CONFORMATION OF SEQUENTIAL POLYPEPTIDE POLY(LEU-LEU-D-PHE-PRO) AND FORMATION OF ION CHANNEL ACROSS BILAYER LIPID MEMBRANE

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ABSTRACT Sequential polypeptide, poly(Leu-Leu-D-Phe-Pro), containing a part of β-turn sequence in gramicidin S, was synthesized and investigated as a model for ion channels. Sequential peptides, Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl[1] (n = 1 – 4), were also synthesized to acquire conformational information about this polypeptide. From the analyses by NMR, CD, and IR measurements, intramolecular hydrogen bonds were found in the sequential peptides with n larger than two and Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl was deduced to adopt a 3_{10}-helical conformation. Poly(Leu-Leu-D-Phe-Pro) was also suggested to have this conformation. With the addition of this polymer to oxidized cholesterol membrane, current-voltage response across the membrane was observed. Stepwise fluctuation of current was recorded under a positive electrtic field to support the channel formation. This polymer might form bundles of 3_{10}-helices across the bilayer lipid membrane to pass through the ion.

INTRODUCTION

The biological significance of ion-channels in biomembranes has been well recognized. Channel-forming molecules are classified into two groups; in one of them the stability of the conducting state is independent of the electric field (or the membrane potential), and in the other it depends on it due to dipole interactions. The former group includes gramicidin A (1), nystatin (2), and amphotericin B (3), and the latter group excitability inducing material (4), hemocyanine (5), and alamethicin (6). In the first group, gramicidin A has been well investigated, and a head-to-head dimer of single-stranded β-helix has been suggested for the structure of the channel formed in membrane (7–9). On the other hand, alamethicin has been reported to have an α-helical conformation (10, 11), and to form an aggregate called "barrel-stave model" under an electric field in the membrane (12–16).

Several kinds of polypeptides have been synthesized to obtain molecules having the ability to form an ion channel. D–L alternative sequential polypeptides (17–19) and α-aminoisobutyric acid-containing polypeptides (20, 21) have been investigated as model peptides for gramicidin A and alamethicin, respectively. On the other hand, Spach (22) showed that bundles of α-helical polypeptides could form channels in membrane.

To clarify the nature of ion channels in terms of molecular conformation, investigation of various regular polypeptides is necessary. In the present investigation, poly(Leu-Leu-D-Phe-Pro), which contains a part of the sequence of gramicidin S and is expected to form a β-turn (23, 24), was synthesized and its conformation and ability of ion-channel formation were investigated. This polymer would not adopt an α-helical conformation because of the presence of strongly helix-breaking Pro residues (25), but it was expected to have a regular structure containing β-turns. A pseudo helicoidal conformation involving successive β-turns has been proposed for poly(Pro-Tyr-Pro-Gln-Gln) (26). A conformation containing successive β-turns would be suitable for channel formation because of a probable formation of a loose spiral having a large pore in the center.

MATERIALS AND METHODS

Synthesis

Synthesis of poly(Leu-Leu-D-Phe-Pro) was carried out in liquid phase method according to Fig. 1. Boc-Leu-Leu-D-Phe-Pro-OBzl Boc-Leu-OSu (1.62 g) and HCl·Leu-D-Phe-Pro-OBzl (2.64 g), which were synthesized by a stepwise elongation to the N-terminal direction, were dissolved in CH_2Cl_2 (10 ml), and triethylamine (0.686 ml) was added. After stirring overnight, the solvent was evaporated and replaced by ethyl acetate, and the solution was washed with 10% citric acid, 4% sodium bicarbonate, and water. The solution was dried with Na_2SO_4 and condensed under reduced pressure. The residue was purified by HPLC using Megapak 201 (Japan Spectroscopic Co., Ltd., Japan), and recrystallized from ethyl acetate/hexane. The yield was 79.2%. Boc-Leu-Leu-D-Phe-Pro-OSu Boc-Leu-Leu-D-Phe-Pro-OBzl was subjected to hydrogenation in methanol using Pd/black as a catalyst. After removal of the catalyst, the solution was evaporated under reduced pressure, and the residue was purified by

[1] *Abbreviations used in this paper*: Boc; t-butyloxycarbonyl, OBzl; benzylester, OSu; succinimideester, HOSu; N-hydroxysuccinimide, DCCI; dicyclohexylcarbodiimide, TFA; trifluoroacetic acid, NEt_3; triethylamine, DMF; dimethylformamide, HPLC; high pressure liquid chromatography.

