BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 44, 158—161 (1971)

Studies of Peptide Antibiotics. XXIV. Synthesis of 4,4'-D-Alanine-gramicidin S

Sannamu LEE, Reiko OHKAWA[1], and Nobuo IZUMIYA[2]

Laboratory of Biochemistry, Faculty of Science, Kyushu University, Hakozaki, Fukuoka

(Received June 22, 1970)

In order to investigate the contribution to antibacterial activity of the D-phenylalanine residues at 4- and 4'-position in gramicidin S, 4,4'-D-alanine-gramicidin S (XX) and 4-D-alanine-semigramicidin S (XXI) were prepared and tested for antibacterial properties. XX exhibited recognizable activity though weaker than that of gramicidin S, whereas XXI showed no activity on any of the microorganisms tested.

We reported that 4,4'-glycine-gramicidin S possessed weak antibacterial activity toward several microorganisms.[3] On the other hand, 4,4'-D-valine- and 4,4'-D-leucine-gramicidin S were as active as natural gramicidin S (GS) (Fig. 1);[4] the results indicated that the aromatic side chains of D-phenylalanine residues of 4- and 4'-position can be replaced by the bulky aliphatic side chains without influencing the activity. From these findings, it seemed of interest to investigate the antibacterial properties of an analog of GS with D-amino acid residues at 4- and 4'-position which are larger than glycine and smaller than valine or leucine.

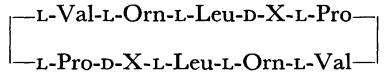

Fig. 1. Structure of GS (X=Phe) and 4,4'-D-Ala-GS (X=Ala).

This paper will describe the synthesis and antibacterial properties of 4,4'-D-alanine-GS besides those of a cyclic pentapeptide, 4-D-alanine-semigramicidin S.

The desired 4,4'-D-Ala-GS (XX) was obtained as a crystalline dihydrochloride by hydrogenolysis of a benzyloxycarbonyl-substituted cyclic decapeptide (XVII) wherein δ-amino functions of ornithine residues were protected with benzyloxycarbonyl groups. The protected cyclic decapeptide (XVII) was prepared from a corresponding decapeptide *p*-nitrophenyl ester trifluoroacetate (XVI) by treatment with pyridine. The trifluoroacetate (XVI) was obtained from *p*-methoxybenzyloxycarbonyl-decapeptide nitrophenyl ester (XV) which was derived from an acyl-decapeptide acid (XIV) and di-*p*-nitrophenyl sulfite.

Synthesis of the protected cyclic decapeptide (XVII) was attempted by possible dimerization reaction of a linear pentapeptide active ester (XVIII). Thus, treatment of the active ester with pyridine afforded a mixture of the protected monomer (XIX) and dimer (XVII) which was prepared as described before; the ratio in weight of XIX and XVII in the mixture was found to be 23 : 77. Separation of the two components was achieved by a Sephadex LH-20 column with methanol, the monomer (XIX) being obtained as a pure material. Hydrogenolysis of XIX in the presence of hydrogen chloride yielded a crystalline monohydrochloride of cyclic pentapeptide (XXI).

The antibacterial activities of the cyclic penta- and decapeptides (XXI and XX) toward several microorganisms were examined. Both compounds exhibited no activity toward Gram negative microorganisms (*e. g.*, *E. coli*). It was also found that 4-D-alanine-semiGS (XXI) possessed no activity toward Gram positive microorganisms (*St. aureus* and *B. subtilis*), whereas 4,4'-D-alanine-GS (XX) possessed recognizable activity though its degree was weaker than that of GS (Table 1). The results indicate that D-alanines can replace D-phenylalanines without drastic drop in activity. The optical rotatory dispersion (ORD) curves of the cyclic decapeptide (XX) and GS were measured with ethanol as a solvent.[5] Both decapeptide afforded a similar shape to a trough at approximately 232 mμ (Fig. 2). Since 4,4'-D-alanine-GS contains no aromatic amino acid residues, the only chromophores in the molecule are amide carbonyls and the shape of the ORD curve will reflect the spatial arrangement of chromophores in the molecule. The results suggest that 4,4'-D-alanine-

1) Present address: Division of Entomology, University of California, Berkeley, Calif., U.S.A.

