Studies of Peptide Antibiotics. XXXVII.[1] Synthesis of [1,1′,3,3′-L-Alanine]-Gramicidin S

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An analog containing alanyl residues in place of valyl and leucyl residues in gramicidin S, namely [1,1′,3,3′-L-alanine]-gramicidin S, was synthesized to investigate the influence of the bulky side chains of the amino acid residues for antibacterial activity and conformation. The analog has the small aliphatic side chains of alanyl residues on one side of the ring frame in the gramicidin S-structure. In the experiments of optical rotatory dispersion and circular dichroism, this analog gave curves similar in shape to these of gramicidin S, and thus should have a similar conformation to that of gramicidin S. The analog showed, however, no antibacterial activity. Thus the four methyl side chains of alanyl residues in the analog are not sufficient for the substance to exhibit biological activity.

Various analogs of gramicidin S (GS, Fig. 1) have been studied to investigate the relationship between chemical structure and biological activity. Particularly, some analogs with other amino acid residues in place of either the valyl or leucyl residues have been prepared by Izumiya and his collaborators. Some of them, such as [1,1′-Ala]-[2] and [1,1′-Leu]-GS,[3] exhibited substantial antibacterial activities. On the other hand, very weak or no activity was observed for the other analogs, that is, [1,1′-Gly]-,[2] [3,3′-Gly]-,[4] and [3,3′-Ala]-GS.[4] In Table 1 are listed the antibacterial activities of the 1,1′- or/and 3,3′-position substituted GS analogs. As shown in Table 1, the activity of 3,3′-position substituted GS decreases significantly upon decreasing the bulkiness of the side chains of 3,3′-residues, whereas the bulkiness of the side chains in 1,1′-position substituted GS is not so important for the activity. For the conformation of GS, several models have been proposed. One of them is based on the β-pleated sheet structure shown in Fig. 2, which seems to be the most likely model.[5] In this specific model, the four hydrophobic side chains of valyl and leucyl residues are located on one side of the ring frame consisting of the cyclic peptide chain, while the two hydrophilic side chains of ornithyl residue are placed on the opposite side.

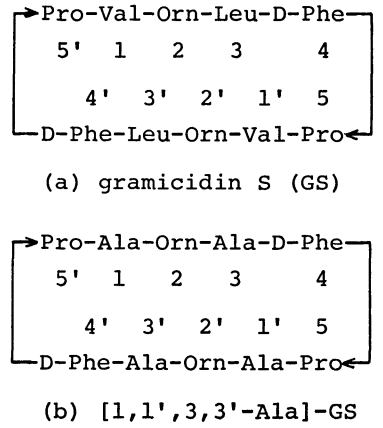

Fig. 1. Structure of GS and [1,1′,3,3′-Ala]-GS.

TABLE 1. ANTIBACTERIAL ACTIVITIES OF 1,1′- OR/AND 3,3′-POSITION SUBSTITUTED GS ANALOGS[a]

Substituted residues	*S. aureus*	*B. subtilis*	Reference No.
1,1′-Gly	100	100	2
1,1′-Ala	5	5	2
1,1′-Val (GS)[b]	2—5	2—5	—
1,1′-Leu	5	10	3
3,3′-Gly	>100	>100	4
3,3′-Ala	50	20	4
3,3′-Leu (GS)[b]	2—5	2—5	—
1,1′,3,3′-Ala	>100	>100	This work

a) This table shows the minimum inhibitory concentration (μg/ml) with a Bouillon agar medium. b) The natural GS used as the control.

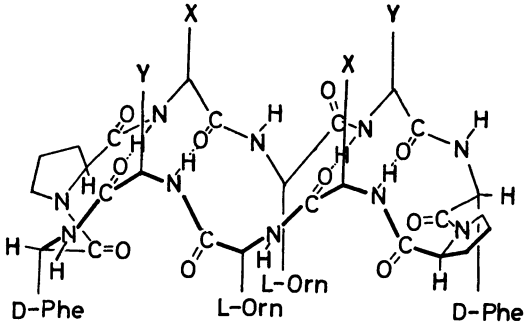

Fig. 2. Possible model of GS (X=Val, Y=Leu) and [1,1′,3,3′-Ala]-GS (X, Y=Ala).

