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Studies of Peptide Antibiotics. XV. The Synthesis of *cyclo*-(L-Valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl-glycyl-glycyl)

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A cyclic heptapeptide, *cyclo*-(L-Val-L-Orn-L-Leu-D-Phe-L-Pro-Gly-Gly-), was synthesized in order to investigate a contribution for an antibacterial activity of a ring size of a cyclic peptide. A linear heptapeptide *p*-nitrophenyl ester, in which an δ-amino function of ornithine residue was protected with benzyloxycarbonyl group, was transformed to the cyclic benzyloxycarbonyl-substituted heptapeptide which was hydrogenated to afford the desired cyclic heptapeptide as hydrochloride. The cyclic peptide thus obtained showed no activity against any of microorganisms tested.

In studies of the relationship between chemical structure and biological activity of antibacterial cyclic peptides, the cyclic penta- and hexapeptide, cyclosemigramicidin S[4] and *cyclo*-(L-Val-L-Orn-L-Leu-D-Phe-L-Pro-Gly-)[5], which contain the partial amino acid sequence found in gramicidin S and tyrocidines, were prepared, and their biological activities examined. However, in spite of its apparent resemblance to those natural antibiotics, the cyclic penta- and hexapeptide were found to be inactive. Furthermore, a cyclic hexapeptide designated as gramicidin J_2 was synthesized, and the peptide showed no activity.[6] Although the reason for the inactivity is not yet clear, other structural features such as appropriate ring sizes may be necessary in this type of molecule for the exhibition of the activity. Therefore, it became of interest to determine whether or not a larger cyclic peptide with an appropriate amino acid sequence would exhibit the antibacterial properties.

This paper is concerned with the synthesis and an antibacterial assay of a cyclic heptapeptide,

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4) M. Waki and N. Izumiya, *J. Amer. Chem. Soc.*, **89**, 1278 (1967); This Bulletin, **40**, 1687 (1967).

5) T. Kato, M. Kondo, M. Ohno and N. Izumiya, This Bulletin, **38**, 1202 (1965).

6) T. Kato and N. Izumiya, *J. Biochem.*, **59**, 629 (1966); This Bulletin, **39**, 2242 (1966).

