Conformation and Complexation of a Cyclic Dodecapeptide [Cyclo(L-Leu-L-Phe-L-Pro)$_4$] with Alkaline Earth Metal Ions in Acetonitrile

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The cyclic dodecapeptide, cyclo(L-Leu-L-Phe-L-Pro)$_4$, was synthesized, and its conformation and complexation with metal ions in acetonitrile were investigated by c.d. and n.m.r. spectroscopy. Cyclo(L-Leu-L-Phe-L-Pro)$_4$ was found to complex selectively with alkaline earth metal ions. The binding constant of the dodecapeptide with Ba^{2+} was larger than that of the cyclic hexapeptide, cyclo(L-Leu-L-Phe-L-Pro)$_2$. In a free state, the skeletal conformation of the cyclic dodecapeptide is non-symmetrical. When complexed with Ba^{2+}, the conformation changes to a C_4-symmetrical one having all peptide bonds in a *trans* configuration and four β-turn structures containing transannular hydrogen bonds. Formation of hydrogen bonds on complexation with the cation accounts for the large binding constant of the cyclic dodecapeptide.

Valinomycin, which was discovered by Brockman and Schmidt-Kastner[1] and synthesized by Shemyakin *et al.*,[2] is a cyclic dodecadepsipeptide antibiotic. The antibacterial action was interpreted in terms of the selective transport of potassium ions through biological membranes in the form of a hydrophobic complex.[3,4] The character of the hydrophobic complex is determined by effective shielding of the captured cation from the aqueous phase by isopropyl groups of the hydroxy acid and the amino acid residues, which form the external wall of the bracelet-type structure of valinomycin. Analyses by *X*-ray crystallography[5] and n.m.r. spectroscopy[6] showed the cation sitting inside the cyclic skeleton, surrounded symmetrically by six ester carbonyl groups and that the complex is enveloped by a wall of hydrophobic side-chains. This structure is stabilized by six intramolecular hydrogen bonds.

We have synthesized a series of lipophilic cyclic octapeptides containing Pro[7] or Sar[8] residues. Since the Pro or Sar residues appeared to alternate in the sequence of the cyclic octapeptides, they tended to adopt a C_4-symmetric conformation, and were found to complex efficiently with Ba^{2+} and Ca^{2+} ions as a result of the effective co-operation of four or eight carbonyl groups. Thus, the binding constants of the cyclic octapeptides were generally larger than those of cyclic hexapeptides. With these experimental results in the background, we extended our interest to cyclic dodecapeptides having a C_4-symmetry both in free and complexed states, and examined their ability of complexation.

A large number of synthetic cyclic dodecapeptides[9,10] and cyclic dodecadepsipeptides[11,12] have been reported as potassium ionophores. However, they were designed to adopt a C_3-symmetric conformation similar to valinomycin. In this study, cyclic dodecapeptide, cyclo(L-Leu-L-Phe-L-Pro)$_4$, which has four repetitions of a tripeptide sequence, was synthesized. We chose this sequence because a C_4-symmetric conformation is expected to be adopted and the cyclic hexapeptide containing hydrophobic Leu, Phe, and Pro residues, cyclo(L-Leu-L-Phe-L-Pro)$_2$, has been previously synthesized and its complexation with metal ions analysed.[13]

Results and Discussion

Complexation with Metal Ions.—Circular dichroism (c.d.) spectra of cyclo(L-Leu-L-Phe-L-Pro)$_4$ in acetonitrile with or without added alkaline earth metal ions are shown in Figure 1. With the addition of $Ba(ClO_4)_2$ and $Ca(ClO_4)_2$, the positive Cotton effect at 230 nm and the negative Cotton effect at 200 nm increased their intensities. In contrast, the addition of potassium or sodium ions did not cause a change in the c.d. spectrum. Analysis of the c.d. spectra revealed that cyclo(L-Leu-L-Phe-L-Pro)$_4$ formed a 1:1 complex with Ba^{2+}, and that the binding constant, which was calculated on the basis of the molar ellipticities in the presence of different amounts of Ba^{2+} ions, was 3.1×10^4 mol^{-1} dm^3. This value is far larger than that of cyclo(L-Leu-L-Phe-L-Pro)$_2$/Ba^{2+} (4.5×10^2 mol^{-1} dm^3).[13]

