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Chemical Studies on Chlorine-containing Peptide—One of the Toxic Metabolites of *P. islandicum* Sopp. I. Structure and Synthesis of Dehydrochlorinated-Peptide Amide[1,2]

Motomu Sato and Takashi Tatsuno

The Institute of Physical and Chemical Research[3]

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An attempt was made to synthesize dehydrochlorinated–peptide amide (II), which is obtained by treating the chlorine–containing peptide (I) isolated from *Penicillium islandicum* Sopp. with concentrated ammonia water. It was concluded from the results of the present investigation that the structure of dehydrochlorinated–peptide amide (II) should be presented as α-pyrrolecarboxylyl-L-α-amino-*n*-butyryl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide (XIX).

Since 1954 we have been continuing the toxicological studies[4,5a–d] on metabolites of *Penicillium islandicum* Sopp, the mold of islandia yellow rice, and have isolated the chlorine–containing peptide (I) from its water soluble fraction.[4,5a] It has been shown [5a] that 5 μg/10 g of this peptide injected subcutaneously to mice could kill them in a very short time and produce such violent symptoms as the necrosis and the vacuolation of liver cells, especially in the peripheral region of lobulus, erythrocytes invading the liver cells, and development of blood–lake, *etc.* We confirmed [5a] that this substance was acyclic peptide containing two chlorine atoms, and that it consisted of L–serine, L–α–amino-*n*–butyric acid, L-β-amino-β-phenylpropionic acid[6] and an unknown chlorine–containing component.

It is shown that the dehydrochlorinated–peptide amide (II) was obtained by treating chlorine–containing peptide (I) with concentrated ammonia water, and that the both peptide (I, II) contained such amino acids as α-amino-*n*-butyric acid and β-amino-β-phenylpropionic acid which are not ordinarily found as constituent amino acids in natural proteins. The chlorine atom seem to play an important role in exhibiting the mortal toxicity of the peptide (I), since the dehydrochlorinated–peptide amide (II) possessed less than 1/100 the toxicity of the parent peptide (I).

On the other hand, independently, Marumo isolated a toxic substance[7] from the cultured broth of *P. islandicum* Sopp, and named it 'islanditoxin' (III) proposing its chemical structure as cyclo-(L-dichloroprolyl-D-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyryl-L-seryl-L-serine) shown in Chart 1.

1) This investigation was supported in part by Cancer Research Grants (1966) given by the Ministry of Welfare to Professor Emeritus K. Uraguchi, Department of Pharmacology, University of Tokyo.

2) This work was presented at the Local Meeting of the Pharmaceutical Society of Japan, Tokyo, June, 1967 and also at the 5th Symposium on Peptide Chemistry, Kyoto, Nov. 1967 (Symposium Abstract (in Japanese), p. 79).

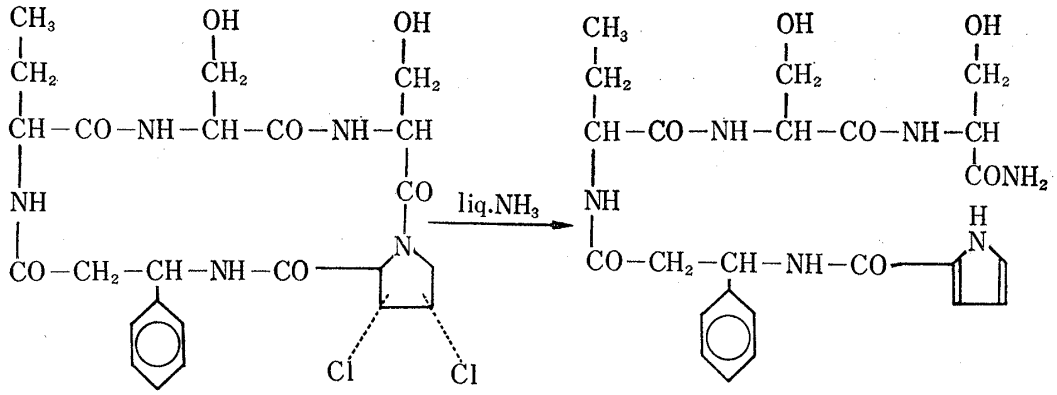
3) Location: *Yamato-Machi. Saitama.*

4) T. Tatsuno, M. Tsukioka, Y. Sakai, Y. Suzuki and Y. Asami, *Chem. Pharm. Bull.* (Tokyo), 3, 476 (1955).

5) *a*) K. Uraguchi, T. Tatsuno, F. Sakai, M. Tsukioka, Y. Sakai, O. Yonemitsu, H. Ito, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiko, *Japan. J. Exp. Med.*, 31, 19 (1961); *b*) K. Uraguchi, T. Tatsuno, Y. Ueno and I. Ueno, *Folia Pharmacol. Japon.*, 56, 166 § (1960); *c*) K. Uraguchi, T. Tatsuno, Y. Ueno and I. Ueno, *ibid.*, 57, 156 § (1961); *d*) T. Tatsuno, Y. Ueno, I. Ueno and K. Nakamura, *J. Japan. Biochem. Soc.*, 35, 38 (1963).

6) The details of the configuration of β-amino-β-phenylpropionic acid will be reported in a separate paper of this Bulletin.

7) S. Marumo, *Bull. Agr. Chem. Soc. Japan*, 19, 258, 262 (1955); 23, 428 (1959).



islanditoxin (III) dehydrochloroislanditoxic amide (IV)

Chart 1

Although the chemical and physical characters of islanditoxin (III) is in very similar with our peptide (I), and the chemical and physical characters of Marumo's dehydrochloroislanditoxic amide (IV), which was obtained by treating islanditoxin (III) with liquid ammonia, were in good agreement with those of our dehydrochlorinated–peptide amide (II), we could not identify Marumo's substances (III, IV) with ours (I, II). Then, in order to determine the amino acid sequence of chlorine–containing peptide (I), we first attempted to synthesize dehydrochloroislanditoxic amide (α-pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyryl-L-seryl-L-serine amide, IV), and to compare the chemical and physical characters with those of our dehydrochlorinated–peptide amide (II).