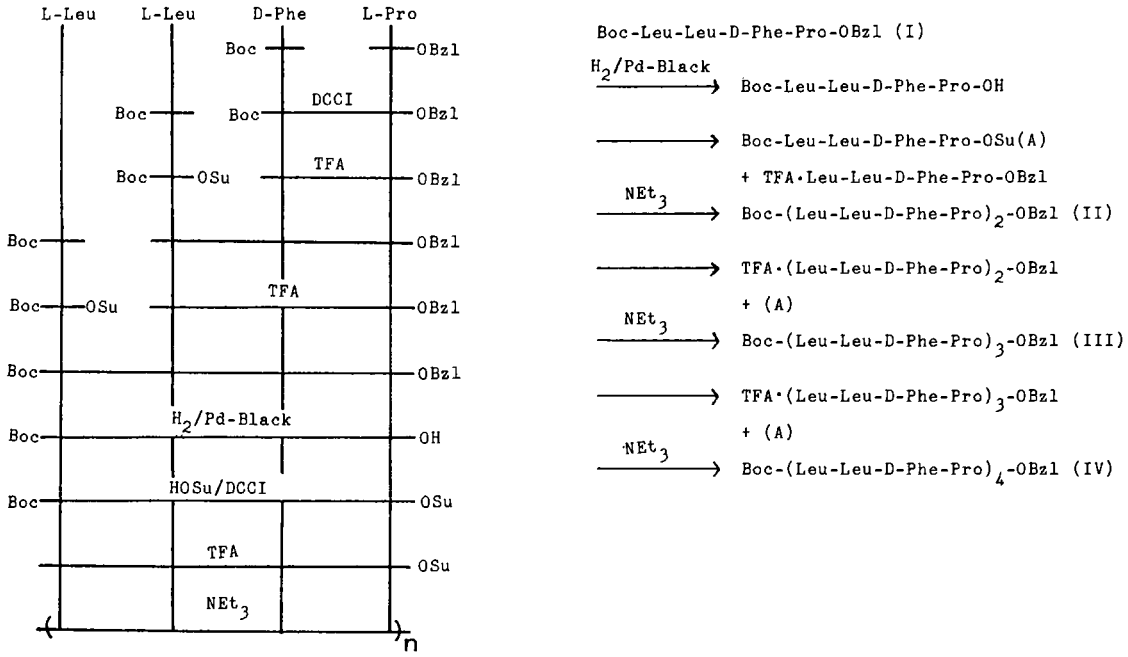


FIGURE 1 Synthetic route of poly(Leu-Leu-D-Phe-Pro) and Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl (n = 1–4).

HPLC using Megapak 201, and precipitated by hexane. Boc-Leu-Leu-D-Phe-Pro-OH (1.50 g) and HOSu (0.44 g) were dissolved in DMF, and DCCI (0.58 g) was added at 0°C. After stirring overnight at room temperature, the solvent was evaporated and replaced by ethyl acetate, and the precipitate was filtered off. The filtrate was washed with 4% sodium bicarbonate and water, and dried with Na_2SO_4. The product was purified by HPLC using Megapak 201. The yield was 52.0%. Poly(Leu-Leu-D-Phe-Pro) TFA (7 ml) and anisole (0.5 ml) were added to Boc-Leu-Leu-D-Phe-Pro-OSu (1.0 g) at 0°C. After 30 min, the solution was concentrated under reduced pressure and ether was added to crystallize the product. TFA·Leu-Leu-D-Phe-Pro-OSu was dissolved in DMF (2.5 ml), and NEt_3 (0.4 ml) was added. To avoid the viscosity increase of the solution during the polymerization, additional DMF was added. After 3 d, methanol (50 ml) was added to precipitate the polymeric product. The product was reprecipitated by the addition of methanol/ether.

Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl (n = 1 – 4) were synthesized by the fragment condensation method in liquid phase according to the scheme described in Fig. 1.

Table I shows the elemental analyses of synthetic products.

Spectroscopic Measurements

CD, IR, 400 MHz proton NMR, 300 MHz proton NMR, and 22.5 MHz carbon-13 NMR spectra were measured on a JASCO spectropolarimeter (model J-20; JASCO, Tokyo, Japan), Digilab (model FTS-15E; Digilab, Tokyo, Japan), JEOL (model JNM-GX400; JEOL, Tokyo, Japan), Nicolet (model QE300; Nicolet, Japan), and JEOL (model FX90Q; JEOL, Tokyo, Japan), respectively.

