Aspochracin, a New Insecticidal Metabolite of *Aspergillus ochraceus*

Part I. Isolation, Structure and Biological Activities

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A new insecticidal substance named aspochracin was isolated from the culture filtrate of *Aspergillus ochraceus*, a pathogenic fungus causing muscardine on insects. The compound was found to be a novel cyclotriptide, composed of N-methyl-L-alanine, N-methyl-L-valine and L-ornithine, containing an octatrienoic acid side chain. Isolation procedure, structural elucidation and biological activities are described in detail.

Insect diseases caused by fungi are generally called muscardines, and some of these microbes have been known to excrete toxins for insects in synthetic culture media. In 1961 insecticidal depsipeptides named destruxins A and B were isolated by Kodaira¹⁾ from the culture filtrate of *Metarrhizium anisopliae* (Metsch.) Sorokin, a pathogenic fungus for silkworm, and their structures were elucidated by Tamura *et al.*²⁾ through the degradation experiments as well as the total syntheses. During further investigations to search for toxic metabolites of muscardine fungi, the presence of a new insecticidal substance named aspochracin (I) in the culture

filtrate of *Aspergillus ochraceus* Wilhelm was found, and the isolation and structure of the toxin was preliminarily reported.³⁾ In this paper we wish to present our experimental results leading to structural elucidation of the toxin in details together with its biological activities.

Asp. ochraceus was cultured in the Czapek-Dox medium containing yeast extract with aeration and agitation. The cultured broth was filtered, and the filtrate was extracted with ethyl acetate. Evaporation of the solvent yielded a brown powder, which was chromatographed on a silicic acid column using a solvent system of benzene-ethyl acetate-acetone (45 : 40 : 15). The active principle thus obtained was further purified by silicic acid chromatography with a solvent system of chloroform-acetone (98 : 2). The toxin was finally obtained as a pale yellow powder, which was confirmed

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1) Y. Kodaira, *Agr. Biol. Chem.*, **26**, 36 (1962).

2) S. Tamura, S. Kuyama, Y. Kodaira and S. Higashikawa, *ibid.*, **28**, 137 (1964); S. Kuyama and S. Tamura, *ibid.*, **29**, 168 (1965); A. Suzuki, S. Kuyama, Y. Kodaira and S. Tamura, *ibid.*, **30**, 517 (1966).

3) R. Myokei, A. Sakurai, C.-F. Chang, Y. Kodaira, N. Takahashi and S. Tamura, *Tetrahedron Letters*, **1969**, 695.

FIG. 1. Isolation Procedure of Aspochracin from the Culture Filtrate of *Aspergillus ochraceus*.

to be homogeneous by silicic acid thin layer chromatography (t.l.c.). The isolation procedure is shown in Fig. 1.

I is a neutral compound showing $[\alpha]_D^{25} -76.0^\circ$ ($c=1.0$, MeOH) and UV absorption maximum at 297 m μ (ϵ 29,500). The molecular formula of I was not available from elemental analysis, since the compound is unstable at room temperature even under nitrogen atmosphere. In the IR spectrum (Fig. 2), the bands at 3300, 1630 and 1520 cm⁻¹ suggest the presence of peptide linkages. This is also indicated by positive coloration of I with the

chlorine-*o*-toluidine-potassium iodide reagent.⁴¹ Hydrolysis of I with hydrochloric acid gave N-methylalanine, N-methylvaline and ornithine which were detected by paper chromatography (p.p.c.) and paper electrophoresis. Moreover, these amino acids were isolated as crystals by preparative p.p.c. and identified by comparisons with the authentic samples. Optical rotation measurement revealed that each amino acid has L-configuration. However, no fragments indicating the chromophore of the molecule of I could be isolated from the hydrolysate.

On catalytic hydrogenation over platinum oxide in ethanol, I consumed three moles of hydrogen to yield hexahydroaspochracin (II), which was recrystallized from ethyl acetate-*n*-hexane to give colorless crystals melting at 137°C. It is stable enough to be characterized, and the molecular formula C₂₃H₄₂O₄N₄ was deduced from elemental analysis and high resolution mass spectrometry. II shows no

FIG. 2. IR Spectrum of Aspochracin (Nujol mull).

4) O. J. Schmid, *J. Chromatog.*, **3**, 499 (1960).

absorption maximum above $210\text{ m}\mu$. The IR spectrum reveals the bands at 3350 , 1640 and 1510 cm^{-1} corresponding to peptide linkages.

On acid hydrolysis, II gave the same amino acids as those obtained from I. Besides, ppc of the ether extract of the hydrolysate indicated

FIG. 3. NMR Spectrum of Aspochracin.