The method used for the preparation is shown in Chart 2. The chain lengthening was performed by starting from the carboxyl end, serine amide, according to the azide method, when the racemization was possible, and according to the mixed anhydride and *p*–nitrophenyl ester methods, when the racemization was impossible.

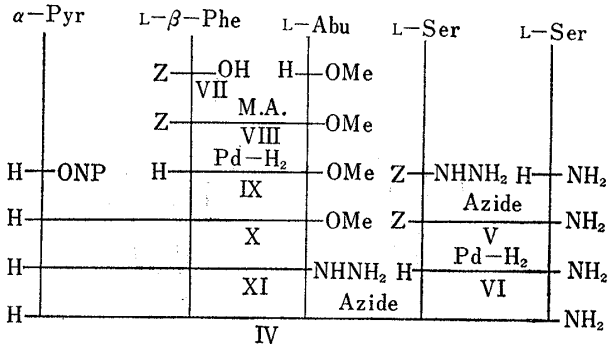


Chart 2

α-Pyr=α-pyrrolecarboxylic acid Abu=α-amino-*n*-butyric acid
β-Phe=β-amino-β-phenylpropionic acid
ONP=*p*-nitrophenyl ester M.A.=mixed anhydride

Benzyloxycarbonyl-L-serine azide prepared from the corresponding hydrazide[8] was allowed to react with L–serine amide hydrochloride[9] to give benzyloxycarbonyl-L-seryl-L-serine amide (V), which was hydrogenated over palladium black with formation of L–seryl-L-serine amide hydrochloride (VI). Benzyloxycarbonyl-L-β-amino-β-phenylpropionic acid (VII) prepared from L-β-amino-β-phenylpropionic acid[10,11] in the usual manner was coupled with methyl L–α–amino–*n*–butyrate hydrochloride[12] by means of the mixed anhydride method using isobutyl chloroformate to yield methyl benzyloxycarbonyl-L-β-amino-β-phenylpropio-

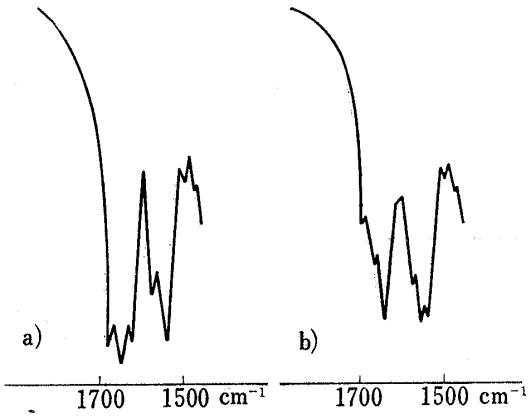
8) J.S. Fruton, *J. Biol. Chem.*, **146**, 463 (1942); E. Baer, J. Maurukas and D.D. Clarke, *Can. J. Chem.*, **34**, 1182 (1956).
9) S.A. Bernhand, A. Berger, J.H. Carter, E. Katchalski, M. Sela and Y. Shalitin, *J. Am. Chem. Soc.*, **84**, 2421 (1962); E.L. Smith and D.H. Spackman, *J. Biol. Chem.*, **212**, 271 (1955).
10) J. Evans and T.B. Johnson, *J. Am. Chem. Soc.*, **52**, 4993 (1930); T.B. Johnson and J.E. Livak, *ibid.*, **58**, 299 (1936).
11) E. Fischer, H. Scheibler and R. Groh, *Chem. Ber.*, **43**, 2020 (1910).
12) E. Kliger and H. Gibian, *Ann.*, **649**, 183 (1961).

nyl-L-α-amino-*n*-butyrate (VIII). Hydrogenolysis of this benzyloxycarbonyl–dipeptide ester (VIII) over palladium black gave methyl L–β-amino-β-phenylpropionyl-L-α-amino-*n*-butyrate hydrochloride (IX). This hydrochloride (IX) was then coupled with *p*-nitrophenyl α-pyrrolecarboxylate prepared from α-pyrrolecarboxylic acid,[13] *p*-nitrophenol and N,N'-dicyclohexylcarbodiimide to yield methyl α-pyrrole carboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyrate (X). This tripeptide ester (X) was allowed to react with hydrazine hydrate in methanol to give its hydrazide (XI).

α-Pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyric acid azide prepared from the corresponding hydrazide (XI) was coupled with L-seryl-L-serine amide hydrochloride (VI) to yield α-pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyryl-L-seryl-L-serine amide (dehydrochloroislanditoxic amide, IV). The crude pentapeptide amide (IV) was purified by reprecipitation from dimethylformamide–ethyl acetate.

The purified material (IV) produced a single sharp spot with the same *Rf* value as that of the natural dehydrochlorinated–peptide amide (II) on the thin–layer chlomatogram. The elemental analysis of this material (IV) was matched with the calculated value, and its acid hydrolysate contained the constituent amino acids in the ratios expected theoretically.

However, the synthetic pentapeptide amide (IV), when compared with the natural dehydrochlorinated–peptide amide (II), showed some different properties, for example in infrared (IR) and nuclear magnetic resonance (NMR) spectra, partial acid hydrolysis, mp, *etc.* The infrared spectrum of the synthetic pentapeptide amide (IV) was different in the range of 1700–1500 cm^{-1} from that of the natural amide (II), as shown in Fig. 1. The pattern of the nuclear



a) dehydrochlorinated-peptide amide (II) *b*) synthetic dehydrochloroislanditoxic amide (IV)

