BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 40 963—967 (1967)

Studies of Peptide Antibiotics. VIII. Synthesis of Gly$^{4,4'}$-gramicidin S

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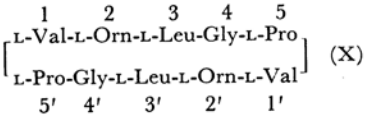
(Received October 22, 1966)

Gly$^{4,4'}$-gramicidin S dihydrochloride, cyclo-(L-valyl-L-ornithyl-L-leucylglycyl-L-prolyl)$_2$·2HCl, which is an analog of gramicidin S, was synthesized for the purpose of comparing its antibacterial activity with that of gramicidin S. The cyclization reaction *via* the linear decapeptide active ester or *via* the linear pentapeptide active ester yielded the same cyclic benzyloxycarbonyl-substituted decapeptide; this was then converted to Gly$^{4,4'}$-gramicidin S dihydrochloride by hydrogenolysis. The effect of the Gly$^{4,4'}$-gramicidin S on bacterial growth was tested; the peptide exhibited weak antibacterial activity against *Bacillus Subtilis*.

For a study of the relationship between the chemical structure and the biological activity of gramicidin S, various analogs wherein one or two amino acid residues in gramicidin S are replaced by other amino acids have been synthesized and their biological activities tested in several laboratories.[1] The present authors have supposed that the D-configurations at the 4 and 4′ positions in gramicidin S are essential in showing the antibacterial activity; the authors observed that D-Leu$^{4,4'}$- and D-Val$^{4,4'}$-gramicidin S, wherein D-phenylalanine residues in the 4 and 4′ positions were replaced by D-leucines and D-valines respectively, were as active as gramicidin S against several microorganisms.[2] On the contrary, L-Phe$^{4,4'}$-gramicidin S was found to exert no activity.[2] Therefore, it appeared of interest to synthesize Gly$^{4,4'}$-gramicidin S, wherein the D-phenylalanines were replaced by glycines, in order to determine how the D-phenylalanine moieties contribute to the biological activity. The present paper will describe the synthesis and antibacterial properties of this cyclic decapeptide (X).



(X)

The sequence of reactions employed for the synthesis of X is shown in Fig. 1 and Fig. 2.

The acylpentapeptide hydrazide (V) was obtained in a yield of 94% by the use of 20 equivalents of hydrazine hydrate for the acylpentapeptide ester (IV) and by letting the reaction mixture stand for 7 days at 30°C. It was observed that the formation of V was not sufficient under the conditions which the authors usually adopted, that is, the use of several equivalent of hydrazine and letting the mixture stand for only 2 days at room temperature. The condensation of the azide derived from V with the free pentapeptide (VII) gave acyldecapeptide (VIII) in a yield of 45%. The treatment of VIII with an excess of di-*p*-nitrophenyl sulfite gave an amorphous acyldecapeptide *p*-nitrophenyl ester. The *p*-methoxybenzyloxycarbonyl group of the ester was removed by the action of trifluoroacetic acid, and the decapeptide *p*-nitrophenyl ester trifluoroacetate thus obtained was treated with a large amount of hot pyridine for the cyclization reaction. The benzyloxycarbonyl-substituted cyclic peptide (IX) obtained in a yield of 43% (from VIII) was found to be a monomer from the results of the molecular-weight determination. The final product (X·2HCl) was obtained as colorless crystals containing six moles of water of crystallization. Its homogeneity was demonstrated by carboxymethylcellulose (CMC) column chromatography (Fig. 3) and paper electrophoresis (Fig. 4).

The synthesis of the protected cyclic decapeptide (IX) was also attempted by the dimerization reaction of the pentapeptide active ester, as is shown in Fig. 2. The treatment of the pentapeptide active ester trifluoroacetate with pyridine gave a crude product with the character of benzyloxycarbonyl(Z)-substituted cyclic peptide. It was recognized that the crude product contains Z-substituted cyclic decapeptide (IX) exclusively through the experiments of CMC column chromatography and a paper electrophoresis with the hydrogenated material of the crude product. The protected cyclic decapeptide (IX) was obtained easily in a pure state by the recrystallization of the crude product in a yield of 18% (from VI).

