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Studies of Peptide Antibiotics. IV. The Synthesis of Tyrocidine A*

By Motonori OHNO, Tetsuo KATO, Satoru MAKISUMI and Nobuo IZUMIYA

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

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The synthesis of a cyclic decapeptide, cyclo-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl, which has an amino acid sequence determined by Craig et al. for natural antibiotics, tyrocidine A, has been described. The chemical and biological properties of the synthetic product have been shown to be in agreement with those of the natural tyrocidine A.

Tyrocidine A is a basic polypeptide isolated as a hydrochloride by Battersby and Craig,[1] using a technique of countercurrent distribution, from the family of closely-related substances present in tyrocidine which is produced in autolyzed cultures of *Bacillus brevis*. Its structure has been determined by Craig and his colleagues[2,3] by means of a

* A part of this work has been briefly communicated: M. Ohno and N. Izumiya, *J. Am. Chem. Soc.*, **88**, 376 (1966).

1) A. R. Battersby and L. C. Craig, ibid., **74**, 4019 (1952).

2) A. R. Battersby and L. C. Craig, ibid., **74**, 4023 (1952).

3) A. Paladini and L. C. Craig, ibid., **76**, 688 (1954).

partial hydrolysis and by means of a study of the countercurrent distribution. Tyrocidine A is, like gramicidin S,[4,5] a cyclic decapeptide; it is constructed from three molecules of phenylalanine and one each of asparagine, glutamine, tyrosine, valine, ornithine, leucine and proline. All amino acid residues except two molecules of D-phenylalanine have a L-configuration. Figure 1 indicates

L-Phe-D-Phe-L-Asp(NH_2)-L-Glu(NH_2)-L-Tyr
↑ ↓
L - Pro - D - Phe - L - Leu - L - Orn - L - Val

Fig. 1. Constructional formula of tyrocidine A.

the constructional formula of tyrocidine A. The pentapeptide sequence, L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl, is also found in gramicidin S, tyrocidine B[6] and tyrocidine C.[7] The syntheses of the linear penta-[8] and decapeptide derivatives[9] related to tyrocidine A have been reported by Schwyzer et al., but the complete synthesis of tyrocidine A has not yet been accomplished. We, therefore, have here attempted to synthesize this cyclic decapeptide. This paper will describe the synthesis of the cyclic decapeptide hydrochloride (I_s) having the amino acid sequence of tyrocidine A (Fig. 1) and will attempt to establish the identity of the synthetic product (I_s) with natural tyrocidine A hydrochloride (I_n) in chemical and biological properties.

The cyclization of a linear decapeptide was carried out by a *p*-nitrophenyl ester method. Di-*p*-nitrophenyl sulfite was used as a reagent of the *p*-nitrophenyl esterification reaction.[10] It has been observed that, when *N*-protected peptide is treated with di-*p*-nitrophenyl sulfite in an attempt to obtain a corresponding *p*-nitrophenyl ester, the *C*-terminal amino acid residue is subjected to partial racemization, with the exception of glycine and proline residues.[10,11] For this reason, the proline residue was selected as the *C*-terminal amino acid in a linear decapeptide to be activated to the *p*-nitrophenyl ester (see the compound XXI in Fig. 5).[12]

p-Methoxybenzyloxycarbonyl[13] and benzyloxycarbonyl groups were employed for the selective blocking of the *N*-terminal amino group and the δ-amino group of ornithine residue respectively.

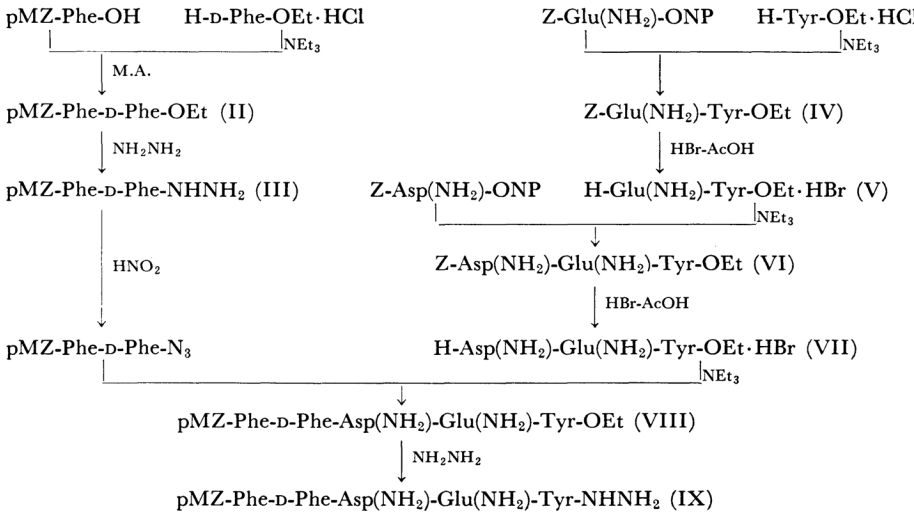

Fig. 2. Synthesis of pMZ-pentapeptide hydrazide (IX): prefix L for amino acids of L-configuration is omitted; pMZ, *p*-methoxybenzyloxycarbonyl; Z, benzyloxycarbonyl; Et, ethyl; NP, *p*-nitrophenyl; NEt_3, triethylamine; AcOH, acetic acid; M. A., mixed anhydride method.

4) R. Consden, A. H. Gordon, A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **41**, 596 (1947); A. R. Battersby and L. C. Craig, *J. Am. Chem. Soc.*, **73**, 1887 (1951).
5) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).
6) T. P. King and L. C. Craig, *J. Am. Chem. Soc.*, **77**, 6627 (1955).
7) M. A. Ruttenberg, T. P. King and L. C. Craig, *Biochemistry*, **4**, 11 (1965).
8) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **42**, 972 (1959).
9) R. Schwyzer, E. Surbeck-Wegmann and H. Dietrich, *Chimia*, **14**, 366 (1960).
10) B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, **43**, 1760 (1960).
11) M. Ohno and N. Izumiya, This Bulletin, **38**, 1831 (1965).
12) In the synthesis of evolidin, natural cyclic heptapeptide, a leucine residue was selected as a *C*-terminal amino acid of a linear heptapeptide to be activated to the *p*-nitrophenyl ester by using di-*p*-nitrophenyl sulfite, in spite of the presence of a proline residue as a constituent. Nevertheless, the resulting cyclic heptapeptide was proved to be homogeneous with regard to the leucine residue; it was identical to the natural product in many properties (R. O. Studer and W. Lergier, *Helv. Chim. Acta*, **48**, 460 (1965)).
13) F. Weygand and H. Hunger, *Chem. Ber.*, **95**, 1 (1962).

