BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 39 1747—1753 (1966)

Studies of Peptide Antibiotics. V. Syntheses of Cyclic Penta- and Decapeptides with the L-Valyl-L-ornithyl-L-leucyl-D-phenylalanylsarcosyl Sequence

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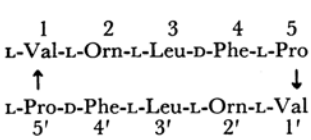
(Received November 15, 1965)

A cyclic decapeptide dihydrochloride cyclo-(L-valyl-L-ornithyl-L-leucyl-D-phenylalanylsarcosyl)$_2$·2HCl, which is an analog of gramicidin S and a cyclic pentapeptide monohydrochloride cyclo-L-valyl-L-ornithyl-L-leucyl-D-phenylalanylsarcosyl·HCl, were synthesized for the purpose of comparing their antibacterial activities with gramicidin S. The cyclic decapeptide dihydrochloride was obtained by hydrogenolysis in the presence of hydrogen chloride of a cyclic benzyloxycarbonyl-substituted decapeptide, which had been prepared by the cyclization of a corresponding linear decapeptide active ester. The cyclization reaction of a linear pentapeptide active ester yielded a mixture of a cyclic benzyloxycarbonyl-substituted pentapeptide as a major component, and a cyclic decapeptide and an unidentified cyclic peptide as minor components. The effects of two cyclic peptide hydrochlorides on bacterial growth were tested. The cyclic decapeptide was as active as gramicidin S; however, the cyclic pentapeptide showed no activity in relation to any of the microorganisms tested.

In connection with a series of studies in this laboratory on the significance of the chemical functional groups of gramicidin S (I) to its biological activities, the authors reported, in a previous paper, the synthesis of an analog of gramicidin S wherein proline residues in the 5 and 5′ positions of the molecule were replaced by glycines.[1] This 5, 5′-glycine-gramicidin S was found to possess approximately 10 times the antibacterial activity of natural gramicidin S in the test with a synthetic medium, while it possessed about half the activity of gramicidin S with a bouillon agar medium. In the light of the considerable antibacterial potency of this analog, the decision was made to investigate the effect of replacing the proline residues by sarcosines. The present paper will describe the syntheses and the antibacterial properties of this cyclic decapeptide, 5, 5′-sarcosine-gramicidin S (II), and of the cyclic pentapeptide, cyclo-L-valyl-L-ornithyl L-leucyl-D-phenylalanylsarcosyl (III), related to I and II.



(I)

The structure of gramicidin S, with numbers indicating the positions of the individual amino acid residues.

1) H. Aoyagi, T. Kato, M. Ohno, M. Kondo, M. Waki, S. Makisumi and N. Izumiya, This Bulletin, **38**, 2139 (1965); H. Aoyagi, T. Kato, M. Ohno and N. Izumiya, *J. Am. Chem. Soc.*, **86**, 5700 (1964).

L-Val-L-Orn-L-Leu-D-Phe-Sar
↑ ↓
Sar-D-Phe-L-Leu-L-Orn-L-Val
(II)

L-Val-L-Orn-L-Leu-D-Phe-Sar
↑ |
|
(III)

The sequence of reactions employed for the synthesis of II is shown in Fig. 1. The condensation of the azide derived from acylpentapeptide hydrazide (XII) with pentapeptide (XI) gave acyldecapeptide (XIII) in a yield of 73%. The treatment of XIII with an excess of di-*p*-nitrophenyl sulfite gave an amorphous acyldecapeptide *p*-nitrophenyl ester (XIV). The *p*-methoxybenzyloxycarbonyl group of XIV was removed by the action of trifluoroacetic acid, and the decapeptide *p*-nitrophenyl ester trifluoroacetate (XV) thus obtained was treated with a large amount of hot pyridine for the cyclization reaction. The benzyloxycarbonyl-substituted cyclic peptide obtained in a yield of 45% (from XIII) was found to be a monomer, shown as XVI, from the result of the molecular weight determination. In this connection, it is of interest to note that the cyclization reaction of the decapeptide active ester yields monomer, cyclic decapeptide.[1,2] The final product (II·2HCl) was obtained as colorless

Fig. 1.