2) To whom reprint requests should be addressed.

3) R. Nagata, M. Waki, M. Kondo, T. Kato, S. Makisumi, and N. Izumiya, This Bulletin, **40**, 963 (1967).

4) H. Aoyagi, T. Kato, M. Waki, O. Abe, R. Okawa, S. Makisumi, and N. Izumiya, *ibid.*, **42**, 782 (1969).

5) Several papers have given ORD curves on GS and its analogs: *e. g.*, D. Balasubramanian, *J. Amer. Chem. Soc.*, **89**, 5445 (1967); T. Kato, M. Waki, S. Matsuura, and N. Izumiya, *J. Biochem.* (Tokyo), **68**, 751 (1970).

GS possesses a conformation similar to GS. Further studies of the ORD measurements on other GS analogs are in progress.

Table 1. Inhibitory activity of the compounds on microorganisms

Minimum inhibitory concentration, μg/ml

	Staphylococcus aureus	*Bacillus substilis*
GS	6.25	3.13
4,4'-D-Ala-GS	50	25
4-D-Ala-semiGS	>100	>100

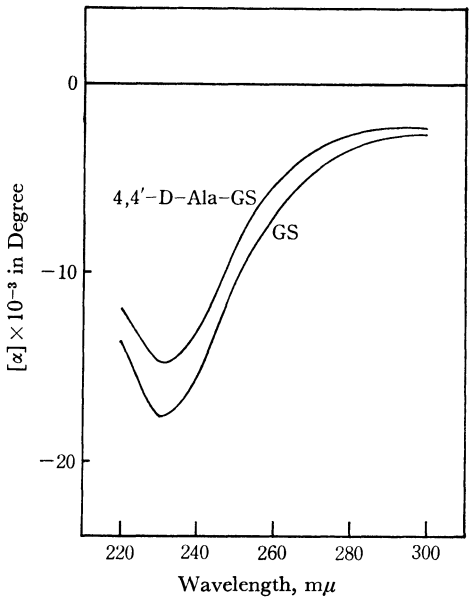

Fig. 2. ORD curves of GS and 4,4'-D-Ala-GS.

Experimental

All melting points are uncorrected. Thin layer chromatography was performed 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. Paper chromatography was performed with the following solvent system: R_f^3, the same solvent used for R_f^1.

Z-D-Ala-Pro-OEt (I).[6] To a chilled solution of Z-D-Ala-OH (2.32 g, 10 mmol)[7] and TEA (1.4 ml, 10 mmol) in tetrahydrofuran (20 ml), isobutyl chloroformate (1.31 ml, 10 mmol) was added. After 10 min, a mixture of H-Pro-OEt·TsOH[8] (3.15 g, 10 mmol), TEA (1.4 ml, 10 mmol) and chloroform (20 ml) was added to the solution. The reaction mixture was allowed to stand overnight and then evaporated to dryness *in vacuo*. After the residual oil was dissolved in ethyl acetate, the solution was washed successively with water, 2% hydrochloric acid, 4% sodium bicarbonate and water, dried over sodium sulfate, and then evaporated. The product was obtained as oil; yield, 2.51 g (72%); R_f^1 0.98.

H-D-Ala-Pro-OEt·HCl (II). A solution of I (2.51, g 7.2 mmol) dissolved in 0.3N methanolic hydrogen chloride (28 ml) was hydrogenated in the presence of palladium black. The filtrate from the catalyst was evaporated to dryness; yield of oil, 1.80 g (100%); R_f^1 0.80.