Conformation in Acetonitrile.—Conformations of cyclo(L-Leu-L-Phe-L-Pro)$_4$ and the cyclo(L-Leu-L-Phe-L-Pro)$_4$/Ba^{2+} complex in CD_3CN were examined by n.m.r. spectroscopy. The ^{13}C n.m.r. spectrum of the cyclic dodecapeptide in a free state

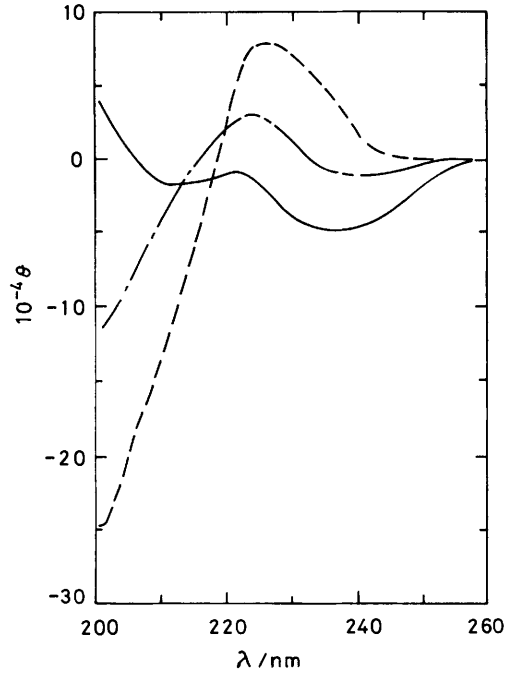

Figure 1. C.d. spectra of cyclo(L-Leu-L-Phe-L-Pro)$_4$ in MeCN with and without the presence of metal ions [$Ba(ClO_4)_2$ and $Ca(ClO_4)_2$]. Peptide concentration was 1.0×10^{-4}M: ——, without metal ions; – – –, with $Ba(ClO_4)_2$ (100 equiv.); — · —, with $Ca(ClO_4)_2$ (100 equiv.)