It would be of interest to note that the cyclization

1) T. Kato, M. Ohno, S. Makisumi and N. Izumiya, *Nippon Kagaku Zasshi, (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **87**, 493 (1966); R. Schwyzer, Symposium of Amino Acids and Peptides with Antimetabolic Activity, CIBA Foundation (1958), p. 171.

2) N. Izumiya *et al.*, to be published.

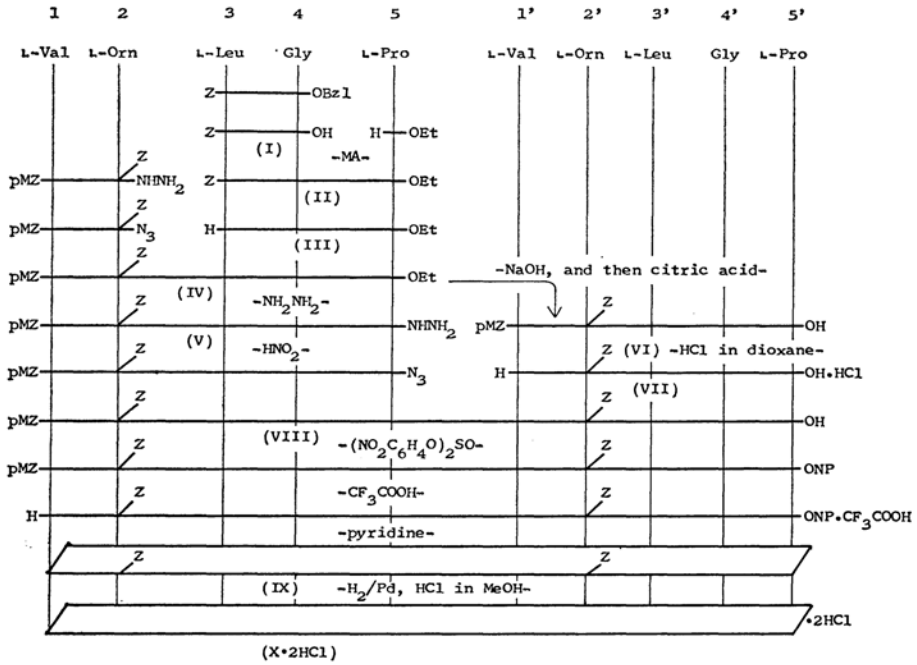


Fig. 1. Scheme of cyclization of linear decapeptide active ester. Z, benzyloxycarbonyl; pMZ, *p*-methoxybenzyloxycarbonyl; ONP, *p*-nitrophenoxy; MA, mixed anhydride method.

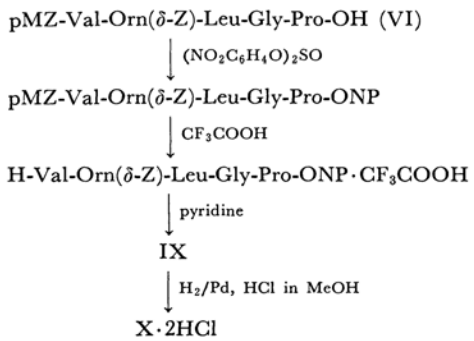


Fig. 2. Scheme of cyclization of linear pentapeptide active ester.

reaction of the active esters of Gly-L-Orn(δ-Z)-L-Leu-D-Phe-Gly[3] (or -L-Pro[2]) and L-Orn(δ-Z)-L-Leu-D-Phe-Gly-Gly[3] yielded a Z-substituted cyclic pentapeptide exclusively. Furthermore, it was observed that the cyclization reaction of the active ester of L-Val-L-Orn(δ-Z)-L-Leu-D-Phe-Gly[4] (or -Sar[5]) yielded a mixture of Z-substituted cyclic penta- and decapeptide.

In order to ascertain whether or not Gly$^{4,4'}$-gramicidin S (X) possesses antibacterial activities, the effect of various levels of X on the growth of several microorganisms was examined (Table 1). It was found that compound X indeed exhibits a weak activity against *Bacillus Subtilis*, but no activity against *Staphylococcus aureus* and several other microorganisms at as high a level as 100 μg per m*l* of the assay medium. This finding shows that the replacement of the D-phenylalanine residues by glycines in the sequence of gramicidin S reduces the activity profoundly.