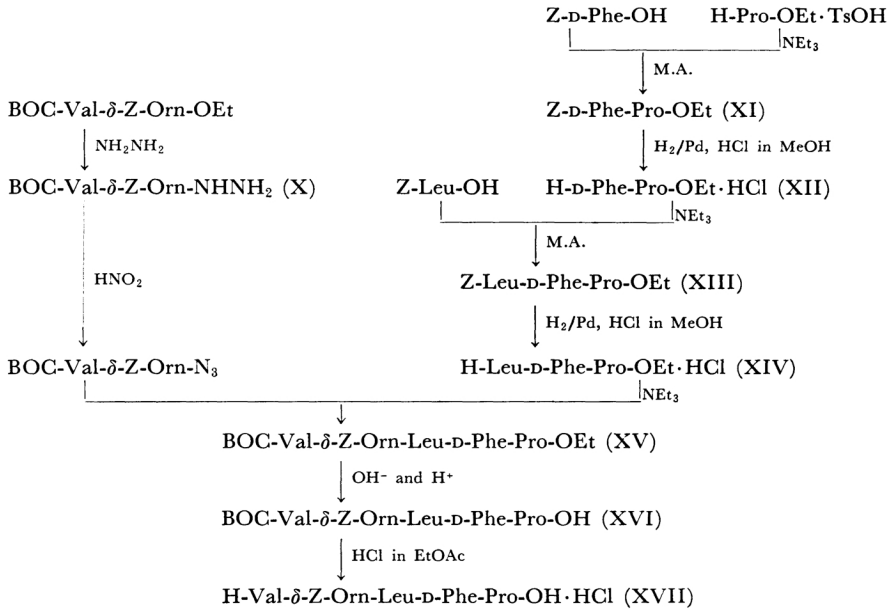

Fig. 3. Synthesis of pentapeptide hydrochloride (XVII): BOC, *t*-butyloxycarbonyl; TsOH, *p*-toluenesulfonic acid; MeOH, methanol; EtOAc, ethyl acetate.

As has been described in a previous paper, the *p*-methoxybenzyloxycarbonyl group is so available that it is released easily with the action of anhydrous trifluoroacetic acid, without hurt to the sensitive *p*-nitrophenyl ester group.[11]

The scheme for the synthesis of the *N*-terminal pentapeptide derivative, *p*-methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosine hydrazide (IX), is presented in Fig. 2. *p*-Methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanine ethyl ester (II) was prepared by the reaction of *p*-methoxybenzyloxycarbonyl-L-phenylalanine[13] with D-phenylalanine ethyl ester by means of the mixed anhydride procedure. The subsequent treatment of II with twenty equivalents of hydrazine in dimethylformamide gave the hydrazide (III) in a 96% yield. The *C*-terminal tripeptide derivative (VI) was prepared by step-by-step elongation by means of the *p*-nitrophenyl ester method. The reaction of benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester with tyrosine ethyl ester gave the crystalline dipeptide derivative (IV). This was treated with hydrogen bromide in acetic acid and then allowed to react with benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester. VI was thus obtained in a crystalline form. The azide, which had been derived from III, was made to react with the tripeptide ester (VII) prepared from VI with the action of hydrogen bromide in acetic acid; the protected pentapeptide ester (VIII) was thus obtained in a 75% yield. VIII was converted to the hydrazide (IX) in a 86% yield by treatment with hydrazine.

Figure 3 indicates the scheme for the synthesis of the *C*-terminal pentapeptide derivative, L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline hydrochloride (XVII). *t*-Butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithine hydrazide (X) was obtained in a 83% yeild by the treatment of the *t*-butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithine ethyl ester[14] with hydrazine. The tripeptide derivative (XIII) was synthesized by a step-by-step reaction by means of the mixed anhydride method. The reaction of the anhydride prepared from benzyloxycarbonyl-D-phenylalanine and isobutyl chloroformate with L-proline ethyl ester afforded the oily dipeptide derivative (XI) in a 86% yield. This was hydrogenolyzed in the presence of palladium black and then allowed to react with the anhydride derived from benzyloxycarbonyl-L-leucine. XIII was thus obtained in a 95% yield as an oily substance. The catalytic hydrogenolysis of XIII also gave the oily tripeptide ester hydrochloride (XIV), which was found to be homogeneous in paper chromatography. The azide, which had been derived from X, was made to react with XIV to give the pure crystalline, protected pentapeptide ester (XV) in a 69% yield. The saponification of XV afforded the crystals of the protected pentapeptide (XVI). The subsequent treatment of XVI with twenty equivalents of hydrogen chloride in ethyl acetate yielded the pentapeptide hydrochloride (XVII) in a crystalline form. On the other hand, the same treatment of XV afforded the crystalline pentapeptide ester hydrochloride (XVIII) (see Fig. 4).

Two approaches were applied to the synthesis

14) N. Izumiya, T. Kato, Y. Fujita, M. Ohno and M. Kondo, This Bulletin, **37**, 1809 (1964).

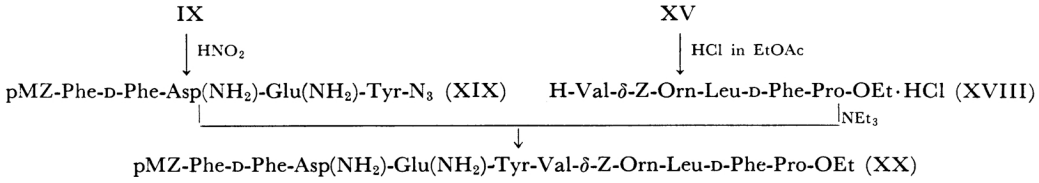

Fig. 4. Synthesis of pMZ-decapeptide ester (XX).

