Cyclic Peptides. I. Cyclo(tri-L-prolyl) and Derivatives. Synthesis and Molecular Conformation from Nuclear Magnetic Resonance

Charles M. Deber,[1a] Dennis A. Torchia,[1b] and Elkan R. Blout* [1a]

Contribution from Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts 02115, and Bell Telephone Laboratories, Inc., Murray Hill, New Jersey 07974.
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Abstract: The syntheses of the cyclic tripeptides cyclo(L-Pro-L-Pro-L-Hyp), cyclo(L-Pro-L-Pro-L-Hyp) acetate, and cyclo(L-Pro-L-Pro-L-Hyp) benzoate are reported. From both the experimentally obtained and computer-simulated 220-MHz nmr spectra of the hydroxyprolyl ring protons in the benzoate, all ring vicinal couplings are determined. Using these couplings and a Karplus-type function to estimate dihedral angles, a ring conformation for the cyclic peptide in solution is deduced in which the four ring carbons are almost coplanar, but the peptide nitrogen lies above this plane. The set of angles describing this conformation is $\chi_1 \approx 30°$, $\chi_2 \approx -10°$, $\chi_3 \approx -5°$, and $\chi_4 \approx 25°$. Vicinal couplings obtained from a computer simulation of the cyclo(tri-L-prolyl) spectrum indicate that the prolyl rings in the parent compound have the same geometry. It is suggested on the basis of model building studies that the deduced ring geometry is a consequence of planar cis peptide bonds in the nine-membered cyclic peptide backbone.

Unusual among peptides in its restricted conformation, cyclo(tri-L-prolyl) affords an opportunity for the study of the geometry of the prolyl residue by relating spectral parameters to structural features. Cyclo(tri-L-prolyl) consists of three symmetrically disposed prolyl residues joined by cis peptide bonds.[2,3] Its synthesis, first accomplished by Rothe, *et al.*,[4] *via* cyclization of tri-L-prolyl-*p*-nitrophenyl ester, is apparently facilitated by the property of linear tri-L-prolyl chains to exist in solution as mixed cis–trans conformers at each peptide junction.[5] Recently, Dale and Titlestad have reported[6] the synthesis of another cyclotripeptide, cyclo(trisarcosyl), isolated along with cyclo(hexasarcosyl) from the cyclization of a trisarcosyl active ester.

We have prepared several derivatives of cyclo(tri-L-prolyl), differing from the parent compound in that one prolyl residue has been replaced by a hydroxyprolyl[7] residue which was subsequently modified. These derivatives have made possible a conformational analysis of the pyrrolidine rings of cyclo(tri-L-prolyl) in solution by means of 220-MHz nmr spectroscopy.

Synthesis of Materials

Cyclo(tri-L-prolyl) (I) was synthesized by a variation of the method of Rothe, *et al.*;[4] the synthesis of cyclo(L-Pro-L-Pro-L-Hyp) was formally similar and is described in detail in the Experimental Section below. The L-Hyp residue was introduced by mixed anhydride coupling of *tert*-butyloxycarbonyl-L-Pro-L-Pro-OH with HCl·H-Hyp benzyl ester, as outlined in Chart I. The resulting *tert*-butyloxycarbonyl-L-Pro-L-Pro-L-Hyp benzyl ester was treated with 10% Pd/C under hydrogenation conditions to generate the corresponding free acid. Reaction with *p*-nitrophenol in the presence of dicyclohexylcarbodiimide (DCCI) provided the active *p*-nitrophenyl ester, whose N-protecting group was then removed with HCl–ethyl acetate. The resulting material, HCl·H-L-Pro-L-Pro-L-Hyp *p*-nitrophenyl ester (IIc), was cyclized at high dilution in pyridine to provide cyclo(L-Pro-L-Pro-L-Hyp) (III), in 12% yield. The corresponding acetate IV and benzoate V were then prepared through reaction with acetic anhydride and benzoyl chloride, respectively, in pyridine.