Z, benzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; pMZ, *p*-methoxybenzyloxycarbonyl; ONP, *p*-nitrophenoxy ester; MA, mixed anhydride method. An amino acid residue except Sar and D-Phe is of L-configuration.

2) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957); **41**, 1582 (1958).

crystals containing seven moles of the water of crystallization. Its homogeneity was demonstrated by CM-cellulose column chromatography (Fig. 3) and paper electrophoresis (Fig. 4).

The synthesis of the protected cyclic decapeptide (XVI) was also attempted by the dimerization reaction of the pentapeptide active ester (XVIII). As is shown in Fig. 2, the treatment of the pentapeptide active ester (XVIII) gave a product (XX) with the character of benzyloxycarbonyl-substituted cyclic peptide in a fairly good yield. After the experiments of CM-cellulose column chromatography and paper electrophoresis (Figs. 3 and 4) with a hydrogenated material (XXI) of the product, this product (XX) was found to be a mixture of three components, namely, a protected cyclic pentapeptide (XIX) as a major component, and the protected cyclic decapeptide (XVI) and an unidentified cyclic peptide as minor components.

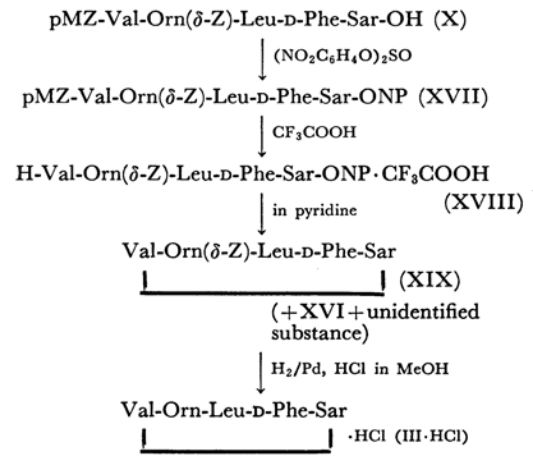


Fig. 2.

It was determined further, by analysis by means of the CM-cellulose column chromatography of the material (XXI) that the molar ratio between XIX and XVI was 92 : 8. The protected cyclic pentapeptide (XIX) in a pure state was obtained easily by several recrystallizations from methanol-ether of the product (XX); XIX was less soluble in methanol than XVI or the unidentified cyclic peptide. The fact that this major product is a monomer, shown as XIX, was denomstrated by the result of the molecular weight determination. The hydrogenation of XIX in the presence of equivalent hydrogen chloride yielded a cyclic pentapeptide hydrochloride (III·HCl) as colorless crystals, with three moles of the water of crystallization.

In a previous paper,[1] we observed that the cyclization reaction of the L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl- D - phenylalanylglycine active ester yielded a large amount of the pyridine-insoluble cyclic peptide, besides a small amount of the desired protected cyclic decapeptide. This pyridine-insoluble substance appears, from the results of the experiment of CM-cellulose chromatography, to be a cyclic benzyloxycarbonyl-substituted pentapeptide.[3] In this connection, it would be of interest to note that two reports have described that cyclization reaction of pentapeptide active esters, wherein *C*-terminal amino acid residues were glycine and isoleucine, yields a monomer cyclic pentapeptide.[4] On the contrary, several experiments have shown that the pentapeptide active ester, wherein the partial sequence at the *C*-terminal part is D - phenylalanyl - L - proline, yielded cyclic decapeptide by dimerization reaction.[5]

In order to ascertain whether or not the cyclic peptides, II and III, possess antibacterial activities, the effect of various levels of II and III on the growth of several microorganisms was examined (Table I). It was found that II was as active as gramicidin S in affecting several microorganisms. This result demonstrates further that the side chain of proline of gramicidin S is not absolutely required for its full activity. On the other hand, III exhibited no antibacterial activity in relation to any of the microorganisms tested. A communication from this laboratory has also described that a cyclic hexapeptide related to gramicidin S, cyclo-L-valyl-L-ornithyl- L - leucyl-D-phenylalanyl-L-prolylglycyl, showed no such activity either.[6] These results suggest that a certain ring size of a molecule, besides a specific amino acid sequence, will be necessary for the exhibition of activity.