Z-Leu-D-Ala-OEt (III). To a solution of Z-Leu-OH·DCHA[9] (6.69 g, 15 mmol) and H-D-Ala-OEt·HCl (1.96 g, 15 mmol)[10] in chloroform (100 ml) was added DCC (3.09 g, 15 mmol) at 0°C. The mixture was stirred for 3 hr at 0°C, and then kept overnight at room temperature. The mixture was evaporated and ethyl acetate was added to the residue. After dicyclohexylurea was filtered off, the filtrate was washed successively with water, 2% hydrochloric acid, 4% sodium bicarbonate, and water, and dried over sodium sulfate. The filtrate was evaporated and the residue was crystallized by addition of ether and petroleum ether. It was recrystallized from ethyl acetate-petroleum ether; yield, 4.45 g (83%); mp 93—94°C; $[\alpha]_D^{20}$ +3.3° (*c* 1, methanol); R_f^1 0.99.

Found: C, 62.49; H, 7.79; N, 7.73%. Calcd for $C_{19}H_{28}O_5N_2$: C, 62.62; H, 7.74; N, 7.73%.

Z-Leu-D-Ala-NHNH$_2$ (IV). A solution of III (4.02 g, 11 mmol) and hydrazine hydrate (5 ml) in DMF (20 ml) was allowed to stand at room temperature for one day. The solution was evaporated, and then water (300 ml) was added to the residue. The resulting crystals were collected by filtration; yield, 3.66 g (95%); mp 171—174°C; $[\alpha]_D^{20}$ −13.8° (*c* 1, DMF); R_f^1 0.87.

Found: C, 58.41; H, 7.58; N, 15.89%. Calcd for $C_{17}H_{26}O_4N_4$: C, 58.27; H, 7.48; N, 15.99%.

Z-Leu-D-Ala-Pro-OEt (V). (*a*) Z-Leu-OH·DCHA (3.2 g, 7.2 mmol) and II (1.8 g, 7.2 mmol) were condensed with DCC (1.48 g, 7.2 mmol) as described for the preparation of III; yield of oil, 2.6 g (77%); R_f^2 0.83.

(*b*) To a solution at −5°C of IV (3.5 g 10 mmol) in DMF (20 ml) containing 2.8N hydrogen chloride in dioxane (7.5 ml), isoamyl nitrite (1.4 ml, 10 mmol) was added.[11] After 10 min, TEA (2.8 ml, 20 mmol) was added. To the solution was added a mixture of H-Pro-OEt·TsOH (3.15g 10 mmol) and TEA (1.4 ml) in DMF (20 ml). The mixture was stirred for 3 days at 0°C and evaporated. The residue was dissolved in ethyl acetate, and the solution was washed successively with 2% hydrochloric acid, 4% sodium bicarbonate and water, dried over sodium sulfate, and then evaporated; yield of oil, 2.6 g (56%); R_f^2 0.83.

Z-Leu-D-Ala-Pro-OH (VI). To a solution of V (2.57 g, 5.6 mmol) in methanol (20 ml), N sodium hydroxide (9 ml) was added. The solution was allowed to stand for 3 hr at room temperature. After the addition of water (20 ml), the solution was evaporated to remove methanol. After the solution was extracted with ethyl acetate, the aqueous layer was acidified with 2N hydrochloric acid. The mixture was extracted with ethyl acetate and the organic layer was dried over sodium sulfate. The filtrate was evaporated to dryness; yield of a semi solid, 2.15 g (89%); R_f^2 0.56.

H-Leu-D-Ala-Pro-OH (VII). A solution of VI (2.15 g, 5 mmol) in a mixture of acetic acid (15 ml), methanol (12 ml) and water (2 ml) was hydrogenated. The filtrate was evaporated to dryness; yield of a semi solid, 1.39 g (93%); R_f^1 0.70.

H-Leu-D-Ala-Pro-OEt·HCl (VIII). This was obtained from V (1.84 g, 4 mmol) as described for the preparation

6) Abbreviations; Z, benzyloxycarbonyl; Z(OMe), *p*-methoxybenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; TEA, triethylamine: DMF, dimethylformamide; DCHA, dicyclohexylamine; CMC, carboxymethyl cellulose. Amino acid symbol denotes L configration unless otherwise noted.

7) C. S. Smith and A. E. Brown, *J. Amer. Chem. Soc.*, **63**, 2605 (1941).