Results and Discussion

The Conformation of the Hydroxyprolyl Ring in Cyclo(L-Pro-L-Pro-L-Hyp) Benzoate. The 220-MHz spectrum of cyclo(tri-L-prolyl) is shown in Figure 1a. In attempting to solve such a spectrum, one is confronted with a seven-spin system in the proline ring, in which several protons have nearly overlapping chemical shifts, resulting in a non-first-order spectrum. It was realized that the introduction of a hydroxyl group into one of the proline rings would serve at least three beneficial functions: (1) one of the γ-hydrogens would be eliminated, thereby reducing the number of coupled protons, with accompanying simplification of the nmr spectrum; (2) the electronegative oxygen atom would cause a general downfield shift of all Hyp ring protons; and (3) the hydroxyl group would open synthetic pathways to a variety of functional derivatives. The nomenclature which has been employed to designate proline ring protons throughout this report is shown in the following diagram.[8]

Nmr studies on CPOH, III, were hampered by its limited solubility; its partial spectrum in trifluoroacetic

(1) (a) Harvard Medical School; (b) Bell Telephone Laboratories.
(2) C. M. Venkatachalam, *Biochim. Biophys. Acta*, **168**, 397 (1968).
(3) C. M. Deber, A. Scatturin, V. M. Vaidya, and E. R. Blout in "Peptides: Chemistry and Biochemistry," B. Weinstein and S. Lande, Ed., Marcel Dekker, New York, N. Y., 1970, pp 163–173.
(4) M. Rothe, K. D. Steffen, and I. Rother, *Angew. Chem., Int. Ed. Engl.*, **4**, 356 (1965).
(5) C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout, *J. Amer. Chem. Soc.*, **92**, 6191 (1970).
(6) J. Dale and K. Titlestad, *Chem. Commun.*, 656 (1969).
(7) In this work the L-hydroxyproline used was the naturally occurring isomer with the hydroxyl group in the 4 position (γ) and trans to the carboxyl group.
(8) Abbreviations used herein: CP = cyclo(tri-L-prolyl) (I); CPOH = cyclo(L-Pro-L-Pro-L-Hyp) (III); CPOAc = cyclo(L-Pro-L-Pro-L-Hyp) acetate (IV); and CPOCOPh = cyclo(L-Pro-L-Pro-L-Hyp) benzoate (V).

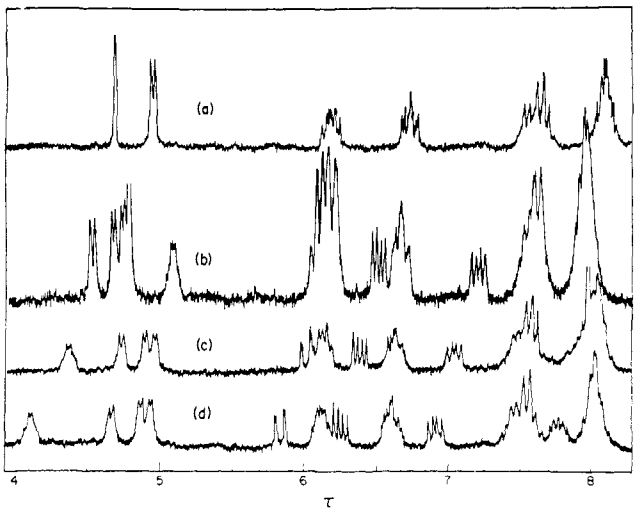
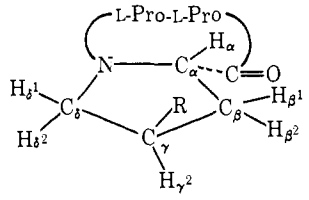


Figure 1. 220-MHz nmr spectra of: (a) cyclo(tri-L-prolyl) (I) in CD_2Cl_2 (solvent peak: τ 4.65); (b) cyclo(L-Pro-L-Pro-L-Hyp) (III) in trifluoroethanol-d_3 (solvent peaks: τ 4.8, 6.15); (c) cyclo(L-Pro-L-Pro-Hyp) acetate (IV) in $CDCl_3$; (d) cyclo(L-Pro-L-Pro-L-Hyp) benzoate (V) in $CDCl_3$. Chemical shifts are in τ units downfield from tetramethylsilane.

acid has been reported.[9] The complete spectrum is shown in Figure 1b, recorded in trifluoroethanol-d_3; residual nondeuterated solvent obscures the spectrum near τ 6.2. CPOAc (IV) and CPOCOPh (V) were



I, R = $H\gamma^1$ IV, R = $OC(=O)CH_3$
III, R = OH V, R = $OC(=O)Ph$

readily soluble in deuteriochloroform; their 220-MHz spectra were recorded in this solvent and appear in Figures 1c and d, respectively. It is seen that the presence of the Hyp residue in III, IV, and V dramatically alters the spectrum as compared with the parent cyclic tripeptide.