Experimental

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

Sarcosine Ethyl Ester *p*-Toluenesulfonate (IV). —This compound was prepared according to the general procedure of Kato et al.[7] A solution of sarcosine (4.0 g.) and *p*-toluenesulfonic acid monohydrate (9.4 g.) in a mixture of ethanol (20 ml.) and carbon tetrachloride (100 ml.) was refluxed, and the water thus liberated was removed as an azeotropic mixture. The reaction mixture was concentrated in vacuo, and the residual oil was treated with ether and petroleum ether. The product was very hygroscopic; yield, 12.0 g.

3) H. Aoyagi and N. Izumiya, unpublished.
4) G. W. Kenner and J. M. Turner, *Chem. & Ind.*, **1955**, 602; G. W. Kenner, P. J. Thomson and J. M. Turner, *J. Chem. Soc.*, **1958**, 4148; K. Isono and R. W. Curtis, *Phytochemistry*, **3**, 277 (1964).
5) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 2186 (1958); German Patent, *Chem. Abstr.*, **57**, 949 (1962).
6) T. Kato, M. Kondo, M. Ohno and N. Izumiya, This Bulletin, **38**, 1202 (1965).
7) T. Kato, S. Makisumi, M. Ohno and N. Izumiya, *J. Chem. Soc. Japan, Pure Chem. Sect. (Nippon Kagaku Zasshi)*, **83**, 1151 (1962).

(92%); R_f 0.61.[8]

Benzyloxycarbonyl - D - phenylalanylsarcosine Ethyl Ester (V).—Into a solution of benzyloxycarbonyl-D-phenylalanine (6.71 g.) and IV (6.5 g.) in chloroform (80 ml.) there were stirred triethylamine (3.14 ml.) and dicyclohexylcarbodiimide[9] (4.61 g.) at 0°C. After it had been permitted to stand overnight at 0°C, the mixture was evaporated in vacuo and the residue was diluted with ethyl acetate; then the dicyclohexylurea thus precipitated was filtered off. The filtrate was washed successively with 4% sodium bicarbonate, 3% hydrochloric acid and water, and dried over anhydrous sodium sulfate. The filtered solution was evaporated in vacuo. The product was obtained as an oil; yield, 7.68 g. (86%); R_f 0.73.[8]

D-Phenylalanylsarcosine Ethyl Ester Hydrochloride (VI).—V (7.68 g.) was subjected to hydrogenolysis in the presence of palladium black and 0.44 N methanolic hydrogen chloride (46.5 ml.). The filtrate from the catalyst was evaporated to dryness in vacuo; yield of oil, 5.80 g. (100%); R_f 0.70.[8]

Benzyloxycarbonyl - L - leucyl - D - phenylalanylsarcosine Ethyl Ester (VII).—To a chilled solution of benzyloxycarbonyl-L-leucine (5.11 g.) and triethylamine (2.70 ml.) in tetrahydrofuran (40 ml.), isobutyl chloroformate (2.52 ml.) was added. After 15 min. a mixture of VI (5.80 g.), triethylamine (2.70 ml.) and chloroform (40 ml.) was added to the solution. The mixture was left overnight at room temperature, and then evaporated in vacuo. The residual oil was dissolved in ethyl acetate and treated as has been described in connection with the preparation of V. The oily residue wieghed 6.45 g. (66%); R_f 0.88.[8]

L-Leucyl-D-phenylalanylsarcosine Ethyl Ester Hydrochloride (VIII).—VII (2.96 g.) was subjected to hydrogenolysis in the presence of palladium black and 0.40 N methanolic hydrogen chloride (16.6 ml.), and then treated as has been described in connection with the preparation of VI. The yield of oily product was 2.28 g. (91%); R_f 0.59[8] and 0.62.[10]

