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Studies of Peptide Antibiotics. XXVII. Synthesis of an Analog of Gramicidin S Containing a Glycine Residue in Place of a Proline[1]

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[Gly^5]-Gramicidin S in which one L-proline residue at 5-position in gramicidin S is replaced with glycine was synthesized and tested for antibacterial properties. The cyclic decapeptide obtained was as active as natural gramicidin S. It was suggested that the mode of antibacterial activity of [Gly^5]-gramicidin S and its conformation are similar to those of GS.

In the studies of synthetic analogs of biologically active peptides, several hybrid analogs which contain two peptide portions suggested from different natural peptides have been synthesized. As such an analog, oxypressin containing the cyclic hexapeptide portion of vasopressin and the linear tripeptide portion of oxytocin was synthesized. The hybrid peptide exhibited unique biological activities compared with the parent hormones.[2] From the results it occurred to us to prepare a gramicidin S analog which contain two peptide portions and to investigate its antibacterial properties.

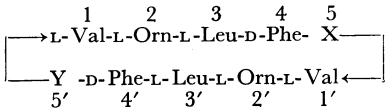

Fig. 1. Structure of GS (X, Y=L-Pro), [Gly^5]-GS (X=Gly, Y=L-Pro) and [Gly$^{5,5'}$]-GS (X, Y=Gly).

In order to understand the relationship between the chemical structure and antibacterial activity of gramicidin S (GS) (Fig. 1), various analogs of GS in which two amino acid residues are substituted with two other amino acids have been synthesized. An analog, [Gly$^{5,5'}$]-GS, was synthesized in this laboratory originally[3] and also in other laboratories,[4] and its strong antibacterial activity has been observed. However, a GS analog in which one amino acid residue is replaced with other amino acid has not yet been synthesized.

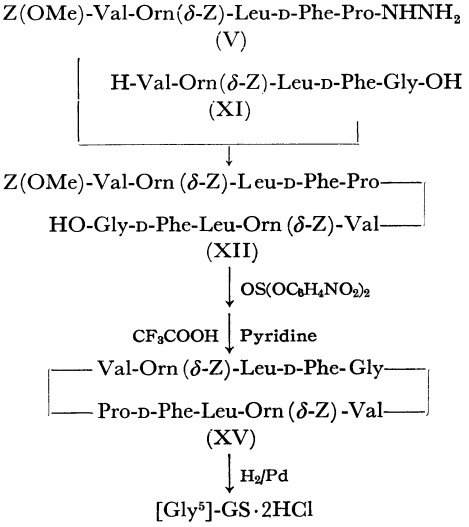

Fig. 2. Synthesis of [Gly^5]-GS.

1) Presented at the 23rd Annual Meeting of the Chemical Society of Japan, Tokyo, April, 1970.

2) R. A. Boissonnas, S. Guttmann, P.-A. Jaquenoud, and J.-P. Waller, *Helv. Chim. Acta*, **39**, 1421 (1957); J. Rudinger, J. Honzl, and M. Zaoral, *Collection Czech. Chem. Commun.*, **21**, 770 (1956); P.G. Katsoyannis, *J. Amer. Chem. Soc.*, **79**, 109 (1957).

3) H. Aoyagi, T. Kato, M. Ohno, M. Kondo, M. Waki, S. Makisumi, and N. Izumiya, This Bulletin, **38**, 2139 (1965).

4) J. Halstrom and H. Klostermeyer, *Ann.* **715**, 208 (1968); A. A. Kiryushkin, Y. A. Ovchinnikov, I. V. Kozhevnikova, and M. M. Shemyakin, "Peptides," North-Holland Pub. Co., Amsterdam (1967), p. 100.

It thus seemd of interest to prepare [Gly^5]-GS (Fig. 1) which is composed of two peptide portions, -Val-Orn-Leu-D-Phe-Pro- from GS and -Val-Orn-Leu-D-Phe-Gly- from [Gly$^{5,5'}$]-GS. This paper describes the synthesis, antibacterial properties and optical rotatory dispersion (ORD) measurement of [Gly^5]-GS.

