Studies on β-Turn of Peptides. XII.[1] Synthetic Confirmation of Weak Activity of [D-Pro$^{5,5'}$]-Gramicidin S Predicted from β-Turn Preference of Its Partial Sequence

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[D-Pro$^{5,5'}$]-Gramicidin S was synthesized to confirm a prediction that D-D sequence at the corner position had low preference for β-turn. The analog showed much weaker activity than gramicidin S and also showed different CD spectra from those of gramicidin S. The results indicate that the analog can not take stable, gramicidin S-like, β-sheet conformation.

In the previous papers, we reported that the CD spectra of *N*-(2,4-dinitrophenyl)tetrapeptide *p*-nitroanilides reflected well the β-turn preference of the tetrapeptide sequences.[2-4] β-Turn preferences of the tetrapeptides related to the β-turn part sequences of gramicidin S (GS), *cyclo*(-Val$^{1,1'}$-Orn$^{2,2'}$-Leu$^{3,3'}$-D-Phe$^{4,4'}$-Pro$^{5,5'}$-)$_2$, [5] which was well known to take β-sheet conformation with two β-turns (Fig. 1),[6-8] had good correlation with antibiotic activities of the GS analogs having similar tetrapeptides sequences at their β-turn parts.[1,3] The GS analogs having D-D sequences at 4—5 and 4′—5′ positions were predicted not to take GS-like β-sheet conformation from the result that Dnp-Leu-D-Ala-D-Leu-Val-pNA showed much weaker CD bands than Dnp-Leu-D-Ala-Leu-Val-pNA did.[3] To confirm this prediction, we synthesized [D-Pro$^{5,5'}$]-GS which had D-Phe-D-Pro sequences at 4—5 and 4′—5′ positions.

Results and Discussion

Synthesis of [D-Pro$^{5,5'}$]-GS (8). [D-Pro$^{5,5'}$]-GS was synthesized according to a scheme shown in Fig. 2. In a cyclization reaction, a pentapeptide with D-Pro at the C-terminus was chosen as a precursor, because many GS analogs had been synthesized in satisfactory results by a similar strategy.[9,10] Boc-Val-Orn(Z)-Leu-D-Phe-D-Pro-OH (**4**) was prepared by azide coupling of Boc-Val-Orn(Z)-Leu-N_2H_3[11] and H-D-Phe-D-Pro-OH (**3**), and converted to its active ester (**5**). Cyclization of H-Val-Orn(Z)-Leu-D-Phe-D-Pro-OSu (**6**) derived from **5** by acid treatment was carried out in anhydrous pyridine at the final concentration of 3 mM (1 M=1 mol dm^{-3}). After usual work-up,[12] a mixture of the crude cyclized peptides was applied to a column of Sephadex LH-20 (Fig. 3). The product obtained from the major peak (peak A) was determined to be cyclic dimer by FAB-MS measurement (M^+, 1141) after hydrogenolysis. Peak-B fractions afforded cyclic trimer (M^+, 1711) after hydrogenolysis. It is interesting that cyclization of pentapeptide active ester af-

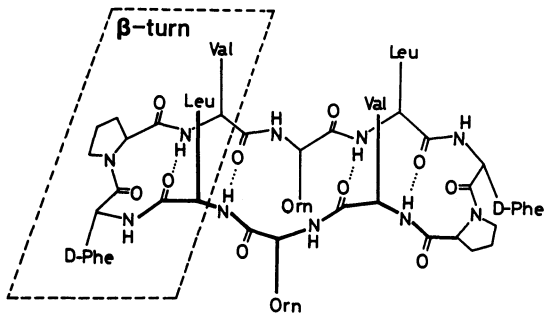

Fig. 1 β-Sheet conformation of gramicidin S.