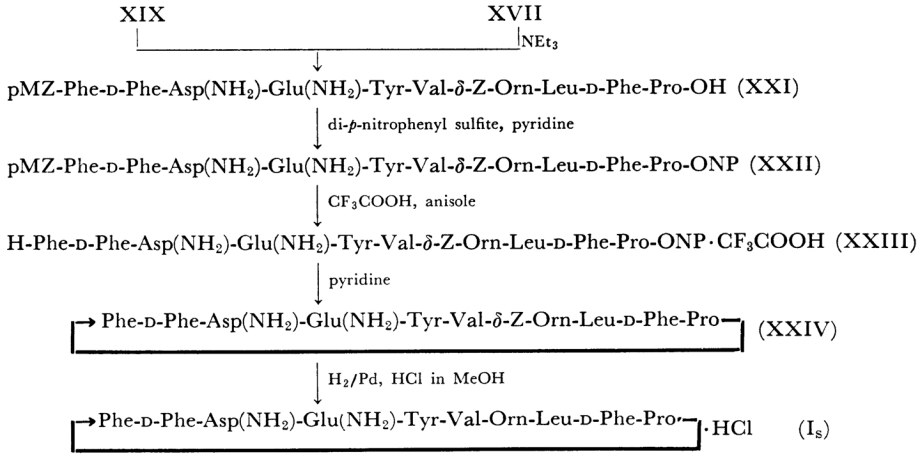

Fig. 5. Synthesis of cyclic decapeptide hydrochloride (I_s).

of the decapeptide derivative (XXI). The one route, via the synthesis of the protected decapeptide ester (XX), is presented in Fig. 4. The protected pentapeptide azide (XIX), which had been derived from IX, was allowed to react with XVIII in the presence of an equivalent amount of triethylamine. XX was thus obtained as an analytically-pure, crystalline product in a 70% yield. However, this approach was not feasible because the saponification of the ester (XX) by alkali was unsuccessful.

Figure 5 indicates another route for the synthesis of the protected decapeptide (XXI) and the subsequent synthesis of the cyclic decapeptides (XXIV and I_s). The reaction of the azide (XIX) with XVII in the presence of two equivalents of triethylamine gave the pure crystalline, protected decapeptide (XXI) in an yield of 75%. XXI was converted to the protected decapeptide *p*-nitrophenyl ester (XXII) with the action of ten equivalents of di-*p*-nitrophenyl sulfite in the presence of pyridine. The removal of the *p*-methoxybenzyloxycarbonyl group from XXII by treatment with trifluoroacetic acid yielded the amorphous decapeptide *p*-nitrophenyl ester trifluroacetate (XXIII) in an excellent yield. The cyclization reaction of XXIII in a large amount of pyridine gave the crude benzyloxycarbonyl-substituted cyclic peptide (XXIV), which was then purified by passing it through columns of strongly acidic and weakly basic ion exchangers. The yield of XXIV from XXI was excellent—60% after the recrystallization. The homogeneity of XXIV was established by the criteria of thin-layer and paper chromatographies and by elemental analysis. The molecular weight determination using the vapor pressure osmometer demonstrated that the molecular size of XXIV corresponded to that of cyclic decapeptide. The catalytic hydrogenolysis of XXIV in the presence of an equivalent amount of hydrogen chloride in methanol afforded the cyclic decapeptide hydrochloride (I_s) in a 74% yield. Although Battersby and Craig[2] found that the treatment of tyrocidine A hydrochloride (I_n) with excess hydrogen chloride in methanol formed its transformation product by the conversion of one primary amide group of asparagine or the glutamine residue to the methyl ester group, it has been shown, from the detection of two moles of ammonia in the amino acid analysis of the acid hydrolysate of I_s, that no such reaction occurred under the above conditions.

The synthetic cyclic decapeptide hydrochloride (I_s) was then compared with the natural product (I_n). In addition to having the same R_f values in paper and thin-layer chromatographies and having indistinguishable paper electrophoretic patterns (Fig. 6), I_s and I_n had superimposable infrared and well-agreeing ultraviolet spectra (Figs. 7 and 8), identical behaviors in the carboxymethylcellulose column (Fig. 9), and the same value in optical rotation. Furthermore, very close similarities were seen in the antibacterial activities

of the two compounds with regard to microorganisms (see Table I). These results show that the synthetic product is identical to natural tyrocidine A. The structure of tyrocidine A offered by Craig et al. has been further confirmed by the present synthesis.

Experimental

The melting points were not corrected. The optical rotations were measured on a Yanagimoto Photometric Polarimeter, OR-20 type. The paper chromatography was carried out on Toyo Roshi No. 52 chromatography paper. The thin-layer chromatography was carried out on Merck silica gel G. The developing solvents commonly used were *n*-butanol - acetic acid - pyridine - water (4 : 1 : 1 : 2 v/v) and *t*-butanol - formic acid - water (75 : 15 : 10 v/v). Spots of materials possessing a free amino group on a thin-layer plate were detected by spraying ninhydrin, and those of the amino group-blocked materials, by spraying 47% hydrobromic acid and then ninhydrin. Prior to analysis, the compounds were dried over phosphorus pentoxide at 100°C and 2 mmHg to a constant weight except for the compound I_8.

***p*-Methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanine Ethyl Ester (II).**—To a mixed anhydride prepared at −5°C from 6.58 g. (20 mmol.) of *p*-methoxybenzyloxycarbonyl-L-phenylalanine,[13] 2.60 ml. (20 mmol.) of isobutyl chloroformate and 2.80 ml. (20 mmol.) of triethylamine in 60 ml. of tetrahydrofuran, a chilled solution of 4.60 g. (20 mmol.) of D-phenylalanine ethyl ester hydrochloride dissolved in a mixture of 2.80 ml. (20 mmol.) of triethylamine and 60 ml. of chloroform was added. The reaction mixture was then allowed to stand overnight and then evaporated in vacuo. The oily residue was dissolved in 80 ml. of ethyl acetate, and the solution was washed successively with 0.5 M citric acid, a 3% sodium bicarbonate solution and water, dried over sodium sulfate, and then evaporated in vacuo. The oily residue was solidified by adding petroleum ether (8.68 g.). The product was recrystallized from ethanol - ether - petroleum ether. Yield, 8.0 g. (79%); m. p. 126—128°C; $[\alpha]_D^{20}$ −2.8° (*c* 4.4, dimethylformamide).

