOBt give rise to yields which are far from quantitative. The presence of type 2 impurities suggests that Fmoc group deprotection occurs in parallel with segment coupling during the long and repetitive coupling reactions. This could occur in the peptide of length n that is already anchored to the resin or in the protected monomer that has not yet been coupled to the $n - 1$ sequence. An alternative possibility is that the free imidazoles of the growing peptide chain might become *N*-acylated by the monomers and that these might subsequently undergo transfer to the N^{α}-amino function upon removal of its Fmoc protecting group.

In the present communication we describe a convergent synthesis of the *N*-terminal domain of the γ-zein protein in which the formation of the various side-products described above are avoided. There are two main improvements that have been introduced into the synthetic strategy for the synthesis of these oligomers. Firstly, the fine tuning of the protecting group of His and of the handle used to anchor the peptide to the resin has made it possible to work with the side-chains of His protected throughout the synthesis. Secondly, for the first time, the use of the newer coupling reagents based on azabenzotriazole[9] has been investigated in CSPPS. The use of these reagents allows higher coupling yields to be achieved in order to reduce type 1 impurities, and the shorter reaction times necessary for coupling reduce the amount of Fmoc-group deprotection which in turn reduces the amount of type 2 impurities that might be formed.

Results and Discussion

Synthesis and Purification of the Protected Segment. The protected segment Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH was synthesized on a solid phase using a combination of the base-labile Fmoc group for the α-amino protection, the acid-labile trityl group for the protection of the side-chain of histidine, and the highly acid-labile 4-(4-(hydroxymethyl)-3-methoxyphenoxy)butyric acid (HMPB) handle developed by Riniker *et al.*[10] which allows the cleavage of protected segment from the resin by treatment with 1% TFA in CH_2Cl_2 with complete retention of the amino acid side-chain protecting groups. The stepwise synthesis of the protected segment proceeded smoothly starting with a 4-methylbenzhydrylamine resin by incorporation of three residues of phenylalanine as internal reference amino acid (IRAA).[11] The HMPB handle was coupled with DIPCDI in the presence of HOBt. The first amino acid, Fmoc-Pro-OH was incorporated with DIPCDI and catalytic amounts of DMAP, double coupling for 1 h each. As described in a previous paper from this laboratory,[7] the incorporation of the third amino acid in the synthesis of this peptide is problematic because the sequence Pro-Pro is unusually prone to give diketopiperazines (DKPs).[12,13] Thus, to avoid the intramolecular cyclization in this synthesis the second and third amino acids were incorporated as Fmoc-Pro-Pro-OH with HOBt and DIPCDI in DMF for 2 h at 25 °C. The possible loss of the chirality in the dipeptide *C*-terminal amino acid after this coupling was studied by HPLC. The results showed that less than 1% epimerization took place during the incorporation of dipeptide. The remaining amino acids were assembled by standard solid-phase procedures as described in the Experimental Section. The ninhydrin[14] or chloranil[15] tests were used to monitor the progress of the couplings, and although in all cases only a single coupling was necessary, acetylation reactions were carried out after the incorporation of each amino acid to ensure the absence of deletion peptides. The amino acid analysis of the peptide-resin after the synthesis gave satisfactory values.

An important feature of this synthetic strategy is the possibility of obtaining the same peptide sequence at different levels of protection by varying the cleavage program used, as shown in Figure 1. The cleavage of the protected peptide **1** from the resin was carried out by acidolysis, treating the peptide-resin with 1% TFA in CH_2Cl_2 alternated with neat CH_2Cl_2 for 20 s each. The cleavage yield was 91%.

Peptide **2**, protected only at the *N*-terminal amino group, was prepared by acidolytic cleavage with TFA–CH_2Cl_2 (1:1) for 90 min at 25 °C. To obtain peptide **3** with the side-chain of histidine protected with the trityl group and the *N*-terminal amino group unprotected, cleavage

(4) Prat, S.; Cortadas, J.; Puigdomènech, P.; Palau, J. *Nucleic Acids Res.* **1985**, *13*, 1493–1504.
(5) Boronat, A.; Martínez, M. C.; Reina, M.; Puigdomenèch, P.; Palau, J. *Plant Sci.* **1986**, *47*, 95–102.
(6) Geli, M. I.; Torrent, M.; Ludevid, D. *Plant Cell* **1994**, *6*, 1911–1922.
(7) Celma, C.; Albericio, F.; Pedroso, E.; Giralt, E. *Peptide Res.* **1992**, *5*, 62–71.
(8) Milton, R. C. L.; Milton, S. C. F.; Adams, P. A. *J. Am. Chem. Soc.* **1990**, *112*, 6039.
(9) Carpino, L. A.; El-Faham, A.; Minor, C. A.; Albericio, F. *J. Chem. Soc., Chem. Commun.* **1994**, 201.
(10) (a) Flörsheimer, A.; Riniker, B. In *Peptides 1990*; Giralt, E., Andreu, D., Eds., ESCOM Science Publishers B. V.; Barcelona, Spain, 1990; pp 131–133. (b) Riniker, B.; Flörsheimer, A.; Fretz, H.; Sieber, P.; Kamber, B. *Tetrahedron* **1993**, *49*, 9307–9320.
(11) Atherton, E.; Clive, D. L.; Sheppard, R. C, *J. Am. Chem. Soc.* **1975**, *97*, 6584–6585.
(12) Gisin, B. F.; Merrifield, R. B. *J. Am. Chem. Soc.* **1972**, *94*, 3102–3106.
(13) Pedroso, E.; Grandas, A.; de las Heras, X.; Giralt, E. *Tetrahedron Lett.* **1986**, *24*, 743–746.
(14) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.
(15) Christensen, T. *Acta Chem. Scand. B* **1979**, *33*, 763–766.