Fig. 1. Infrared Spectra of II and IV in 1700—1500 cm^{-1}

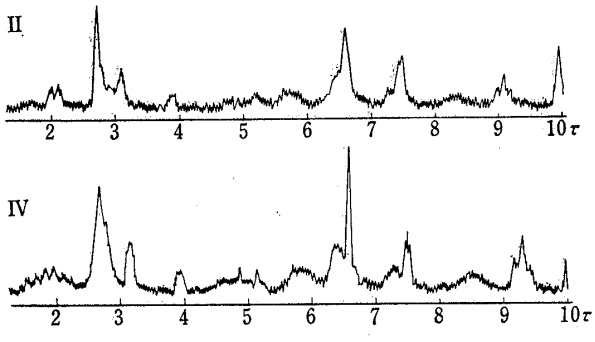


Fig. 2. Nuclear Magnetic Resonance Spctra of Dehydrochlorinated–Peptide Amide (II) and Synthetic Dehydrochloroislanditoxic Amide (IV) in DMSO–d_6 at 60 Mc

magnetic resonance spectrum of the synthetic pentapeptide amide (IV) differed from that of the natural amide (II), especially regarding the chemical shift of the methyl signal of L-α-amino-*n*-butyric acid containing in both peptide amides (synthetic (IV): τ=9.33, natural (II): τ=9.15), as shown in Fig. 2. The melting point of the synthetic pentapeptide amide (IV) was 254—256° and that of the natural amide (II) was 270—273°. The results of the partial acid hydrolyses[14] of these two peptide amides (II, IV) by concentrated hydrochloric acid were different on the thin–layer chromatogram, as shown in Fig. 3. Each spot to obtain by the partial acid hydrolysis was detected qualitatively and quantitatively, for example N–terminal and C-terminal amino acids, and completely acid hydrolysis, *etc.* Two spots corresponding

13) E. Banberger and G. Djierdjian, *Chem. Ber.*, **33**, 536 (1900); R.M. Silverstein, E.E. Ryskiewicz and C. Willand, "Organic Syntheses," John Wiley & Sons Inc., New York, N.Y., Vol. 36, 1956, p. 74.
14) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951); F. Sanger and E.O.P. Thompson, *ibid.*, **53**, 353 (1953).

to α-pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyric acid and L-serine were detected with the synthetic pentapeptide amide (IV), but four spots corresponding to α-pyrrolecarboxylyl-L-α-amino-*n*-butyric acid, L-seryl-L-β-amino-β-phenylpropionic acid, L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide and L-serine were detected with the natural amide (II).

It is concluded from these results that the amino acid sequence of our dehydrochlorinated-peptide amide (II) not coincided with dehydrochloroislanditoxic amide (IV), but it should be α-pyrrolecarboxylyl-L-α-amino-*n*-butyryl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide (XIX), as shown in Chart 3.

Then we attempted the synthesis of dehydrochlorinated–peptide amide (II) according to our amino acid sequence (XIX). The method used for the preparation is shown in Chart 4. The chain lengthening was performed by the fragment condensation according to the azide method, when the racemization was possible, and according to the mixed anhydride and 2,4-dinitrophenyl ester methods,[15] when the racemization was impossible.

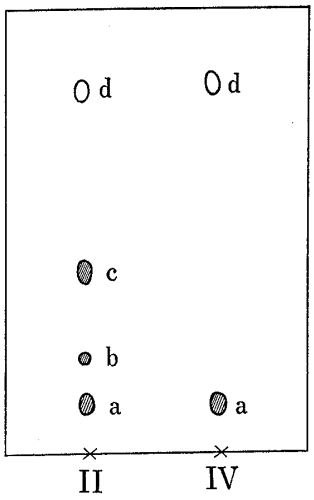
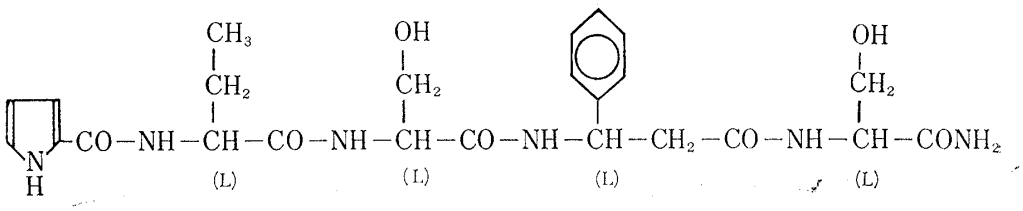


Fig. 3. Thin–layer Chromatogram of Partial Acid Hydrolysates of Dehydrochlorinated-peptide Amide (II) and Synthetic Dehydrochloroislanditoxic Amide (IV) (Silica Gel G, *n*-BuOH–AcOH–H_2O (4:1:2, v/v)

IIa, IVa: L-Ser IIb: L-Ser-L-β-Phe-L-SerNH$_2$
IIc: L-Ser-L-β-Phe IId: α-Pyr-L-Abu
IVb: α-Pyr-L-β-Phe-L-Abu



XIX

Chart 3

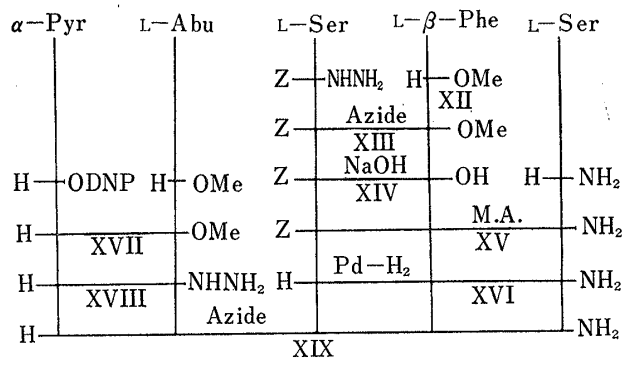


Chart 4

α-Pyr=α-pyrrolecarboxylic acid Abu=α-amino-*n*-butyric acid
β-Phe=β-amino-β-phenylpropionic acid
ODNP=2,4-dinitrophenyl ester M.A.=mixed anhydride

Benzyloxycarbonyl-L-serine azide prepared from the corresponding hydrazide[8] was allowed to react with methyl L-β-amino-β-phenylpropionate hydrochloride (XII) to give methyl benzyloxycarbonyl-L-seryl-L-β-amino-β-phenylpropionate (XIII), which was saponified with formation of benzyloxycarbonyl-L-seryl-L-β-amino-β-phenylpropionic acid (XIV). This benzyloxycarbonyl-dipeptide (XIV) was coupled with L-serine amide hydrochloride[9] by means of the mixed anhydride method using isobutyl chloroformate to yield benzyloxycarbonyl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide (XV). Hydrogenolysis of this benzyloxycarbonyl–tripeptide amide (XV) over palladium black gave L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide hydrochloride (XVI).