Bilayer Lipid Membrane

The bilayer lipid membrane was formed on an aperture of 0.5 mm diameter separating two electric cell chambers, each of which was filled with 3 ml of KCl solution. The n-decane solution of oxidized cholesterol was used to form the membrane. The polypeptide dissolved in trifluoroethanol (a few μl of 6.8 × 10^{-4} M solution) was added initially to the negative side of membrane potential.

RESULTS AND DISCUSSION

Conformation of Poly(Leu-Leu-D-Phe-Pro)

From vapor pressure osmometry, the molecular weight of this polymer was estimated to be 15,000. This polypeptide is soluble in chloroform, 1,2-dichloroethane, and trifluoroethanol, but insoluble in methanol and water.

Fig. 2 shows the N–H stretching region of IR spectra of poly(Leu-Leu-D-Phe-Pro) and peptides (I), (II), (III), and (IV) in chloroform. Peptides (II), (III), and (IV) and poly(Leu-Leu-D-Phe-Pro) showed an absorption at ~3,300 cm^{-1}, which is ascribed to hydrogen-bonded N–H (27). This absorption was not found in peptide (I). The concentration dependence of this absorption was examined for peptide (IV). When the concentration was raised to a higher value than 10^{-2} M, the IR spectra changed, indicating the occurrence of intermolecular aggregation. Since the spectra in Fig. 2 were measured at the concentration of

TABLE I
ELEMENTAL ANALYSES OF SYNTHESIZED PEPTIDES

		C	H	N
Boc-Leu-Leu-D-Phe-Pro-OBzl	Found	67.46	8.10	8.49
	Calcd	67.23	8.02	8.25
Boc-(Leu-Leu-D-Phe-Pro)$_2$-OBzl · 1/2H_2O	Found	66.29	8.13	9.70
	Calcd	66.46	7.93	9.69
Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl · H_2O	Found	65.90	8.08	10.32
	Calcd	66.07	8.01	10.27
Boc-(Leu-Leu-D-Phe-Pro)$_4$-OBzl · 3/2H_2O	Found	65.74	8.29	10.58
	Calcd	65.79	8.14	10.51
poly(Leu-Leu-D-Phe-Pro) · 1/2H_2O	Found	65.14	8.11	11.78
	Calcd	65.11	8.19	11.68

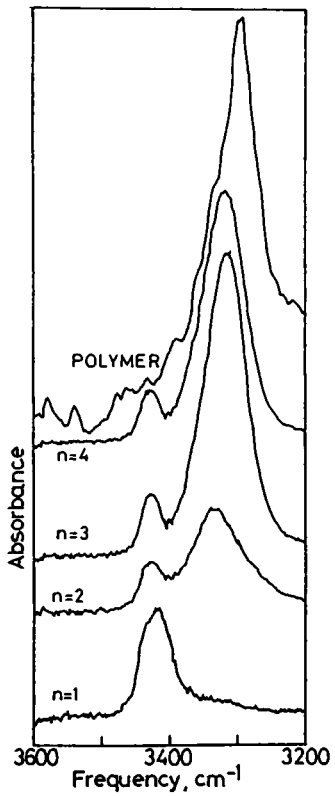


FIGURE 2 N–H stretching absorptions in IR spectra of poly(Leu-Leu-D-Phe-Pro) and Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl (n = 1–4) in chloroform. The concentrations in fragment units of peptides are $\sim 1 \times 10^{-3}$ M.

1×10^{-3} M, the hydrogen bonds found in poly(Leu-Leu-D-Phe-Pro)and peptides (II), (III), and (IV) must be intramolecular.

The effect of TFA on the conformation of poly(Leu-Leu-D-Phe-Pro) in chloroform was measured by 90 MHz proton NMR (Fig. 3). The addition of TFA caused changes of the spectrum in the region of amide protons and C^α protons. This observation suggests that a regular structure of poly(Leu-Leu-D-Phe-Pro) involving intramolecular hydrogen-bonds exists in chloroform that were broken by the addition of TFA.

Further evidence for this polypeptide having a regular structure is obtained from measurement of C^α chemical shift of Pro residue. This signal has been reported to shift to a lower magnetic field when it is involved in a helically folded structure (28). In the case of peptide (I), which is not believed to adopt any regular structure, this signal appeared at a higher magnetic field than 60 ppm (at 58.9 ppm). In the case of peptide (III), two of three C^α signals of Pro appeared at lower magnetic fields than 60 ppm, indicating that these two Pro residues are involved in a helical structure. A C^α signal of Pro residue in poly(Leu-Leu-D-Phe-Pro) appeared at 61.6 ppm, indicating a helical conformation of this polypeptide.