***p*-Methoxybenzyloxycarbonyl-L-valyl - δ - benzyloxycarbonyl-L-ornithyl-L - leucyl - D - phenylalanyl - sarcosine Ethyl Ester (IX).**—The following operations were carried out in a cold room. To a chilled solution *p*-methoxybenzyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl - L - ornithine hydrazide[6] (1.47 g.) in glacial acetic acid (30 ml.) there were stirred N hydrochloric acid (4.1 ml.) and sodium nitrite (210 mg.) in water (1 ml.). After 6 min., cold water (200 ml.) 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 desiccator over phosphorus pentoxiede and potassium hydroxide. The azide was added to a solution of VIII (1.12 g.) and triethylamine (0.38 ml.) in dimethylformamide (20 ml.). The mixture was stirred for 3 days at 0°C and evaporated in vacuo. The precipitate which formed upon the addition of water (30 ml.) to the syrup was collected, wahsed with 4% sodium bicarbonate, 10% citric acid and water, and dried. It was recrystallized from methanol - ether - petroleum ether; yield, 1.82 g. (75%); m. p. 143—146°C; $[\alpha]_D^{26}$ —28.4° (*c* 0.5, acetic acid); R_f 0.73.[8]

Found: C, 62.79; H, 7.52; N, 9.70. Calcd. for $C_{47}H_{64}O_{11}N_6 \cdot {}^1/_2H_2O$: C, 62.86; H, 7.30; N, 9.40%.

***p*-Methoxybenzyloxycarbonyl- L -valyl- δ -benzyloxycarbonyl - L - ornithyl-L-leucyl-D-phenylalanyl-sarcosine (X).**—To a solution of IX (1.16 g.) in a mixture of methanol (30 ml.) and dioxane (10 ml.), 2 N sodium hydroxide (2 ml.) was added, and the solution was allowed to stand for 4 hr. at room temperature. After the addition of 10% citric acid (15 ml.) under cooling, the solution was concentrated in vacuo at a low temperature and the residue treated with water (100 ml.). After it had been stored in a refrigerator for several hours, the precipitate was collected by filtration, washed with water, and dried. The product was recrystallized from methanol - ether - petroleum ether; yield, 0.96 g. (86%); m. p. 151—153°C; $[\alpha]_D^{26}$ −26.8° (*c* 0.5, acetic acid); R_f 0.75.[8]

Found: C, 61.90; H, 7.20; N, 9.73. Calcd. for $C_{45}H_{60}O_{11}N_6 \cdot {}^1/_2H_2O$: C, 62.12; H, 7.07; N, 9.66%.

L-Valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylsarcosine Hydrochloride (XI).—To a solution of X (1.64 g.) in dioxane (20 ml.), there was added 4.0 N hydrogen chloride in dioxane (20 ml.). After it had been permitted to stand for 2 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, 1.38 g. (98%); m. p. 162°C; $[\alpha]_D^{20}$ −15.4° (*c* 0.5, acetic acid); R_f 0.67.[8]

Found: C, 58.69; H, 7.47; N, 11.31. Calcd. for $C_{36}H_{53}O_8N_6Cl$: C, 58.96; H, 7.28; N, 11.46%.

An attempt to crystallize the HCl-free pentapeptide failed.

***p*-Methoxybenzyloxycarbonyl- L -valyl- δ -benzyloxycarbonyl- L -ornithyl- L -leucyl- D -phenylalanyl-sarcosine Hydrazide (XII).**—To a solution of IX (2.30 g.) in dimethylformamide (10 ml.), hydrazine hydrate (2.5 ml.) was added and the solution was allowed to stand for 2 days at 30°C. The reaction mixture was then evaporated in vacuo. The hydrazide which precipitated upon the addition of water (30 ml.) was collected by filtration and dried; yield, 2.19 g. (98%); m. p. 172—175°C; $[\alpha]_D^{26}$ −32.8° (*c* 0.5, acetic acid); R_f 0.74.[8]

Found: C, 60.76; H, 7.18; N, 12.55. Calcd. for $C_{45}H_{62}O_{10}N_8 \cdot H_2O$: C, 60.52; H, 7.22; N, 12.55%.