15) R. Rocchi, F.M. Marchiori and E. Scoffone, *Gazz. Chim. Ital.*, 93, 823, 834 (1963).

2,4-Dinitrophenyl α-pyrrolecarboxylate prepared from α-pyrrolecarboxylic acid,[13] 2,4-dinitrophenol[15] and N,N'-dicyclohexylcarbodiimide was allowed to react with methyl L-α-amino-*n*-butyrate hydrochloride[12] to give methyl α-pyrrolecarboxylyl-L-α-amino-*n*-butyrate (XVII), which was purified by chromatography on silica gel. This compound (XVII) was allowed to react with hydrazine hydrate in methanol to give its hydrazide (XVIII).

α-Pyrrolecarboxylyl-L-α-amino-*n*-butyric acid azide prepared from the corresponding hydrazide (XVIII) was coupled with L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide hydrochloride (XVI) to yield α-pyrrolecarboxylyl-L-α-amino-*n*-butyryl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide (XIX). The crude pentapeptide amide (XIX) was purified by recrystallization from *n*-butanol–acetic acid–water (4:1:2, v/v).

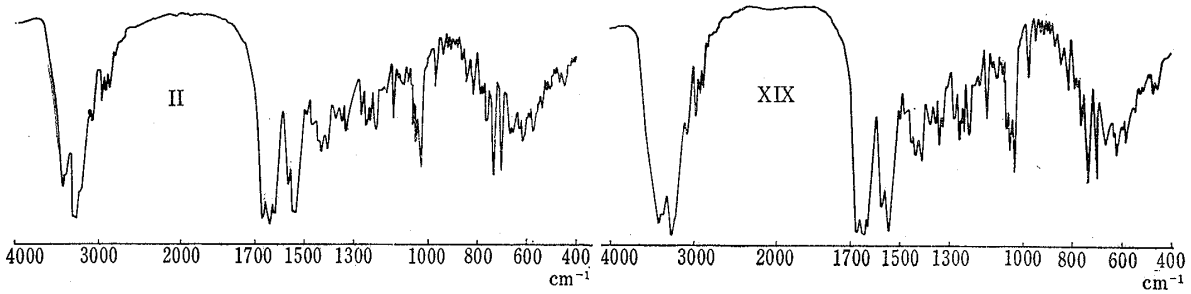


Fig. 4. Infrared Spectra of Dehydrochlorinated-peptide Amide (II) and Synthetic Pentapeptide Amide (XIX)

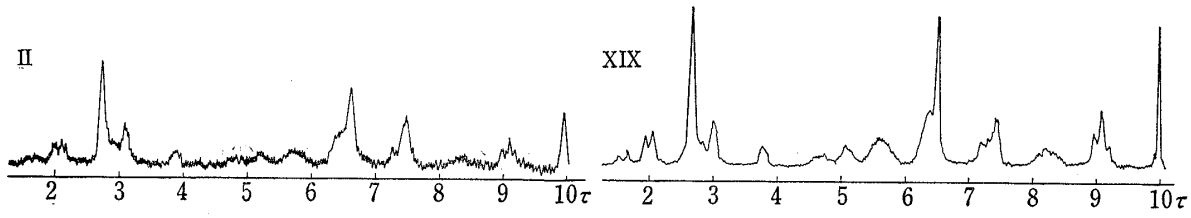


Fig. 5. Nuclear Magnetic Resonance Spectra of Dehydrochlorinated-peptide Amide (II) and Synthetic Pentapeptide Amide (XIX) in DMSO-d_6 at 60 Mc

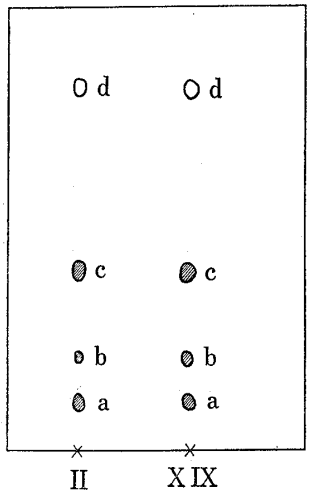


Fig. 6. Thin-layer Chromatogram of Partial Acid Hydrolysates of Dehydrochlorinated-peptide Amide (II) and Synthetic Pentapeptide Amide (XIX) (Silica Gel G, *n*-BuOH–AcOH–H_2O (4:1:2, v/v))

IIa, XIXa: L-Ser
IIb, XIXb: L-Ser-L-β-Phe-L-SerNH_2
IIc, XIXc: L-Ser-L-β-Phe
IId, XIXd: α-Pyr-L-Abu

The purified material (XIX) produced a single sharp spot with the same *Rf* value as that of the natural dehydrochlorinated-peptide amide (II) on the thin-layer chromatogram. The elemental analysis of this material (XIX) was matched with the calculated value, and its acid hydrolysate contained the constituent amino acids in the ratios expected theoretically.

The infrared spectrum of the synthetic pentapeptide amide (XIX) was completely identical with that of the natural amide (II), as shown in Fig. 4. The pattarn of nuclear magnetic resonance spectrum of the synthetic pentapeptide amide (XIX) was completely identical with that of the natural amide (II), as shown in Fig. 5. The melting points of both peptide amides (II, XIX) were same. The results of the partial acid hydrolyses[14] of these two peptide amides (II, XIX) by concentrated hydrochloric acid also gave completely identical patterns on the thin-layer chromatogram, as shown in Fig. 6. Each spot to obtain by the partial acid hydrolysis was detected qualitatively and quanti-

tatively, for example N-terminal and C-terminal amino acids, and completely acid hydrolysis, *etc.* Four spots corresponding to α-pyrrolecarboxylyl-L-α-amino-*n*-butyric acid, L-seryl-L-β-amino-β-phenylpropionic acid, L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide and L-serine were detected with both peptide amides (II, XIX).