For the purpose of investigating the effect of the hydrophobic side chains on the activity, it seemed of interest to synthesize an analog having alanyl residues in place of both the valyl and leucyl residues in GS. The analog has the small aliphatic side chains on one side of the ring frame in the GS-structure. The present paper will describe the synthesis of [1,1′,3,3′-L-analine]-gramicidin S ([1,1′,3,3′-Ala]-GS) (as shown in Fig. 1), the ORD and CD measurements, and the antibacterial properties.

The synthesis of [1,1′,3,3′-Ala]-GS (**10**) is outlined in Fig. 3. Acyldipeptide ester (**1**) was prepared by means of the MA method,[6] and then converted into a hydrazide (**2**). Acyltripeptide ester (**3**) was prepared by stepwise elongation by means of the DCC method as a crystalline solid. It had previously been reported as an oil.[4]

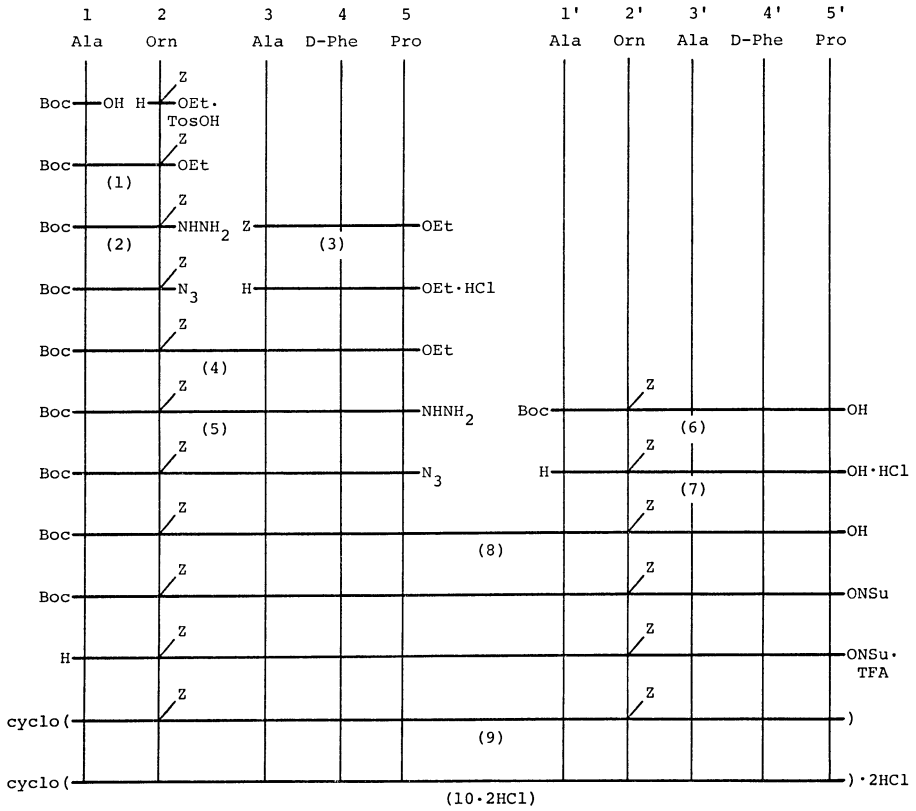

Fig. 3. Synthesis of [1,1′,3,3′-Ala]-GS.

Its hydrogenolysis gave a tripeptide ester. Acylpentapeptide ester (**4**) was prepared by condensation of the azide derived from **2** with the tripeptide ester mentioned above. The ester (**4**) was converted into a hydrazide (**5**), while a part of **4** was saponified to afford an acylpentapeptide (**6**). Compound **6** was converted into a pentapeptide (**7**) by treatment with HCl in formic acid. Acyldecapeptide (**8**) was prepared by condensation of the azide derived from **5** with **7**. For its cyclization, the *N*-hydroxysuccinimide ester of **8** was prepared as an active ester, and its Boc-group was removed by the action of TFA at low temperature. The decapeptide active ester was treated with a large amount pyridine, and the resulting Z-substituted cyclic decapeptide (**9**) was purified by passing it through a column of resins. For the synthesis of the final [1,1′,3,3′-Ala]-GS, the Z-substituted cyclodecapeptide (**10**) was subjected to hydrogenolysis, and the desired analog was obtained as a crystalline dihydrochloride (**10·2HCl**). The homogeneity of **10** was confirmed by paper and thin-layer chromatography, paper electrophoresis, and elemental analysis.