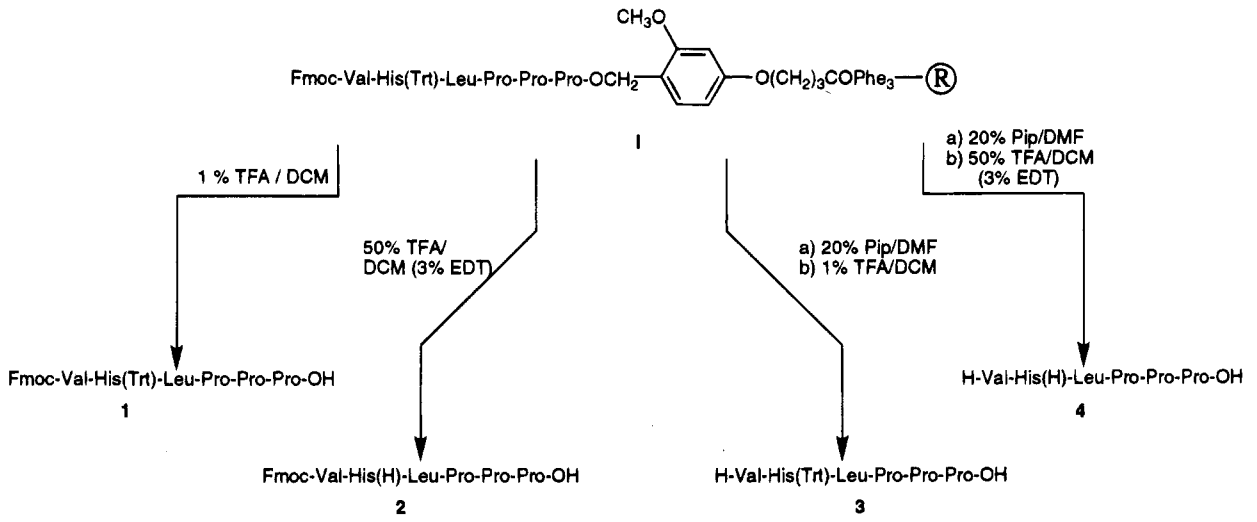

Figure 1. Cleavages of the peptide-resin I.

was carried out in two steps: (a) removal of the Fmoc group of the peptide-resin I by treatment with piperidine–DMF (1:4) and (b) cleavage with 1% TFA in CH_2Cl_2. The same peptide sequence without protecting groups, **4**, was obtained by acidolytic cleavage with TFA–CH_2Cl_2 (1:1) for 1.5 h at 25 °C after removal of the Fmoc-group with piperidine–DMF (1:4). The yields of these cleavages were >90% in all cases, as judged by amino acid analysis of the peptide-resins. Analytical HPLC of the crude peptides are shown in Figure 2. FAB mass spectrometric data for all these peptides are in accord with the calculated values.

One of the most serious problems associated with convergent solid-phase methodology is the low solubility often displayed by protected peptide segments. Fortunately, Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH (**1**) was very soluble in acetonitrile and it was possible to purify it by semipreparative reversed-phase MPLC without using DMF as solvent.[16] However, initial attempts at purification were not successful because of problems associated with the stability of the trityl group. When standard acetonitrile–water–TFA eluents were used (Figure 3a), a significant amount of peptide lacking the trityl group was observed after chromatography and lyophilization. Loss of the trityl group was observed even using acetonitrile–water without carboxylic acid modifier in the eluents (Figure 3c).

This undesired premature imidazole deprotection was solved by addition of pyridine (0.1%) to the eluents (Figure 3d). The presence of the Fmoc group on the protected segment allowed monitoring of the purification under these conditions by UV absorbance at 279 nm, where pyridine does not absorb strongly. Using these conditions, purification of this protected segment was straightforward and efficient (Figure 4). Purity of **1** was confirmed by amino acid analysis, analytical RPHPLC, and FABMS.

Peptide Synthesis by Segment Condensations. Figure 5 shows the general strategy for the synthesis of the H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH oligomers. The first step of this synthesis involved the preparation of the monomer-resin II. Aminomethylated polystyrene resin, incorporating three residues of phenylalanine as internal standard, was the solid support used for building this peptide-resin. The handle 3-(4-(hydroxymethyl)-