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

9) E. Klieger, E. Schröder, and H. Gibian, *Liebigs Ann. Chem.*, **640**, 157 (1961).

10) D. A. Rowlands and G. T. Young, *J. Chem. Soc.*, **1950**, 3159.

11) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961); E. Wünsch and A. Zwick, *Chem. Ber.*, **99**, 101 (1966).

of II; yield of oil, 1.46 g (100%); R_f^1 0.87.

Z-(OMe)-Val-Orn(δ-Z)-Leu-D-Ala-Pro-OH (IX). The azide[12] derived from Z(OMe)-Val-Orn(δ-Z)-$NHNH_2$ (XXII) (2.49 g, 4.4 mmol) was added to a solution of VII (1.19 g, 4 mmol) and TEA (1.12 ml, 8 mmol) in DMF (50 ml). The mixture was stirred for 2 days at 0°C and evaporated. The residue was triturated with 0.5M citric acid, and the precipitate was collected by filtration. It was recrystallized from methanol - ether; yield, 2.26 g (68%); mp 137—139°C; $[\alpha]_D^{20}$ −35.5° (*c* 1, methanol).

Found: C, 58.13; H, 7.24; N, 9.77%. Calcd for $C_{41}H_{58}O_{11}N_6 \cdot H_2O$: C, 58.14; H, 7.61; N, 9.92%.

Z-(OMe)-Val-Orn(δ-Z)-Leu-D-Ala-Pro-OEt (X). The azide[12] derived from XXII (2.49 g, 4.4 mmol) was condensed with VIII (1.50 g, 4.1 mmol) as described for the preparation of V. The precipitate which formed upon addition of water was collected, washed successively with 4% sodium bicarbonate, 0.5M citric acid and water; yield, 3.96 g (85%); mp 163—165°C; $[\alpha]_D^{20}$ −20.7° (*c* 1, DMF); R_f^1 0.98.

Found: C, 61.18; H, 7.45; N, 10.26%. Calcd for $C_{43}H_{62}O_{11}N_6$: C, 61.55; H, 7.45; N, 10.02%.

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Ala-Pro-$NHNH_2$ (XI). A solution of X (4.12 g, 5 mmol) and hydrazine hydrate (2 ml) in DMF (20 ml) was allowed to stand for 5 days at 30°C. After evaporation, the hydrazide which precipitated upon addition of water (400 ml) was collected and recrystallized from dioxane - ether; yield, 1.98g (48%); mp 158—160°C; $[\alpha]_D^{20}$ −20.1° (*c* 1, DMF); R_f^1 0.65.

Found: C, 60.01; H, 7.40; N, 13.69%. Calcd for $C_{41}H_{60}O_{10}N_8$: C, 59.69; H, 7.33; N, 13.59%.

H-Val-Orn(δ-Z)-Leu-D-Ala-Pro-OH·HCl (XII). To a mixture of IX (2.05 g, 2.5 mmol) and anisole (0.3 ml), 2.7N hydrogen chloride in dioxane (20 ml) was added at room temperature. After 2 hr, the solution was evaporated, and the residue was triturated with ether; yield, 1.75 g (97%); mp 147—149°C (decomp.); $[\alpha]_D^{20}$ −36.3° (*c* 1, DMF); R_f^1 0.73.

Found: C, 53.39; H, 7.66; N, 11.52%. Calcd for $C_{32}H_{51}O_8N_6Cl \cdot 2H_2O$: C, 53.43; H, 7.56; N, 11.69%.

Z(OMe)-Val-Orn(δ-Z)-Leu-D-Ala-Pro-Val-Orn(δ-Z)-Leu-D-Ala-Pro-OH (XIV). The azide (XIII) was prepared from XI (2.06 g, 2.5 mmol), N hydrochloric acid (6 ml) and sodium nitrite (0.175 g, 2.5 mmol) in acetic acid (20 ml) as described for the preparation of the azide[12] from XXII. XIII was condensed with XII (1.73 g, 2.4 mmol) as described for the preparation of IX. The product was recrystallized from methanol - ether-petroleum ether; yield, 3.19 g (88%); mp 185—187°C; $[\alpha]_D^{20}$ −59.9° (*c* 1, methanol); R_f^1 0.92, R_f^2 0.66.