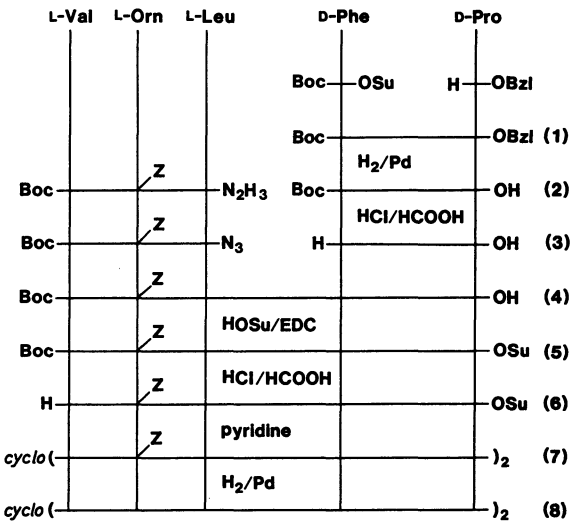

Fig. 2. Synthetic scheme of [D-Pro$^{5,5'}$]-GS (**8**).

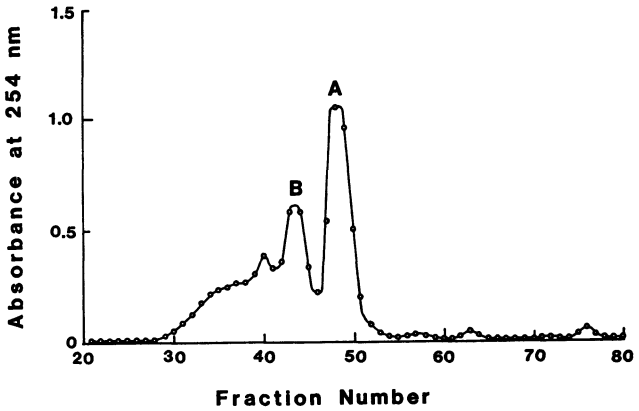

Fig. 3. Elution profile of crude cyclized peptides. For detail, see experimental section.

Table 1. Antibiotic Activity of GS and Its Analog (Minimum Inhibitory Concentration, μg/ml)

Strain	GS	[D-Pro$^{5,5'}$]-GS
Staphylococcus aureus MCI-1380	3.13	100
Bacillus subtilis marfurg 168	1.56	25
Escherichia coli C-600	>100	>100

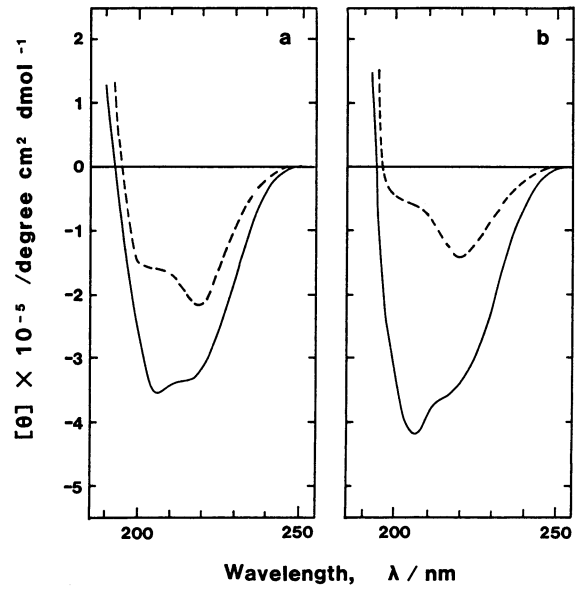

Fig. 4. CD spectra of GS and its analog measured at 25°C in (a) H_2O and in (b) MeOH solutions.
——: GS, ----: [D-Pro$^{5,5'}$]-GS.

forded much polymeric products and almost no cyclic monomer, since there are few papers which reported the absence of cyclic monomerization of pentapeptides.[10,13]. The results suggest that the sequence of [D-Pro$^{5,5'}$]-GS is unsuitable for compact, folded conformation.