In the acetate IV the resonances of five out of the six Hyp ring protons are shifted downfield sufficiently so as to emerge from the complex resonances due to the two unsubstituted prolyl residues (themselves now slightly nonequivalent). The methyl group is seen as a singlet near τ 8. In the benzoate there is an even larger downfield shift of the Hyp protons, and all six Hyp ring resonances are observed. The β proton hidden (in the acetate) under the multiplet at τ 8 is now visible at 7.8, along with the other β proton at 6.9, the two δ protons at 5.8 and 6.3, the α proton at 4.7, and the γ proton at 4.1. The downfield resonances due to the phenyl hydrogens (not shown) display a typical benzoate pattern with ortho, para, and meta protons at τ 2.0, 2.4, and 2.6, respectively. All assignments were confirmed by spin decoupling. Because of the clarity of the Hyp proton resonances, the cyclo(L-Pro-L-Pro-L-Hyp) benzoate was chosen for the study described below.

In the spectrum of cyclo(tri-L-prolyl), Figure 1a, one doublet at τ 4.95 is observed for the three equivalent α protons; in the three Hyp derivatives the α protons occur as three separate doublets in each spectrum. The appearance of a doublet for the α-proton resonance is noteworthy because in proline oligomers[5] and in poly-L-proline,[10] the α protons appear as quartets or triplets. Since the Hyp α-proton resonances constitute the X portions of AMX (first order) systems in each of the compounds III, IV, and V (the β protons are now separated by about 1 ppm), the occurrence of a doublet implies that one of the coupling constants $J_{\alpha\beta^1}$ or $J_{\alpha\beta^2}$ is close to 0.[11]

Spin-decoupling experiments with the benzoate spectrum (Figure 1d) confirmed the near-zero coupling constant; the α-H doublet at τ 4.7 collapses to a singlet on irradiation of the H_β resonance at 7.8, but is not affected by similar irradiation of the H_β resonance at 6.9. The fact that α-proton doublets prevail throughout all of these spectra suggests that introduction of γ substituents has not appreciably affected the fundamental cyclo(tri-L-prolyl) conformation.

In order to obtain a complete set of vicinal coupling constants, it was necessary to produce a computer-simulated spectrum of the protons of the benzoylated hydroxyprolyl residue. Vicinal and geminal couplings were, when possible, obtained from the observed splittings; since one $J_{\alpha\beta}$ and one $J_{\beta\gamma}$ were not directly measurable, initial estimates of these couplings were made and then varied until the simulation was satisfactory. The comparison of the experimentally obtained and computer-simulated spectra is shown in Figure 2, along with a summary of the chemical shifts and couplings used to generate the simulated spectrum. The neglect of long-range couplings (through four or more bonds) in simulating the spectrum should not affect the results obtained since such couplings are expected to be small[12-14] (*ca.* 1 Hz) relative to the measured line widths (2–2.5 Hz).

The specific assignments of the β and δ protons shown in Figure 2 were made by assuming that the dependence of vicinal couplings on the dihedral angles has the functional form, $A \cos^2 \phi_d + B$, predicted by Karplus.[15-17] Employing usual values[18] for A (7–12 Hz) and B (0–2 Hz), only the assignments shown allow a ring structure which is not strained.

Having obtained the complete set of vicinal coupling constants for the Hyp ring protons, the Karplus relation (eq 1 and 2) was used to estimate values of the

(9) C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout in "Proceedings of the 10th European Peptide Symposium," E. Scoffone, Ed., North-Holland Publishing Co., Amsterdam, 1971, pp 189–299.

(10) D. A. Torchia and F. A. Bovey, *Macromolecules*, **4**, 246 (1971).

(11) This is not necessarily true for the parent compound, where the α proton is part of a five-spin system. Even in three-spin systems, *i.e.*, ABX, observed splittings in the X portions are not always related in a simple first-order fashion to coupling constants. For a discussion see F. A. Bovey, "Nuclear Magnetic Resonance Spectroscopy," Academic Press, New York, N. Y., 1969, pp 105–113.

(12) R. J. Abraham, K. A. McLauchlan, S. Dalby, G. W. Kenner, R. C. Sheppard, and L. F. Burroughs, *Nature (London)*, **192**, 1150 (1961).

(13) R. J. Abraham and K. A. McLauchlan, *Mol. Phys.*, **5**, 195 (1962).

(14) H. Booth, *Progr. Nucl. Magn. Resonance Spectros.*, **5**, 149 (1969).

(15) M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959).

(16) M. Karplus, *ibid.*, **33**, 1842 (1960).