Figure 2 indicates the route for the synthesis of [Gly^5]-GS.[5] Condensation of the azide derived from Z(OMe)-pentapeptide hydrazide (V) with a neutral pentapeptide (XI) gave an acyldecapeptide acid (XII). Pentapeptide (XI) obtained previously from Z(OMe)-pentapeptide acid[3] was prepared alternately from Boc-pentapeptide acid (X) by the action of formic acid. Z(OMe)-decapeptide acid (XII) was converted into an acyldecapeptide nitrophenyl ester (XIII), and its Z(OMe) group was removed with trifluoroacetic acid. Decapeptide trifluoroacetate (XIV) thus obtained was treated with pyridine for the cyclization reaction. The reaction mixture yielded a pure Z-substituted cyclic decapeptide (XV) which was hydrogenated to afford [Gly^5]-GS as a crystalline dihydrochloride.

TABLE 1. ANTIBACTERIAL ACTIVITY OF THE COMPOUNDS
(Minimum inhibitory concentration, μg/ml)

Compound	*Echerichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*
	(Bouillon agar medium, pH 7.0)		
GS	20	2	2
[Gly^5]-GS	20	5	5
[Gly$^{5,5'}$]-GS	20	5	5
Mixture of GS and [Gly$^{5,5'}$]-GS[a]	20	2—5	2—5
	(Synthetic medium, pH 7.0)		
GS	20	5	5
[Gly^5]-GS	20	5	5
[Gly$^{5,5'}$]-GS	20	5	2—5
Mixture of GS and [Gly$^{5,5'}$]-GS[a]	20	5	5

a) A mixture was prepared with 1:1 weight ratio of GS and [Gly$^{5,5'}$]-GS.

The antibacterial activities of [Gly^5]-GS and several reference compounds toward microorganisms were tested (see Table 1). Three compounds, [Gly^5]-GS, GS and [Gly$^{5,5'}$]-GS, exhibited approximately the same activities for all the microorganisms tested. Erlanger and Goode observed that some linear decapeptides, *e.g.* H-(Val-Lys-Leu-D-Phe-Pro)$_2$-OH, revealed synergistic activity with GS against *E. coli*. They noted that the mode of antibacterial action of such linear peptides might differ from that of GS.[6] As shown in Table 1, a mixture of GS and [Gly$^{5,5'}$]-GS possessed activity in the same degree with each compound GS, [Gly$^{5,5'}$]-GS and [Gly^5]-GS; no synergistic activity of [Gly$^{5,5'}$]-GS on GS was observed. We thus assumed that the mode of action of [Gly$^{5,5'}$]-GS, and then [Gly^5]-GS, is similar to that of GS. This assumption is favored by comparison of ORD curves (Fig. 3) of the compounds referred to above with that of [β-Ala$^{5,5'}$]-GS which possessed no antibacterial activity.[7] The three compounds, [Gly^5]-GS, GS, and [Gly$^{5,5'}$]-GS, afforded a similar shape with a trough at approximately 232 mμ, while [β-Ala$^{5,5'}$]-GS afforded a shape of simple dispersion curve with no trough. The results of ORD measurements suggest that [Gly^5]-GS possesses a conformation similar to that of GS.

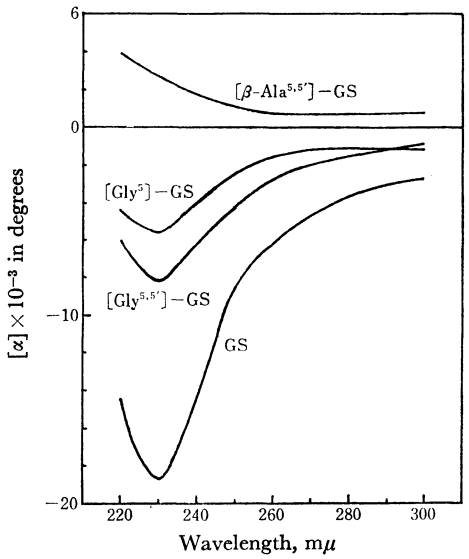

Fig. 3. ORD curves of the four compounds in ethanol. The curve of [β-Ala$^{5,5'}$]-GS is talen from a pre^{4}ious paper.[7]

Experimental

Thin layer chromatography was carried out on Merck silica gel G with the following solvent systems: R_f^1, *n*-butanol-acetic acid-pyridine-water (4:1:1:2, *v/v*); R_f^2, chloroform-methanol (5:1, *v/v*).