It is concluded from these results that the amino acid sequence of our dehydrochlorinated-peptide amide (II) is α-pyrrolecarboxylyl-L-α-amino-*n*-butyryl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide (XIX).

From these results, we suppose that the chlorine-containing peptide (I) has same amino acid sequence, but the position and configuration of two chlorine atoms are not yet determined. We are trying to approach that problem.

Experimental

All melting point were uncorrected. The optical rotations were measured with a Yanagimoto direct reading polarimeter OR-20 (Na-D line). The infrared spectra were recorded with a Hitachi infrared spectrophotometer EPI-G_2 in KBr disks. The nuclear magnetic resonance spectra were obtained by a Japan Electron Optics Laboratory 3H-60 spectrometer operating at 60 Mc in DMSO-6d solution *vs.* Me_4Si as internal reference. The ultraviolet absorption spectra were obtained with a Hitachi recording spectrophotomrter EPS-3A. The amino acid composition of acid hydrolysates was determined by the DNP method.[16] Dinitrophenylation of acid hydrolysates was carried out in the usual manner. The DNP-amino acids were applied to the thin-layer chromatography on Silica Gel G with the system of benzene–pyridine–acetic acid (80:20:4, v/v). The following abbreviations are used; Abu=α-amino-*n*-butyric acid; β-Phe=β-amino-β-phenylpropionic acid; Pyr=α-pyrrolecarboxylic acid.

A) Dehydrochlorinated-peptide Amide (II)

Chlorine-containing peptide (I) was isolated from the cultured broth of *P. islandicum* Sopp,[4] mp 251°. Chlorine-containing peptide I (51.9 mg) was dissolved in concentrated ammonia water (6.0 ml) and stored at room temperature for 3 day. The precipitate was collected by filtration, washed with water, acetone and methanol successively and dried at 100°; colorless needles, yield 24.7 mg, mp 270—273° (decomp.), UV λ_{max}^{EtOH} 268 mμ ($\varepsilon=1.42\times10^4$), IR: cm^{-1}; 3430, 3270, 1670, 1645, 1620, 1540. *Anal.* Calcd. for $C_{24}H_{32}O_7N_6$: C, 55.81; H, 6.24; N, 16.27. Found: C, 55.74; H, 6.20; N, 16.29. Amino acid ratios in an acid hydrolysate $Ser_{1.72}Abu_{0.89}\beta\text{-}Phe_{1.00}$.

This amide II (3.0 mg) was dissolved in concentrated hydrochloric acid (3.0 ml),[14] and the solution was incubated at 37° for 20 hr and then evaporated to dryness *in vacuo*. The thin-layer chromatogram of this partial acid hydrolysate in the system of *n*-butanol–acetic acid–water (4:1:2, v/v) on Silica Gel G revealed three ninhydrin positive spots, *Rf* 0.11 (L-serine), *Rf* 0.21 (L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide) and *Rf* 0.42 (L-seryl-L-β-amino-β-phenylpropionic acid), and one ninhydrin negative spot, *Rf* 0.84 (α-pyrrolecarboxylyl-L-α-amino-*n*-butyric acid) and no spot corresponding to this amide (II) was detected, as shown in Fig. 3.

B) Synthesis of Dehydrochloroislanditoxic Amide——α-Pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyryl-L-seryl-L-serine Amide (IV)

Benzyloxycarbonyl-L-seryl-L-serine Amide (V)——A solution of $NaNO_2$ (0.50 g) in cold water (2.0 ml) was added dropwise to an ice-cold solution of benzyloxycarbonyl-L-serine hydrazide[8] (1.27 g) in 10% HCl (2.75 ml), acetic acid (1.05 ml) and water (10 ml). After 10 min, the mixture was adjusted with 30% K_2CO_3 aqueous solution to pH 7.5, and the azide was extracted with ethyl acetate (40 ml) and then dried over Na_2SO_4 for 30 min at 0°. This azide solution was added to an ice-cold solution of L-serine amide hydrochloride[9] (0.70 g) and triethylamine (0.70 ml) in dimethylformamide (10 ml). The mixture was stirred for 2 day at 4°. The precipitate was collected by filtration, washed with ethyl acetate and water, and recrystallized from dimethylformamide–ethyl acetate to give colorless powder; yield 0.97 g (59.5%), mp 238—240°. IR cm^{-1}: 3230, 1670, 1630, 1540, $[\alpha]_D^{23}$ +36.1° ($c=0.632$, DMF). *Anal.* Calcd. for $C_{14}H_{19}O_6N_3$: C, 51.69; H, 5.89; N, 12.92. Found: C, 51.75; H, 5.73; N, 12.93.

L-Seryl-L-serine Amide Hydrochloride (VI)——The protected dipeptide amide V (1.138 g) in methanol (25 ml) and 0.4 N HCl-methanol (9.0 ml) was hydrogenated over palladium black (0.15 g) for 13 hr in the usual manner. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo* to give hygroscopic colorless powder; yield 0.762 g (96.5%). This hydrochloride (VI) was used without further purification.

16) A.L. Levy, *Nature*, **174**, 126 (1954); H. Fraenkel-Conrat, J.I. Harris and A.L. Levy, "Methods of Biochemical Analysis," ed. by D. Glick, Academic Press Inc., New York, N.Y., Vol. 2, 1955, p. 359.

L-β-Amino-β-phenylpropionic Acid——DL-β-Amino-β-phenylpropionic acid was prepared by the method described by J.B. Johnson, *et al.*[10] L-β-Amino-β-phenylpropionic acid was prepared by the method of E. Fischer, *et al.*[11] Colorless plates, mp 239—241° (decomp.), $[\alpha]_D^{25}$ +6.62° (c=0.756, H_2O), (lit. mp 234—235° (decomp.), $[\alpha]_D^{20}$ +6.9° (c=1.09, H_2O),[11] mp 234°, $[\alpha]_D^{20}$ +6.8° (c=0.8, H_2O)[17]).