Found: C, 59.36; H, 7.26; N, 10.73%. Calcd for $C_{73}H_{106}O_{18}N_{12} \cdot 2H_2O$: C, 59.41; H, 7.51; N, 11.30%.

cyclo-*(Val-Orn(δ-Z)-Leu-D-Ala-Pro-)$_2$ (XVII). (a) From XIV.* To a solution of XIV (729 mg, 0.5 mmol) in pyridine (10 ml), di-*p*-nitrophenyl sulfite (1.61 g, 5 mmol) was added. After 8 hr at room temperature, the mixture was evaporated. The residual solid was collected by filtration with the aid of a mixture of ether-petroleum ether (1 : 1, v/v); yield of acyldecapeptide *p*-nitrophenyl ester (XV), 752 mg. To XV (745 mg) thus obtained, anisole (0.5 ml) and trifluoroacetic acid (5 ml) were added at 0°C. After 40 min, the solution was evaporated and the residual powder was collected by filtration with the aid of ether. Decapeptide *p*-nitrophenyl ester trifluoroacetate (XVI) (520 mg) thus obtained was dissolved in DMF (10 ml) and acetic acid (0.1 ml). The solution was added dropwise into pyridine (20 ml) at 50—60°C for 4 hr and stirring was continued for additional 2 hr. After the solvent was removed, the residue was dissolved in a mixture of methanol (40 ml) and water (10 ml). The solution was passed through the columns of Dowex 1 and 50. The effluent was evaporated, and the product was collected by filtration with the aid of water. It was recrystallized from methanol - ether; yield, 151 mg (23% from XIV); mp 234—236°C (decomp.); $[\alpha]_D^{20}$ −199° (*c* 1, methanol); R_f^1 0.95.

Found: C, 56.68; H, 7.61; N, 12.55%; mol wt 1240.[13] Calcd for $C_{64}H_{96}O_{14}N_{12} \cdot 3H_2O$: C, 58.60; H, 7.65; N, 12.81%; wt 1311.

(b) From IX. Acylpentapeptide acid (IX) (405 mg, 0.5 mmol) was converted to pentapeptide *p*-nitrophenyl ester trifluoroacetate (XVIII) (372 mg) as described for the preparation of XV and XVI. XVIII thus obtained was added to pyridine (150 ml) at 60°C as described above. After evaporation, the residue was treated with columns of Dowex 1 and 50, the effluent was evaporated, and the product was collected by filtration with the aid of water; yield of the crude product (XXIII), 110 mg. The solution of XXIII (100 mg) in methanol (5 ml) was applied to a column (2×110 cm) with Sephadex LH-20, and the development continued with methanol; a 2 ml fraction was collected in each test tube. The peak of XVII appeared from the test tube number 61 to 70 and the peak of XIX from 80 to 90. The fractions 61—70 were evaporated, and the product was collected by filtration with the aid of water (yield, 64 mg). It was recrystallized from methanol - ether; yield, 52 mg; mp 240—242°C; $[\alpha]_D^{20}$ −192° (*c* 1, methanol); R_f^1 0.95.

cyclo-*(Val-Orn(δ-Z)-Leu-D-Ala-Pro-) (XIX).* The fractions 80—90 were evaporated to afford an oily residue which was crystallized after several days. It was collected by the aid of a mixture of methanol and ether; yield, 19 mg; mp 130—133°C; $[\alpha]_D^{20}$ −64.6° (*c* 0.5, methanol); R_f^1 0.93.

Found: C, 59.49; H, 7.65; N, 13.01%; mol wt 596.[13] Calcd for $C_{32}H_{48}O_7N_6 \cdot H_2O$: C, 59.22; H, 7.74; N, 12.99%; mol wt 647.

cyclo-*(Val-Orn-Leu-D-Ala-Pro-)$_2$·2HCl (4,4'-D-Ala-GS·2HCl) (XX·2HCl).* A solution of XVII (50 mg, 0.038 mmol) in 0.01N methanolic hydrogen chloride (8.4 ml) was hydrogenated and the filtrate was evaporated. The product was recrystallized from methanol - ether - petroleum ether; yield, 31 mg (73%); mp 190—191°C (decomp.); $[\alpha]_D^{20}$ −189° (*c* 1, methanol); R_f^1 0.89.