***p* - Methoxybenzyloxycarbonyl -L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L - leucyl - D - phenylalanyl-sarcosyl-L-valyl-δ- benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylsarcosine (XIII).** — Into a chilled solution of XII (0.79 g.) in a mixture of dimethylformamide (10 ml.) and glacial acetic acid (10 ml.), 1.2 N hydrochloric acid (2.4 ml.) and sodium nitrite (70 mg.) in water (4 ml.) were stirred. After 5 min., cold water (200 ml.) was added to the solution. The azide which thereupon precipitated was collected by

8) The R_f 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.

9) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

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

filtration and treated as has been described in connection with the preparation of IX. The azide was added to a solution of XI (0.66 g.) in a mixture of dimethylformamide (20 ml.) and triethylamine (0.26 ml.), and the mixture was stirred for 3 days at 0°C. The insoluble material was removed by filtration, and the filtrate was evaporated in vacuo. The precipitate which formed upon the addition of 10% citric acid (30 ml.) was collected, washed with 10% citric acid and water and dried. Recrystallization from dioxane-ether gave 1.02 g. (73%) of XIII; m. p. 208—209°C; $[\alpha]_D^{26}$ −45.6° (*c* 0.5, acetic acid); R_f 0.76.[8]

Found: C, 62.44; H, 7.53; N, 10.82. Calcd. for $C_{81}H_{110}O_{18}N_{12}\cdot H_2O$: C, 62.45; H, 7.25; N, 10.79%.

Cyclo-(L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylsarcosyl)$_2$ (XVI).—To a solution of XIII (467 mg.) in pyridine (6 ml.), di-*p*-nitrophenyl sulfite[11] (972 mg.) was added, and the reaction mixture was allowed to stand for 24 hr. at room temperature. After evaporation, the oily 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 collected by filtration, washed with a mixture of ether and petroleum ether, and dried. It weighed 444 mg. A small portion of this product (1.1 mg.) was dissolved in 25 ml. of dimethylformamide - N sodium hydroxide (1 : 1) (2.64×10^{-5} M/l.); the *p*-nitrophenyl ester content was estimated by measuring the optical density of the solution at 412 mμ.[2] The purity of the compound was estimated to be 88%.

To the *p*-nitrophenyl ester (XIV) (442 mg.), anisole (0.5 ml.) and trifluoroacetic acid (3.5 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 (XV) was collected, washed with ether, and dissolved in dimethylformamide (9 ml.) containing glacial acetic acid (0.1 ml.). The solution was stirred, drop by drop, into pyridine (150 ml.) kept at 55—60°C over a period of 3.5 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 (100 ml.) and water (20 ml.). An insoluble substance was removed by filtration, and the filtrate was passed successively through columns of Dowex 1 (OH^- form, 2×10 cm.) and Dowex 50 (H^+ form, 2×7 cm.). The columns were washed with the same solvent (150 ml.), the combined effluent was evaporated to dryness in vacuo, and the product was suspended in water (50 ml.), collected by filtration, and dried (219 mg.). The product was recrystallized from methanol - ether - petroleum ether; yield, 186 mg. (45% from XIII); m. p. 165°C; $[\alpha]_D^{26}$ −139° (*c* 0.3, acetic acid); R_f 0.93.[8]

Found: C, 62.76; H, 7.61; N, 12.24. Calcd. for $C_{72}H_{100}O_{14}N_{12}\cdot H_2O$: C, 62.86; H, 7.47; N, 12.22%.

The molecular weight of XVI was determined by a Model 301 A Osmometer, Mechrolab Inc. (solvent: methanol).[12]

Found: 1240. Calcd. for $C_{72}H_{100}O_{14}N_{12}\cdot H_2O$: 1376.