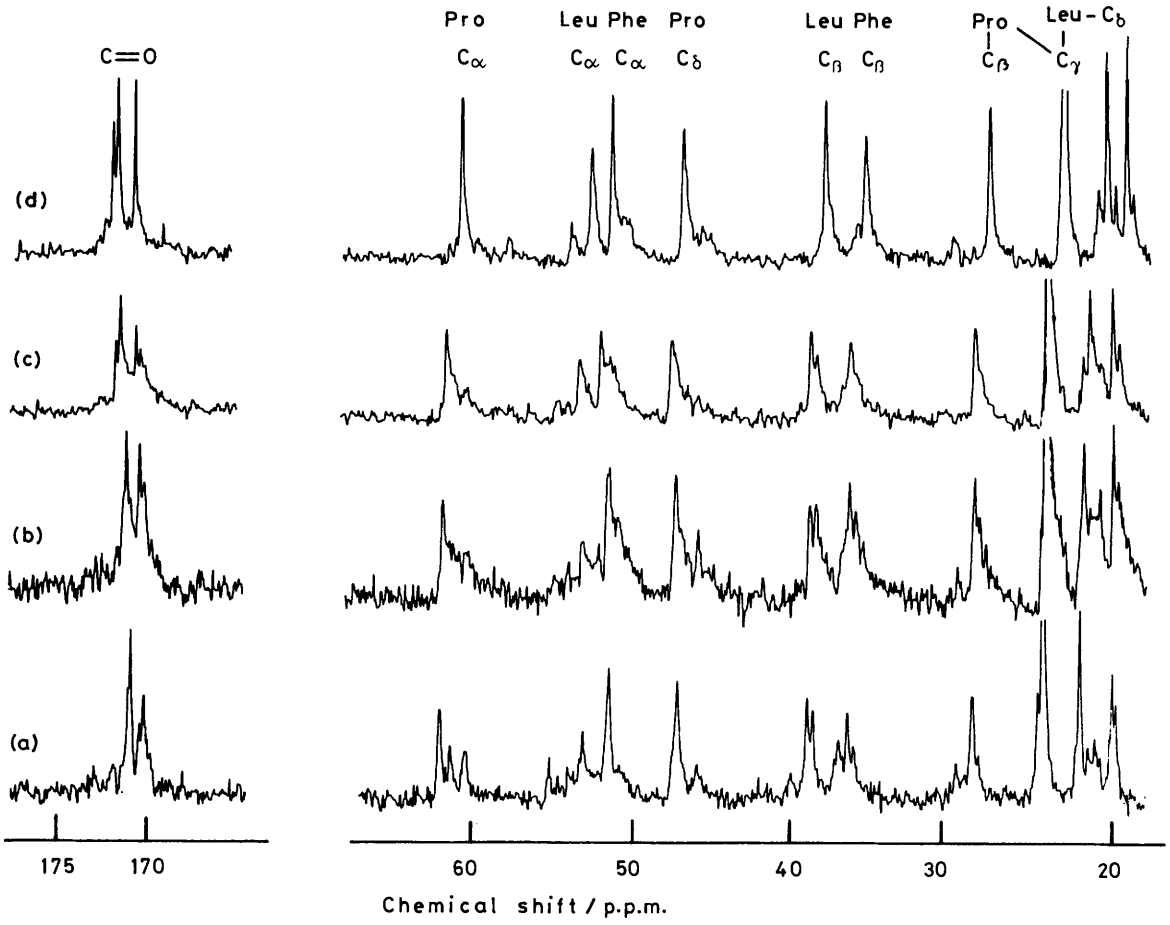

Figure 2. ^{13}C N.m.r. spectra of cyclo(L-Leu-L-Phe-L-Pro)$_4$ in CD_3CN with and without $Ba(ClO_4)_2$. Molar ratios of salt against peptide were (a), 0; (b), 0.2; (c), 0.5; (d), 1. The peptide concentration was 0.03M

[Figure 2(a)] shows several signals for a carbon atom in a given amino acid residue. The ^1H n.m.r. spectrum showed many amide NH signals in the region δ 5.5—9.0 (data not shown). These results indicate that cyclo(L-Leu-L-Phe-L-Pro)$_4$ takes a non-symmetric conformation and/or several different conformations in a free state.

Conformation of the Ba^{2+} *Complex.*—Figures 2(b)—(d) show the ^{13}C n.m.r. spectra of cyclo(L-Leu-L-Phe-L-Pro)$_4$ in CD_3CN in the presence of $Ba(ClO_4)_2$. The assignment of signals was based on their chemical shifts.[14] A single signal appeared for a carbon atom involved in the Leu, Phe, and Pro residues in the presence of an excess of Ba^{2+} salt, indicating that adoption of a C_4-symmetric conformation occurs on complexation with Ba^{2+}. The chemical shifts of Pro C^β and C^γ atoms in the ^{13}C n.m.r. spectrum were δ 29.18 and 24.47 p.p.m., respectively. Correlation of the chemical shifts of the C^β and C^γ atoms of the Pro residue with the *cis* and *trans* configuration of the X-Pro bond[15,16] indicates a *trans* configuration of L-Phe-L-Pro peptide bond.

The ^1H n.m.r. spectrum of the cyclic dodecapeptide/Ba^{2+} complex showed two NH signals at δ 7.51 and 6.49, supporting a C_4-symmetric conformation of the complex. The temperature dependences of the chemical shift of the amide NH protons in CD_3CN were 1.1×10^{-3} p.p.m./deg for Leu NH and 8.3×10^{-4} p.p.m./deg for Phe NH, which were smaller than that for solvent exposed amide protons. Furthermore, amide protons of Leu and Phe residues retained their intensities in the presence of CD_3OD (80 equiv.) for 12 hours. These amide protons are, therefore, shielded from the solvent.