In order to clarify the effectiveness of the D-phenylalanine residues further, the synthesis of D-Ala$^{4,4'}$-gramicidin S is now in progress in this laboratory.

Experimental

All melting points are uncorrected. Prior to analysis, the compounds were dried to a constant weight, over phosphorus pentoxide at 80°C and 2 mmHg, except in the case of X·2HCl.

Benzyloxycarbonyl-L-leucylglycine (I). To a solution of the benzyloxycarbonyl-L-leucylglycine benzyl ester[6] (2.06 g) in methanol (15 m*l*), N sodium hydroxide (5.5 m*l*) was added; the solution was then allowed to stand for 1 hr at room temperature. After concentration *in vacuo*, water (15 ml) was added. The solution was then acidified with N hydrochloric acid (6.0 m*l*), and the mixture was extracted with ethyl acetate. After it had been dried over anhydrous sodium sulfate, the filtered solution was evaporated *in vacuo*; the residual oil solidified after petroleum ether had been added.

3) M. Kondo, H. Aoyagi, T. Kato and N. Izumiya, This Bulletin, **39**, 2234 (1966).
4) H. Aoyagi, T. Kato, M. Ohno, M. Kondo, M. Waki, S. Makisumi and N. Izumiya, *ibid.*, **38**, 2139 (1965).
5) H. Aoyagi and N. Izumiya, *ibid.*, **39**, 1747 (1966).
6) H. Voss, *Zeit. für Naturforschung*, **20b**, 122 (1965).

The product was recrystallized from ethyl acetate - petroleum ether; yield, 1.1 g (69%); mp 116—118°C; $[\alpha]_D^{23}$ −14.0° (*c* 1, ethyl acetate).

Clayton *et al.* prepared this compound (I) through the coupling of the benzyloxycarbonyl-L-leucine *p*-nitrophenyl thiolester and glycine; mp 117—118°C.[7]

Benzyloxycarbonyl-L-leucylglycyl-L-proline Ethyl Ester (II). To a chilled solution of I (967 mg) and triethylamine (0.42 m*l*) in tetrahydrofuran (6 m*l*), isobutyl chloroformate (0.39 m*l*) was added at −5°C. After 15 min, a mixture of L-proline ethyl ester *p*-toluenesulfonate[8] (1.04 g) and triethylamine (0.46 m*l*) in chloroform (6 m*l*) was added, and the mixture was left to stand overnight at room temperature. After the removal of the solvent by evaporation *in vacuo*, the residual oil was dissolved in ethyl acetate; then the solution was washed successively with 4% sodium bicarbonate, 2% hydrochloric acid, and water, and dried over sodium sulfate. The filtered solution was evaporated *in vacuo;* yield of oil, 996 mg (74%); R_f 0.77.[9]

L-Leucylglycyl-L-proline Ethyl Ester Hydrochloride (III). II (0.94 g) was subjected to hydrogenolysis in the presence of palladium black and 0.35 N methanolic hydrogen chloride (7.3 m*l*). The filtrate from the catalyst was then evaporated to dryness *in vacuo;* yield of oil, 0.73 g (99%); R_f 0.61.[9]

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucylglycyl-L-proline Ethyl Ester (IV).** The following operations were carried out in a cold room. Into a chilled solution of *p*-methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithine hydrazide[10] (1.09 g) in glcacial acetic acid (20 m*l*), there were stirred N hydrochloric acid (4.4 m*l*) and sodium nitrite (145 mg) in water (2 m*l*). After 6 min, cold water (100 m*l*) was added to the solution. The azide which thereupon precipitated as a white mass was collected by filtration and washed with water, 4% sodium bicarbonate, and water, and then dried under a vacuum in a desciccator over phosphorus pentoxide and potassium hydroxide. The azide was added to a solution of III (0.70 g) and triethylamine (0.28 m*l*) in dimethylformamide (10 m*l*). The mixture was stirred for 3 days at 0°C and then evaporated *in vacuo*. The precipitate which formed upon the addition of water was collected, washed with 4% sodium bicarbonate, 10% citric acid, and water, and dried. It was recrystallized from dioxane - ether-petroleum ether; yield, 1.32 g (80%); mp 150—152°C; $[\alpha]_D^{22}$ −48.6° (*c* 0.7, acetic acid); R_f 0.81,[9] R_f of the hydrogenolyzed product of IV 0.76[11]

Found: C, 60.75; H, 7.43; N, 10.15%. Calcd for $C_{42}H_{60}N_6O_{11}$: C, 61.14; H, 7.33; N, 10.19%.