Biological Activity. The antibiotic activity of the synthesized analog was compared with that of GS. The minimum concentration of the compound necessary for the complete inhibition of growth of several microorganisms was determined by a dilution method using a nutrient agar (Table 1). The analog showed much weaker activity than GS indicating that Pro at 5, 5′ position could not be replaced with D-Pro without affecting on its activity.

CD Spectra. In many GS analogs, good correlation was observed between their CD spectra and activities.[11,14] CD spectra of [D-Pro$^{5,5'}$]-GS and GS were measured in H_2O and in MeOH solutions (Fig. 4). The analog showed different CD patterns from those of GS in both solutions and also showed larger solvent dependence of CD intensities than GS did. These results suggested that the analog did not take stable,

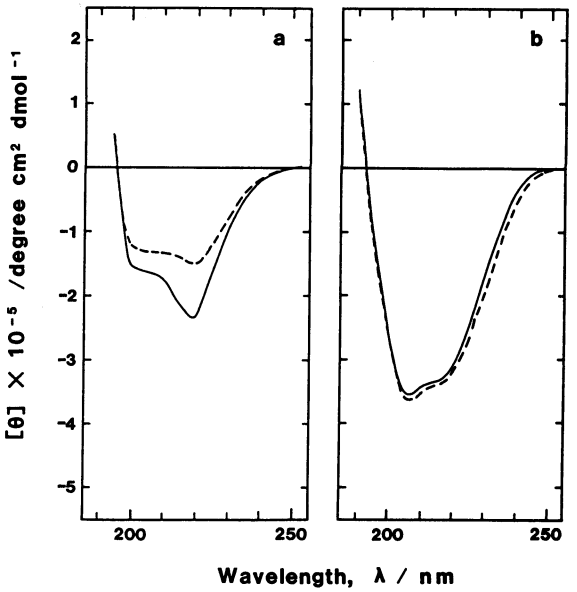

Fig. 5. Temperature dependence of CD spectra of (a) [D-Pro$^{5,5'}$]-GS and (b) GS in H_2O solution.
——: 10°C, ----: 85°C.

GS-like, β-sheet conformation. The large temperature dependence of CD spectra of the analog also suggested that the analog had flexible conformation (Fig. 5).

Structure-Activity Relationship. The decreased activity of the analog is considered to be caused by the conformational change as described above. And it is quite reasonable to consider that the conformational change is caused by the reluctance of D-Phe-D-Pro sequence to take stable, GS-like, β-turn conformation.

Present study proves that CD spectra of the chromophoric derivatives of tetrapeptides are useful for predicting the conformation and activity of bioactive peptides such as GS in which β-turn conformation plays an important role for its activity.

Experimental

All the melting points were uncorrected. Thin-layer chromatographies were carried out on Merck silica gel 60 F_{254} plates with the following solvent systems, the ratio in parentheses after the solvent system being indicated by vol: R_f^1, $CHCl_3$–MeOH (5:1); R_f^2, $CHCl_3$–MeOH–AcOH (95:5:1); R_f^3, *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2); R_f^4, *n*-BuOH–AcOH–H_2O (4:1:5, organic). Optical rotations were measured on a JASCO DIP-360 digital polarimeter. FAB-MS spectra were measured on a JEOL HX-100 mass spectrometer.

Boc-D-Phe-D-Pro-OBzl (1). To a chilled solution of H-D-Pro-OBzl·HCl (1.21 g, 5 mmol) in $CHCl_3$ (20 ml) were added TEA (0.7 ml, 5 mmol) and Boc-D-Phe-OSu (1.81 g, 5 mmol). After being stirred overnight at room temperature, the solution was evaporated under reduced pressure. The residue was dissolved in EtOAc and washed succesively with 10% citric acid, 4% $NaHCO_3$, and H_2O, and the solution was dried over anhydrous Na_2SO_4. After evaporation of the

solvent, the residue was crystallized by addition of ether and recrystallized from EtOAc-ether; yield, 1.76 g (78%); mp 104—108 °C; $[\alpha]_D^{24}$ 50.2° (*c* 0.7, MeOH); R_f^1=0.83, R_f^2=0.73, R_f^3=0.86, R_f^4=0.87.