Nuclear Overhauser effect-correlated two-dimensional ^1H n.m.r. spectroscopy (NOESY) of the Ba^{2+} complex was measured to obtain precise information about the conformation. Figure 3 shows the NOESY spectrum of the cyclo(L-Leu-L-Phe-L-Pro)$_4$/Ba^{2+} (1:1) complex in CD_3CN. Assignment of signals was carried out by proton decoupling and correlation ^1H n.m.r. spectroscopy (COSY). Nuclear Overhauser effect (n.O.e.)-correlated signals were observed for the proton pairs of (Pro $C^\alpha H$, Leu $C^\alpha H$) and (Pro $C^\delta H$, Phe $C^\alpha H$). Protons in these pairs might be close to each other.

Leu^1 $C^\alpha H$ can be close to $C^\alpha H$ of either Pro^3 or Pro^{12}. But, since the amide bond of Pro^{12}-Leu^1 is in a *trans* form, Leu^1 $C^\alpha H$ cannot be close to Pro^{12} $C^\alpha H$ to give the observed n.O.e. To explain the observed n.O.e., it is reasonable that Leu^i $C^\alpha H$ and Pro^{i+2} $C^\alpha H$ should be close to each other. Similarly, the Phe^{i+1} $C^\alpha H$ should be close to Pro^{i+2} $C^\delta H$. From these results and with the aid of a space-filling molecular model, the conformation of the cyclo(L-Leu-L-Phe-L-Pro)$_4$/Ba^{2+} complex is constructed as shown in Figure 4. Four C=O groups from the Pro residues point towards the interior of the cavity of the cyclic compound and interact with the Ba^{2+} ion. A Phe-Pro-Leu-Phe sequence forms a β-turn structure with the Pro residue occurring at position 2, and with a hydrogen bond formed between Phe^{i+1} C=O and Phe^{i+4} NH. The hydrophobic side-chains of the Phe, Leu, and Pro residues point towards the exterior of the hydrophilic cavity of the cyclic compound. In this conformation, the Phe NH protons are involved in intramolecular hydrogen bonding and the Leu NH protons are buried within the molecular architecture, shielded from solvent by the side-chains of Leu and Pro residues. This conformation is consistent with