H-D-Phe-OEt·TsOH (I). This was prepared according to the procedure for the L isomer.[8] Yield, 88%; mp 153—154°C; $[\alpha]_D^{20}$ −20.0° (*c* 1, ethanol) (Found: C, 59.18; H, 6.48; N, 3.83%). Reported values for the L isomer;[8] mp 153°C, $[\alpha]_D$ +15.5° (DMF).

Z-Leu-D-Phe-OEt (II). This was prepared from Z-Leu-OH·DCHA (21.8 g) and I (17.8 g) with DCC (10 g) in the same way as for Z-Leu-D-Phe-OBzl.[9] Yield, 18.0 g (84%); mp 106—107°C; $[\alpha]_D^{15}$ −7.8° (*c* 1, methanol); R_f^1 0.98, R_f^2 0.76.

Found: C, 67.89; H, 7.34; N, 6.46%. Calcd for $C_{25}H_{32}O_5N_2$: C, 68.16; H, 7.32; N, 6.36%.

Z-Leu-D-Phe-NHNH$_2$ (III). This was prepared from II (17.2 g) and hydrazine hydrate (38 ml) in the same way as for the same product from Z-Leu-D-Phe-OBzl and hydrazine.[9] Yield, 15.8 g (95%); mp 163°C. No depression

5) Abbreviations: Z, benzyloxycarbonyl; Z(OMe), *p*-methoxybenzyloxycarbonyl; Boc, *t*-butyloxycarbonyl; ONp, *p*-nitrophenoxy; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; TEA, triethylamine; TsOH, *p*-toluenesulfonic acid; DMF, dimethylformamide. Amino acid symbols except D-Phe and Gly denote the L-configuration.

6) B. F. Erlanger and L. Goode, *Science*, **131**, 669 (1960).

7) S. Matsuura, M. Waki, S. Makisumi, and N. Izumiya, This Bulletin, **43**, 1197 (1970).

8) T. Kato, S. Makisumi, M. Ohno, and N. Izumiya, *Nippon Kagaku Zasshi*, **83**, 1151 (1962).

9) K. Kuromizu and N. Izumiya, This Bulletin, **43**, 2199 (1970).

of melting point was observed on a mixture of III with the product[9] prepared before. III was used for the preparation of Z-Leu-D-Phe-Pro-OEt[9] and Z(OMe)-Val-Orn(δ-Z)-Leu-D-Phe-Pro-OEt (IV)[10] as described in literature.[9,10]

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Phe-Pro-NHNH$_2$ (V). A solution of IV (7.65 g, 8.4 mmol) and hydrazine hydrate (8.3 ml, 167 mmol) in DMF (15 ml) was allowed to stand at room temperature for 7 days. The reaction mixture was poured into water (1000 ml), and the crystals formed were collected by filtration; yield, 7.01 g (93%); mp 131—132°C; $[\alpha]_D^{15}$ −32.8° (*c* 0.5, DMF); R_f^1 0.64.

Found: C, 61.77; H, 7.23; N, 12.13%. Calcd for $C_{47}H_{64}O_{10}N_8 \cdot H_2O$: C, 61.42; H, 7.24; N, 12.19%.

Z(OMe)-D-Phe-Gly-OBzl (VI). This was obtained from Z(OMe)-D-Phe-OH·DCHA[11] (15.32 g) and H-Gly-OBzl·TsOH (10.11 g) with DCC (6.18 g) in the same way as for Z(OMe)-Trp-D-Phe-OBzl.[9] Yield, 11.72 g (82%); mp 115—116°C; $[\alpha]_D^{20}$ +14.2° (*c* 1, ethanol); R_f^1 0.95.

Found: C, 68.12; H, 6.19; N, 5.98%. Calcd for $C_{27}H_{28}O_6N_2$: C, 68.05; H, 5.92; N, 5.88%.

Z-Leu-D-Phe-Gly-OBzl (VII). To a mixture of VI (16.2 g, 34 mmol) and anisole (10 ml) was added 2 N hydrogen chloride in dioxane (240 ml). After 3 hr at room temperature, the solution was evaporated to dryness (yield of a syrup, 11.9 g; R_f^1 0.75). The syrup (H-D-Phe-Gly-OBzl·HCl) was condensed with Z-Leu-OH·DCHA (15.2 g, 34 mmol) and DCC (7.02 g) in chloroform (150 ml) as in the case of Z-Leu-D-Phe-OBzl.[9] Yield, 13.1 g (69%); mp 106—107°C; $[\alpha]_D^{20}$ +13.8° (*c* 1, methanol); R_f^1 0.95.