Fig. 4 shows the CD spectra of poly(Leu-Leu-D-Phe-Pro)and peptides (I), (II), (III), and (IV) in trifluoroethanol. Negative Cotton effects were observed at 207 and 220 nm. This pattern of spectrum resembles those of an α-helix, a 3_{10}-helix (29), and gramicidin S (30). According to Sudha et al., the chiroptical distinction between α-helix and 3_{10}-helix is uncertain (29). As the length of peptides increased from (I) to (IV), the CD spectra became similar to that of poly(Leu-Leu-D-Phe-Pro). From the carbon-13 NMR measurement peptides (I) and (II) were found to contain the *cis* peptide bond of Pro (31), but peptide (III) and poly(Leu-Leu-D-Phe-Pro) were found to contain only *trans* peptide bonds (spectra are not shown). Combination of the CD, IR, and carbon-13 NMR data leads to an interpretation that peptides (III) and (IV) take the same conformation as that of poly(Leu-Leu-D-Phe-Pro). Therefore, the regular structure of poly(Leu-Leu-D-Phe-Pro) could be deduced from the conformational analyses of peptide (III) or (IV).

400 MHz proton NMR measurement was carried out for peptide (III) in $CDCl_3$. Fig. 5 shows the assignment of each signal on the basis of decoupling experiments. Two signals of the amide proton of Leu residues appeared distinctly at higher magnetic field than others. One of them located at 6.3 ppm could be assigned to the Leu residue adjacent to a urethane group from its chemical shift. The other amide proton signal appearing at 4.9 ppm might be a result of aromatic ring current effects of the adjacent Phe residue.

The effect of methanol addition (1–5%) on the amide proton region of the NMR spectrum of peptide (III) in $CDCl_3$ is shown in Fig. 6 *A*. The addition of a small amount of methanol did not have any influence on the conformation of peptide, which was confirmed by the invariable coupling constants. A part of amide proton signals is not shown in Fig. 6 *A* because of overlapping with signals of Phe residues. However, as a whole, the signals appearing at ~7.5 ppm were not influenced very much by the methanol addition. On the other hand, two amide protons of Leu residues at higher magnetic fields shifted to a considerably lower magnetic field. Furthermore, the rate of H–D exchange with CD_3OD of these two amide protons was faster than others. Therefore, two amide protons of Leu residues were suggested to be exposed to solvent, and the others are involved in intramolecular hydrogen-bonding and shielded from solvent. This conclusion is supported by the examination of NH stretching region of IR spectrum (Fig. 2), which shows a large degree of intramolecular hydrogen-bonding with only a small contribution from free amide protons.

Information about whether or not the carbonyl groups are involved in hydrogen-bonding can be acquired by the examination of the effect of methanol addition on the chemical shifts of carbonyl carbons (Fig. 6 *B*) (32). A signal at 157 ppm, which has been assigned to a carbonyl group of the urethane group, did not shift very much by the