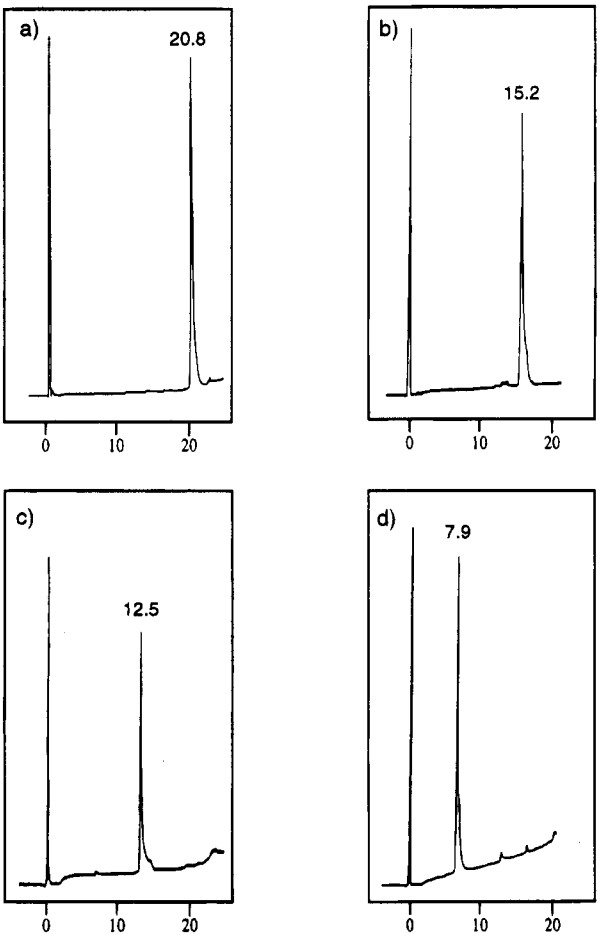

Figure 2. Analytical RP-HPLC on a C_{18} column of crude Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH (a); Fmoc-Val-His(H)-Leu-Pro-Pro-Pro-OH (b); H-Val-His(Trt)-Leu-Pro-Pro-Pro-OH (c), and H-Val-His(H)-Leu-Pro-Pro-Pro-OH (d). Elution conditions: A: H_2O–0.045% TFA; B: CH_3CN–0.036% TFA; linear gradient from 10% to 100% B in 25 min; flow rate, 1 mL/min; λ = 220 nm.

phenoxy)propionic acid was attached to the support as its 2,4,5-trichlorophenyl ester[17] in the presence of HOBt using DMF as solvent. The amino acids of the sequence were then incorporated stepwise using the same protocols

(16) Gairí, M.; Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Int. J. Pept. Protein Res.* **1995**, in press.

(17) Albericio, F.; Barany, G. *Int. J. Pep. Protein Res.* **1985**, *26*, 92–97.

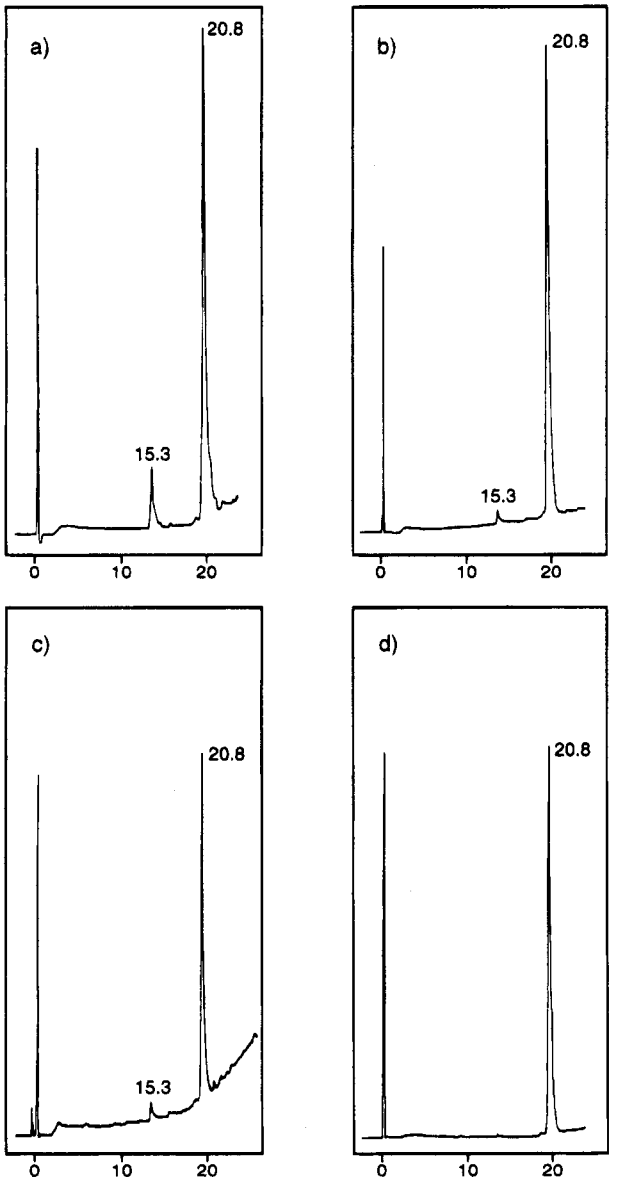

Figure 3. Analytical HPLC on reversed-phase C_{18} of the protected peptide segment Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH after purification. The peak at t_R 15.3 min corresponds to the detrityled peptide Fmoc-Val-His(H)-Leu-Pro-Pro-Pro-OH. Elution conditions as in Figure 2.

already described for the synthesis of the protected segment **1**. In order to avoid diketopiperazine formation the second and third amino acids were incorporated as the dipeptide Fmoc-Pro-Pro-OH. Amino acid analysis of the peptide-resin after synthesis gave the correct values.

Next, a study was carried out in order to find the best conditions to couple the protected peptide **1**. Coupling of Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH with H-Val-His(Trt)-Leu-Pro-Pro-Pro-resin was taken as a model. This coupling, using DIPCDI/HOBt (5:5 equiv) or Bop-Cl/HOBt/DIEA (5:5:10 equiv, 15 min preactivation) as coupling reagents, was not successful as judged by amino acid analysis of the peptide-resins and HPLC of the crude peptides, with yields of the 40 and 48%, respectively. After 72 h of reaction, incomplete incorporation was detected using both these coupling reagents. However, it was important to verify that the only products observed in the crude peptides by HPLC were the desired dimer and the unreacted monomer, and not the $n + 1$ oligomers formed in the previously reported synthesis of these compounds[7] as discussed in the introduction.[18,19] The