Cyclo-(L-valyl-L-ornithyl-L-leucyl-D-phenylalanylsarcosyl)$_2$ Dihydrochloride (II·2HCl).—XVI (41 mg.), dissolved in methanol (0.6 ml.) and 0.24 N methanolic hydrogen chloride (0.3 ml.), was subjected to hydrogenolysis in the presence of palladium black. The solution, after being filtered from the calalyst, was evaporated to dryness in vacuo. The powder which remained was collected with the aid of ether; the yield of the air-dried product, 32 mg. (92%); m. p. 212—214°C; $[\alpha]_D^{20}$ −157° (*c* 0.3, ethanol); R_f 0.68,[8] 0.89,[10] 0.71[13] and 0.88.[14]

Found: C, 52.51; H, 7.87; N, 12.50. Calcd. for $C_{56}H_{90}O_{10}N_{12}Cl_2\cdot 7H_2O$: C, 52.20; H, 8.12; N, 13.05%.

The molecular weight was determined by the procedure described above (solvent: methanol).

Found: 510.[15] Calcd. for (1/3) × ($C_{56}H_{90}O_{10}N_{12}Cl_2\cdot 7H_2O$): 429.

The air-dried product lost 1.5% of its weight when it was left in a desiccator over calcium chloride at room temperature. Calcd. for H_2O: 1.4%. The air-dried product lost 7.4% of its weight after drying over phosphorus pentoxide for 3 hr. at 80°C (2mmHg), Calcd. for $5H_2O$: 7.0%. The product was treated with dinitrofluorobenzene in the manner described by Sanger et al.[16] After the hydrolysis of the dinitrophenylated compound (DNP-II), only one DNP-amino acid, identified as δ-DNP-ornithine, was detected on a paper chromatogram; R_f 0.68.[8]

Cyclo-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanylsarcosyl (XIX).—To a solution of X (610 mg.) in pyridine (5 ml.), di-*p*-nitrophenyl sulfite (920 mg.) was added. The reaction mixture was allowed to stand for 24 hr. at room temperature and then treated as has been described in connection with the preparation of XVI. The product (XVII) obtained weighed 605 mg. The purity of the compound was estimated to be 107%. To the *p*-nitrophenyl ester (XVII) (600 mg.), anisole (0.5 ml.) and trifluoroacetic acid (3.5 ml.) were then added. The solution was evaporated in vacuo, and the residue was triturated with ether. The pentapeptide *p*-nitrophenyl ester trifluoroacetate (XVIII) was collected by filtration and dissolved in dimethylformamide (10 ml.) containing glacial acetic acid (0.1 ml.). The solution was then stirred, drop by drop, into pyridine (180 ml.) kept at 55—60°C over a period of 5 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 (100 ml.) and water (20 ml.). An insoluble substance was removed by filtration, and the filtrate was treated with the columns of Dowex 1 and 50 described in connection with the preparation of XVI. It weighed 183 mg. (XX). A few mg. of XX were subjected to hydrogenolysis; the hydrogenated material was designated as

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

12) We are indebted to Mr. M. Waki of this laboratory for the molecular-weight determination.

13) The R_f value of the thin-layer chromatography with Merck silica gel G refers to the *t*-butanol - formic acid - water (75 : 15 : 10, v/v) system.

14) The R_f value refers to the paper chromatography on Toyo Roshi No. 52 with the *t*-butanol - formic acid - water (75 : 15 : 10, v/v) system.

15) This compound appears to dissociate into three molecules under the conditions under which the determination of the molecular weight was carried out.

16) F. Sanger and E. O. P. Thompson, *Biochem. J.*, **53**, 353 (1953).

XXI. XXI was found to be a mixture of II, III and an unidentified substance (XXII) (Figs. 3 and 4). Several recrystallizations of XX from methanol-ether gave 95 mg. (20% from X) of pure XIX; m. p. 224—227°C; $[\alpha]_D^{26}$ +0.7° (*c* 0.3, acetic acid); R_f 0.93.[8]

Found: C, 62.80; H, 7.50; N, 12.44. Calcd. for $C_{36}H_{50}O_7N_6 \cdot {}^1/_2H_2O$: C, 62.86; H, 7.47; N, 12.22%.