Found: C, 69.18; H, 6.13; N, 5.66. Calcd. for $C_{29}H_{32}O_6N_2$: C, 69.03; H, 6.39; N, 5.55%.

***p*-Methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanine Hydrazide (III).**—A solution of 6.06 g. (12 mmol.) of II and 12 ml. (240 mmol.) of hydrazine hydrate in 18 ml. of dimethylformamide was allowed to stand at room temperature for 2 days. The solution was then evaporated in vacuo in order to remove the excess hydrazine, after which 180 ml. of water was added to the residual solution; the resulting crystals were collected by filtration (5.80 g.). The crude hydrazide was purified by recrystallization from dimethylformamide - ether - petroleum ether. Yield, 5.64 g. (96%); m. p. 194—195°C; $[\alpha]_D^{20}$ −3.4° (*c* 5.0, dimethylformamide).

Found: C, 66.21; H, 6.03; N, 11.23. Calcd. for $C_{27}H_{30}O_5N_4$: C, 66.10; H, 6.16; N, 11.42%.

Benzyloxycarbonyl-L-glutaminyl-L-tyrosine Ethyl Ester (IV).—To a solution of 7.37 g. (30 mmol.) of L-tyrosine ethyl ester hydrochloride dissolved in a mixture of 8.40 ml. (60 mmol.) of triethylamine and 40 ml. of dimethylformamide, a solution of 12.0 g. (30 mmol.) of benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester in 40 ml. of dimethylformamide was added. After the solution had been allowed to stand for 4 hr. at room temperature, it was diluted with 1.5 l. of water. The crystalline precipitate was collected by filtration and washed successively with 2 N hydrochloric acid, a 3% sodium bicarbonate solution, and water (12.0 g.). It was recrystallized from ethanol-ether. Yield, 9.96 g. (70%); m. p. 197—199°C; $[\alpha]_D^{20}$ +1.8° (*c* 4.5, dimethylformamide).

Found: C, 61.18; H, 6.27; N, 8.81. Calcd. for $C_{24}H_{29}O_7N_3$: C, 61.13; H, 6.20; N, 8.91%.

L-Glutaminyl-L-tyrosine Ethyl Ester Hydrobromide (V).—To a suspension of 5.66 g. (12 mmol.) of IV in 24 ml. of acetic acid, 12 ml. of 6 N dry hydrogen bromide in acetic acid was added. After it had stood for one hour, the solution was evaporated to dryness in vacuo, and then ether was added. The oily residue was further washed with ether by decantation and dried over calcium chloride and sodium hydroxide. It weighed 4.61 g. (94%).

Benzyloxycarbonyl-L-asparaginyl-L-glutaminyl-L-tyrosine Ethyl Ester (VI).—To a solution of 4.61 g. (11.3 mmol.) of V in 20 ml. of dimethylformamide, 3 ml. of triethylamine was added, followed by a solution of 3.87 g. (10 mmol.) of the benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester in 10 ml. of dimethylformamide. After it had stood for 6 hr. at room temperature, the solution was diluted with 400 ml. of water. The crystalline product deposited was collected by filtration and washed successively with 2 N hydrochloric acid, a 3% sodium bicarbonate solution, and water (4.51 g.). It was then recrystallized from dimethylformamide-ether. Yield, 4.22 g. (72%); m. p. 224—225°C (decomp.), $[\alpha]_D^{20}$ −11.6° (*c* 2.45, dimethylformamide).

Found: C, 56.74; H, 6.33; N, 11.53. Calcd. for $C_{28}H_{35}O_9N_5 \cdot 1/2H_2O$: C, 56.56; H, 6.27; N, 11.78%.

L-Asparaginyl-L-glutaminyl-L-tyrosine Ethyl Ester Hydrobromide (VII).—To a suspension of 2.93 g. (5 mmol.) of VI in 12 ml. of acetic acid, 6.3 ml. of 6 N dry hydrogen bromide in acetic acid was added. After it had stood for one hour, the solution was evaporated to dryness in vacuo. The oily residue was then treated as has been described in the previous paragraph. The oil weighed 2.61 g. (98%).

***p*-Methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosine Ethyl Ester (VIII).**—A solution of 1.96 g. (4 mmol.) of III dissolved in a mixture of 15 ml. of dimethylformamide and 24 ml. of acetic aicd was cooled to −5°C. To this, 8.8 ml. (8.8 mmol.) of N hydrochloric acid was added, followed by 2.2 ml. (4.4 mmol.) of 2 N sodium nitrite. After it had sood for 5 min. at this temperature, the solution was diluted with 200 ml. of cold water. The precipitated azide was collected by filtration, washed with a 3% sodium bicarbonate solution and water, and then dried in vacuo at 0°C. To a chilled (−5°) solution of 2.24 g. (4.2 mmol.) of VII dissolved in 15 ml. of dimethylformamide, 2 ml. of triethylamine was added, followed by the azide crystals prepared above. The solution was stirred for 2 days at 0°C and then diluted with 200

ml. of water. The precipitate was collected by filtration and washed successively with 0.5 M citric acid, a 3% sodium bicarbonate solution and water (3.05 g.). The product was recrystallized from dimethylformamide-ether. Yield, 2.73 g. (75%); m. p. 206—209°C (decomp.); $[\alpha]_D^{20}$ −10.4° (*c* 2.9, dimethylformamide).

Found: C, 62.58; H, 6.35; N, 11.00. Calcd. for $C_{47}H_{55}O_{12}N_7$: C, 62.03; H, 6.09; N, 10.78%.

***p*-Methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosine Hydrazide (IX).**—This compound was prepared by the same procedure as has been described for the preparation of III. One and eighty-two hundredths grams (2 mmol.) of VIII and 2.0 ml. (40 mmol.) of hydrazine hydrate gave 1.64 g. of the crude product. It was recrystallized from dimethylformamide-ether. Yield, 1.54 g. (86%); m. p. 225—227°C (decomp.); $[\alpha]_D^{20}$ −26.0° (*c* 2.05, dimethylformamide).