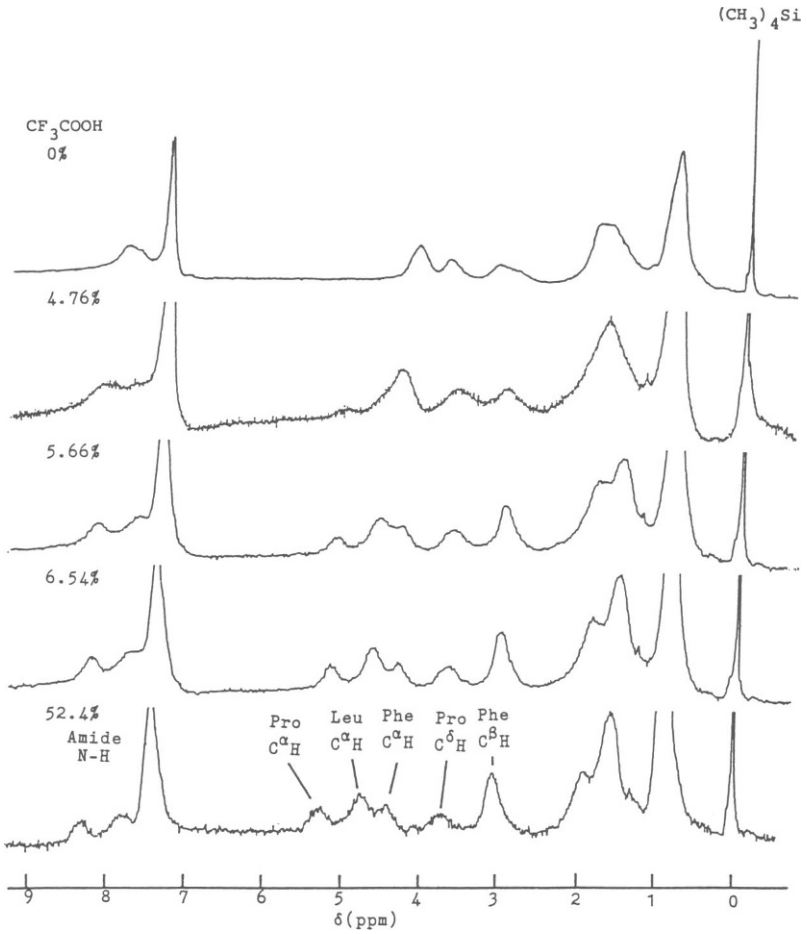


FIGURE 3 Effect of TFA on 90 MHz proton magnetic resonance of poly(Leu-Leu-D-Phe-Pro) in $CDCl_3$.

addition of methanol compared with other carbon signals. For comparison, the chemical shift of the urethane carbonyl carbon in Boc-Leu-Leu-D-Phe-Pro-OBzl, which is exposed to solvent, was 155.72, 155.98, and 156.05 ppm in the presence of 0, 5, and 8% methanol, respectively. Therefore, this carbonyl group of the polymer is considered to be involved in intramolecular hydrogen bonding.

Table II summarizes the pattern of intramolecular hydrogen-bonds for each of possible regular structures of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl. The agreement with the results obtained from NMR measurements is obtained only with peptide (III) having a 3_{10}-helical conformation.

3_{10}-helical structure has been found in short-chain peptides containing α-aminoisobutyric acid (28, 33, 34). On the other hand, long-chain peptides containing α-aminoisobutyric acid tend to adopt an α-helical conformation (10, 35). In the case of peptide (III), the presence of Pro residue, which is the strong α-helix breaker (25), makes the occurrence of α-helical conformation difficult. On the other hand, Pro residue can be incorporated into a 3_{10}-helical conformation as found in Z-(Aib-Pro)$_4$-OMe (28).

The experimental results and these speculations suggest that peptide (III) should adopt a 3_{10}-helical structure.

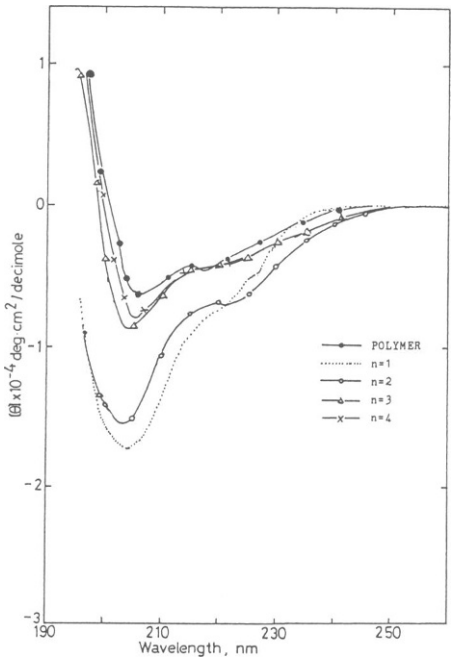


FIGURE 4 CD spectra of poly(Leu-Leu-D-Phe-Pro) and Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl (n = 1–4) in trifluoroethanol. 0.3 mg/ml.