(17) M. Karplus, *J. Amer. Chem. Soc.*, **85**, 2870 (1963).

(18) A. A. Bothner-by, *Advan. Nucl. Magn. Resonance*, **1**, 195 (1965).

$$J_{H,H} = A \cos^2 \phi_d + B \qquad 0° \leq \phi_d \leq 90° \quad (1)$$

$$J_{H,H} = A' \cos^2 \phi_d + B \qquad 90° \leq \phi_d \leq 180° \quad (2)$$

dihedral angles ϕ_d, between the ring protons. In applying eq 1 and 2 the following values of A, A', and B were employed: $A = 8.5$ Hz, $A' = 10.5$ Hz, and $B = 1.4$ Hz, for couplings not involving the Hyp γ proton (set X); $A = 7.0$ Hz, $A' = 9.0$ Hz, and $B = 1.1$ Hz, for couplings involving the Hyp γ proton (set Y).[19]

To produce the geometry required by $J_{\alpha\beta^2} = 1.4$ Hz (that is, a dihedral angle of near 90° between H_α–C_α–C_β–H_{β^2}), a downward puckering of the α-carbon atom relative to the β-carbon atom is necessary (that is, $\chi_1 \approx 30°$).[21] The dihedral angles calculated from the nmr data also require a small upward puckering of the β carbon relative to the γ carbon ($\chi_2 \approx -10°$), and, in turn, a further small upward puckering of the γ carbon relative to the δ carbon ($\chi_3 \approx -5°$). As pointed out,[17] the accuracy of dihedral angles obtained from this type of analysis is estimated to be about ±5°.

It should be noted that only three vicinal couplings are needed to determine χ_1, χ_2, χ_3, whereas the nmr analysis yields all six vicinal couplings. These additional couplings were used to check the internal consistency of the Karplus relation and the geometry obtained.

Prolyl ring geometries in which the γ-carbon lies out of the $C_\delta NC_\alpha C_\beta$ plane have been found in linear Hyp peptides in the solid state by X-ray methods[22] and for hydroxyproline itself in solution by nmr methods.[12,23,24] With the values found in the present investigation, (χ_1, χ_2, χ_3) = (30, −10, −5°) the four ring carbon atoms are almost coplanar, and the peptide nitrogen is displaced from this plane, with the $C_\delta NC_\alpha$ plane and the plane of the four carbons making an angle of $\chi_4 \approx 25°$. A Dreiding model of the cyclo(L-Pro-L-Pro-L-Hyp) benzoate, containing *planar* cis peptide bonds, and in which the α-proton contact distance is held at 1.95 Å, has the Hyp ring conformation that is predicted by the nmr analysis. The model indicates that this geometry can be significantly altered only by introducing valence angle strain in the ring or by allowing nonplanar cis peptide bonds. Thus the ring geometry predicted by the nmr data is apparently due to the presence of planar cis bonds in the nine-membered cyclic peptide backbone. This conclusion is not in agreement with that of Venkatachalam,[2] who suggested on the basis of stereochemical considerations that in cyclo(tri-L-prolyl) the three cis peptide bonds might be distorted as much as 25–30° away from planarity. Additional evidence for peptide bond planarity is seen in the infrared spectra of cyclo(tri-L-prolyl) compounds, which consistently display amide I carbonyl bands near 1630 cm^{-1}, about 20 cm^{-1} lower than those shown by typical L-Pro-L-Pro peptide bonds in linear peptides.[25] If nonplanar peptide bonds were present in the cyclic compounds, an *increase* in their carbonyl frequencies would be expected.