Found: C, 68.36; H, 6.73; N, 7.62%. Calcd for $C_{32}H_{37}O_6N_3$: C, 68.67; H, 6.66; N, 7.51%.

Z-Leu-D-Phe-Gly-OH (VIII). To a solution of Z-Leu-D-Phe-Gly-OEt[3] (7.0 g, 14.1 mmol) in methanol (70 ml) was added N sodium hydroxide (16.9 ml). The solution was left for 2 hr at room temperature, and N hydrochloric acid (17 ml) was added. The solution was evaporated and the resulting crystals were recrystallized from ethyl acetate-petroleum ether; yield, 5.43 g (82%); mp 116—117°C; $[\alpha]_D^{20}$ +9.3° (*c* 1, methanol); R_f^1 0.75.

Found: C, 62.87; H, 6.96; N, 8.85%. Calcd for $C_{25}H_{31}O_6N_3 \cdot 1/2H_2O$: C, 62.74; H, 6.74; N, 8.78%.

H-Leu-D-Phe-Gly-OH (IX). *(a) From VII*: A solution of VII (13.1 g, 23.4 mmol) in a mixture of acetic acid (70 ml), methanol (35 ml) and water (12 ml) was hydrogenated in the presence of palladium black. The filtrate was evaporated and the resulting crystals were collected with the aid of a mixture of ethanol and acetone; yield, 5.89 g (75%); mp 231—232°C (decomp.); $[\alpha]_D^{20}$ +32.5° (*c* 0.5, acetic acid); R_f^1 0.67.

Found: C, 60.83; H, 7.69; N, 12.30%. Calcd for $C_{17}H_{25}O_4N_3$: C, 60.88; H, 7.51; N, 12.53%.

(b) From VIII. VIII (4.69 g, 10 mmol) was hydrogenated as described above; yield, 2.58 g (77%); mp 230—232°C (decomp.); $[\alpha]_D^{20}$ +32.2° (*c* 0.5, acetic acid).

Boc-Val-Orn(δ-Z)-Leu-D-Phe-Gly-OH (X). Boc-Val-Orn(δ-Z)-NHNH$_2$ (9.69 g, 20.2 mmol)[12] was dissolved in DMF (200 ml) and 2N hydrogen chloride in dioxane (20.2 ml). To the solution at −30°C was added isoamyl nitrite (3.11 ml, 22.2 mmol). After some 10 min, disappearance of the hydrazide was recognized by the detection method of Ertel and Horner.[13] To the reaction mixture was added TEA (3.11 ml, 22.2 mmol). After 5 min at 0°C, a suspension of IX (6.77 g, 20.2 mmol) in DMF (450 ml) and dimethyl sulfoxide (50 ml) containing dicyclohexylamine (4.02 ml, 20.2 mmol) was added to the mixture. After 3 days at 4°C, the reaction mixture was evaporated to approximately 200 ml. To this was added 0.01 M citric acid (5000 ml). The resulting solid was collected and recrystallized from methanol-ether; yield, 12.02 g, (76%); mp 182—183°C; $[\alpha]_D^{25}$ +3.8° (*c* 1, DMF); R_f^1 0.82.

Found: C, 59.52; H, 7.60; N, 10.51%. Calcd for $C_{40}H_{58}O_{10}N_6 \cdot 1/2H_2O$: C, 59.31; H, 7.59; N, 10.38%.

H-Val-Orn(δ-Z)-Leu-D-Phe-Gly-OH (XI). A solution of X (3.9 g, 5 mmol) in 98% formic acid (80 ml)[14] was allowed to stand for 3 hr at room temperature. The solution was evaporated, and a mixture of TEA (20 ml) and methanol (100 ml) was added. The solution was evaporated and the resulting crystals were collected by filtration with the aid of water; yield, 2.59 g (76%); mp 192—193°C; $[\alpha]_D^{25}$ −4.3° (*c* 0.2, acetic acid); R_f^1 0.80, R_f^2 0.55 (Found as monohydrate: C, 59.86; H, 7.44; N, 12.20%). The same compound (XI) was prepared from Z(OMe)-Val-Orn(δ-Z)-Leu-D-Phe-Gly-OH with the action of trifluoroacetic acid;[3] mp 191—192°; $[\alpha]_D^{25}$ −4.2° (acetic acid).