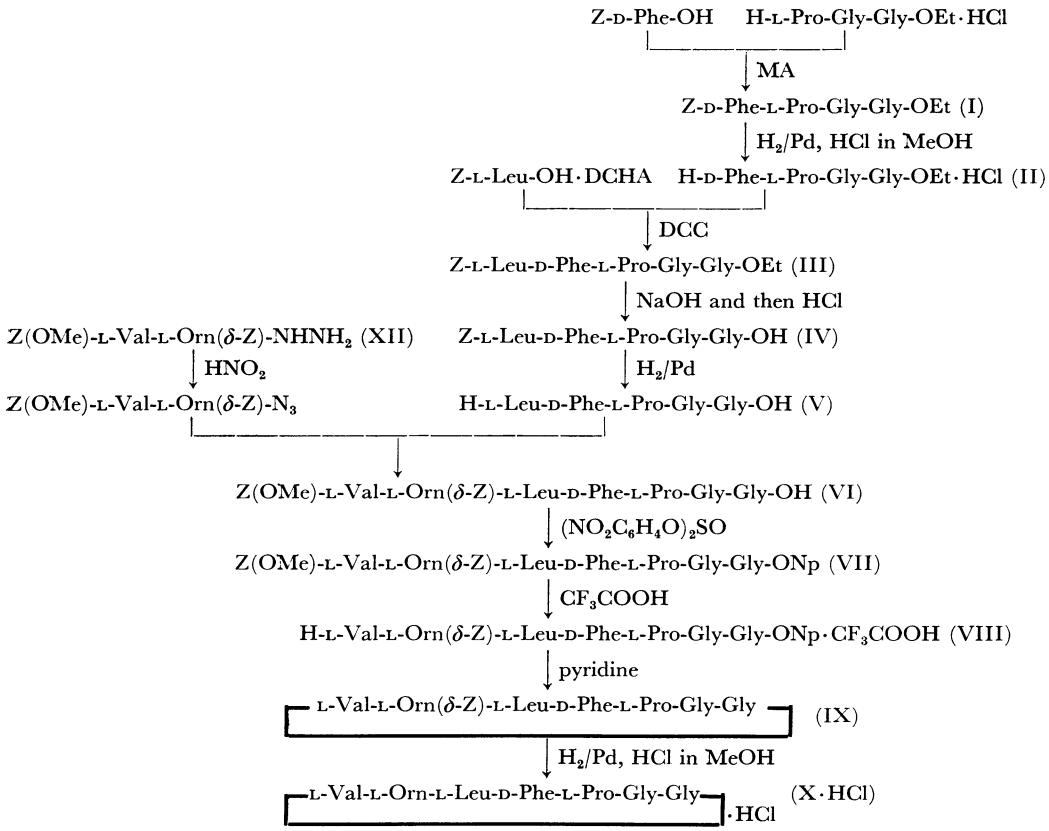

Fig. 1. Cyclization of linear heptapeptide active ester

cyclo-(L-Val-L-Orn-L-Leu-D-Phe-L-Pro-Gly-Gly-), which contains the partial sequence -L-Val-L-Orn-L-Leu-D-Phe-L-Pro- found in the natural antibiotics mentioned above.

Figure 1 indicates the route for the synthesis of the cyclic heptapeptide (X·HCl). *p*-Methoxybenzyloxycarbonyl-heptapeptide acid (VI) was prepared by the coupling reaction of the acyl-dipeptide azide derived from XII with the neutral pentapeptide (V), and then VI was converted to the benzyloxycarbonyl-substituted heptapeptide *p*-nitrophenyl ester trifluoroacetate (VIII). The cyclization reaction of the trifluoroacetate in pyridine gave the benzyloxycarbonyl-substituted cyclic peptide (IX) which was purified by passing it through columns of Dowex 50 and Dowex 1. The molecular-weight determination demonstrated that the molecular size of IX corresponds to that of cyclic heptapeptide. The final product, X·HCl, was obtained upon the hydrogenolysis of IX in the presence of one equivalent of hydrogen chloride as crystals with five moles of water; its homogeneity was demonstrated by several chromatographic techniques.

The antibacterial activity of the cyclic peptide (X) toward several microorganisms was examined. The compound X showed no activity against any of the microorganisms even at 100 μg per m*l* of the assay medium. Hence, it appears that even the presence of seven amino acid residues in a cyclic peptide does not satisfy the requirements for antibacterial activity.

Experiments on the preparations and the properties of several cyclic peptides with larger ring sizes, such as a cyclic octapeptide, are in progress in this laboratory in order to extend this study further.

Experimental

All the melting points are uncorrected. Prior to analysis, the compounds were dried over phosphorus pentoxide to a constant weight at 80°C and 2 mmHg, except for the cyclic peptide (X·HCl·5H_2O).

Z-D-Phe-Pro-Gly-Gly-OEt (I).[7] To a solution of benzyloxycarbonyl-D-phenylalanine (2.57 g, 8.5 mmol), H-Pro-Gly-Gly-OEt·HCl[8] (2.54 g, 8.6 mmol) and triethylamine (1.2 m*l*) in tetrahydrofuran (40 m*l*) was

7) The following abbreviations are used according to *Biochemistry*, **5**, 2485 (1966); Z-, benzyloxycarbonyl; Z(OMe)-, *p*-methoxybenzyloxycarbonyl; -ONp, *p*-nitrophenyl ester; -OEt, ethyl ester; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide. Amino acid symbols except D-Phe denote the L configuration.

8) H. N. Rydon and P. W. G. Smith, *J. Chem. Soc.*, **1956**, 3642.

added DCC (1.77 g, 8.6 mmol) at 0°C. After the stirring had been continued for 2 hr at 0°C, the mixture was left to stand for overnight at room temperature. The mixture was then evaporated *in vacuo*, and ethyl acetate was added to the residue. After the dicyclohexylurea was filtered off, the filtrate was washed successively with 4% sodium bicarbonate solution and 2% hydrochloric acid and dried over sodium sulfate. The filtrate was evaporated *in vacuo*. The residual foam weighed 4.07 g (88%); R_f 0.95.[9]

H-D-Phe-Tro-Gly-Gly-OEt·HCl (II). A solution of I (4.07 g) in 0.4N methanolic hydrogen chloride (20.8 m*l*) was subjected to hydrogenolysis in the presence of palladium black. The filtrate from the catalyst was evaporated to dryness *in vacuo*; yield of oil, 3.37 g (102%); R_f 0.80[9] and 0.95.[10]