The ORD curves of the analog (**10**) in ethanol as well as of GS are shown in Fig. 4-a. The analog has a very similar to that which curve with a negative trough at 232 nm, GS possesses. The CD measurements of cyclic and linear peptides relating to GS or its analogs are interesting in connection with the elucidation of antibacterial activities and cyclization reactions. Thus, the CD curves of the analog and GS were measured with methanol as a solvent (Fig. 4-b). The CD curves of both peptides have nearly the same

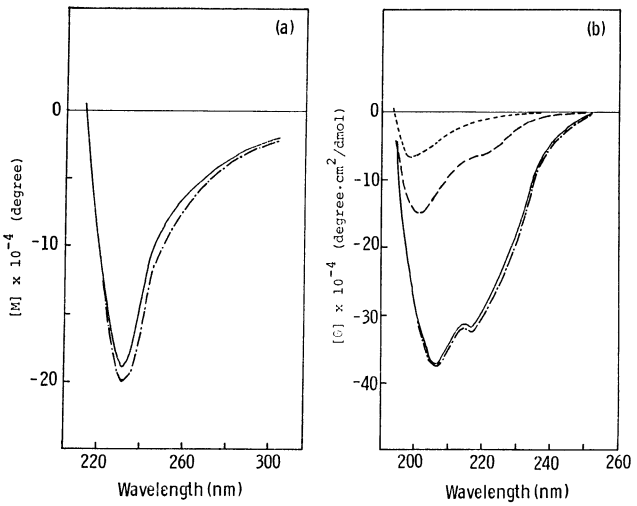

Fig. 4. ORD (a) and CD (b) curves of the cyclic peptides and the Boc-peptides. Solvent: a, ethanol; b, methanol. GS: ——, [1,1′,3,3′-Ala]-GS (**10**): ——, Boc-pentapeptide (**6**): ----, Boc-decapeptide (**8**): ———.

pattern, in which the two troughs are at 207 and 217 nm. The CD curves of Boc-peptides (**6** and **8**) were also measured: the trough of the Boc-pentapeptide (**6**) is at 198 nm, whereas that of the Boc-decapeptide (**8**) is at 202 nm. The troughs of the linear peptides (**6** and **8**) are shallower than those of cyclic peptides (GS and **10**).

The results of ORD and CD measurements indicate

that the conformation of the analog (**10**) is similar to that of GS. On the other hand, the CD curve of the Boc-pentapeptide (**6**) shows that **6** has a random structure, and that of the Boc-decapeptide (**8**) indicates that **8** lies in a transition state toward the β-sheet structure such as GS. In a number of experiments, it has been found that a linear decapeptide in the cyclization reaction gives rise to only the cyclic decapeptide, whereas a linear pentapeptide gives a mixture of the cyclic penta- and decapeptides. In the present study, the decapeptide active ester derived from **8** afforded a cyclic monomer in good yield as the sole product; this result can be explained by the suggestion that **8** possesses a spatial conformation resembling that of GS.

At the beginning of this study, we expected that [1,1′3,3′-Ala]-GS (**10**) might possess appreciable antibacterial activity, like [3,3′-Ala]-GS, because [1,1′-Ala]-GS possesses the same strong activity as GS. It seemed that this expectation was supported by the results of the ORD and CD experiments of **10** and GS. Compound **10** showed, however, no activity against any of Gram positive microorganisms even at 100 μg/ml of assay medium. These results indicate that analogs with two methyl side chains in place of either of the two bulky side chains of $Val^{1,1'}$ or $Leu^{3,3'}$ show some activity, but that the analog (**10**) with four methyl side chains loses its activity completely because of its inability to interact with the surface of a Gram positive microorganism.