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucylglycyl-L-proline Hydrazide (V).** To a solution of IV (413 mg) in dimethylformamide (3 m*l*), hydrazine hydrate (0.5 m*l*) was added, and the solution was allowed to stand for 7 dyas at 30°C. The reaction mixture was then evaporated *in vacuo*. The hydrazide which precipitated upon the addition of water was collected by filtration and dried; yield, 382 mg (94%); mp 165—167°C; $[\alpha]_D^{25}$ −52.4° (*c* 0.5, acetic acid); R_f 0.72,[9] R_f of the hydrogenolyzed product of V 0.57.[11]

Found: C, 58.73; H, 7.10; N, 13.29%. Calcd for $C_{40}H_{58}N_6O_{10}\cdot 1/2H_2O$: C, 58.59; H, 7.25; N, 13.67%.

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucylglycyl-L-proline (VI).** To a solution of IV (1.24 g) in a mixture of methanol (10 m*l*) and dioxane (10 m*l*), N sodium hydroxide (4.5 m*l*) was added; the solution was then allowed to stand for 24 hr at room temperature. After the addition of 0.5 M citric acid (9 m*l*) under cooling, the solution was concentrated *in vacuo* at a low temperature, and the residue was treated with water (100 m*l*). After it had been stored in a refrigerator for several hours, the precipitate was collected by filtration, washed with water, and dried. Recrystallization from methanol - ether - petroleum ether gave 0.80 g (67%); mp 144—147°C; $[\alpha]_D^{22}$ −37.1° (*c* 0.7, acetic acid); R_f 0.72.[9]

Found: C, 60.46; H, 7.13; N, 10.68%. Calcd for $C_{40}H_{56}N_6O_{11}$: C, 60.28, H, 7.08, N, 10.55%.

L-Valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucylglycyl-L-proline Hydrochloride (VII). To a solution of VI (398 mg) in dioxane (5 m*l*), 3.8 N hydrogen chloride in dioxane (4.8 m*l*) was added. After it had then been permitted to stand for 6 hr at room temperature, the solution was evaporated *in vacuo*. The residue was triturated with ether and washed repeatedly with ether by decantation. The crystalline product was collected by filtration with the aid of ether; yield, 322 mg (96%); mp 160—162°C; $[\alpha]_D^{25}$ −41.3° (*c* 0.3, acetic acid); R_f 0.68.[9]

Found: C, 52.29; H, 7.59; N, 12.27%. Calcd for $C_{31}H_{49}N_6O_8Cl\cdot 2H_2O$: C, 52.79; H, 7.58; N, 11.92%.

***p*-Methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucylglycyl-L-prolyl-L-valyl-δ-benzyloxy-carbonyl-L-ornithyl-L-leucylglycyl-L-proline (VIII).** Into a chilled solution of V (382 mg) in glacial acetic acid (15 m*l*), N hydrochloric acid (1 m*l*) and sodium nitrite (36 mg) in water (1.5 m*l*) were stirred. After 6 min, cold water (100 m*l*) was added to the solution. The azide which thereupon precipitated was collected by filtration and treated as has been described in connection with the preparation of IV. The azide was added to a solution of VII (314 mg) in a mixture of dimethylformamide (10 m*l*) and triethylamine (0.13 m*l*), and the mixture was stirred for 3 days at 0°C. The reaction mixture was then evaporated *in vacuo*. The precipitate which formed upon the addition of 10% citric acid was collected, washed with 10% citric acid, and water, and dried. Recrystallization from methanol - petroleum ether gave 300 mg (45%); mp 223—225°C; $[\alpha]_D^{25}$ −76.7° (*c* 0.3, acetic acid); R_f 0.78.[9]

Found: C, 59.18; H, 7.59; N, 11.33%. Calcd for

7) D. W. Clayton, J. A. Farrington, G. W. Kenner and J. M. Turner, *J. Chem. Soc.*, **1957**, 1398.