Z-Leu-D-Phe-Pro-Gly-Gly-OEt (III). To a solution of benzyloxycarbonyl-L-leucine dicyclohexylamine salt[11] (3.35 g, 7.5 mmol) and II (4.03 g) in chloroform (50 m*l*) was added DCC (1.45 g, 7.5 mmol) at 0°C. The reaction mixture was treated as has been described for the preparation of I. The residual foam weighed 4.61 g (94%); R_f 0.94.[9]

Z-Leu-D-Phe-Pro-Gly-Gly-OH (IV). To a solution of III (4.37 g, 6.7 mmol) in methanol (50 m*l*), N sodium hydroxide (13.4 m*l*) was added. After the solution had been allowed to stand two days at room temperature, N hydrochloric acid (27 m*l*) was added and the solution was evaporated *in vacuo*. The oily product was extracted with ethyl acetate and the solution was dried over sodium sulfate. The filtrate was evaporated, and the residue was crystallized by the addition of ether. It was recrystallized from ethanol-ether; yield, 2.97 g (71%); mp 178—180°C; $[\alpha]_D^{20}$ −4.4° (*c* 1, MeOH).

Found: C, 61.49; H, 6.69; N, 10.98%. Calcd for $C_{32}H_{41}O_8N_5$: C, 61.62; H, 6.63; N, 11.23%.

H-Leu-D-Phe-Pro-Gly-Gly-OH (V). A solution of IV (2.50 g, 4.01 mmol) in a mixture of acetic acid-methanol-water (6 : 4 : 1, 40 m*l*) was subjected to hydrogenolysis in the presence of palladium black. The filtrate was evaporated to dryness *in vacuo*, and the residual crystals were collected with the aid of ether. It was recrystallized from ethanol-ether; yield, 1.76 g (90%); mp 158—159°C (dec.); $[\alpha]_D^{20}$ −20.0° (*c*, 1, MeOH); R_f 0.64.[9]

Found: C, 57.07; H, 7.42; N, 13.47%. Calcd for $C_{24}H_{35}O_4N_5 \cdot H_2O$: C, 56.78; H, 7.36; N, 13.80%.

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Phe-Pro-Gly-Gly-OH (VI). The azide[5] derived from Z(OMe)-Val-Orn(δ-Z)-$NHNH_2$ (XII) (1.63 g, 3 mmol) was added to a solution of V (1.47 g, 3 mmol) and triethylamine (0.42 m*l*) in DMF (50 m*l*). The mixture was stirred for 3 days at 0°C and evaporated *in vacuo*. The resideu was triturated with 10% citric acid, and the precipitate was collected by filtration and washed with water. It was recrystallized from methanol-ether-petroleum ether; yield, 2.71 g (90%); mp 119—120°C $[\alpha]_D^{20}$ −8.0° (*c* 1, DMF); R_f 0.75.[9]

Found: C, 59.26; H, 6.82; N, 10.85%. Calcd for $C_{51}H_{68}O_{13}N_8 \cdot 2H_2O$: C, 59.05; H, 7.01; N, 10.80%.

***cyclo*-(Val-Orn(δ-Z)-Leu-D-Phe-Pro-Gly-Gly-) (IX).** To a solution of VI (0.50 g, 0.5 mmol) in pyridine (25 m*l*), di-*p*-nitrophenyl sulfite[12] (1.62 g, 5 mmol) was added, and the reaction mixture was then allowed to stand for 24 hr at room temperature. After evaporation of the solvent, the product was triturated with petroleum ether and washed repeatedly with a mixture of ether and petroleum ether (1 : 1) by decantation (yield of VII, 578 mg). The *p*-nitropheynl ester content was estimated to be 90% by measuring the optical density at 412 mμ.[13]