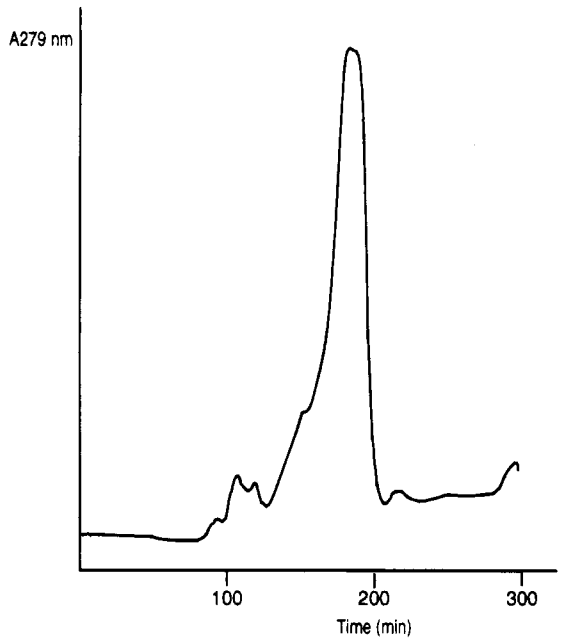

Figure 4. MPLC profile on reversed-phase C_8 of the protected peptide segment Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH. Linear gradient starting from CH_3CN-H_2O (35:65 with 0.1% pyridine) (600 mL) to CH_3CN (0.1% pyridine) (600 mL); flow rate 5 mL/min; $\lambda = 279$ nm.

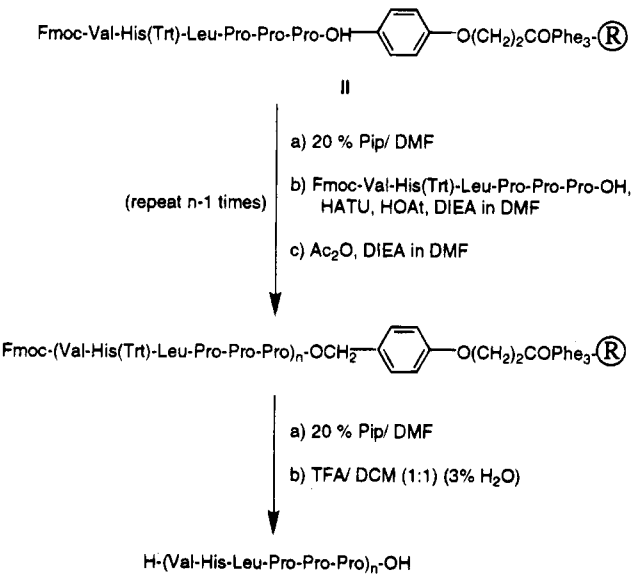

Figure 5. Convergent solid-phase peptide synthesis of H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH.

recently developed 7-aza-1-hydroxybenzotriazole-based reagents[9,20] HATU and HAPyU were tested for the coupling of the protected peptide. Using HATU/HOAt/DIEA and HAPyU/HOAt/DIEA coupling yields of over 80% were obtained after 4 h using a excess of 4 equiv of protected peptide and the coupling reagents. These results indicated that the azabenzotriazole-based coupling reagents are much more efficient for the coupling

(18) In the case of Bop-Cl, in spite of the fact that preactivation was used, formation of a phosphinamidate by reaction of unreacted Bop-Cl with the primary amine in the peptide resin cannot be excluded. This side product, in the subsequent cleavage step by treatment with $TFA-CH_2Cl_2-H_2O$, could be hydrolyzed to regenerate the starting monomer observed by HPLC.

(19) (a) Van der Auwera, C.; Van Damme, S.; Anteunis, M. J. O. *Int. J. Pept. Protein Res.* **1987**, *29*, 464–471. (b) Colucci, W. J.; Tung, R. D.; Petri, J. A.; Rich, D. H. *J. Org. Chem.* **1990**, *55*, 2895–2903.

(20) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397–4398.

of this protected peptide, and that the HATU/HOAt/DIEA combination of reagents offered the best conditions for coupling.

A progressive improvement of the synthesis conditions led us to verify that the segment couplings were completed in 15 min with a 1.5 equiv excess of the reagents. Convergent synthesis of the *N*-terminal domain of γ-zein was carried out on the peptide-resin II by repetitive coupling of the protected peptide Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH (**1**) (1.5 equiv) using HATU, HOAt and DIEA (1.5 equiv each), and DMF as solvent. Single couplings for 15 min at 25 °C were carried out until the $n = 8$ oligomer was obtained. After each segment coupling an acetylation with Ac_2O and DIEA was carried out to cap any unreacted amino group, allowing the deletion sequences to be differentiated more easily. The *N*-terminal Fmoc group was removed prior to each coupling by treatment with piperidine-DMF (1:4). Resin samples were removed after each coupling step, and the progress of the synthesis was monitored by amino acid analysis of these and by ion-exchange HPLC of the crude peptides after cleavage from the resin. The cleavage of the H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH sequence and the other oligomers from the resin was carried out by treatment with TFA−CH_2Cl_2−H_2O (48:49:3) for 90 min, after Fmoc group removal.