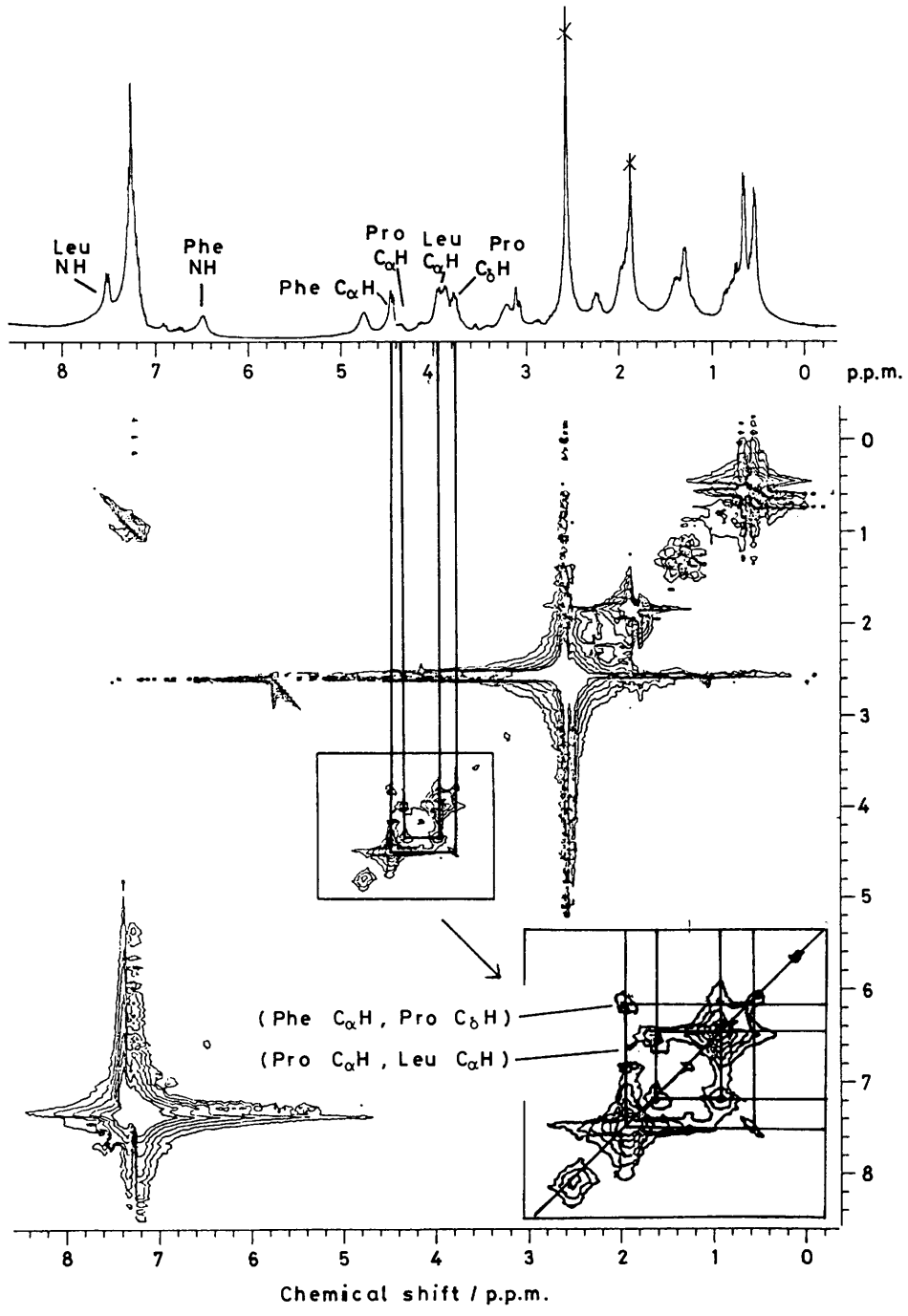

Figure 3. NOESY spectrum of cyclo(L-Leu-L-Phe-L-Pro)$_4$/Ba^{2+} complex in MeCN

the observations of low temperature dependence of the chemical shift and slow H–D exchange rate of NH protons. Furthermore the dihedral angles of the NH–C$^{\alpha}$H atoms of the Leu and Phe residues in this conformation correspond to the coupling constants observed in n.m.r. spectrum.

Figure 5 shows the proposed structure of the cyclo(L-Leu-L-Phe-L-Pro)$_4$/Ba^{2+} complex in acetonitrile using the Corey–Pauling–Koltun (CPK) molecular model. Four carbonyl groups of the Pro residues form a hydrophilic cavity at the centre of the cyclic structure and the side-chains of the Leu, Phe, and Pro residues form a hydrophobic wall outside the cyclic skeleton. The arrangement of the carbonyl groups in the hydrophilic cavity is similar to that in the metal ion complex of cyclic octapeptides, cyclo(X-Pro)$_4$ [7] and cyclo(X-Sar)$_4$.[8]

The conformation of the complex is composed of four β-turns with hydrogen bonding between Phe^{i+1} C=O and Phe^{i+4} NH. The peptide bond of the L-Phe-L-Pro sequence is *trans*, and the Pro residue takes the position 2. For cyclo(L-Leu-L-Phe-L-Pro)$_2$, the cyclic skeleton adopts a C_2-symmetric conformation, the Phe-Pro peptide bond either in a free state or in a complex state is *cis*, and the molecule comprises two β-turns. Thus, the smaller, less flexible cyclic hexapeptide forces the Phe-Pro peptide bond to take a *cis* configuration.

On complexation of the cyclic dodecapeptide with the Ba^{2+} ion, intramolecular hydrogen bonds are formed. These should stabilize the complex to result in a large binding constant. In contrast, for the cyclic hexapeptide, cyclo(L-Leu-L-Phe-L-Pro)$_2$, intramolecular hydrogen bonds are broken upon complexation.