Found: C, 59.44; H, 5.96; N, 13.62. Calcd. for $C_{45}H_{53}O_{11}N_9$: C, 60.32; H, 5.96; N, 14.07%.

***t*-Butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithine Hydrazide (X).**—This compound was also prepared by the same procedure as has been described for the preparation of III. Seven and forty hundredths grams (15 mmol.) of *t*-butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithine ethyl ester[14] and 15 ml. (300 mmol.) of hydrazine hydrate afforded 6.35 g. of the crude product. It was recrystallized from ethanol-ether-petroleum ether. Yield, 5.96 g. (83%); m. p. 175°C; $[\alpha]_D^{20}$ −14.1° (*c* 2.35, dimethylformamide).

Found: C, 57.40; H, 7.94; N, 14.34. Calcd. for $C_{23}H_{37}O_6N_5$: C, 57.60; H, 7.78; N, 14.60%.

Benzyloxycarbonyl-D-phenylalanyl-L-proline Ethyl Ester (XI).—To a mixed anhydride prepared at −5°C from 4.0 g. (16.7 mmol.) of benzyloxycarbonyl-D-phenylalanine, 2.16 ml. (16.7 mmol.) of isobutyl chloroformate and 2.36 ml. (16.7 mmol.) of triethylamine in 25 ml. of chloroform, a chilled solution of 6.60 g. (20.9 mmol.) of L-proline ethyl ester *p*-toluenesulfonate[15] dissolved in a mixture of 2.93 ml. (20.9 mmol.) of triethylamine and 25 ml. of chloroform was added. After the reaction mixture had been allowed to stand overnight, it was evaporated in vacuo. The residue was dissolved in 80 ml. of ethyl acetate, and the solution was washed with 2 N hydrochloric acid, a 3% sodium bicarbonate solution and water, dried over sodium sulfate, and then evaporated to dryness in vacuo. The residual oil weighed 6.08 g. (86%). All attempts to crystallize this oil failed, so it was used as such for the next step.

D-Phenylalanyl-L-proline Ethyl Ester Hydrochloride (XII).—A solution of 5.80 g. (13.7 mmol.) of XI dissolved in a mixture of 12.7 ml. (15.0 mmol.) of 1.18 N dry hydrogen chloride in methanol and 30 ml. of methanol was hydrogenolyzed in the presence of palladium black. After 5 hr. the filtrate from the catalyst was evaporated to dryness in vacuo. The oil weighed 4.36 g. (98%). The homogeneity of this oil was certified by paper chromatography, in which only one spot was detected, R_f 0.88.[16]

Benzyloxycarbonyl-L-leucyl-D-phenylalanyl-L-proline Ethyl Ester (XIII).—This compound was prepared by the same anhydride method as has been described for the preparation of XI. Three and forty-four hundredths grams (13 mmol.) of benzyloxycarbonyl-L-leucine and 4.36 g. (13.4 mmol.) of XII gave 6.64 g. (95%) of XIII as an oily product.

L-Leucyl-D-phenylalanyl-L-proline Ethyl Ester Hydrochloride (XIV).—This compound was prepared by the same procedure as has been described for the preparation of XII. The hydrogenolysis of 3.37 g. (6.26 mmol.) of XIII in the presence of a 1.1 equivalent of hydrogen chloride afforded 2.59 g. (94%) of XIV as an oil. This gave only one spot in paper chromatography, R_f 0.92.[16] It was used for the next reaction without further purification.

***t*-Butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline Ethyl Ester (XV).**—A solution of 2.88 g. (6 mmol.) of X dissolved in a mixture of 8 ml. of dimethylformamide and 45 ml. of acetic acid was cooled to −5°C. To this, 6.6 ml. (6.6 mmol.) of N hydrochloric acid was added, followed by 2.2 ml. (6.6 mmol.) of 3 N sodium nitrite and then 6.6 ml. (6.6 mmol.) of N hydrochloric acid. After it had stood for 5 min. at this temperature, with occasional stirring, the solution was diluted with 250 ml. of water. The azide separated as a viscous mass was twice extracted with 40-ml. portions of ethyl acetate, and the combined ethyl acetate solution was washed thoroughly with a cold 3% sodium bicarbonate solution and cold water. After it had been dried over sodium sulfate for 10 min. at 0°C, this solution was added to a chilled (−5°C) solution of 2.70 g. (6.13 mmol.) of XIV dissolved in a mixture of 0.86 ml. (6.13 mmol.) of triethylamine and 15 ml. of dimethylformamide. After it had then been stirred for 2 days at 0°C, the solution was diluted with 20 ml. of ethyl acetate and washed successively with 0.5 M citric acid, a 3% sodium bicarbonate solution and water, dried over sodium sulfate, and then evaporated in vacuo. The resulting crystals were collected by filtration with the aid of petroleum ether (4.07 g.). The product was recrystallized from ethyl acetate-ether-petroleum ether. Yield, 3.53 g. (69%); m. p. 167—170°C; $[\alpha]_D^{20}$ −32.1° (*c* 2.05, dimethylformamide), R_f 0.96.[17]

Found: C, 63.28; H, 7.76; N, 10.17. Calcd. for $C_{45}H_{66}O_{10}N_6$: C, 63.51; H, 7.82; N, 9.88%.

***t*-Butyloxycarbonyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline (XVI).**—To a solution of 3.40 g. (4 mmol.) of XV dissolved in 30 ml. of methanol, 8 ml. (8 mmol.) of N sodium hydroxide was added. Saponification was carried out at room temperature, and the progress of the reaction was checked by thin-layer chromatography. The reaction was complete after 6 hr. The solution was evaporated in vacuo to a volume of about 12 ml., after which the residual solution was diluted with 40 ml. of water and then acidified with 10 ml. (10 mmol.) of M citric acid. The precipitated crystalline solid was taken up twice with 25-ml. portions of ethyl acetate, and the combined ethyl acetate solution was washed with water, dried over sodium sulfate, and then evaporated in vacuo.

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

16) The R_f value refers to the paper chromatography with the solvent system of *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2 v/v).