Owing to the difficult separation of the peptides corresponding to the possible deletion sequences of this synthesis, H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH and Ac-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH where $n \leq 7$, and the target peptide H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH, it was very difficult to verify the purity of the final product. Attempts to separate H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH and Ac-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH ($n = 2-8$) oligomers completely by reversed-phase HPLC and capillary electrophoresis were not satisfactory. The oligomers with $n > 4$ show very similar retention times by RPHPLC under a variety of conditions. Varying the TFA percentage in the standard eluents acetonitrile−water, the pH, the temperature, and the reversed-phase did not lead to appreciable improvement in separation. Differentiation of the various products was possible by cation-exchange HPLC analysis. The retention times of the $n = 5-7$ oligomers are indicated by arrows in the chromatogram shown in Figure 6. The crude octamer H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH obtained after acidolysis was also analyzed by ESMS; a molecular weight value (5145) was found in accordance with the calculated value (5144) (Figure 7). Combining the information from ion-exchange HPLC and mass-spectrometry the only impurities that could be detected in the crude peptide were traces of acetylpentamer (less than 4%) and traces of heptamer (less than 4%).

In summary, we have established an efficient protocol for the synthesis of the *N*-terminal proline-rich repetitive domain of the γ-zein using the convergent solid-phase approach and coupling reagents derived from HOAt. The synthetic strategy employed to obtain the protected peptide Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH and the same peptide sequence at different levels of protection simply by varying the cleavage program used demonstrate that the Riniker handle is very versatile and is completely compatible with the use of the trityl group for protection of the side chain of histidine. The use of a small quantity of pyridine to purify the protected peptide by RPMPLC does not affect the Fmoc group present in the peptide and avoids premature imidazole detritylation.

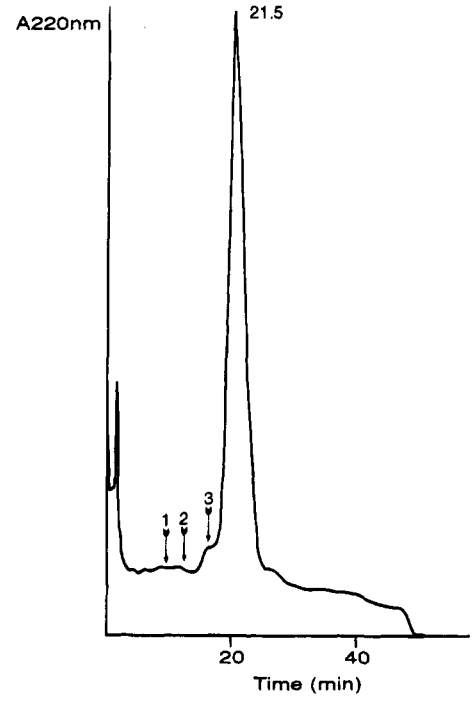

Figure 6. Cation-exchange HPLC of the crude peptide H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH on a Protein-Pak SP-8HR (5 × 100 mm) column. Arrows indicate the retention times of Ac-(Val-His-Leu-Pro-Pro-Pro)$_7$-OH (1), Ac-(Val-His-Leu-Pro-Pro-Pro)$_6$-OH (2), and Ac-(Val-His-Leu-Pro-Pro-Pro)$_5$-OH (3). Elution conditions: A: 20 mM sodium citrate buffer (pH 3); B: 20 mM sodium citrate buffer (pH 3) containing 0.5 M NaCl. Isocratic elution at 30% B for 10 min and from 30−40% B over 30 min and from 40−100% B over 10 min; flow rate, 0.5 mL/min; $\lambda = 227$ nm.

We believe that this purification method can be used to solve the problems found with the stability of the other trityl protected peptides.

Cation-exchange HPLC and mass spectrometry have been shown to be the best analytical methods for the separation and characterization of H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH and Ac-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH oligomers. From our results it is clear that the protection of the side chain of histidine throughout the synthesis together with the use of azabenzotriazole-based coupling reagents solved the problem of the $n - 1$, $n + 1$, and $n + 2$ oligomer formation. The purity of the target compounds was the same when long segment coupling times were used. Taken together our results indicate that *N*-acylation of nonprotected histidine rather than premature Fmoc cleavage was the origin of the $n + 1$ and $n + 2$ oligomer formation in the previous synthesis.

Finally, this synthesis demonstrates the suitability of the convergent solid-phase approach for the synthesis of peptides with repetitive sequences.

Experimental Section

Protected amino acids were from NovaBiochem AG (Läufelfingen, Switzerland) and Advanced ChemTech (Maidenhead, England). Fmoc-Pro-Pro-OH·HCl was obtained from the H-Pro-Pro-OH. HCl (Bachem Feinchemikalien AG, Bubendorf, Switzerland) and Fmoc-Cl in basic medium according to the method described by Carpino and Han.[21] The handles 4-(4-(hydroxymethyl)-3-methoxyphenoxy)butyric acid and 3-(4-(hydroxymethyl)phenoxy)propionic acid were from NovaBiochem AG. DIPCDI and HOBt were from Fluka Chemika (Buchs,

(21) Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404−3409.