Experimental

Melting points are uncorrected. TLC was carried out on silica gel G (Merck) with the solvent system: R_f^1, 1-butanol-pyridine-water (4 : 1 : 1 : 2, v/v). Paper chromatography was carried out on Toyo Roshi No. 52 with the above solvent, R_f^2, being used.

*Boc–Ala–Orn(Z)–OEt (**1**).* To a solution of Boc–Ala–OH (3.78 g, 20 mmol) and TEA (2.80 ml, 20 mmol) in THF (40 ml) was added isobutyl chloroformate (2.62 ml, 20 mmol) at −5 °C. After 15 min, a mixture of H–Orn(Z)–OEt TosOH[7] (9.33 g, 20 mmol) and TEA (2.80 ml, 20 mmol) in chloroform (40 ml) was added. The mixture was left to stand overnight at room temperature, evaporated *in vacuo*, and the oily residue was dissolved in ethyl acetate (80 ml). The solution was washed successively with 4% $NaHCO_3$, 10% citric acid, and water, and then dried (Na_2SO_4). The filtrate which separated from the salt was concentrated *in vacuo*. The product which precipitated upon the addition of ether and petroleum ether was collected and washed with the same solvent. It was obtained as very hygroscopic crystals: yield, 5.33 g (57%); mp 49—52 °C; $[\alpha]_D^{29}$ −14.2° (*c* 1, DMF); R_f^1 0.87.

Found: C, 58.81; H, 7.74; N. 8.91%. Calcd for $C_{23}H_{35}O_7N_3 \cdot 1/3H_2O$: C, 58.58; H, 7.62; N, 8.91%.

*Boc–Ala–Orn(Z)–NHNH$_2$ (**2**).* A solution of **1** (5.12 g, 11 mmol) and hydrazine hydrate (10.7 ml, 220 mmol) in DMF (50 ml) was allowed to stand at room temperature for 3 days. The excess hydrazine was evaporated *in vacuo*, and then water (60 ml) was added to the residue. The resulting solid was collected by filtration: yield, 4.18 g (84%); mp 174—175 °C; $[\alpha]_D^{29}$ −27.3° (*c* 1, AcOH).

Found: C, 55.92; H, 7.45; N, 15.48%. Calcd for $C_{21}H_{33}O_6N_5$: C, 55.86; H, 7.37; N, 15.51%.

*Z–Ala–D-Phe–Pro–OEt (**3**).* To a solution of Z–Ala–OH (7.39 g, 33.1 mmol) in chloroform (60 ml) was added DCC (6.83 g, 33.1 mmol) at −5 °C. After several minutes, a solution of H–D-Phe–Pro–OEt·HCl[8] (10.8 g, 33.1 mmol) and TEA (4.63 ml, 33.1 mmol) in chloroform (60 ml) was added to this solution. The mixture was left to stand for 25 h at room temperature, evaporated *in vacuo*, and the oily residue was dissolved in ethyl acetate (150 ml). The solution was washed successively with 4% $NaHCO_3$, 2% HCl, and water, dried (Na_2SO_4), and the filtrate was evaporated. The oily residue solidified upon the addition of ether and petroleum ether. The product was obtained in solid form, though a previous paper[4] had reported it as an oily substance: yield, 10.3 g (63%); mp 93—94 °C; $[\alpha]_D^{20}$ −33° (*c* 1, DMF); R_f^1 0.94.

Found: C, 65.17; H, 6.95; N, 8.44%. Calcd for $C_{27}H_{33}O_6N_3$: C, 65.44; H, 6.71; N, 8.48%.