17) The R_f value refers to the thin-layer chromatography using a solvent system of *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2 v/v).

The resulting crystalline product was collected by filtration with the aid of a mixture of ether and petroleum ether (1 : 2) (3.06 g.). It was recrystallized from ethyl acetate - ether - petroleum ether. Yield, 2.92 g. (86%); m. p. 122—125°C; $[\alpha]_D^{20}$ −39.0° (*c* 2.05, dimethylformamide), R_f 0.82.[17]

Found: C, 62.42; H, 7.57; N, 10.14. Calcd. for $C_{43}H_{62}O_{10}N_6$: C, 62.75; H, 7.59; N, 10.21%.

L-Valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline Hydrochloride (XVII).*—To a suspension of 1.24 g. (1.5 mmol.) of XVI in 5 ml. of ethyl acetate, 12 ml. (30 mmol.) of 2.5 N dry hydrogen chloride in ethyl acetate was added; meanwhile the material dissolved to form a clear solution. The solution was then allowed to stand at room temperature, and the reaction was followed by thin-layer chromatography. After 2 hr. the solution was evaporated in vacuo to afford crystals, which were then filtered off with the aid of ether and washed thoroughly with ether. Yield, 1.09 g. (95%); m. p. 195—200°C (decomp.); $[\alpha]_D^{20}$ −22.5° (*c* 2.12 dimethylformamide), R_f 0.66.[17]

Found: N, 10.89. Calcd. for $C_{88}H_{54}O_8N_6 \cdot HCl$: N, 11.07%. This product was used as such for the next reaction.

L-Valyl-δ-benzyloxycarbonyl-L-ornithyl-D-phenylalanyl-L-leucyl-L-proline Ethyl Ester Hydrochloride (XVIII).—To a suspension of 1.02 g. (1.2 mmol.) of XV in 6 ml. of ethyl acetate, 6 ml. (24 mmol.) of 4 N dry hydrogen chloride in ethyl acetate was added. The reaction mixture formed a clear solution. The solution was then allowed to stand at room temperature, and the progress of the reaction was checked by thin-layer chromatography. After 5 hr. the solution was evaporated to dryness in vacuo. The resulting oily residue was crystallized by the addition of ether (0.882 g.). The product was recrystallized from ethanol-ether. Yield, 0.811 g. (86%); m. p. 183—185°C; $[\alpha]_D^{20}$ −19.0° (*c* 2.1, dimethylformamide), R_f 0.73.[17]

Found: C, 60.65; H, 7.63; N, 10.78. Calcd. for $C_{40}H_{58}O_8N_6 \cdot HCl$: C, 61.01; H, 7.55; N, 10.67%.

***p*-Methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline Ethyl Ester (XX).**—A solution of 0.488 g. (0.5 mmol.) of IX dissolved in 6 ml. of dimethylformamide was cooled to −5°C. To this, 1.4 ml. (1.4 mmol.) of N hydrochloric acid was added, followed by 0.55 ml. (0.55 mmol.) of N sodium nitrite. After it had stood for 5 min. at −5°C, the solution was diluted with 50 ml. of cold water. The precipitated azide (XIX) was collected by filtration, washed with a cold 3% sodium bicarbonate solution and cold water, and then dried in vacuo at 0°C. The dried azide was added to a chilled (−5°C) solution of 0.394 g. (0.5 mmol.) of XVIII dissolved in a mixture of 0.07 ml. (0.5 mmol.) of triethylamine and 8 ml. of dimethylformamide; the solution was stirred for 2 days at 0°C, and then diluted with 80 ml. of water. The crystalline precipitate was collected by filtration and washed thoroughly with 0.5 M citric acid, a 3% sodium bicarbonate solution and water (0.596 g.). It was recrystallized from dimethylformamide-methanol-ether. Yield, 0.564 g. (70%); m. p. 242—243°C (decomp.); $[\alpha]_D^{20}$ −27.9° (*c* 0.62, dimethylformamide). The homogeneity was certified by thin-layer chromatography with regard to the hydrogenolyzed product, R_f 0.86.[17]

Found: C, 63.30; H, 6.80; N, 11.37. Calcd. for $C_{89}H_{107}O_{19}N_{13}$: C, 63.30; H, 6.56; N, 11.29%.

***p*-Methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline (XXI).**—The azide (XIX), which had been freshly prepared from 0.896 g. (1.0 mmol.) of IX by the procedure described just above, was added to a chilled (−5°C) solution of 0.911 g. (1.2 mmol.) of XVII dissolved in a mixture of 0.34 ml. (2.4 mmol.) of triethylamine and 8 ml. of dimethylformamide. The solution was then stirred for 2 days at 0°C, and the small amount of insoluble materials was filtered off. The filtrate was evaporated in vacuo, and the resulting semi-solid mass was triturated with a mixture of 15 ml. of ethyl acetate and 5 ml. of ether, and collected by filtration with the aid of ether. The solid mass crystallized when it was treated with a citric acid solution. The crystals were collected by filtration, and further washed with a dilute citric acid solution and then water (1.24 g.). The product was recrystallized from dimethylformamide-ether. Yield, 1.19 g. (75%); m. p. 233—236°C (decomp.); $[\alpha]_D^{20}$ −31.6° (*c* 0.58, dimethylformamide). The homogeneity of this product was established by thin-layer chromatography after hydrogenolysis, R_f 0.80.[17]

Found: C, 62.58; H, 6.57; N, 11.63. Calcd. for $C_{83}H_{103}O_{19}N_{13}$: C, 62.82; H, 6.57; N, 11.48%.

***p*-Methoxybenzyloxycarbonyl-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline *p*-Nitrophenyl Ester (XXII).**—To a solution of 0.300 g. (0.189 mmol.) of XXI dissolved in 3.5 ml. of dimethylformamide, 5 ml. of anhydrous pyridine was added, followed by 0.612 g. (1.89 mmol.) of di-*p*-nitrophenyl sulfite. The faintly yellow solution was allowed to stand for 16 hr. at room temperature and then evaporated in vacuo. The oily residue was triturated with a mixture of ether and petroleum ether (1 : 1). The solid mass was collected by filtration and washed with a mixture of ether and petroleum ether (1 : 1) unitil the yellow color could not be discerned upon the addition of N sodium hydroxide to the filtrate. A faintly yellow product was thus obtained (0.325 g.). The *p*-nitrophenyl ester content of this product was estimated to be 110% by means of the method described by Schwyzer and Sieber,[5] except that a mixture of dimethylformamide and N sodium hydroxide (1 : 1) was used as the solvent and the absorption measurements were performed at the wavelength of 411 mμ. This product was used for the next reaction without further purification.