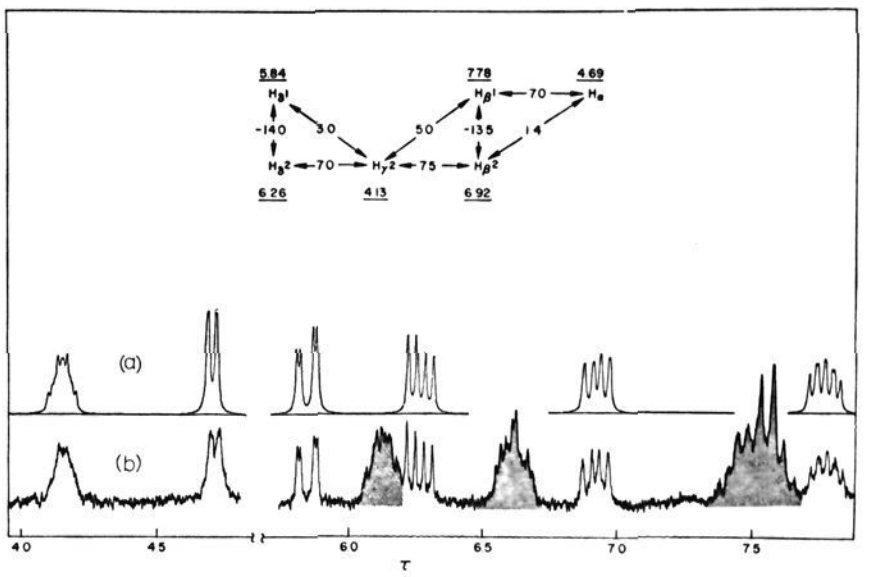


Figure 2. Comparison of 220-MHz spectra of Hyp ring protons of cyclo(L-Pro-L-Pro-L-Hyp) benzoate (V): (a) computer-simulated spectrum; (b) measured spectrum, Hyp resonances, nonshaded; Pro resonances, shaded. At the top is a schematic summary of the coupling constants (hertz) and chemical shifts (underlined) used in the simulation; uncertainty in vicinal couplings listed, ±0.5 Hz. Line width assumed in simulation, 2.5 Hz.

The Conformation of Cyclo(tri-L-prolyl). Approximate values of δ proton geminal and vicinal couplings in the CP spectrum were obtained by spin decoupling the γ protons. Values of vicinal couplings between β and γ protons which were not available from spin decoupling were obtained initially by *assuming* that the prolyl rings in CP had the same conformation as that deduced for the Hyp residue in CPOCOPh. Equations 1 and 2 were then used to approximate vicinal couplings, using values of A, A', and B given in set X. Geminal couplings of the β and γ protons were initially assumed to be −13 Hz.

Chemical shifts of the protons in this seven-spin system were obtained as follows: the α proton is assigned to τ 4.95; the δ protons are readily assigned to the resonances at τ 6.18 and 6.74. H_{δ^1} is assigned to the upfield position because spin decoupling indicated that this proton was coupled to the γ protons with one small (about 4 Hz) and one large (about 10 Hz) coupling constant; assuming the CPOCOPh ring geometry, eq 1 and 2 and coupling set X predict that only H_{δ^1} could have such couplings. In similar fashion the τ 8.1 position was assigned to H_{γ^2}, since irradiation of the spectrum there collapsed the 4-Hz splittings of the H_{δ^1} resonance. The H_{β^1} proton was assigned initially to *ca.* τ 8.1 since irradiation at this position caused the α-proton resonance to collapse to a singlet. The H_{β^2} and H_{γ^1} resonances were tentatively assigned positions in the τ 7.5–7.7 region.

(19) The constants in set X are in close agreement with the empirical set of constants ($A = 9$ Hz, $A' = 11$ Hz, $B = 2$ Hz) proposed[18] from an analysis of vicinal coupling data for six-membered (unstrained) rings. The smaller constants of set Y were used for couplings involving the benzoyl Hyp γ proton, since theoretical[15–17,20] and experimental[14,18] results indicate that vicinal couplings are reduced in the presence of strong electronegative substituents.

(20) M. Barfield and D. M. Grant, *Advan. Nucl. Magn. Resonance*, **1**, 149 (1965).

(21) For explanations of conventions used in dihedral angle nomenclature, see J. T. Edsall, P. J. Flory, J. C. Kendrew, A. M. Liquori, G. Nemethy, G. N. Ramachandran, and H. A. Scheraga, *J. Biol. Chem.*, **241**, 1004 (1966). The χ_n angles used herein may be visualized as the dihedral angles formed between the following pairs of planes: χ_1, $NC_\alpha C_\beta$ and $C_\alpha C_\beta C_\gamma$; χ_2, $C_\alpha C_\beta C_\gamma$ and $C_\beta C_\gamma C_\delta$; χ_3, $C_\beta C_\gamma C_\delta$ and $C_\gamma C_\delta N$; χ_4, $C_\gamma C_\delta N$ and $C_\delta NC_\alpha$.

(22) R. Balasubramanian, A. V. Lakshminarayanan, M. N. Sabesan, G. Tegoni, K Venkatesan, and G. N. Ramachandran, *Int. J. Protein Res.*, **3**, 25 (1971).

(23) R. J. Abraham and K. A. McLauchlan, *Mol. Phys.*, **5**, 513 (1963).

(24) R. J. Abraham and W. A. Thomas, *J. Chem. Soc.*, 3739 (1964).