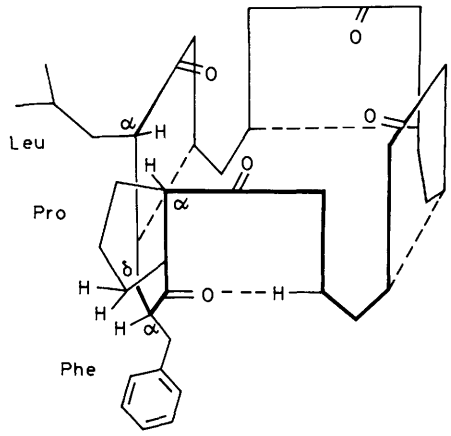

Figure 4. Conformation of cyclo(L-Leu-L-Phe-L-Pro)$_4$/Ba^{2+} complex in MeCN

This difference should explain the larger binding constant of the cyclic dodecapeptide than that of the cyclic hexapeptide.

It is notable that a cyclic dodecapeptide, such as cyclo(L-Leu-L-Phe-L-Pro)$_4$, adopts a C_4-symmetric conformation with four β-turns and that on complexation with metal ions forms intramolecular hydrogen bonds. This structure is similar to the bracelet-type conformation of valinomycin–metal ion complex.

Experimental

Synthesis.—The cyclic dodecapeptide was synthesized by the liquid-phase method (see Figure 6). Boc(L-Leu-L-Phe-L-Pro)$_2$OBzl was synthesized by fragment condensation of the tripeptides using dicyclohexylcarbodi-imide (DCCI) as a coupling reagent. [δ_H(90 MHz; solvent $CDCl_3$; standard Me_4Si) 0.90—1.11 (12 H, d, Leu $C^{\delta}H$), 1.11—1.47 (9 H, s, Boc Me), 1.47—1.75 (2 H, m, Leu $C^{\gamma}H$), 1.75—2.36 (8 H, m, Pro $C^{\beta}H$ and $C^{\gamma}H$), 2.63—3.27 (8 H, m, Leu and Phe $C^{\beta}H$), 3.27—3.86