Found: C, 51.58; H, 7.87; N, 14.05%. Calcd for $C_{48}H_{86}$-

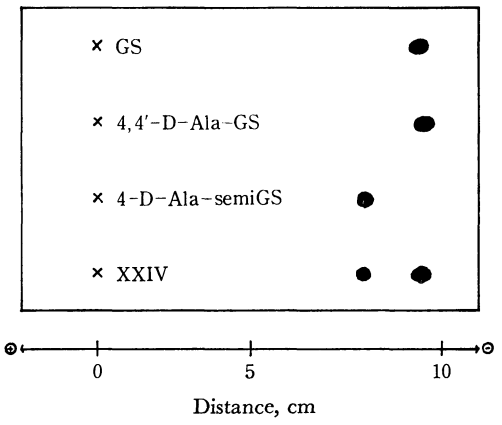

Fig. 3. Paper electrophoresis of the compounds.
XXIV, hydrogenated material after cyclization of pentapeptide active ester trifluoroacetate.

12) O. Abe and N. Izumiya, This Bulletin, **43**, 1202 (1970).

13) Molecular weight was determined on a Hitachi Osmometer type 115, using methanol as a solvent.

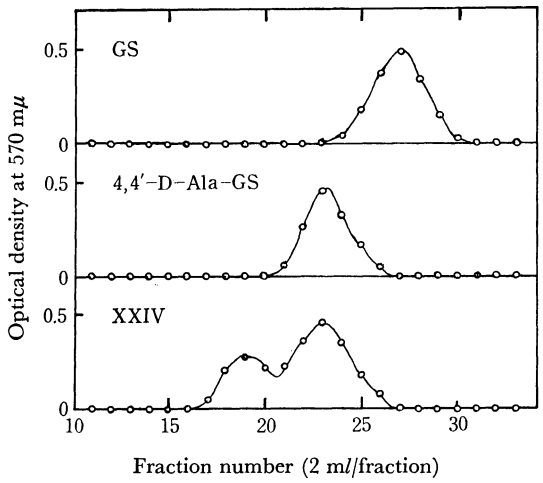

Fig. 4. CMC column chromatography of the compounds. XXIV, see Fig. 3.

$O_{10}N_{12}Cl_2 \cdot 3H_2O$: C, 51.64; H, 8.31; N, 14.33%.

cyclo-*(Val-Orn-Leu-*D*-Ala-Pro-)·HCl (4-*D*-Ala-semiGS·HCl)* *(XXI·HCl).* XIX (15 mg, 0.02 mmol) was treated as described above; yield of a semi solid, 9.8 mg (98%); R_f^1 0.86.

Electrophoresis and CMC Chromatography. The experiments were carried out as described before.[4] A part of the crude product (XXIII) obtained after cyclization reaction of the pentapeptide active ester (XVIII), was hydrogenated and the product was designated as XXIV. As shown in Fig. 3, 4,4'-Ala-GS and 4-D-Ala-semiGS were clearly separated in the electrophoresis, whereas two compounds could be only partly separated with a column (0.9×50 cm) of CMC.

Microbiological Assays[14] *and ORD Measurements.*[15] The minimum amount of the compounds necessary for the complete inhibition of growth was determined by a dilution method using a bouillon agar medium and the results are shown in Table 1. ORD measurements were performed with JASCO Spectropolarimeter model ORD-CD/UV-5 over a wavelength range of 220 to 300 mμ in ethanol on the compound, and ORD curves are shown in Fig. 2.

14) We are indebted to Meiji Seika Co., Ltd. for the microbiological assays.

15) We are indebted to Mr. S. Matsuura and Dr. M. Waki in this laboratory for ORD measurements.