(25) C. M. Deber and E. R. Blout, unpublished observations.

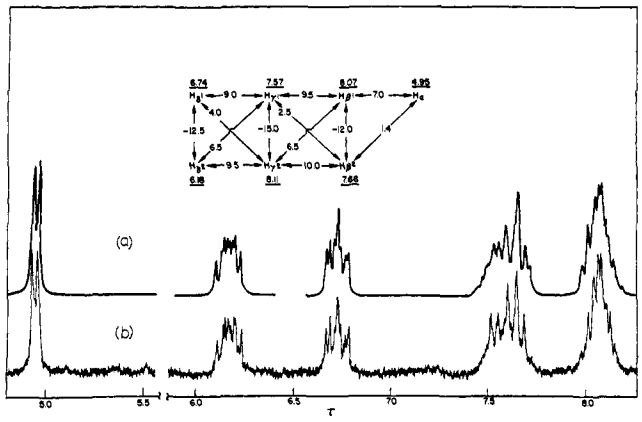


Figure 3. Comparison of 220-MHz spectra of proline ring protons of cyclo(tri-L-prolyl) (I): (a) computer-simulated spectrum; (b) measured spectrum. At the top is a schematic summary of the coupling constants (hertz) and chemical shifts (underlined) used in the simulation; uncertainty in vicinal couplings listed, α and δ protons ± 0.5 Hz, β and γ protons ± 1 Hz. Line width assumed in simulation, 2.5 Hz.

The initial estimates of coupling constants and chemical shifts were than varied until a satisfactory simulation of the CP spectrum was obtained. The final computer-simulated spectrum, along with the experimentally observed spectrum, and the values of J and τ used in the simulation, are all shown in Figure 3.

If eq 1 and 2 and set X are applied to a conformation with the same dihedral angles as those deduced above for the Hyp residue, vicinal couplings are obtained which agree, to within ± 1 Hz, with the vicinal couplings obtained by the computer simulation for cyclo(tri-L-prolyl) itself. Thus it is concluded that the parent compound exists in solution in a conformation consisting of three equivalent prolyl rings with dihedral angles the same as in the Hyp residue of V. A stereo view of the deduced molecular conformation of cyclo(tri-L-prolyl) is shown in Figure 4.

Conclusions

Our results suggest a conformation for cyclo(tri-L-prolyl) in which the nitrogen atom lies out of the plane of the four carbon atoms in each pyrrolidine ring. Such a conformation appears to be a consequence of planar cis peptide bonds in the cyclic tripeptide backbone. A Karplus-type analysis has shown that the proposed conformation predicts all the vicinal couplings in Figures 2 and 3 to within ± 1 Hz. All our results are consistent with the conformation shown in Figure 4; we await the results of X-ray determinations of the structures of cyclo(tri-L-prolyl) and cyclo(L-Pro-L-Pro-L-Hyp) in the solid state.

Experimental Section

Nuclear Magnetic Resonance Spectra. Nmr spectra were obtained using the Varian HA-100 and HR-220 spectrometers at Bell Telephone Laboratories and at Rockefeller University. Homonuclear spin decoupling was accomplished using Muirhead D-890-B and General Radio 1107-A audio oscillators at 100 and 220 MHz, respectively.

Synthesis. The scheme of the synthesis of the CP derivatives is shown in Chart I.

***tert*-Butyloxycarbonyl-L-prolyl-L-prolyl-L-hydroxyprolyl Benzyl Ester (IIa).** A solution of *tert*-butyloxycarbonyl-L-prolyl-L-proline[5] (2.50 g) in chloroform (20 ml) was cooled to $-20°$ in Dry Ice–CCl_4. *N*-Methylmorpholine (0.90 ml) and isobutylchloroformate (1.33 ml)