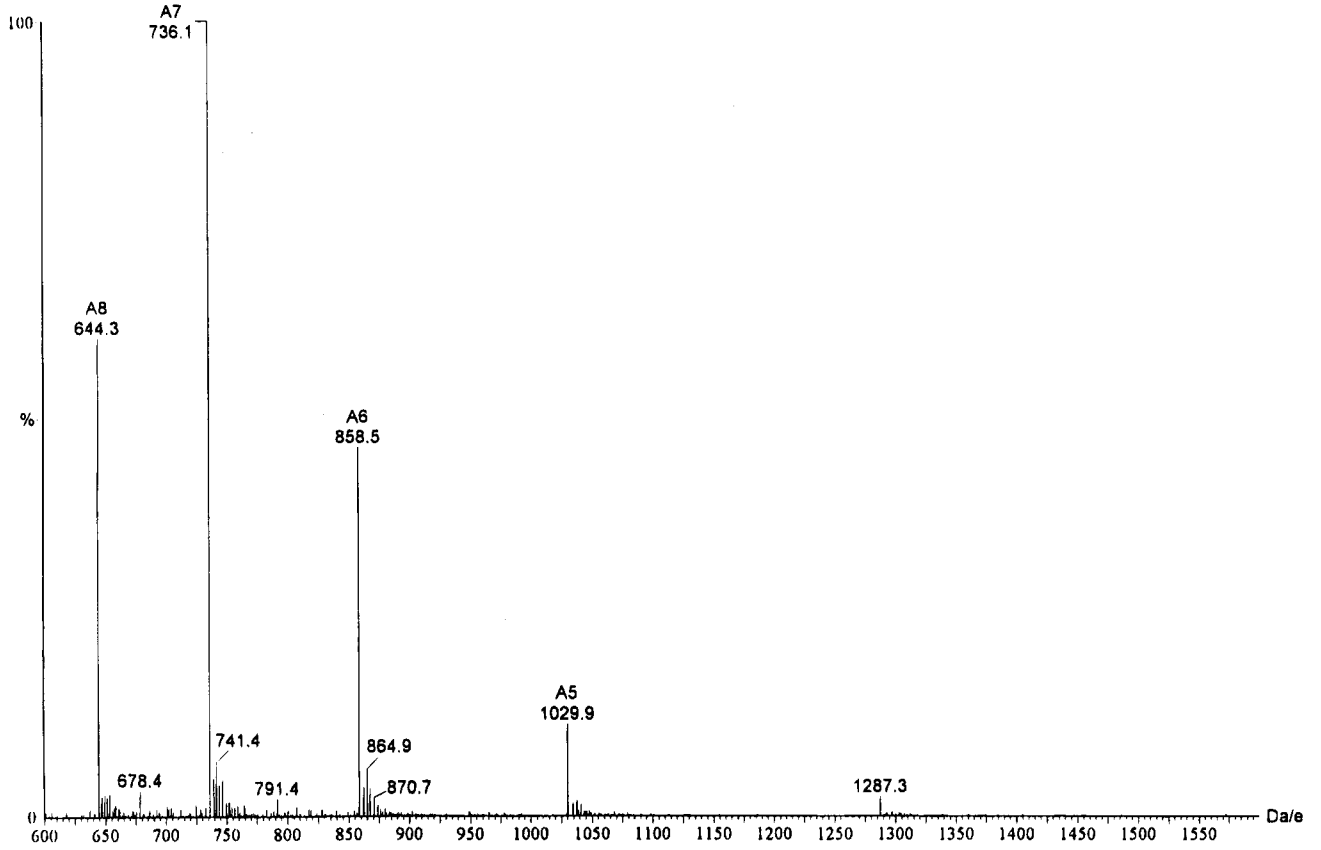

Figure 7. Positive ESMS spectrum of the crude peptide H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH in H_2O-CH_3CN (1:1) with 1% formic acid. Molecular weight calcd 5141.0 (monoisotopic mass), 5144.3 (average mass). Molecular weight found 5146.4 $[M + 8H]^{8+}$ (peak A8), 5145.7 $[M + 7H]^{7+}$ (peak A7), 5145.0 $[M + 6H]^{6+}$ (peak A6), and 5144.5 $[M + 5H]^{5+}$ (peak A5).

Switzerland), and the HBTU was supplied by Richelieu Biotechnologies (St. Hyacinthe, Canada). HATU, HAPyU, and HOAt reagents were from PerSeptive Biosystems (Framingham, MA). These reagents were used without further purification. Acetonitrile was HPLC grade; TFA and DCM were peptide synthesis grade. DMF was from Panreac (Barcelona, Spain) and was bubbled with nitrogen and kept stored over activated 4 Å molecular sieves. Other reagents were used without further purification. 4-Methylbenzhydrylamine resin and aminomethylated polystyrene resin were from NovaBiochem AG. These supports were thoroughly washed to remove any impurities as following: 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_2Cl2 (1:19) and 5 × 30 s CH_2Cl_2. Peptide synthesis were performed manually in a polypropylene syringe fitted with a polyethylene disc.

Peptide-resins were hydrolyzed at 110 °C for 48 h with 12 M HCl/propionic acid (1:1), and peptides were hydrolyzed at 110 °C for 24 h using 6 M aqueous HCl solution. Amino acid analyses were performed on a ion-exchange autoanalyzser. Analytical HPLC was performed on a system comprising two solvent delivery pumps, automatic injector, and variable wavelength detector, using a reversed-phase C_{18} column Nucleosil (25 × 0.4 cm, 10 μm) or a cationic-exchange glass column Protein-Pak SP-8HR (5 × 100 mm). Reversed-phase MPLC was performed on a Lichroprep RP-8 (44 × 3.7 cm; 40–60 μm) using a LDC/Milton Roy pump, a variable wavelength detector and an automatic fraction collector. Mass spectra were recorded on a VG Quattro quadrupole instrument. 1,4-Dithiothreitol–1,4-Dithioerythritol (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.

Synthesis and Purification of Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH. Synthesis. The three residues of Phe were incorporated on a 4-methylbenzhydrylamine resin (1.5 g; 0.8 mmol/g) using the following synthesis 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, 5 × 1 min; (6) Boc-Phe-OH (1.6 g; 6.0 mmol, 5 equiv) in CH_2Cl_2, after 1 min add equivalent amount of DIC, shake 45 min, (7) CH_2Cl_2, 5 × 1 min; (8) DMF, 4 × 1 min.