Benzyloxycarbonyl-L-β-amino-β-phenylpropionic Acid (VII)——Benzyloxycarbonyl-L-β-amino-β-phenylpropionic acid (VII) was prepared from L-β-amino-β-phenylpropionic acid[10,11] and benzyloxycarbonyl chloride in 10% NaOH aqueous solution by the usual method. This (VII) was recrystallized from ethyl acetate to give colorless needles; yield 76.3%, mp 139—140°, IR: cm^{-1}; 3380, 1710, 1690, 1530, $[\alpha]_D^{21}$ +24.4° (c=1.061, EtOH). *Anal.* Calcd. for $C_{17}H_{17}O_4N$: C, 68.22; H, 5.73; N, 4.68. Found: C, 68.37; H, 5.68; N, 4.62.

Methyl Benzyloxycarbonyl-L-β-amino-β-phenylpropionyl-L-α-amino-n-butyrate (VIII)——A mixed anhydride prepared from benzyloxycarbonyl-L-β-amino-β-phenylpropionic acid VII (3.590 g) in tetrahydrofuran (30 ml) with triethylamine (1.68 ml) and isobutyl chloroformate (1.65 g) was added to a solution of methyl L-α-amino-n-butyrate hydrochloride[12] (1.842 g) in chloroform (30 ml) with triethylamine (1.68 ml). The mixture was stirred in an ice bath for 1 hr, and then stored in a refrigerator overnight. The solution was washed succesively with H_2O, saturated $NaHCO_3$ aqueous solution, 1N HCl and H_2O, and dried over Na_2SO_4. The solvent was evaporated to dryness *in vacuo* and the residue was recrystallized from ethyl acetate to give colorless needles; yield 4.11 g (86.0%), mp 149—150°, IR: cm^{-1}; 3320, 1730, 1690, 1640, 1545, $[\alpha]_D^{24}$ −12.9° (c=0.804, MeOH). *Anal.* Calcd. for $C_{22}H_{26}O_5N_2$: C, 66.32; H, 6.58; N, 7.03. Found: C, 66.61; H, 6.41; N, 6.95.

Methyl L-β-Amino-β-phenylpropionyl-L-α-amino-n-butyrate Hydrochloride (IX)——Methyl benzyloxycarbonyl-L-β-amino-β-phenylpropionyl-L-α-amino-n-butyrate VIII (3.19 g) in methanol (20 ml) and 0.4 N HCl-methanol (20 ml) was hydrogenated over palladium black (0.20 g) for 2 hr in the usual manner. The catalyst was removed by filtration. The filtrate was evaporated to dryness *in vacuo* and the residue was recrystallized from methanol–ether to give colorless plates; yield 2.18 g (90.5%), mp 185—188°,. IR: cm^{-1}; 3350, 2950, 1745, 1650, 1550, $[\alpha]_D^{24}$ −37.4° (c=0.848, MeOH). *Anal.* Calcd. for $C_{14}H_{21}O_3N_2Cl$: C, 55.90; H, 7.04; N, 9.31. Found: C, 56.34; H, 6.90; N, 9.57.

Methyl α-Pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-n-butyrate (X)——A solution of p-nitrophenyl α-pyrrolecarboxyrate prepared from α-pyrrole carboxylic acid[13] (0.67 g) and p-nitrophenol (0.92 g) in ethyl acetate (40 ml) with dicyclohexylcarbodiimide (1.28 g)was added to a solution of methyl L-β-amino-β-phenylpropionyl-L-α-amino-n-butyrate hydrochloride IX (1.805 g) in chloroform (20 ml) with triethylamine (0.84 ml). The mixture was stored at room temperature for 3 day. N,N′-Dicyclohexylurea was removed by filtration and the filtrate was washed successively with H_2O, 5% NH_3 aq., 1N HCl and H_2O, and dried over Na_2SO_4. The solution was evaporated to dryness *in vacuo* and the residue was recrystallized from methanol to give colorless needles; yield 0.972 g (45.4%), mp 194—196°, IR: cm^{-1}; 3380, 1735, 1655, 1635, 1530, $[\alpha]_D^{30}$ −45.0° (c=1.102, EtOH). *Anal.* Calcd. for $C_{19}H_{23}O_4N_3$: C, 63.84; H, 6.49; N, 11.76. Found: C, 64.40; H, 6.81; N, 11.88.

α-Pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-n-butyric Acid Hydrazide (XI)——Methyl α-pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-n-butyrate X (0.502 g) was dissolved in ethanol (20 ml) and 85% hydrazine hydrate (1.0 ml) was added. The mixture was kept at room temperature for 3 days. The solution was added to water (50 ml). The precipitate was collected by filtration, washed successively with water and dried to give colorless amorphous powder; yield 0.47 g (94.0%), mp 248—251°, IR: cm^{-1}; 3270, 1680, 1645, 1620, 1530, $[\alpha]_D^{23}$ −41.8° (c=0.267, DMF). *Anal.* Calcd. for $C_{18}H_{23}O_3N_5$: C, 60.49; H, 6.49; N, 19.57. Found: C, 61.75; H, 7.01; N, 18.74.