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Phe-Pro-Val-Orn(δ-Z)-Leu-D-Phe-Gly-OH (XII). V (292 mg, 0.32 mmol) was treated with isoamyl nitrite (0.049 ml, 0.352 mmol) as in the case of X. To the azide solution was added a solution of XI (240 mg, 0.352 mmol) in DMF (4 ml) containing TEA (0.049 ml, 0.352 mmol). After being stirred for 5 days at 4°C, the reaction mixture was added to 0.02 M citric acid (250 ml). The resulting solid was collected and recrystallized from methanol-petroleum ether; yield, 343 mg (69%); mp 128—129°C; $[\alpha]_D^{20}$ −21.0° (*c* 0.5, DMF); R_f^1, 0.98, R_f^2 0.62.

Found: C, 61.39; H, 7.16; N, 10.62%. Calcd for $C_{82}H_{110}O_{18}N_{12} \cdot 3H_2O$: C, 61.32; H, 7.28; N, 10.47%.

cyclo-*(Val-Orn(δ-Z)-Leu-D-Phe-Pro-Val-Orn(δ-Z)-Leu-D-Phe-Gly) (XV).* XII (280 mg, 0.18 mmol) was converted into the corresponding *p*-nitrophenyl ester (XIII) (344 mg) with di-*p*-nitrophenyl sulfite (583 mg, 1.8 mmol). After XIII (340 mg) was converted into a decapeptide nitrophenyl ester trifluoroacetate (XIV), XIV was subjected to the cyclization reaction with pyridine.[3] Yield, 76 mg (31% from XII); mp 156—157°C; $[\alpha]_D^{20}$ −93.5° (*c* 0.25, acetic acid).

Found: C, 62.00; H, 7.36; N, 11.43%; mol wt,[15] 1380. Calcd for $C_{73}H_{100}O_{14}N_{12} \cdot 2H_2O$: C, 61.97; H, 7.48; N, 11.67%; mol wt, 1405.

cyclo-*(Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Gly) Dihydrochloride ([Gly^5]-GS·2HCl).* A solution of XV (36mg, 0.026 mmol) in 0.01 N methanolic hydrogen chloride (5.7 ml) was hydrogenated and the filtrate was evaporated. The resulting crystals were recrystallized from methanol-ether-petroleum ether; yield of an air-dried product, 31.8 mg (94%); mp 205—207°C (decomp.); $[\alpha]_D^{20}$ −88.9° (*c* 0.25, ethanol); R_f^1 0.77, R_f^2 0.02. Paper electrophoresis and carboxymethylcellulose column chromatography of this product[7] revealed a single spot or peak, which was at the same position as that by natural GS. Amino acid ratio in acid hydrolysate; Pro 1.08, Gly 0.98, Val 2.11, Leu 2.10, Phe 1.98, Orn 2.05.

Found: C, 52.88; H, 7.91; N, 12.63%. Calcd for $C_{57}H_{88}O_{10}N_{12} \cdot 2HCl \cdot 7H_2O$: C, 52.64; H, 8.06; N, 12.92%.

10) M. Waki and N. Izumiya, This Bulletin, **40**, 1687 (1967).
11) M. Waki and N. Izumiya, *ibid.*, **41**, 1909 (1968).
12) T. Kato, M. Kondo, M. Ohno, and N. Izumiya, *ibid.*, **38**, 1202 (1965).
13) H. Ertel and L. Horner, *J. Chromatog.*, **7**, 268 (1962).
14) B. Halpern and D. E. Nitecki, *Tetrahedron Letters*, **1967**, 3031.
15) Molecular weight was determined by a Hitachi Osmometer, Type 115, using methanol as a solvent.

Microbiological Assays[16] *and ORD Measurements.*[17] The minimum inhibitory concentration was determined by a dilution method. The results are shown in Table 1. ORD measurements were performed as described previously.[7] The curves are shown in Fig. 3.

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

17) We are indebted to the members of Prof. Funatsu's Laboratory, Faculty of Agriculture, for help in ORD measurements.