Found: C, 68.97; H, 7.11; N, 6.37%. Calcd for $C_{26}H_{32}O_5N_2$: C, 69.01; H, 7.13; N, 6.19%.

Boc-D-Phe-D-Pro-OH (2). Compound **1** (769 mg, 1.7 mmol) dissolved in MeOH (20 ml) was hydrogenated in the presence of Pd-black (ca. 200 mg) for 5 h at room temperature. After filtration of catalyst, the filtrate was evaporated to leave an colorless oil which was used for the next reaction without further treatment; yield was quantitative; R_f^1=0.36, R_f^2=0.21, R_f^3=0.58, R_f^4=0.65.

H-D-Phe-D-Pro-OH·HCl (3·HCl). Compound **2** (616 mg, 1.7 mmol) was dissolved in 0.1 M hydrogen chloride in formic acid (26 ml). The solution was allowed to stand at room temperature for 30 min and evaporated. The desired product was collected by filtration with the aid of ether and used for the next reaction without further treatment; yield was quantitative; R_f^3=0.48, R_f^4=0.38.

Boc-Val-Orn(Z)-Leu-D-Phe-D-Pro-OH (4). To a solution of Boc-Val-Orn(Z)-Leu-N_2H_3[11] (541 mg, 0.91 mmol) in DMF (5 ml) were added 2 M hydrogen chloride in EtOAc (1.4 ml) and isopentyl nitrite (0.14 ml, 1.0 mmol) at −60 °C. After being left to stand at −20 °C for 10 min, the solution was cooled again to −60 °C and neutralized with TEA (0.38 ml, 2.73 mmol). To the mixture was added a chilled solution of **3**·HCl (508 mg, 1.7 mmol) and TEA (0.48 ml, 3.4 mmol) in DMF (3 ml). The reaction mixture was stirred at 5 °C for 2 d and then evaporated. Addition of 10% citric acid to the residue gave a white precipitate. The crude product (794 mg, >100%) dissolved in MeOH (2 ml) was applied to a column (3×170 cm) of Sephadex LH-20 and eluted with MeOH, each 12 ml fraction being collected. The fractions (No. 49—55) containing the desired product detected by UV absorption and TLC were collected and evaporated, and the residue was recrystallized from MeOH-ether; yield, 518 mg (69%); mp 116—120 °C; $[\alpha]_D^{24}$ −15.6° (*c* 0.6, MeOH); R_f^1=0.30, R_f^2=0.66, R_f^3=0.70, R_f^4=0.77.

Found: C, 62.11; H, 7.67; N, 9.98%. Calcd for $C_{43}H_{62}O_{10}N_6 \cdot 0.5H_2O$: C, 62.08; H, 7.63; N, 10.10%.

Boc-Val-Orn(Z)-Leu-D-Phe-D-Pro-OSu (5). To a chilled solution of **4** (411 mg, 0.5 mmol) and HOSu (115 mg, 1 mmol) in DMF (4 ml) was added a solution of EDC·HCl (192 mg, 1 mmol) in $CHCl_3$ (1 ml). After being stirred at 5 °C overnight, the solution was evaporated under reduced pressure. The precipitate fromed by the addedion of chilled H_2O was collected by filtration and dried in vacuo over P_2O_5. The product was used for next reaction without further treatment; yield 441 mg (96%); R_f^1=0.79, R_f^2=0.21.

H-Val-Orn(Z)-Leu-D-Phe-D-Pro-OSu·HCl (6·HCl). Compound **5** (441 mg, 0.48 mmol) was dissolved in 0.1 M hydrogen chloride in formic acid (7.2 ml). The solution was allowed to stand at room temperature for 30 min and evaporated to leave an oil which was crystallized by addition of ether. The product was used for the next reaction without further treatment; yield 390 mg (90%); R_f^1=0.25.