*Boc–Ala–Orn(Z)–Ala–D-Phe–Pro–OEt (**4**).* A solution of **2** (4.06 g, 9 mmol) dissolved in DMF (50 ml) was cooled to −20 °C. To this solution, 3.70 M HCl in dioxane (4.86 ml) and isopentyl nitrite (1.22 ml, 9 mmol) were added. After 20 min, the solution was neutralized with TEA (2.52 ml, 18 mmol). To this solution was added a chilled solution of H–Ala–D-Phe–Pro–OEt·HCl[4] (3.58 g, 9 mmol) and TEA (1.26 ml, 9 mmol) in DMF (20 ml). The reaction mixture was stirred at 0 °C for 3 days and evaporated. The residual solid was triturated with petroleum ether (30 ml) and water (30 ml), and allowed to stand in a refrigerator. The solid was collected by filtration and washed successively with 4% $NaHCO_3$, 10% citric acid, and water. It was recrystallized from ethanol-ether: yield, 4.92 g (70%); mp 190—192 °C; $[\alpha]_D^{18}$ −27.6° (*c* 1, DMF); R_f^1 0.94.

Found: C, 61.34; H, 7.22; N, 10.89%. Calcd for $C_{40}H_{56}O_{10}N_6$: C, 61.52; H, 7.23; N, 10.76%.

*Boc–Ala–Orn(Z)–Ala–D-Phe–Pro–NHNH$_2$ (**5**).* This hydrazide was obtained from **4** (1.56 g, 2 mmol) as described for the preparation of **2**: yield, 1.44 g (93%); mp 203—206 °C; $[\alpha]_D^{18}$ −75.0° (*c* 1, AcOH).

Found: C, 59.23; H, 7.08; N, 14.21%. Calcd for $C_{38}H_{54}O_9N_8$: C, 59.52; H, 7.10; N, 14.61%.

*Boc–Ala–Orn(Z)–Ala–D-Phe–Pro–OH (**6**).* To a solution of **4** (1.95 g, 2.5 mmol) in a mixture of methanol (10 ml) and DMF (15 ml), 1 M NaOH (3 ml) was added, and the solution was allowed to stand at 5 °C for 1 day. After the addition of 1 M citric acid (5 ml), the solution was evaporated to dryness. The residual oil was dissolved in ethyl acetate and the solution was concentrated *in vacuo*. The product which precipitated upon the addition of ether was collected by filtration: yield, 1.69 g (90%); mp 126—130 °C; $[\alpha]_D^{18}$ −35.6° (*c* 1, DMF); R_f^1 0.74.

Found: C, 60.23; H, 6.92; N, 10.77%. Calcd for $C_{38}H_{52}O_{10}N_6$: C, 60.62; H, 6.96; N, 11.16%.

*H–Ala–Orn(Z)–Ala–D-Phe–Pro–OH·HCl (**7**).* Compound **6** (1.51 g, 2 mmol) was dissolved in 0.164 M HCl in formic acid (15 ml). After being left to stand at room temperature for 20 min, the solution was evaporated to dryness, and the resulting solid was collected by filtration with the aid of ether. The product was a very hygroscopic powder: yield, 1.47 g (107%); mp 131—135 °C; $[\alpha]_D^{18}$ −31.0° (*c* 1, DMF); R_f^1 0.71.

Found: C, 53.27; H, 7.50; N, 11.15%. Calcd for $C_{33}H_{47}O_8N_6Cl \cdot 3H_2O$: C, 53.18; H, 7.17; N, 11.28%.

*Boc–Ala–Orn(Z)–Ala–D-Phe–Pro–Ala–Orn(Z)–Ala–D-Phe–Pro–OH (**8**).* The azide derived from **5** (1.30 g, 1.7 mmol) was coupled with **7** (1.17 g, 1.7 mmol) as described for the preparation of **4**. The product was obtained as a crystalline solid: yield, 2.02 g (93%); mp 134—136 °C;

$[\alpha]_D^{18}$ −39.0° (*c* 1, DMF); R_f^1 0.75.