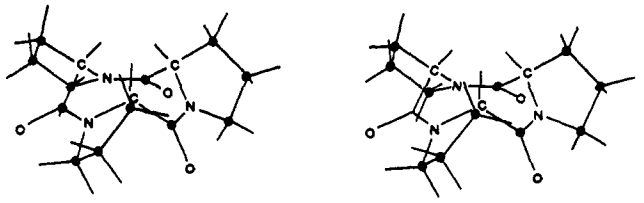
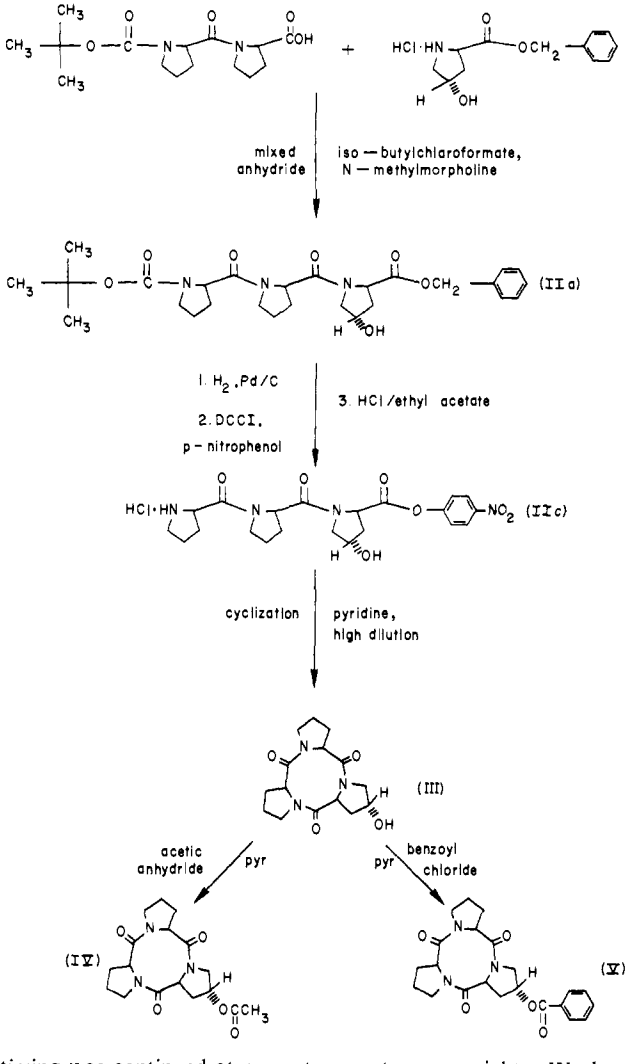


Figure 4. A stereoview of the molecular conformation deduced for cyclo(tri-L-prolyl) with the α-carbon, peptide nitrogen, and carbonyl oxygen atoms denoted by the letters C, N, and O, respectively.

were then added in succession with stirring. After 20 min at $-20°$ L-4-hydroxyproline benzyl ester hydrochloride (2.06 g) was stirred into the reaction mixture, followed by an additional 1 equiv (0.9 ml) of *N*-methylmorpholine. The reaction mixture was allowed to warm to room temperature slowly as the Dry Ice evaporated and

Chart I. Scheme of Synthesis of Cyclo(tri-L-prolyl) Derivatives



stirring was continued at room temperature overnight. Work-up was accomplished by dilution with 20 ml of chloroform and then by extraction successively with 40-ml portions of water, 5% aqueous sodium bicarbonate, and saturated sodium chloride. The chloroform layer was separated and dried over sodium sulfate, and the solvent was evaporated to yield *tert*-butyloxycarbonyl-L-prolyl-L-prolyl-L-hydroxyprolyl benzyl ester (IIa) (3.6 g, 88%) as a pale yellow syrup which could not be crystallized. The material had appropriate ir and nmr spectra and chromatographic (tlc) behavior.

***tert*-Butyloxycarbonyl-L-prolyl-L-prolyl-L-hydroxyproline (IIb).** The benzyl ester IIa (3.0 g) was dissolved in 15 ml of *tert*-butyl alcohol, treated with a catalytic amount of 10% Pd/C, and hydrogenated at 20 psi for 24 hr at room temperature. The catalyst was then removed by filtration through Celite, and the solvent was

evaporated. The semisolid residue could not be crystallized, and was obtained after overnight treatment *in vacuo* as a colorless amorphous foam (2.3 g). The product displayed appropriate ir and nmr spectra and chromatographic behavior.