The Phe$_3$-resin was then allowed to stand overnight with the HMPB handle (0.4 g; 1.8 mmol), HOBt (0.25 g; 1.8 mmol), and DIPCDI (0.3 mL; 1.8 mmol) in DMF. After the usual washings with DMF and CH_2Cl_2, the ninhydrin test was negative. The first Pro residue was incorporated as Fmoc-Pro-OH (2.0 g; 6.0 mmol), using DIPCDI (0.9 mL; 6.0 mmol) and DMAP (74 mg; 0.6 mmol) in DMF. After double coupling of 1 h each the resin was washed with DMF (4 × 1 min) and CH_2Cl_2 (4 × 1 min), and the amino acid analysis gave a >98% yield of incorporation. The possible remaining hydroxyl groups on the resin were blocked by acetylation with acetic anhydride (1.2 mL; 12.0 mmol) and DMAP (0.15 g; 1.2 mmol) in CH_2Cl_2 (1 × 10 min).

The rest of the amino acids were incorporated following the program sequence:

Program II (Fmoc group removal): (1) CH_2Cl_2 (4 × 1 min); (2) DMF (4 × 1 min); (3) piperidine–DMF (2:8) (1 × 1 min + 3 × 3 min); (4) DMF (4 × 1 min); (5) CH_2Cl_2 (4 × 1 min).

Program III (amino acid coupling, except for the second and third amino acids): (1) Fmoc amino acid (6.0 mmol; 5 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; (2) DMF (4 × 1 min); (3) CH_2Cl_2 (4 × 1 min). The second and third amino acids were incorporated in the form of a dipeptide by the following program: (1) CH_2Cl_2 (4 × 1 min); (2) DMF (4 × 1 min); (3) Fmoc-Pro-Pro-OH (1.0 g; 2.4 mmol), HOBt (0.16 g; 1.2 mmol), and DIPCDI (0.4 mL; 2.4 mmol) in DMF (1 × 2 h); (4) DMF (4 × 1 min); (5) CH_2Cl_2 (4 × 1 min).

Coupling efficiencies were monitored using the ninhydrin or chloranil tests. At the end of each coupling an acetylation was carried out in order to cap unreacted amino groups: (1)

CH_2Cl_2 (4 × 1 min); (2) Ac_2O (10 equiv) in pyridine–CH_2Cl_2 (1:19) (1 × 15 min); (3) CH_2Cl_2 (4 × 1 min). For all Fmoc-amino acids only one coupling was necessary. The amino acids analysis of the peptide-resin after N-terminal deprotection and hydrolysis gave: $Phe_{3.0}$(IRAA), $Pro_{2.88}$, $Val_{0.83}$; $Leu_{0.88}$, $His_{0.82}$.

Cleavage. (a) Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH. A 470 mg amount of the peptide-resin I was preswollen with washes of the CH_2Cl_2 (3 × 10 min), and the cleavage was carried out by alternating washes of 1% TFA in CH_2Cl_2 and CH_2Cl_2 for 20 s (2.5 mL of solution each time, total volume of 50 mL). This operation was repeated three times. The solvent of the combined cleavage portions was evaporated and the protected peptide Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH, precipitated in water, was filtered and dried in vacuum over P_2O_5 to give 132 mg (90% yield) of a white powder which was about 97% pure by analytical HPLC. t_R 20.8 min (Nucleosil C_{18} column, HPLC profile is shown in Figure 2a). FAB-MS [M + H] calcd 1123.6; found, 1123.7.

The same peptide sequence at different levels of protection was obtained by varying the treatment of the peptide-resin I using the following cleavage procedures:

(b) Fmoc-Val-His(H)-Leu-Pro-Pro-Pro-OH. A 50 mg amount of the peptide-resin I was preswollen in CH_2Cl_2 (3 × 10 min) and suspended in TFA–CH_2Cl_2 (1:1) (2 mL) containing 3% of EDT. After 1.5 h the cleaved peptide-resin was filtered and washed with TFA–CH_2Cl_2 (1:1) (2 mL) and CH_2Cl_2 (3 × 2 mL). The combined filtrates and washings were evaporated to dryness at 25 °C, chased with ether (3 × 40 mL), and then dissolved in water and lyophilized to give the crude peptide in >99% yield by amino acid analysis. t_R 15.2 min (Nucleosil C_{18} column, HPLC profile is shown in Figure 2b). FAB-MS [M + H] calcd 881.5; found, 881.9.

(c) H-Val-His(Trt)-Leu-Pro-Pro-Pro-OH. A 50 mg amount of the peptide-resin I, after washes with CH_2Cl_2 and DMF, was deprotected in the amino terminal group as described in the program II. The cleavage was carried out by washing for 20 s with 1% TFA in CH_2Cl_2 (2 mL of solution each time, total volume of solution 10 mL) alternating with CH_2Cl_2 washings. The solutions were filtered into methanol containing 200 μL of pyridine. This operation was repeated three times. The combined fractions were evaporated to dryness, chased with ether (3 × 30 mL), dissolved in water, and lyophilized to give the crude peptide in >99% of yield by amino acid analysis. t_R 12.5 min (Nucleosil C_{18} column, HPLC profile is shown in Figure 2c). FAB-MS [M + H] calcd 901.5; found, 901.8.

(d) H-Val-His(H)-Leu-Pro-Pro-Pro-OH. A 50 mg amount of the peptide-resin I were washed with CH_2Cl_2 and DMF, and deprotected with piperidine–CH_2Cl_2 (1:4) according to program II. Then, the peptide-resin was suspended in TFA–CH_2Cl_2 (1:1) (2 mL) containing 3% of EDT. After 1.5 h the cleavaged peptide-resin was treated by the proceeding used in item b described above. Yield by amino acid analysis was 98.5% of the crude peptide. t_R 7.9 min (Nucleosil C_{18} column, HPLC profile is shown in Figure 2d). FAB-MS [M + H] calcd 659.4; found, 659.6.