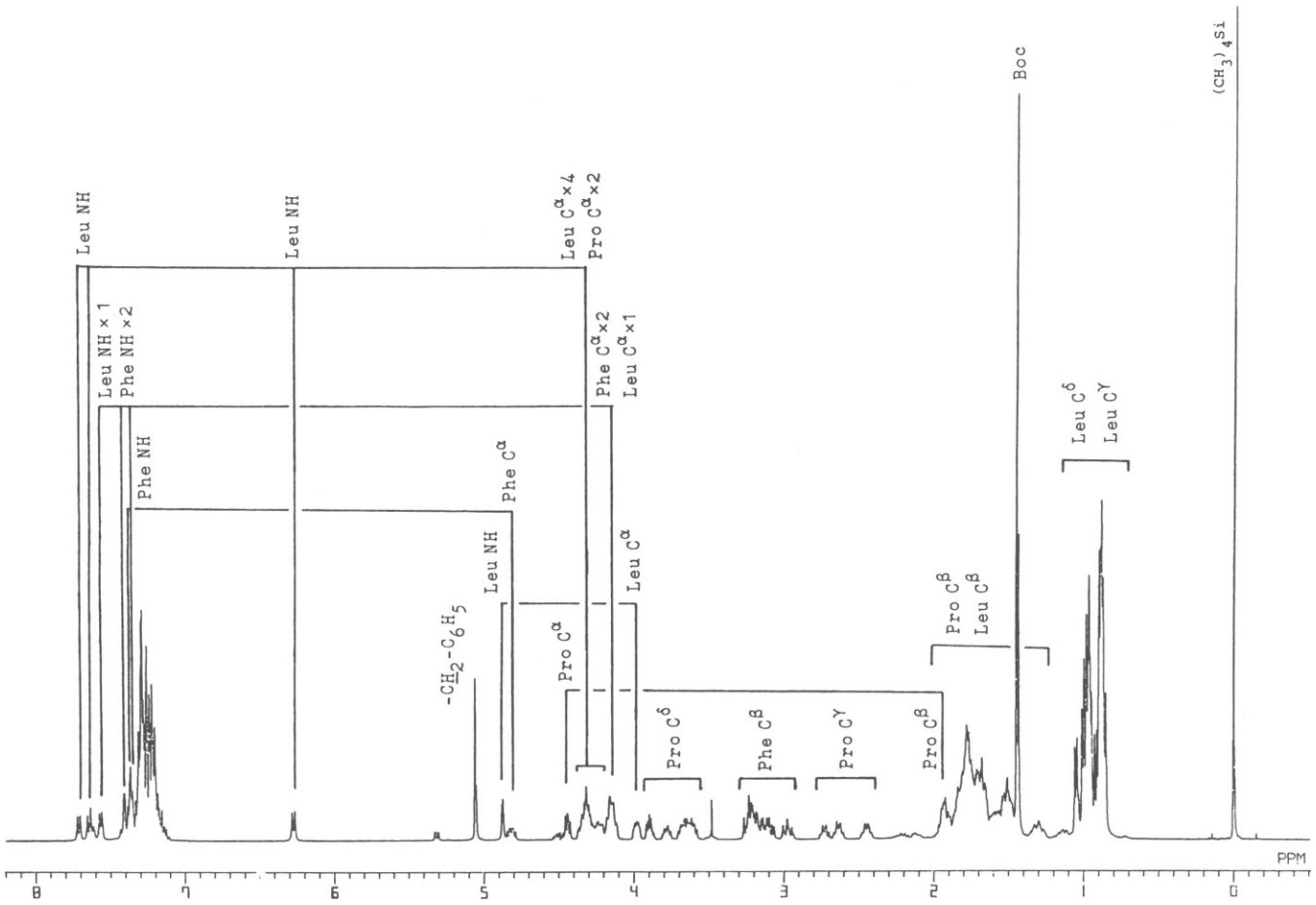


FIGURE 5 400 MHz proton magnetic resonance spectrum of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl in $CDCl_3$.