Found: C, 59.02; H, 6.78; N, 11.30%. Calcd for $C_{71}H_{94}O_{17}N_{12}\cdot 3H_2O$: C, 59.15; H, 6.99; N, 11.66%.

cyclo-[*Ala-Orn(Z)-Ala-*D*-Phe-Pro-*]$_2$ (**9**). To a solution of **8** (770 mg, 0.555 mmol) in ethyl acetate (25 ml), DCC (124 mg, 0.66 mmol) and HONSu (69 mg, 0.66 mmol) were added at 0 °C. After being left to stand at 0 °C for 3 h, the dicyclohexylurea which precipitated was filtered off, and the filtrate was evaporated *in vacuo* at 0 °C. The residual solid was washed with ether by decantation and dried. To this acyldecapeptide *N*-hydroxysuccinimide ester, anisole (0.6 ml) and trifluoroacetic acid (6 ml) were added at −5 °C. After being left to stand for 25 min, the solution was evaporated *in vacuo* at 0 °C, and the residual oil was dissolved in DMF (15 ml). The solution was added dropwise into pyridine (300 ml) at room temperature for 2 h; the stirring was continued for an additional 2 h. After the solvent was removed, the residue was dissolved in a mixture of dioxane, methanol, and water (250 ml, 2 : 1 : 1, v/v). The solution was passed through the columns of Dowex 1 (OH^- form) and 50 (H^+ form). The columns were washed with the same solvent (300 ml), and the effluent was evaporated to dryness. The residual solid was collected by filtration with the aid of water. It was recrystallized from ethanol-dioxane-petroleum ether: yield, 246 mg (35%); mp 288—292 °C (dec); $[\alpha]_D^{24}$ −243° (*c* 0.3, DMF); R_f^1 0.95.

Found: C, 59.63; H, 6.76; N, 12.72%; mol wt 1290.[9] Calcd for $C_{66}H_{84}O_{14}N_{12}\cdot 3H_2O$: C, 59.85; H, 6.85; N, 12.69%; mol wt 1325.

cyclo-*(Ala-Orn-Ala-*D*-Phe-Pro-)*$_2$*·2HCl ([1,1',3,3'-Ala]-GS·2HCl)* (**10·2HCl**). A solution of **9** (127 mg, 0.1 mmol) in 0.102 M HCl in ethanol (2.35 ml) was hydrogenated in the presence of Pd black. After removal of the catalyst, the filtrate was evaporated, and the resulting crystals were collected by filtration with the aid of ether. It was recrystallized from methanol-ether; yield, 90 mg (84%); mp 246—249 °C (dec); $[\alpha]_D^{24}$ −365° (*c* 0.2, MeOH); R_f^1 0.70, R_f^2 0.87.

Found: C, 52.80; H, 7.14; N, 14.42%. Calcd for $C_{50}H_{74}O_{10}N_{12}Cl_2\cdot 4H_2O$: C, 52.40; H, 7.20; N, 14.66%.

Electrophoresis. Electrophoresis on Toyo Roshi No. 52 paper was carried out with the solvent system: formic acid-acetic acid-methanol-water (1 : 3 : 6 : 10, v/v; pH 1.8) for 3 h at 600 V/30 cm. The mobility of **10** was comparable with that of GS; the ratio of the mobility of **10** *vs.* GS was 1.04.

ORD and CD Measurements. The measurements of ORD and CD were performed with Jasco Models J-20 and J-40, respectively. In Fig. 4-a are shown the ORD curves of **10** and GS, and in Fig. 4-b are shown the CD curves of Boc-pentapeptide (**6**), Boc-decapeptide (**8**), **10**, and GS.

Microbiological Assays. The microorganisms employed were *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Bacillus subtilis*. The minimum amount of the compounds necessary for the complete inhibition of growth was determined by a dilution method in nutrient agar medium. GS was examined as a reference compound. Compound **10** exhibited no antibacterial activity against the microorganisms tested even at 100 μg/ml, whereas the minimum concentration of GS for inhibition were 2—5 μg/ml on *S. aureus* and 1—2 μg/ml on *B. subtilis*.

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6) Abbreviations: Z, benzyloxycarbonyl; Boc, *t*-butoxycarbonyl; HONSu, *N*-hydroxysuccinimide; MA, mixed anhydride method; DCC, dicyclohexylcarbodiimide; TosOH, *p*-toluenesulfonic acid; TEA, triethylamine; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide. Amino acid symbols, except D-Phe, denote the L-configuration.

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