L-Prolyl-L-prolyl-L-hydroxyprolyl *p*-Nitrophenyl Ester Hydrochloride (IIc). The preparation of acid IIb was repeated on a scale which led to 10.0 g of material. Thus *tert*-butyloxycarbonyl-L-prolyl-L-prolyl-L-hydroxyproline (10.0 g) was treated with dicyclohexylcarbodiimide (4.86 g) and *p*-nitrophenol (3.33 g) in a minimum amount of chloroform with overnight stirring at 0°. The chloroform was evaporated, a few milliliters of acetone was added, and the insoluble urea was removed by filtration. Two drops of acetic acid were added, the mixture was kept at 0° for 1 hr, and additional urea was removed by filtration. Evaporation of the solvent provided *tert*-butyloxycarbonyl-L-prolyl-L-prolyl-L-hydroxyprolyl *p*-nitrophenyl ester as an off-white semisolid, which was dissolved directly in 100 ml of 4 *N* hydrochloric acid–ethyl acetate. After 1 hr of stirring at room temperature, the solvents were evaporated; trituration with ether followed by decanting removed free *p*-nitrophenol. The residue was dried overnight *in vacuo*, providing L-prolyl-L-prolyl-L-hydroxyprolyl *p*-nitrophenyl ester hydrochloride (IIc) (11.3 g, 98%) as an off-white foam, which was used directly in the ensuing cyclization procedure.

Cyclo(L-prolyl-L-prolyl-L-hydroxyprolyl) (III). The *p*-nitrophenyl ester hydrochloride (IIc) (11.3 g) was dissolved in 280 ml of dried (over sodium sulfate) dimethylformamide (DMF). (The DMF already contained 5 drops of glacial acetic acid to neutralize any amines present.) This solution was added dropwise, with efficient stirring, to a 5-l. round-bottomed flask containing 2800 ml of spectroquality pyridine preheated to 75°. Addition was complete after 3 hr, and the reaction mixture was maintained at 75° for an additional 17 hr. After cooling, the reaction mixture was concentrated; the dark syrupy residue was treated several times with methanol and the evaporation was repeated to ensure removal of residual pyridine and DMF. The crude product was then dissolved in a minimum volume of acetone, and allowed to stand for a short time at room temperature, when crystallization of the product commenced. Upon filtration cyclo(L-prolyl-L-prolyl-L-hydroxyprolyl) was collected as white crystals, 0.86 g, 12% yield. Crystallization of a sample from methanol gave an analytical sample: mp ~350° with accompanying sublimation; mass spectrum, molecular ion peak 307; ir major bands 1630, 1440, 1300, 1080 cm^{-1}; ninhydrin negative.

Anal. Calcd for $C_{15}H_{21}N_3O_4$: C, 58.61; H, 6.89; N, 13.67. Found: C, 58.47; H, 6.78; N, 13.30.

Cyclo(L-prolyl-L-prolyl-L-hydroxyprolyl) Acetate (IV). A solution of cyclo(L-prolyl-L-prolyl-L-hydroxyprolyl) (III) (15.0 mg) in a minimum volume of pyridine (about 5 ml) was prepared and treated at room temperature with 3 drops of acetic anhydride. The reaction vessel was protected from moisture with Drierite and stirred at room temperature for 36 hr. Evaporation of pyridine and excess acetic anhydride gave a solid residue (amorphous, 13 mg), which was recrystallized from a minimum volume of acetone. Upon filtration, crystals were obtained of cyclo(L-prolyl-L-prolyl-L-hydroxyprolyl) acetate (11 mg): mp 206–210°; mass spectrum, molecular ion peak 349; ir carbonyl region bands, 1730, 1635 cm^{-1}.

Anal. Calcd for $C_{17}H_{23}N_3O_5$: C, 58.44; H, 6.64; N, 12.03. Found: C, 58.46; H, 6.61; N, 11.52.

Cyclo(L-prolyl-L-prolyl-L-hydroxyprolyl) Benzoate (V). A solution of cyclo(L-prolyl-L-prolyl-L-hydroxyprolyl) (III) (25 mg) in a minimum volume of pyridine (about 6 ml) was prepared in a reaction vessel equipped with a nitrogen inlet and vacuum outlet. The air was evacuated from the flask and replaced with nitrogen, and the process repeated several times. Maintaining a positive flow of nitrogen, the flask was opened, two drops of benzoyl chloride was added, and the flask was sealed quickly; the reaction mixture became pale pink in color. Stirring under nitrogen was continued for 4 hr at room temperature. Solvents were then evaporated. The crude residue was swirled with portions of ether, and the ether was decanted; the portions were combined and evaporation provided a crystalline product, cyclo(L-prolyl-L-prolyl-L-hydroxyprolyl) benzoate (V), 20 mg. Crystallization of a portion of this material from ether gave an analytical sample: mp 212–216°; mass spectrum, molecular ion peak 411; ir carbonyl region bands, 1715, 1630 cm^{-1} (slightly split band).

Anal. Calcd for $C_{22}H_{25}N_3O_5$: C, 64.22; H, 6.12; N, 10.21. Found: C, 63.97; H, 6.09; N, 9.86.

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