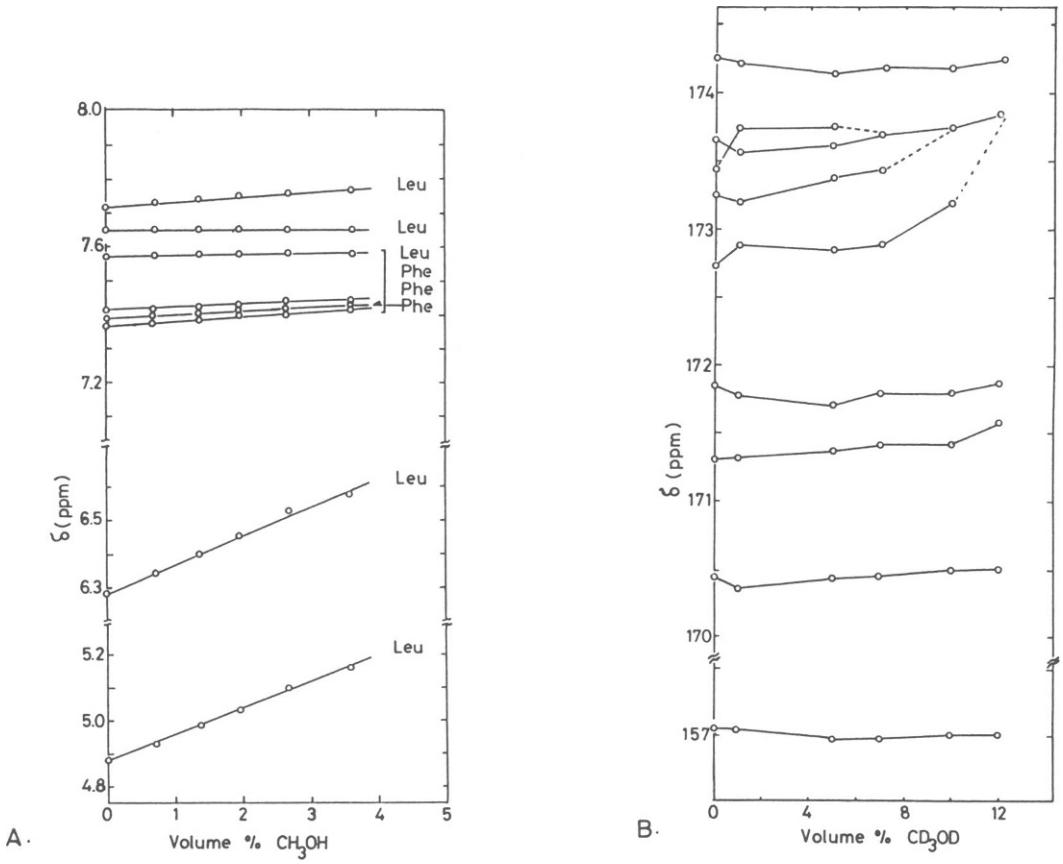


FIGURE 6 (*A*) Effect of methanol addition of NH chemical shift of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl in $CDCl_3$. 10 mg/ml. (*B*) Effect of methanol addition on C═O chemical shift of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl in $CDCl_3$. 50 mg/ml.

TABLE II
HYDROGEN-BONDS CHARACTERISTICS FOR VARIOUS CONFORMATION OF Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl

Conformation	H-bond free NH	Urethane NH	Urethane C=O
α-Helix	Leu^1, Leu^2, D-Phe^3	Not H-Bonded	Not H-Bonded
3_{10}-Helix	Leu^1, Leu^2	Not H-Bonded	H-Bonded
$\beta^{4,1}$-Helix*	Leu^1, Leu^2, D-Phe^3	Not H-Bonded	H-Bonded
$\beta^{6,3}$-Helix‡	Leu^1, Leu^2, D-Phe^3, Leu^5, Leu^6	Not H-Bonded	H-Bonded
$\uparrow\downarrow\beta^{5,6}$-Helix (left hand)	Leu^2	H-Bonded	Not H-Bonded
$\uparrow\downarrow\beta^{5,6}$-Helix (right hand)	Leu^1, D-Phe^3	Not H-Bonded	Not H-Bonded

*6 → 1 Hydrogen-Bonding
‡8 → 1 Hydrogen Bonding

However, the possibility of an intermixing state of an α-helix and a 3_{10}-helix or the others can not be excluded.

The 3_{10}-helix contains a succession of the 4→1 hydrogen-bonds (type III β-turn). Fig. 7 shows the pattern of intramolecular hydrogen-bonds for peptide (III) having a 3_{10}-helix. The 3_{10}-helix is constructed from third to tenth residues. According to the previous conclusion that the conformation of poly(Leu-Leu-D-Phe-Pro) in chloroform is the same as those of peptides (III) and (IV), it is concluded that poly(Leu-Leu-D-Phe-Pro) takes a 3_{10}-helix in chloroform.

Channel Formation of Poly(Leu-Leu-D-Phe-Pro)

The channel formation of poly(Leu-Leu-D-Phe-Pro) in the oxidized cholesterol membrane was investigated. Adding poly(Leu-Leu-D-Phe-Pro) to the negative side of a lipid bilayer and holding the membrane potential at −75 mV, a sharp increase in the current was observed after some tens of minutes. Fig. 8 *A* shows the current-voltage relationship. The maximum current at 75 mV was 55 pA and that at −75 mV was −55 pA. Fig. 8 *B* demonstrates the current change at 75 mV, which can be accounted for by assuming a random fluctuation of the channel population between two conductance states (i.e., closed state and open state). Under the experimental conditions the membrane alone