8) T. Kato, S. Makisumi, M. Ohno and N. Izumiya, *Nippon Kagaku Zasshi, (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **83**, 1151 (1962).

9) The *Rf* value refers 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) T. Kato, M. Kondo, M. Ohno and N. Izumiya, This Bulletin, **38**, 1202 (1965).

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

$C_{71}H_{102}N_{12}O_{18}\cdot 2H_2O$: C, 58.90; H, 7.39; N, 11.61%.

Cyclo-(L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-glycyl-L-prolyl)$_2$ (IX). *IX (from deca).* To a solution of VIII (240 mg) in pyridine (5 ml), di-*p*-nitrophenyl sulfite[12] (1.1 g) was added; the reaction mixture was then allowed to stand for 24 hr at room temperature. After evaporation, the product was triturated with petroleum ether and washed repeatedly with a mixture of ether and petroleum ether (1 : 1) by decantation, until no yellow color could be discerned on the addition of a sodium hydroxide solution to the washings. The product was then filtered, washed with a mixture of ether and petroleum ether (1 : 1), and dried. It weighed 269 mg. A small portion of this product was dissolved in dimethylformamide - N sodium hydroxide (1 : 1), and the *p*-nitrophenyl ester content was estimated by measuring the optical density of the solution at 412 mμ.[13] The purity of the compound was estimated to be 100%. To the compound, anisole (0.2 ml) and trifluoroacetic acid (2 ml) were added at 0°C. The solution was then evaporated *in vacuo* at 0°C, and the residue was triturated with ether. The decapeptide *p*-nitrophenyl ester trifluoroacetate was collected, washed with ether, and dissolved in dimethylformamide (6 ml). The solution was stirred, drop by drop, into pyridine (100 ml) which had been kept at 55—60°C over a period of 2 hr; the stirring was then continued for an additional 2 hr at the same temperature. After the solvent had been removed, the residual oil was dissolved in a mixture of methanol (70 ml) and water (30 ml). The insoluble substance was removed by filtration, and the filtrate was passed successively through columns of Dowex 1 (OH^- form, 2×8 cm) and Dowex 50 (H^+ form, 1.6×8 cm). The columns were then washed with the same solvent (300 ml), the combined effluent was evaporated to dryness *in vacuo*, and the residue was collected by filtration by the aid of water (97 mg). The product was recrystallized from methanol - ether - petroleum ether; yield, 90 mg (43% from VIII); mp 159—161°C; $[\alpha]_D^{24}$ −195° (*c* 0.3, acetic acid); R_f 0.85.[9]

Found: C, 59.63; H, 7.49; N, 13.18%. Calcd for $C_{62}H_{92}N_{12}O_{14}\cdot H_2O$: C, 59.69; H, 7.60; N, 13.48%.

The molecular weight of IX was determined by the use of a Hitachi osmometer, type 115 (solvent: methanol).

Found: 1240. Calcd for $C_{62}H_{92}N_{12}O_{14}\cdot H_2O$: 1257.

IX (from penta). To a VI solution (398 mg) in pyridine (4 ml), di-*p*-nitrophenyl sulfite (1.62 g) was added. The pentapeptide *p*-nitrophenyl ester trifluoroacetate was prepared as has been described above and then dissolved in dimethylformamide (5 ml). The solution was treated with pyridine (150 ml) at 55—60°C for 4 hr, the solvent was evaporated *in vacuo*, and the residue was dissolved in a mixture of methanol (80 ml), dioxane (40 ml), and water (30 ml). The insoluble substance was removed by filtration, and the filtrate was treated with columns of Dowex 1 and Dowex 50. When the combined effluent was evaporated to dryness, the residue weighed 140 mg. A few milligrams of the residue were hydrogenolyzed in the presence of palladium black, after which the hydrogenolyzed material was subjected to assays by CMC column chromatography and paper electrophoresis; the material gave only one peak or spot, which had a position similar to that of the authentic cyclic decapeptide dihydrochloride (X·2HCl). The residue, when recrystallized from methanol-petroleum ether gave 100 mg (18% from VI); mp 159—161°C; mixed melting point with IX from deca, 159—161°C; $[\alpha]_D^{24}$ −193° (*c* 0.3, acetic acid); R_f 0.85.[9]