Purification of Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH. The material of the combined cleavages (300 mg of the crude peptide) was purified by RPMPLC in a Lichroprep RP-8 column using a gradient of 35% CH_3CN in H_2O (containing 0.1% pyridine) (600 mL) to 100% CH_3CN (0.1% pyridine) (600 mL) at a flow rate of 5 mL/min. The volume of each individual fraction collected was 10 mL. Monitoring was carried out by UV detector at 279 nm. The purity of the fractions was verified by analytical HPLC using a Nucleosil C_{18} column and a linear gradient of the 10% to 100% of B in A over 25 min [eluents: A, water (0.045% TFA) and B, acetonitrile (0.036% TFA)]. Under these conditions, the retention time of the peptide is 20.8 min. The fractions of the pure peptide were combined, the organic solvent was removed on a rotatory evaporator with a cool water-bath, and the remaining aqueous solution was lyophilized to provide 160 mg (0.14 mmol, 53% yield) of peptide which was >99% pure by analytical HPLC. Amino acid analysis: $Pro_{3.10}Val_{1.00}Leu_{1.15}His_{1.01}$.

General Procedure for the Preparation of H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH. Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-handle-Phe3-resin II. The Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-handle-Phe_3-resin II used for the coupling segments was prepared on a aminomethylated polystyrene resin (2.0 g; 0.6 mmol/g). Three residues of Phe were incorporated using synthesis program I. After deprotection of Boc-Phe_3-resin and neutralization (steps 1–5 of synthesis program I), the Phe_3-resin was shaken 9 h with 2,4,5-trichlorophenyl 3-(4-(hydroxymethyl)phenoxy)propionate (0.6 g; 1.7 mmol) and HOBt (0.2 g; 1.7 mmol) in DMF, washed with DMF (5 x 30 s) and CH_2Cl_2 (5 × 30 s). The ninhydrin test after this incorporation was negative. The Fmoc amino acids were incorporated according to the procedure described in the synthesis of the protected segment. For the first Fmoc-Pro-OH a second double coupling (5 equiv, 1 h each coupling) was carried out. The second and third Pro residues were incorporated in the form of a dipeptide as described before [Fmoc-Pro-Pro-OH (2 equiv), HOBt (1 equiv), and DIPCDI (2 equiv) in DMF]. Coupling efficiencies were monitored using the ninhydrin or chloranil tests. Amino acids analysis of the Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-resin II after Fmoc deprotection and hydrolysis gave the following: $Phe_{3.00}$(IRAA), $Pro_{2.99}$, $Val_{0.74}$, $Leu_{0.92}$, $His_{0.84}$.

Segment Coupling. Each H-(Val-His-Leu-Pro-Pro-Pro)$_n$-OH oligomer was prepared using procedures essentially identical to those employed in the synthesis of the $n = 2$ analog given below:

H-(Val-His-Leu-Pro-Pro-Pro)$_2$-OH. Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-resin II (12 mg; 0.3 mmol/g) was deprotected using program IV where an extended preswelling step with CH_2Cl_2 and DMF was carried out described below:

Program IV: (1) CH_2Cl_2 (3 × 10 min); (2) DMF (3 × 10 min); (3) piperidine–DMF (2:8) (1 × 1 min + 3 × 3 min); (4) DMF (5 × 30 s) and (5) CH_2Cl_2 (5 × 30 s).

The protected segment Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH (15 mg; 14 μmol) was dissolved in 45 μL of DMF and solutions of HATU in DMF (15 μL of a 2 mM solution), HOAt in DMF (15 μL, solution of a 5.7 mM solution), and DIEA in DMF (15 μL of a 6.7 mM solution) were added, and the mixture (total volume 90 μL) was stirred for 15 min at room temperature. After this time, the resin was washed with DMF (5 × 3 mL, 1 min) and CH_2Cl_2 (5 × 3 mL, 1 min). The ninhydrin test was negative. After coupling, acetylation (6 μL of Ac_2O, 12 μL of DIEA in DMF) was performed to cap unreacted amino groups. A sample of the resultant peptide-resin was cleaved, after Fmoc deprotection according to program IV, with TFA–CH_2Cl_2–H_2O (49:48:3) (2 mL) for 1.5 h at 25 °C by the procedure described above to give the crude peptide with a yield of 98% by amino acid analysis. HPLC: t_R 9.5 min (Nucleosil C18 column; elution conditions as in Figure 2). FAB-MS [M + H] calcd 1300; found, 1300.

H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH. Couplings of the Fmoc-Val-His(Trt)-Leu-Pro-Pro-Pro-OH were carried out according to the protocol described for the synthesis of H-(Val-His-Leu-Pro-Pro-Pro)$_2$-OH. Prior to each coupling step an acetylation reaction was performed using the same procedure described above. A sample of the H-(Val-His-Leu-Pro-Pro-Pro)$_8$-OH-resin was cleaved according to the procedure used for the cleavage of the H-(Val-His-Leu-Pro-Pro-Pro)$_2$-OH to obtain the crude peptide in a 99% of yield as judged by amino acid analysis. The product was characterized by cation-exchange HPLC (t_R 21.5 min; HPLC profile is shown in Figure 6) and ESMS (calcd 5144; found, 5145).

Acknowledgment. This research was supported by funds from the CICYT (Grant PB92-0864) and the Generalitat de Catalunya (Centre de Referència en Biotecnologia). I. D. has a fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brazil).

JO9512012