α-Pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-n-butyryl-L-seryl-L-serine Amide (IV)——A solution of $NaNO_2$ (0.20 g) in cold water (1.0 ml) was added dropwise to an ice–cold solution of α-pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-n-butyric acid hydrazide XI (0.659 g) in 10% HCl (1.6 ml), acetic acid (5.0 ml) and water (3.0 ml). After 10 min, the mixture was adjusted with 30% K_2CO_3 aqueous solution to pH 7.5, and the azide was extracted with ethyl acetate (100 ml) and then dried over Na_2SO_4 for 30 min at 0°. This azide solution was added to an ice-cold solution of L-seryl-L-serine amide hydrochloride VI (0.419 g) and triethylamine (0.26 ml) in dimethylformamide (12 ml). The mixture was stirred for 3 days in an ice bath and stored for 2 days in a refrigerator. The precipitate was collected by filtration, washed with ethyl acetate and water, and reprecipitated from dimethylformamide-ethyl acetate to give colorless amorphous powder; yield 0.129 g (13.6%), mp 254—256°, UV λ_{max}^{EtOH} 268 mμ (ε=1.69×10⁴), IR: cm^{-1}; 3460, 3330, 1690, 1660, 1635, 1555, 1530, $[\alpha]_D^{24}$ −5.7° (c=1.059, DMF). *Anal.* Calcd. for $C_{24}H_{32}O_7N_6$: C, 55.81; H, 6.24; N, 16.27. Found: C, 54.49; H, 6.37; N, 15.80. Amino acid ratios in an acid hydrolysate $Ser_{1.80}Abu_{0.98}\beta\text{-}Phe_{1.00}$.

This peptide amide IV (2.6 mg) was dissolved in concentrated hydrochloric acid (2.0 ml),[11] and the solution was incubated at 37° for 20 hr and then evaporated to dryness *in vacuo*. The thin-layer chromatogram of this partial acid hydrolysate in the system of n-butanol–acetic acid–water (4:1:2, v/v) on Silica Gel

17) E. Graf and H. Boeddeker, *Ann.*, **613**, 111 (1958).

G revealed one ninhydrin positive spot, *Rf* 0.11 (L-serine) and one ninhydrin negative spot, *Rf* 0.82 (α-pyrrolecarboxylyl-L-β-amino-β-phenylpropionyl-L-α-amino-*n*-butyric acid), and no spot corresponding to this amide (IV) was detected, as shown in Fig. 3.

C) Synthesis of α-Pyrrolecarboxylyl-L-α-amino-*n*-butyryl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine Amide (XIX)

Methyl α-Pyrrolecarboxylyl-L-α-amino-*n*-butyrate (XVII)——A solution of 2,4-dinitrophenyl α-pyrrolecarboxylate prepared from α-pyrrolecarboxylic acid[13] (1.111 g) and 2,4-dinitrophenol[15] (2.020 g) in ethyl acetate (40 ml) with dicyclohexylcarbodiimide (2.170 g) was added to a solution of methyl L-α-amino-*n*-butyrate hydrochloride[12] (1.536 g) in chloroform (15 ml) with triethylamine (1.40 ml). The mixture was stored at room temperature for 20 hr, refluxed for 4 hr and cooled in an ice bath for 1 hr. N,N'-Dicyclohexylurea was removed by filtration and the filtrate was washed successively with H_2O, 5% NH_3aq., 1 N HCl and H_2O, and dried over Na_2SO_4. The solution was evaporated to dryness *in vacuo*. The residue was dissolved in benzene–chloroform (1:2, v/v) (50 ml) and the solution was applied to a column of Kiesel gel 0.08 mm (3×15 cm) which was eluted with benzene-chloroform (1:2, v/v). The desired fractions were pooled and evaporated to dryness *in vacuo*. The residue was recrystallized from ethyl acetate–*n*-hexane to give colorless cubes; yield 1.584 g (75.4%), mp 89°, UV λ_{max}^{EtOH} 267 mμ ($\varepsilon=1.89\times10^4$), IR: cm^{-1}; 3350, 1730, 1620, 1565, 1525, $[\alpha]_D^{17}$ −26.7° ($c=1.000$, EtOH). *Anal.* Calcd. for $C_{10}H_{14}O_3N_2$: C, 57.13; H, 6.71; N, 13.33. Found: C, 57.34; H, 6.65; N, 12.69.

α-Pyrrolecarboxylyl-L-α-amino-*n*-butyric Acid Hydrazide (XVIII)——Methyl α-pyrrolecarboxylyl-L-α-amino-*n*-butyrate XVII (0.421 g) was dissolved in methanol (5.0 ml) and 85% hydrazine hydrate (0.32 ml) was added. The mixture was kept at room temperature for 2 days. Then the solution was evaporated to dryness *in vacuo* to give colorless powder, which was used without further purifiction; yield 0.421 g (100%).

Methyl L-β-Amino-β-phenylpropionate Hydrochloride (XII)——L-β-Amino-β-phenylpropionic acid[10,11] (3.535 g) suspended in methanol (30 ml) was added dropwise to thionyl chloride (3.1 ml) with stirring in an ice bath and then the mixture was refluxed for 1 hr. The solution was evaporated to dryness *in vacuo* and the residue was recrystallized from methanol–ether to give colorless needles; yield 4.390 g (95.0%), mp 182—183°, IR: cm^{-1}; 2860, 1725, 1520, $[\alpha]_D^{20}$ −7.22° ($c=1.041$, MeOH). *Anal.* Calcd. for $C_{10}H_{14}O_2NCl$: C, 55.69; H, 6.54; N, 6.49. Found: C, 55.71; H, 6.36; N, 6.50.

Methyl Benzyloxycarbonyl-L-seryl-L-β-amino-β-phenylpropionate (XIII)——A solution of $NaNO_2$ (1.0 g) in cold water (5.0 ml) was added dropwise to an ice–cold solution of benzyloxycarbonyl-L-serine hydrazide[8] (2.533 g) in 10% HCl (5.6 ml), acetic acid (2.2 ml) and water (20 ml). After 10 min, the mixture was adjusted with 30% K_2CO_3 aqueous solution to pH 7.5, and the azide was extracted with ethyl acetate (50 ml) and then dried over Na_2SO_4 for 30 min at 0°. This azide solution was added to an ice-cold solution of methyl L-β-amino-β-phenylpropionate hydrochloride XII (2.157 g) and triethylamine (1.40 ml) in chloroform (20 ml). The mixture was stirred for 3 hr in an ice bath and stored for 4 day in a refrigerator. The solution was washed successively with H_2O, saturated $NaHCO_3$ aqueous solution, 1N HCl and H_2O, and dried over Na_2SO_4. The solvent was evaporated to dryness *in vacuo* and the residue was recrystallized from ethyl acetate to give colorless needles; yield 2.586 g (64.6%), mp 128—129°, IR: cm^{-1}; 3350, 1730, 1710, 1630, 1530, $[\alpha]_D^{17.5}$ −21.75° ($c=1.008$, MeOH). *Anal.* Calcd. for $C_{21}H_{24}O_6N_2$: C, 62.99; H, 6.04; N, 7.00. Found: C, 62.93; H, 6.44; N, 7.03.