The molecular weight of XIX was determined by a Model 301 A osmometer (solvent: methanol).

Found: 750. Calcd. for $C_{36}H_{50}O_7N_6 \cdot {}^1/_2H_2O$: 689.

Cyclo - L - valyl - L - ornithyl- L -leucyl- D -phenylalanylsarcosyl Hydrochloride (III · HCl). — XIX (34.4 mg.) was dissolved in methanol (1.5 ml.) and 0.24 N methanolic hydrogen chloride (0.25 ml.) and treated as has been described in connection with the preparation of II·2HCl; yield, 22.4 mg. (77%); m. p. 198°C; $[\alpha]_D^{20}$ −26.8° (*c* 0.3, ethanol); R_f, 0.69,[8] 0.88,[10] 0.70[13] and 0.87.[14]

Found: C, 53.20; H, 7.94; N, 13.13. Calcd. for $C_{28}H_{45}O_5N_6Cl \cdot 3H_2O$: C, 52.94; H, 8.09; N, 13.23%.

The molecular weight was determined by the procedure described above (solvent: methanol).

Found: 340.[17] Calcd. for $(1/2) \times (C_{28}H_{45}O_5N_6Cl \cdot 3H_2O)$: 318.

The air-dried product lost 5.4% of its weight when it was left in a desiccator over calcium chloride at room temperature. Calcd. for $2H_2O$: 5.7%. The air-dried product lost 8.3% of its weight after having been dried over phosphorus pentoxide for 3 hr. at 80°C, 2 mmHg. Calcd. for $3H_2O$: 8.5%. After the hydrolysis of the dinitrophenylated compound (DNP-III), only δ-DNP-ornithine was detected on a paper chromatogram; R_f 0.68.[10]

Amino Acid Analyses of II and III.—A complete amino acid analysis except for the sarcosine of each compound was carried out according to the procedure described in a previous paper.[1] Amino acid determinations of II and III gave the molar ratios of 1.0 : 1.0 : 1.1 : 1.0 and 1.0 : 0.9 : 1.0 : 1.1 for valine, ornithine, leucine and phenylalanine respectively. In order to determine the sarcosine content, a mixture of sarcosine (0.5 μmol.) and leucine (0.5 μmol.) was dinitophenylated[18] and subjected to thin-layer chromatography.[19] Each band of DNP-sarcosine and DNP-leucine was eluted with methanol, the eluent was evaporated, and the residue was dissolved in 1% sodium bicarbonate (10 ml.) for spectrophotometric determination. It was found that the intensities at 380—385 mμ for DNP-sarcosine and at 360 mμ for DNP-leucine were almost the same. The same operations were carried out for the hydrolysates of II and III; the molar ratios for sarcosine and leucine are of 0.9 : 1.0 and 0.9 : 1.0 respectively.

Chromatography and Electrophoresis of II, III, XXI and Gramicidin S.—Carboxymethylcellulose column chromatography was performed as follows: Each portion (0.5—1 mg.) of hydrochlorides of II, III, XXI and gramicidin S was dissolved in 0.2—0.3 ml. of 0.2 M pyridinium acetate containing 30% methanol (pH 5.0), and then the solution was applied to a column (0.9×50 cm.) of carboxymethyl cellulose (Eastman Organic Chem. 7796). When elution was carried out with the same solvent at 20—25°C, and a flow rate was about 20 ml. per hr., 2 ml. fractions were collected. The peptide content in the fractions was determined by the method described by Yemm and Cocking.[20] Gramicidin S was used as a control. The results are shown in Fig. 3. In order to examine the molar ratio between XIX and XVI in the cyclization product (XX) from the pentapeptide active ester (XVIII), a solution containing 1 μmol. of III and 0.5 μmol. of II was subjected to column chromatography; the ratio of intensities resulting from ninhydrin between III and II was found to be 80 : 100 by calculating the areas on a chromatogram of the optical density-fraction number. By the use of the value obtained above, it was calculated from Fig. 3 that the molar ratio between III and II in XXI was about 92 : 8. Therefore, it was deduced that the cyclization reaction of the pentapeptide active ester (XVIII) gave the cyclic pentapeptide (XIX) predominantly. Their analyses were also carried out by electrophoresis. An appropriate amount of the material was placed on a paper of Toyo Roshi No. 52, and 500 V./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