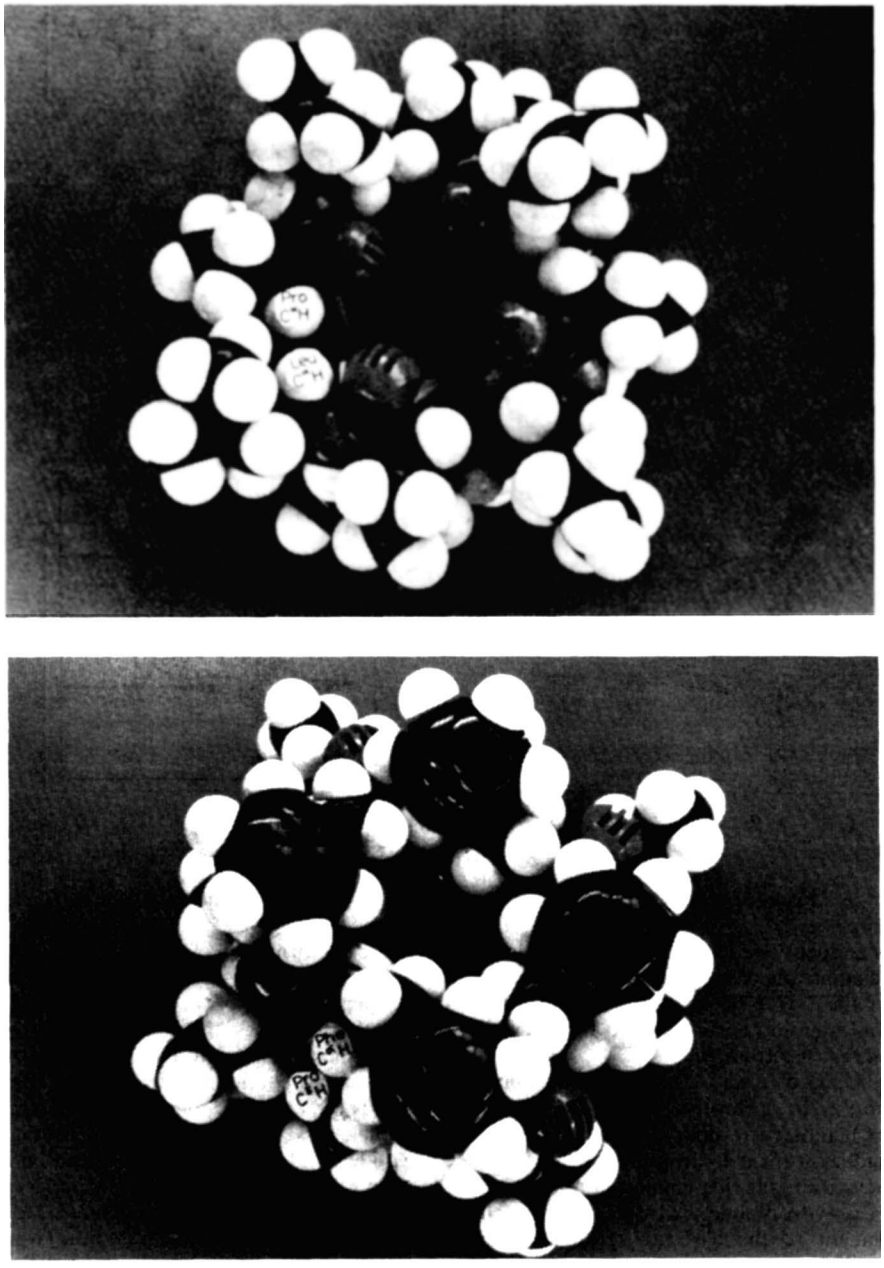

Figure 5. CPK molecular model of cyclo(L-Leu-L-Phe-L-Pro)$_4$/Ba^{2+} complex

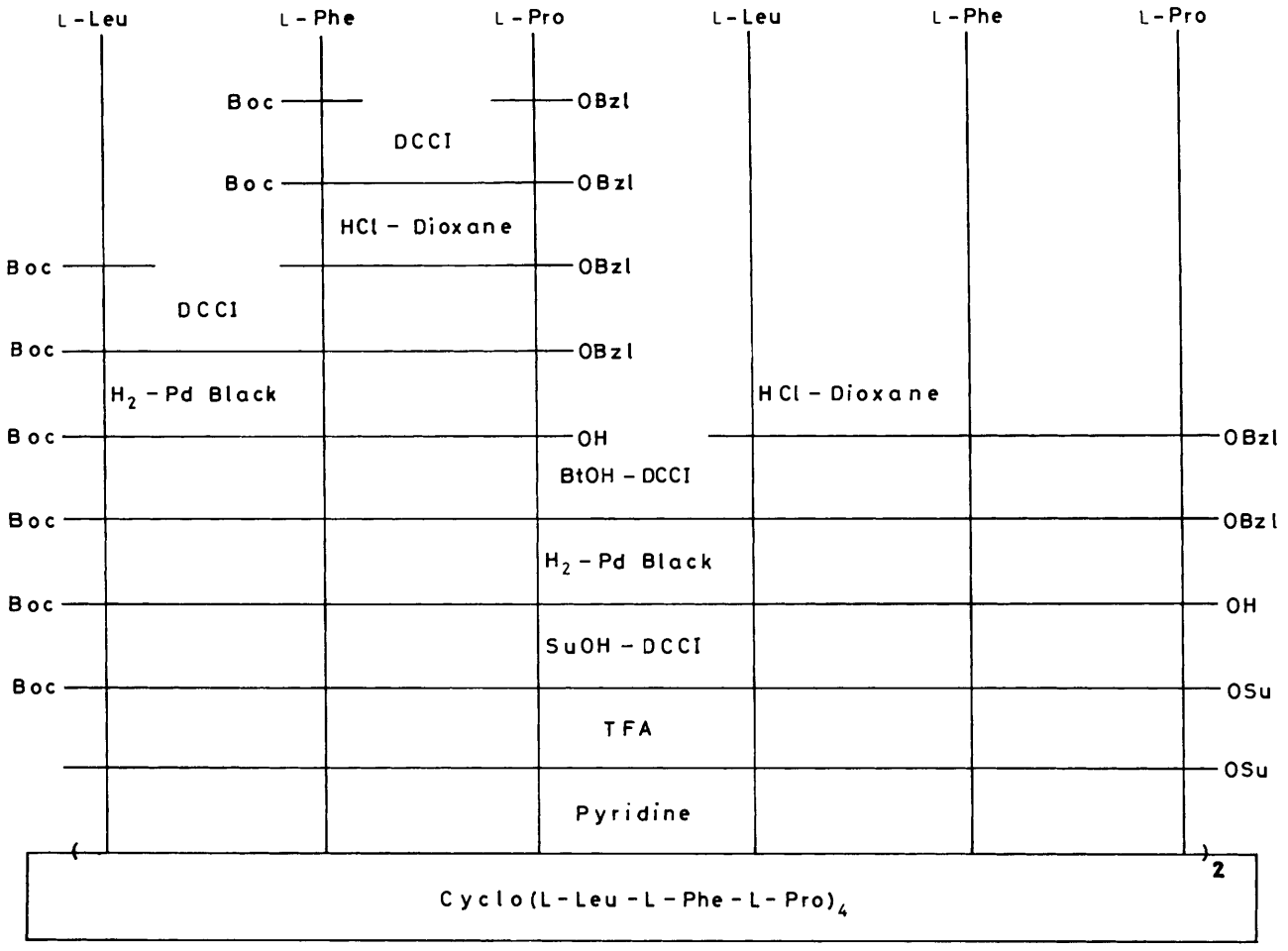

Figure 6. Synthetic route of cyclo(L-Leu-L-Phe-L-Pro)$_4$

(6 H, m, Proc C$^{\alpha}$H and C$^{\delta}$H), 4.23—4.64 (2 H, m, Phe C$^{\alpha}$H), 4.64—5.05 (2 H, m, Leu C$^{\alpha}$H), 5.05—5.20 (2 H, s, OBzl CH_2), 5.85—6.40 (1 H, d, Leu^1 NH), 6.75—7.06 (2 H, d, Phe^2 NH), 7.06—7.48 (10 H, m, 2 × Ph), 8.39—8.85 (1 H, d, Leu^2 NH)]. (Details of the synthesis will be reported elsewhere.[17]) The benzyl ester group was removed by catalytic hydrogenation, and the C-terminal carboxy group was converted into a succinimide ester group. The obtained Boc(L-Leu-L-Phe-L-Pro)$_2$OSu (574 mg) was treated with trifluoroacetic acid at 0 °C for 30 min, and ether was added to solidify the product. After drying over NaOH pellet, the powder was dissolved in dimethylformamide (DMF) (10 ml), and the solution was added dropwise to pyridine (800 ml). The solution was stirred overnight, and condensed under reduced pressure. The crude products were purified by gel permeation chromatography (G.P.C.) with a LH 20 column (2 × 180 cm) using