L-Phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosyl-L-valyl-δ-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline *p*-Nitrophenyl Ester Trifluoroacetate (XXIII).—To a mixture of 0.325 g. of XXII and 0.4 ml. of anisole, 4 ml. of anhydrous trifluoroacetic acid was added at −5°C. When swirled, the reaction mixture formed a solution after 10 min. The solution was then evaporated in vacuo at 0°C, and the resulting oily residue was triturated by

* An attempt to crystallize the HCl-free pentapeptide failed. When an aqueous solution of XVII was neutralized by the addition of triethylamine, the oil separated. This oil did not crystallize on prolonged standing in a refrigerator.

ether. The product was collected by filtration and washed with ether (0.292 g.). The *p*-nitrophenyl ester content of this product was estimated to be 95% by the procedure described in the preceding paragraph. This material was used as such in the next step.

Cyclo-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosyl-L-valyl-∂-benzyloxycarbonyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl (XXIV).—A solution of 0.292 g. of XXIII dissolved in a mixture of 0.5 ml. of acetic acid and 10 ml. of dimethylformamide was stirred, drop by drop, into 100 ml. of anhydrous pyridine kept at 70—75°C over a 3.5-hr. period. The solution was further stirred for 1.5 hr. at this temperature, and then evaporated to dryness in vacuo. The resulting residue was collected by filtration with the aid of water, dried in vacuo (0.235 g.), and then dissolved in 50 ml. of a mixture of dioxane and water (5 : 2). This solution was passed successively through columns (2×4.5 cm.) of Dowex-50 (H^+ form) and Amberlite IR-4B (OH^- form) which had been washed with a mixture of dioxane and water (5 : 2). The columns were then washed with the same solvent. The filtrate and washings were combined (total 150 ml.) and lyophilized to afford the crystalline product (0.175 g.). It was recrystallized from dioxane - ether - petroleum ether. Yield, 0.160 g. (60% from XXI); m. p. 263—265°C (decomp.); $[\alpha]_D^{20}$ −111° (*c* 0.65, dimethylformamide). The homogeneity was certified by thin-layer chromatography, R_f 0.94.[17]

Found: C, 63.42; H, 6.86; N. 12.77. Calcd. for $C_{74}H_{93}O_{15}N_{13}$: C, 63.27; H, 6.67; N, 12.96%.

The molecular weight was measured on a vapor pressure osmometer of Mechrolab, Inc., model 301 A, using methanol as the solvent.* Found: 1422. Calcd. for the benzyloxycarbonyl-substituted cyclic decapeptide: 1405.

Cyclo-L-phenylalanyl-D-phenylalanyl-L-asparaginyl-L-glutaminyl-L-tyrosyl-L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl Hydrochloride (I_s).—A solution of 86.5 mg. (0.062 mmol.) of XXIV dissolved in a mixture of 0.32 ml. (0.064 mmol.) of 0.20 N dry hydrogen chloride in methanol and 3 ml. of methanol was hydrogenolyzed in the presence of palladium black. The progress of the reaction was checked by thin-layer chromatography. After 2.5 hr. the filtrate from the catalyst was concentrated to a small volume (0.1 ml.), and 2 ml. of ether was added to afford crystals; yield, 70 mg. Recrystallization from methanol-ether gave 65 mg. (74%) as the air-dried product (m. p. 239—240°C (decomp.)). Only one spot was obtained in thin-layer chromatography, R_f 0.76.[17] The elemental analysis was carried out using a desiccator-dried sample.

Found: C, 57.12; H, 6.96; N, 12.79. Calcd. for $C_{68}H_{87}O_{13}N_{13}\cdot HCl\cdot 5H_2O$: C, 56.75; H, 7.00; N, 13.03%.

When dried at 110°C (2 mmHg), this desiccator-dried sample lost 6.2% of its weight. Calcd. for $5H_2O$: 6.3%. On the other hand, the air-dried sample lost 8.8% of its weight after being dried for 2 hr. at 110°C (2 mmHg). Calcd. for $7H_2O$: 8.81%.

Amino acid analysis gave the molar ratios: leucine 1.0, phenylalanine 3.0, aspartic acid 1.0, glutamic acid 1.0, tyrosine 0.8, valine 1.0, ornithine 1.0, proline 1.0 and ammonia 2.1.

A Comparison of I_s and Natural Tyrocidine A Hydrochloride (I_n). — *(a) Paper Chromatography.* — This was carried out in two solvents. Both compounds gave a single spot and the same R_f values. R_f values of I_s: 0.92,[16] 0.95.[18] R_f values of I_n: 0.92,[16] 0.95.[18]

(b) Thin-layer Chromatography.—The R_f values of both compounds were exactly the same. R_f value of I_s: 0.76.[17] R_f value of I_n: 0.76.[17]

(c) Paper Electrophoresis.—This was carried out under the following conditions: paper, Toyo Roshi No. 52 chromatography paper; solvent, formic acid - acetic acid - methanol - water (1 : 3 : 6 : 10 v/v) (pH 1.8); voltage gradient, 17 V./cm.; charged period, 2.5 hr. Gramicidin S dihydrochloride was used as the reference compound. Figure 6 demonstrates the electrophoretic patterns of I_s, I_n and gramicidin S dihydrochloride. The mobility of I_s was indistinguishable from that of I_n.

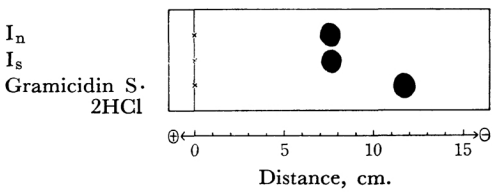

Fig. 6. Paper electrophoreses of I_s, I_n and gramicidin S dihydrochloride.