Cyclo-(L-valyl-L-ornithyl-L-leucylglycyl-L-prolyl)$_2$·2HCl (X·2HCl). IX (49 mg), dissolved in methanol (1 ml) and 0.55 N methanolic hydrogen chloride (0.17 ml), 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 powder which remained was collected with the aid of ether; yield of the air-dried product, 43 mg (94%); mp 239—240°C; $[\alpha]_D^{24}$ −203° (*c* 0.3, ethanol); R_f 0.59,[9] 0.80.[11]

Found: C, 48.66; H, 8.40; N, 14.75%. Calcd for $C_{46}H_{82}N_{12}O_{10}Cl_2\cdot 6H_2O$: C, 48.36; H, 3.30; N, 14.72%.

The air-dried product lost 3.3% of its weight when it was left in a desiccator over calcium chloride at room temperature. Calcd for $2H_2O$: 3.2%. The air-dried product lost 9.6% of its weight after it had been dried over phosphorus pentoxide for 3 hr at 80°C, 2 mmHg. Calcd for $6H_2O$: 9.5%.

Chromatography and Electrophoresis of X and Gramicidin S. CMC column chromatography was performed as follows. Each portion (0.5—1 mg) of hydrochlorides of X and gramicidin S was dissolved in 0.2—0.3 ml of 0.2 M pyridinium acetate containing 30% methanol (pH 5.0); then the solution was applied to a column (0.9×50 cm) of carboxymethylcellulose

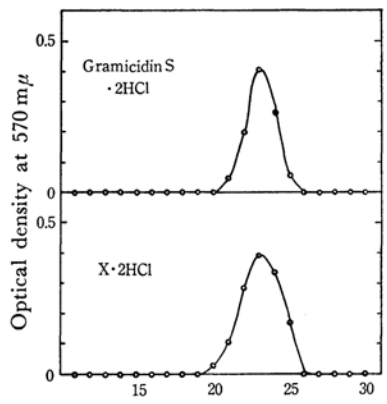


Fig. 3. CMC chromatography of hydrochlorides of gramicidin S and X.

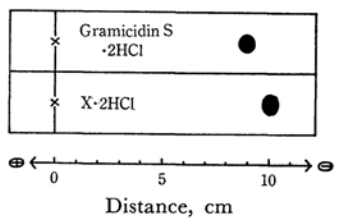


Fig. 4. Paper electrophoresis of hydrochlorides of gramicidin S and X.

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

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

Table 1. Inhibitory of gramicidin S and X on microorganisms minimum inhibitory concentrations, μg/ml

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Micobacterium avium*
Gramicidin S·2HCl	>100	>100	5	5	50
$Gly^{4,4'}$-gramicidin S·2HCl	>100	>100	>100	50	>100

(Eastman Organic Chem. 7796). Elution was carried out with the same solvent at 20—25°C, and a flow rate was about 20 m*l* per hr and 2-m*l* fractions were collected. The peptide content of the fractions was determined by the method described by Yemm and Cocking,[14] using gramicidin S as a control. The results shown in Fig. 3 indicate that the pattern of the chromatogram of X is indistinguishable from that of gramicidin S.

Their analyses were also carried out by electrophoresis. An appropriate amount of the material was placed on a Toyo Roshi No. 52 paper, and 500V/30 cm were applied for 3 hr at room temperature, using a formic acid - acetic acid - methanol - water (1 : 3 : 6 : 10, v/v) system, at pH 1.8 (Fig. 4). Figure 4 shows that X migrates faster toward the cathode than gramicidin S.

Microbiological Assays. The microorganisms employed are shown in Table 1. $Gly^{4,4'}$-gramicidin S (X) appears to exhibit weak antibacterial activity against *Bacillus Subtilis*.

The authors wish to express their thanks to Dr. Motoo Shibata of Takeda Chemical Industries, Ltd., for his microbiological assays. Thanks are also due the Ajinomoto Co., Inc., and the Kyowa Hakko Kogyo Co., Ltd., for supplying some of the amino acids.

14) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).