***Cyclo*[-Val-Orn(Z)-Leu-D-Phe-D-Pro-]$_2$ (7).** A solution of **6**·HCl (390 mg, 0.46 mmol) in DMF (5 ml) was added dropwise into pyridine (148 ml) at room temperature. The final concentration was 3 mM. The reaction mixture was stirred overnight and evaporated. The residue was dissolved in mixture (36 ml) of MeOH-H_2O (5:1, by vol.), and applied to columns (1.6×10 cm each) of Dowex 50 (H^+ form) and Dowex 1 (OH^- form). The columns were washed with the same solvent (100 ml) and the combined effluent was evaporated to leave a white solid, which was collected with the aid of H_2O. The crude product (198 mg, 61%) dissolved in MeOH (2 ml) was applied to a column (3×170 cm) of Sephadex LH-20 and eluted with MeOH, each 12 ml fraction being colleted (Fig. 3). The fractions (No. 47—51) containing the desired product were collected and evaporated. The product was recrystallized from MeOH-EtOAc-ether; yield 65 mg (20%); mp 148—153 °C; $[\alpha]_D^{24}$ −42.6° (*c* 0.2, MeOH); R_f^1=0.76, R_f^2=0.12, R_f^3=0.95, R_f^4=0.92.

Found: C, 63.30; H, 7.34; N, 11.59%. Calcd for $C_{76}H_{104}O_{14}N_{12} \cdot H_2O$: C, 63.14; H, 7.53; N, 11.63%.

***Cyclo*(-Val-Orn-Leu-D-Phe-D-Pro-)$_2$·2HCl ([D-Pro$^{5,5'}$]-GS·2HCl) (8·2HCl).** Compound **7** (48 mg, 0.03 mmol) dissolved in 0.02 M hydrogen chloride in MeOH (3 ml) was hydrogenated in the presence of Pd-black (ca. 100 mg) for 3 h at room temperature. After filtration of catalyst, the filtrate was evaporated and the residue was recrystallized from MeOH-ether; yield, 30 mg (83%); mp 251—255 °C; $[\alpha]_D^{24}$ −40.9° (*c* 0.7, MeOH); R_f^3=0.62, R_f^4=0.15; MS (FAB), *m/z* 1141 ($C_{60}H_{92}O_{10}N_{12}$, M^+).

Found: C, 55.78; H, 7.71; N, 12.95%. Calcd for $C_{60}H_{92}O_{10}N_{12} \cdot 2HCl \cdot 4H_2O$: C, 56.02; H, 7.99; N, 13.07%

Paper Electrophoresis. Electrophoresis was carried out with Whatman No. 1 paper and a solvent system of formic acid-AcOH-MeOH-H_2O (1:3:6:10, by vol., pH 1.8) for 2 h at 500 V/30cm. [D-Pro$^{5,5'}$]-GS showed single spot and the mobility was as follows: [D-Pro$^{5,5'}$]-GS, 7.3 cm; GS, 7.5 cm.

CD Measurements. CD spectra were recorded on a JASCO J-40 spectropolarimeter in 0.1 mM solution using a cell of 1 mm pass length.

Microbiological Assays. The minimum concentration of the compounds necessary for the complete inhibition of growth of several microorganisms was determined by a dilution method using a nutrient agar "Eiken" Sensitivity Test Agar E-MC10.

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5) The abbreviations used in this paper are those recommended by IUPAC-IUB: *Eur. J. Biochem.*, **138**, 9 (1984). Additional abbreviations: EDC-1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; pNA, *p*-nitroanilide; DMF, *N,N*-dimethylformamide; TEA, triethylamine; GS, gramicidin S. Amino acids are of L-configuration unless otherwise mentioned.

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