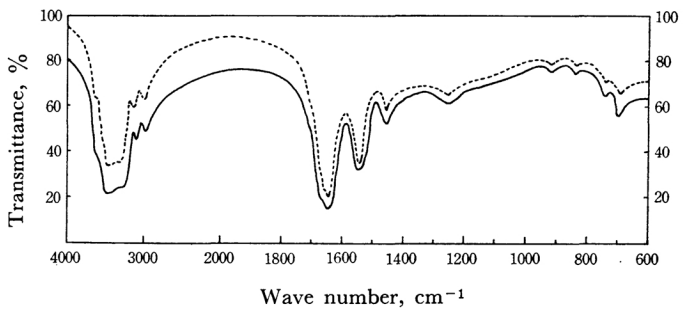

Fig. 7. Infrared spectra of I_s (——) and I_n (----).

* The atuhors are indebted to Mr. Michinori Waki of this laboratory for the molecular weight measurements.

18) The R_f value refers to paper chromatography with the solvent system of *t*-butanol - formic acid - water (75 : 15 : 10 v/v).

Table I. Inhibitory activity of I_S, I_n 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.)
I_S[b]	>100	>100	20	10	>100	>100
I_n[b]	>100	>100	20	10	>100	>100
Gramicidin S·2HCl	>100	>100	5—2	5—2	>100	>100

B. Synthetic medium[c]

	Escherichia coli	*Proteus vulgaris*	*Staphylococcus aureus*	*Bacillus subtilis*	*Mycobacterium avium*	*Mycobacterium avium* (Streptomycin resistant St.)
I_S[b]	>100	>100	20	10	>100	>100
I_n[b]	>100	50	20	20	>100	>100
Gramicidin S·2HCl	>100	>100	5	5	>100	>100

a) Usual bouillon agar medium, pH 7.0.
b) Air-dried samples were employed.
c) 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.

(d) Infrared Spectra.—These were obtained on a Hitachi EPI-2 spectrophotometer. The spectra of both I_S and I_n are presented in Fig. 7. The spectrum of I_S is superimposable on that of I_n.

(e) Ultraviolet Spectra.—These were obtained on a Hitachi EPS-2 spectrophotometer using a pair of 1-cm. quartz cells. Figure 8 indicates the spectra of I_S and I_n in 50% ethanol; here their absorption maxima agree well with each other.

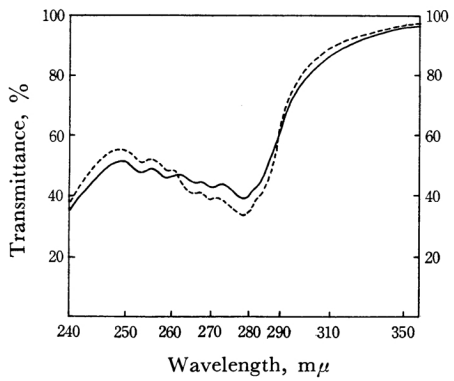

Fig. 8. Ultraviolet spectra of I_S (——) and I_n (----) in 50% ethanol; concentrations of I_S and I_n are 1.74×10^{-4} M and 1.85×10^{-4} M, respectively.

*(f) Carboxymethylcellulose (CMC) Column Chromatography.**—A 0.9×100 cm. CMC column was employed. The solvent used here was a methanol-containing (50%, v/v) 0.2 M pyridine-acetate buffer (pH 5.0). About 0.5 mg. of the sample was placed in 0.4 ml. of this solvent in the column, and the mixture was eluted with the same solvent. One milliliter fractions were collected at a flow rate of 7 ml. per hour. Each of them was kept for one hour at 90°C in order to remove methanol, and then 1 ml. of N sodium hydroxide was added to the residual solution and left to hydrolyze for 3 hr. at 90°C. After the solution had been neutralized by the addition of 0.5 ml. of 30% acetic acid, the components were determined by the Yemm-Cocking method.[19]

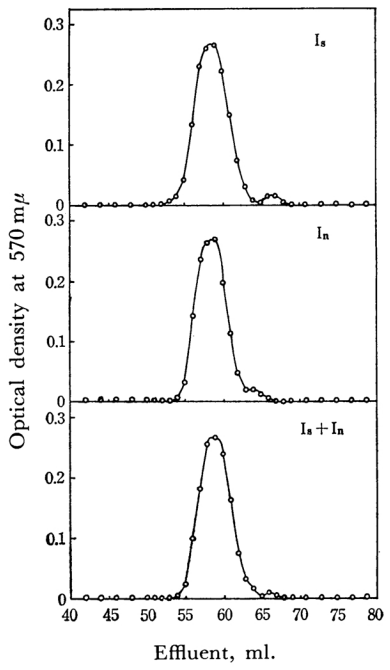

Fig. 9. CMC column chromatography of I_S, I_n and admixture of I_S and I_n.

* The authors are indebted to Mr. Haruhiko Aoyagi of this laboratory, who found the usefulness of the application of the CMC column chromatography in the separation of mixture of basic cyclic peptides.

19) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955); *Biochem. J.*, **59**, xii (1954).

This procedure was applied to I_s, I_n and an equivalent admixture of I_s and I_n respectively. The results are shown in Fig. 9. The same chromatographic pattern was obtained in each of the three samples.

(g) Optical Rotation.—Air-dried samples were employed for the measurement, using 50% ethanol as the solvent: $[\alpha]_D^{20}$ of I_s $-108°$ (*c* 0.141); $[\alpha]_D^{20}$ of I_n $-108°$ (*c* 0.126). Lit.[10] $[\alpha]_D^{25}$ $-111°$ (*c* 1.37, 50% ethanol) for a sample dried at 110°C.

(h) Microbiological Assays.—The microorganisms employed are listed in Table I. The minimum amount of the compound necessary for the complete inhibition of growth was determined by a dilution method with a bouillon agar medium and with a synthetic medium. In addition to I_s and I_n, gramicidin S dihydrochloride was examined as a reference compound. Although, in the synthetic medium, small differences were observed in the effects of I_s and I_n on the *Proteus vulgaris* and *Bacillus subtilis*, the antibacterial spectra of the two compounds can be considered to be in agreement with one other.

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