methanol as eluant. In the elution profile, there were two intensive peaks at 185 ml (peak 1) and 220 ml (peak 2) elution volume, which were ninhydrin negative but I_2 positive, indicating the formation of cyclic peptides. The slower elution component, peak 2, was identified as cyclo(L-Leu-L-Phe-L-Pro)$_2$ (81.4 mg, 17.5%) by F.A.B. mass spectroscopy and elemental analysis; *m/z* 715 (MH^+); (Found: C, 65.06; H, 7.39; N, 11.38. $C_{40}H_{54}N_6O_6$ requires C, 64.88; H, 7.49; N, 11.29%). Consequently, the faster elution component, peak 1, was ascribed to cyclo(L-Leu-L-Phe-L-Pro)$_4$, a product of dimerization–cyclization reactions [78.5 mg, 16.9% on the basis of Boc(L-Leu-L-Phe-L-Pro)$_2$OSu], m.p. 170 °C; (Found: C, 64.86; H, 7.48; N, 11.32. $C_{80}H_{108}N_{12}O_{12}$ requires C, 64.88; H, 7.49; N, 11.29%).

Measurement.—N.m.r. measurements were carried out on a JEOL FX 90Q FT NMR spectrometer (90 MHz for ^{1}H and 22.5 MHz for ^{13}C n.m.r.) and a NICOLET NT-300 spectrometer (300 MHz for ^{1}H n.m.r.). The concentration of peptide was 16.3 mM. C.d. measurements were carried out on a JASCO J-20 spectropolarimeter.

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