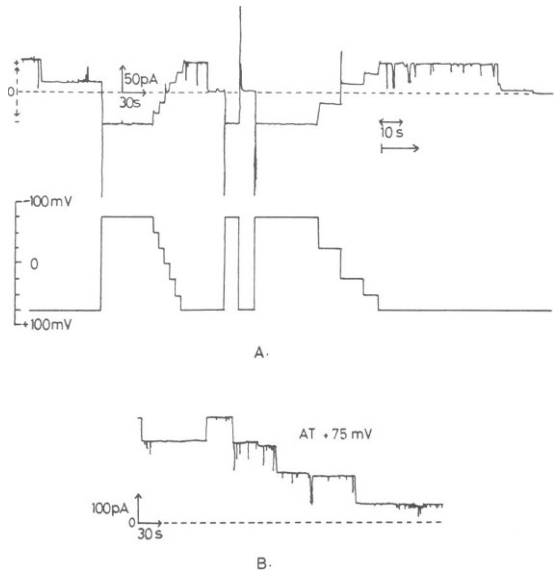
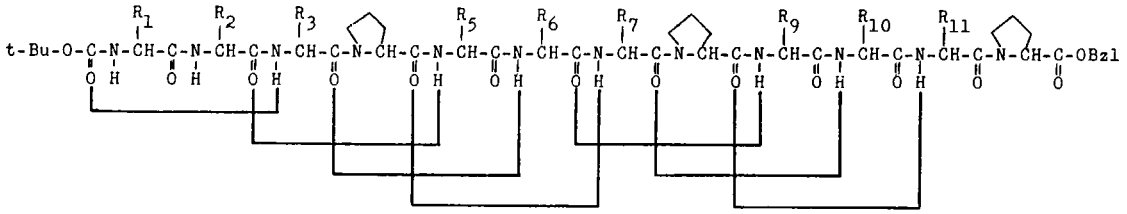


FIGURE 8 (*A*) Current trace through oxidized cholesterol membrane containing poly(Leu-Leu-D-Phe-Pro). 0.5 M KCl, pH 6.8. (*B*) Current fluctuations of oxidized cholesterol membrane containing poly(Leu-Leu-D-Phe-Pro). Applied voltage 75 mV, 1.0 M KCl, pH 6.8.

did not cause current fluctuation even when voltage was continuously applied to the membrane for 1 h. The stepwise current fluctuation was observed only in the presence of poly(Leu-Leu-D-Phe-Pro). Therefore, the observed current fluctuation must be attributed to the interaction of poly(Leu-Leu-D-Phe-Pro) with the lipid membrane. The step size in current was 110 pA in the presence of 1 M KCl, which is twice as large as that in Fig. 8 *A*, because the concentration of KCl was two times greater. Therefore, under the conditions in Fig. 8 *A* only one channel was formed in the membrane, and this state was kept for a long period before the second channel was formed. The current-voltage relationship showed the ohmic behavior between 75 mV and −75 mV, and the steady state conductance was independent of the membrane potential. However, when the applied potential was 75 mV, the current fluctuated and finally the response was lost. A detailed picture of this channel may be elucidated by the precise analysis of the



$R_1, R_2, R_5, R_6, R_9, R_{10} = -CH_2CH(CH_3)_2$

$R_3, R_7, R_{11} = -CH_2C_6H_5$

FIGURE 7 Mode of intramolecular hydrogen-bonds proposed for Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl.

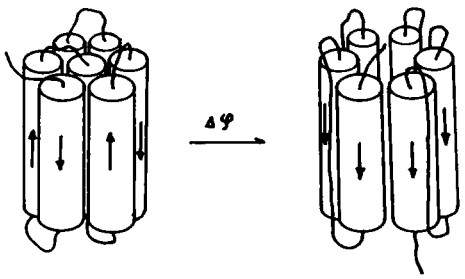


FIGURE 9 Schematic representation of the conformation of poly(Leu-Leu-D-Phe-Pro) in membrane. The rods represent 3_{10}-helices. The details are described in the text.

conductance behavior (concentration dependence, and so on). However, it may be envisioned that the chain of poly(Leu-Leu-D-Phe-Pro) folds back into a bundle of antiparallel segments of 3_{10}-helix, which is energetically favorable. When a membrane potential is applied, the dipoles are oriented parallel to the electric field, leaving a pore in the middle of the intramolecular aggregate, which is illustrated in Fig. 9 (36–39). When an inverse potential was applied, the dipole moment of peptide bonds would be aligned opposite to the electrical gradient, which makes the original state unstable and the structure of channel would be broken to abolish the current response.

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