Benzyloxycarbonyl-L-seryl-L-β-amino-β-phenylpropionic Acid (XIV)——To a solution of benzyloxycarbonyl-dipeptide methyl ester XIII (2.002 g) in methanol (15 ml) was added 1N NaOH (6.0 ml). The mixture was stirred for 2 hr at room temperature. The solution was concentrated *in vacuo* to one half of the volume, added to water (10 ml) and washed with ethyl acetate. The aqueous layer was acidified to pH 2 with 10% HCl and benzyloxycarbonyl-L-seryl-L-β-amino-β-phenylpropionic acid (XIV) was extracted with ethyl acetate and dried over Na_2SO_4. The solvent was evaporated to dryness *in vacuo* and the residue was reprecipitated from chloroform–*n*-hexane to give colorless amorphous powder; yield 2.435 g (62.9%). This (XIV) was used without further purification.

Benzyloxycarbonyl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine Amide (XV)——A mixed anhydride prepared from benzyloxycarbonyl-L-seryl-L-β-amino-β-phenylpropionic acid XIV (1.159 g) in tetrahydrofuran (25 ml) with triethylamine (0.43 ml) and isobutyl chloroformate (0.40 ml) was added to a solution of L-serine amide hydrochloride[9] (0.428 g) in chloroform (5.0 ml) and dimethylformamide (5.0 ml) with triethylamine (0.42 ml). The mixture was stirred in an ice bath for 2 hr and then stored in a refrigerator overnight. The mixture was added to ethyl acetate (20 ml) and the precipitate was collected by filtration, washed with ethyl acetate and water, and dried to give colorless powder; yield 0.611 g (48.5%), mp 254—256°, IR: cm^{-1}; 3350, 3270, 1690, 1660, 1640, 1530, $[\alpha]_D^{23}$ +37.4° ($c=0.953$, DMSO). *Anal.* Calcd. for $C_{23}H_{28}O_7N_4$: C, 58.50; H, 5.97; N, 11.86. Found: C, 58.05; H, 5.92; N, 12.03.

L-Seryl-L-β-amino-β-phenylpropionyl-L-serine Amide Hydrochloride (XVI)——The protected tripeptide amide XV (0.945 g) in methanol (20 ml) and 0.4N HCl-methanol (5.0 ml) was hydrogenated over palladium black (0.10 g) for 15 hr in the usual manner. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo* to give colorless amorphous powder; yield 0.408 g (54.5%). This (XVI) was used without further purification.

α-Pyrrolecarboxylyl-L-α-amino-*n*-butyryl-L-seryl-L-β-amino-β-phenylpropionyl-L-serine Amide (XIX)—— A solution of $NaNO_2$ (0.105 g) in cold water (1.0 ml) was added dropwise to an ice–cold solution of α-pyrrolecarboxylyl-L-α-amino-*n*-butyric acid hydrazide XVIII (0.231 g) in 10% HCl (1.0 ml), acetic acid (2.0 ml) and water (2.0 ml). After 10 min, the mixture was adjusted with 30% K_2CO_3 aqueous solution to pH 7.5, and the azide was extracted with ethyl acetate (30 ml) and then dried over Na_2SO_4 for 30 min at 0°. This azide solution was added to an ice-cold solution of L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide hydrochlode XVI (0.374 g) and triethylamine (0.14 ml) in dimethylformamide (10 ml). The mixture was stirred for 11 hr in an ice bath and stored for 3 days in a refrigerator. The solvent was evaporated to dryness *in vacuo* and the residue was added to *n*-butanol (25 ml) and water (10 ml). The organic layer was separated, washed with H_2O, 1N HCl, saturated $NaHCO_3$ aqueous solution and H_2O, concentrated *in vacuo* to one fourth of the volume, added to ether (15 ml) and stored for 2 hr in an ice bath. The precipitate was collected by filtration, and recrystallized from *n*-butanol–acetic acid–water (4:1:2, v/v) to give colorless needles; yield 0.203 g (39.3%), mp 270—272°, UV λ_{max}^{EtOH} 268 mμ ($\varepsilon = 1.57 \times 10^4$), IR cm^{-1}: 3430, 3270, 1675, 1650, 1620, 1540, $[\alpha]_D^{24}$ +2.5° ($c = 0.787$, DMF). *Anal.* Calcd. for $C_{24}H_{32}O_7N_6$: C, 55.81; H, 6.24; N, 16.27. Found: C, 55.64; H, 5.93; N, 15.98. Amino acid ratios in an acid hydrolysate $Ser_{1.86}Abu_{1.02}\beta\text{-}Phe_{1.00}$.

This peptide amide XIX (3.0 mg) was dissolved in concentrated hydrochloric acid (3.0 ml),[14] and the solution was incubated at 37° for 20 hr and then evaporated to dryness *in vacuo*. The thin-layer chromatogram of this partial acid hydrolysate in the system of *n*-butanol–acetic acid–water (4:1:2, v/v) on Silica Gel G revealed three ninhydrin positive spots, *Rf* 0.11 (L-serine), *Rf* 0.21 (L-seryl-L-β-amino-β-phenylpropionyl-L-serine amide) and *Rf* 0.42 (L-seryl-L-β-amino-β-phenylpropionic acid), and one ninhydrin negative spot, *Rf* 0.84 (α-pyrrolecarboxylyl-L-α-amino-*n*-butyric acid) and no spot corresponding to this amide (XIX) was detected, as shown in Fig. 6.

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