To VII (576 mg) thus obtained were added anisole (1 m*l*) and trifluoroacetic acid (6 m*l*) at 0°C. After 20 min, the solution was evaporated, and the solid was collected with the aid of a mixture of ether and petroleum ether. The heptapeptide *p*-nitrophenyl ester trifluoroacetate (VIII) thus obtained was dissolved in DMF (10 m*l*) with acetic acid (0.5 m*l*). The solution was added dropwise into pyridine (300 m*l*) at 55—60°C during 5 hr and the stirring was continued for additional 2 hr. After removal of the solvent, the residue was dissolved in a mixture of methanol-water (5 : 1, 150 m*l*). The solution was treated with columns (1.8×15 cm) of Dowex 1 (OH^- form) and Dowex 50 (H^+ form). The columns were washed with the same solvent (400 m*l*), and the combined effluent was evaporated to dryness. The residual product was collected by filtration with the aid of water and dried (249 mg). It was recrystallized from dioxane-methanol-ether; yield of air-dried product, 202 mg (50% form VI); mp 149—151°C (decomp.); $[\alpha]_D^{20}$ −4.0° (*c* 1, MeOH); R_f 0.94.[9]

Found: C, 60.69; H, 7.17; N, 13.25%; mol wt, 850.[14] Calcd for $C_{42}H_{58}N_8O_9 \cdot \frac{1}{2}H_2O$: C, 60.91; H, 7.20; N, 13.53%: mol wt, 828.

The air-dried product lost 1.00% of its weight after

Table 1. Inhibitory activity of the compounds on microorganisms

Minimum inhibitory concentration, μg/m*l*

Compound	Synthetic medium[a]		Bouillon agar medium[b]	
	E. coli	*B. subtilis*	*R. coli*	*B. substilis*
The cyclic heptapeptide (X)	>100	>100	>100	>100
Gramicidin S	>100	10	>100	2—5

a) Stephenson-Whetham's medium (modified); K_2HPO_4 0.1%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, Na-glutamate 0.4%, casamino acid 0.2%, yeast-extract 0.05% and agar 2.0%, pH 7.0.

b) Usual boulillon agar medium, pH 7.0.

9) The R_f values refer to the thin-layer chromatography with Merck silica gel G and to the *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, *v/v*) system. Compounds possessing a free amino group were detected by spraying them with ninhydrin, and those with blocked amino groups, by spraying them with 47% hydrobromic acid, and then with ninhydrin.

10) The R_f of the paper chromatography with Toyo Roshi No. 52 refers to the *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, *v/v*) system.

11) E. Klieger, E. Schröder and H. Gibian, *Ann.*, **640**, 157 (1966).

12) B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1760 (1960).

13) R. Schwyzer and P. Sieber, *ibid.*, **40**, 624 (1957).

14) The molecular weight was determined on a Hitachi Osmometer, type 115, using DMF as the solvent.

being dried for 2 hr at 110°C, 2 mmHg. Calcd for $\frac{1}{2}H_2O$: 1.09%.

***cyclo*-(Val-Orn-Leu-D-Phe-Pro-Gly-Gly-)·HCl(X·HCl).** A solution of VII (82 mg; 0.1 mmol) dissolved in 0.126N methanolic hydrogen chloride (0.88 m*l*) was subjected to hydrogenolysis in the presence of palladium black. The solution, after being filtered from the catalyst, was evaporated to dryness *in vacuo*. The crystals were collected with the aid of ether; yield of the air-dried product, 60.8 mg (84%); mp 150—153°C (decomp.); R_f 0.78[9] and 0.94;[10] $R_{gramicidin\ S}$ of paper electrophoresis,[15] 0.76; $[\alpha]_D^{20}$ −18.0° (*c* 1, MeOH); amino acid ratios in acid hydrolysate, $Val_{0.9}Orn_{1.1}$-$Leu_{1.0}Phe_{0.9}Pro_{1.0}Gly_{2.2}$.

Found: C, 50.21; H, 7.44; N, 13.52%. Calcd for $C_{34}H_{52}N_8C_7Cl \cdot 5H_2O$: C, 50.38; H, 7.73; N, 13.83%.

Microbiological Assays.[16] The microorganism employed are listed in Table 1. The minimum amount of the compound necessary for the complete inhibition of growth was determined by a dilution method using a bouillon agar medium and a synthetic medium. As is shown in Table 1, the cyclic heptapeptide was found to be devoid of activity in reaction against any of the microorganisms utilized.

15) Detail of the experiment has been described in the previous publication; H. Aoyagi, T. Kato, M. Waki, O. Abe, R. Okawa, S. Makisumi and N. Izumiya, This Bulletin, **42**, 782 (1969).

16) We are indebted to Dr. M. Shibata of Takeda Chemical Industries, Ltd. for the assay.