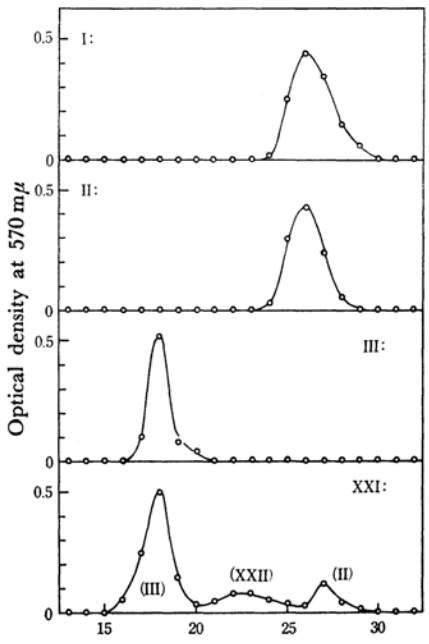


Fig. 3. CM-Column chromatography of hydrochlorides of gramicidin S (I), II, III and hydrogenated material (XXI) of the product (XX) obtained by cyclization reaction of pentapeptide active ester (XVIII). XXII is an unidentified cyclic peptide.

17) This compound appears to dissociate into two molecules under the conditions under which the determination of the molecular weight was carried out.

18) W. A. Schroeder and J. LeGette, *J. Am. Chem. Soc.*, **75**, 4612 (1953).

19) M. Brenner, N. Niederwieser and G. Pataki, *Experientia*, **17**, 145 (1961).

20) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955); *Biochem. J.*, **58**, xii (1954).

TABLE I. INHIBITORY ACTIVITY OF II, III AND GRAMICIDIN S ON MICROORGANISMS

Minimum inhibitory concentrations, μg./ml.

A. Bouillon agar medium[a)]

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Mycobacterium avium*	*Mycobacterium avium* (Streptomycin resistant St.)
II·2HCl	>100	>100	10	5	100	100
III·HCl	>100	>100	>100	>100	>100	>100
Gramicidin S·2HCl	>100	>100	5	5	>100	>100

B. Synthetic medium[b)]

II·2HCl	>100	>100	10	5	100	100
III·HCl	>100	>100	>100	>100	>100	>100
Gramicidin S·2HCl	>100	>100	5	5	>100	>100

a) Usual bouillon agar medium, pH 7.0.
b) 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.

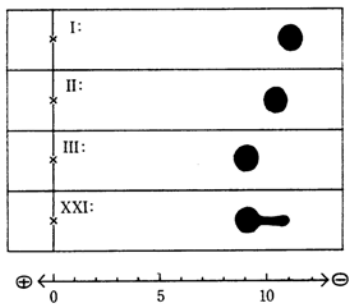


Fig. 4. Paper electrophoresis of hydrochlorides of gramicidin (I), II, III and XXI.

1.8 (Fig. 4). Figure 4 shows also that the cyclization product (XX) contained the cyclic pentapeptide (XIX) as a major component.

Microbiological Assays. — The microorganisms employed are shown in Table I. The minimum amount of the compounds needed for a complete inhibition of growth was determined by a dilution method with a bouillon agar medium and with a synthetic medium. As is shown in Table I, the cyclic decapeptide II was found to be as active as gramicidin S against *B. subtilis* and *Staph. aureus*. II appears to exhibit weak antibacterial activity against *Mycobact. avium* (streptomycin-resistant) and *Mycobact. avium*. The cyclic pentapeptide III exhibited no activity with relation to any of the organisms tested.

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