FIG. 4. NMR Spectrum of Hexahydroaspochracin.

TABLE I. MULTIPLICITY CHANGES AND PRECISE
 CHEMICAL SHIFTS OF PROTONS IN SPIN
 DECOUPLING STUDIES OF ASPOCHRACIN
 AND HEXAHYDROASPOCHRACIN

(a) Aspochracin				
Position of protons,				
Irradiated		Observed		Multiplicity change
H _m	3.34	H _l	4.99	broad t → broad d
H _o	2.79	H _n	4.16	d → changed
H _n	4.16	H _o	2.79	dd → broad d
H _s	4.10	H _t	8.18	d → s
H _t	8.18	H _s	4.10	m → changed

(b) Hexahydroaspochracin				
Position of protons,				
Irradiated		Observed		Multiplicity change
H _m	3.61	H _l	5.10	broad t → dd
H _h	4.25	H _i	6.4 ~ 6.8	m → changed
H _l	5.10	H _m	3.61	d → s
"	"	H _k	7.4 ~ 7.7	m → changed
"	"	H _j	8.3 ~ 8.4	m → changed
H _d	4.91	H _c	7.4 ~ 7.7	m → changed
H _g	5.42	H _f	8.50	d → s
H _c	7.55	H _d	4.91	d → broad s
"	"	H _b	{ 9.09 9.26	d → s d → s
H _u	7.82	H _v	8.3 ~ 8.4	m → changed
H _j	8.42	H _i	6.4 ~ 6.8	m → changed
"	"	H _k	7.4 ~ 7.7	m → changed
"	"	H _l	5.10	broad t → sharp t
H _v	"	H _u	7.82	t → s

4.3) and allyl methyl protons (3H at τ 8.18d) in the spectrum of I disappeared on hydrogenation, while the signals due to caprylic acid residue ($-\text{COCH}_2-$ at τ 7.82 t, $-\text{COCH}_2\text{CH}_2-$ at τ 8.4 m, $-(\text{CH}_2)_4-$ at τ 8.73 s and $-(\text{CH}_2)_4\text{CH}_3$ at τ 9.11 t)* were observed in the spectrum of II. On the other hand, no great difference was recognized between the signals attributable to the amino acids constituting of the both compounds.

The results of double irradiation studies on the NMR spectra of I and II are summarized in Table I. Unexpected splitting patterns are observed in the signals due to the ornithine protons. In the spectrum of II the signal of H_l appears as a broad triplet being coupled with H_m by $J=7.2$ cps. Accordingly, the coupling of H_l with the adjacent methylene protons H_k is non-equivalent ($J=8.0$ and $0\sim 1$ cps). The signals of H_l also showed a complicated multiplet owing to non-equivalent couplings with H_n. These features should be ascribed to the rigid structure of II shown later.

On hydrolysis of II with barium hydroxide, partial hydrolysis occurred, and resulting peptides A and B were isolated from the hydrolysate by preparative p.p.c. and subsequent silicic acid t.l.c.. Acid hydrolysis of

TABLE II. AMINO ACID SEQUENCE OF PEPTIDES A AND B

	Amino acid constitution	N-Terminal	C-Terminal
Peptide A	N-MeAla, N-MeVal, Orn, Caprylic acid	δ -DNP-Orn	N-MeAla
Peptide B	N-MeAla, N-MeVal, Orn	di-DNP-Orn	N-MeAla

the presence of an acid, which was then isolated by preparative ppc and identified as caprylic acid by gas chromatography of its methyl ester.

The isolation of caprylic acid from the hydrolysate of II suggests that the strong UV absorption of I must be due to an octatrienecarboxamide residue. This assumption was confirmed by comparison of the NMR spectra of I and II shown in Figs. 3 and 4. The signals of olefinic protons (6H at τ 2.6~

the peptides followed by p.p.c. revealed that peptide A consists of caprylic acid, ornithine, N-methylalanine and N-methylvaline and that peptide B consists of the same amino acids as those in the former. The N- and C-terminal groups of the both peptides were determined by the DNP method and hydrazinolysis as shown in Table II. Accordingly,

* The abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet and m, multiplet.

FIG. 5. Mass Spectrum of Hexahydroaspochracin.

- I: $R = \text{CH}_3\text{-CH=CH-CH=CH-CH=CH-}$
 II: $R = \text{CH}_3\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$

caprylic acid is considered to be attached to the α -amino group of ornithine. Thus, the cyclic structure of II can be deduced to hexahydroaspochracin and consequently the structure of aspochracin should be represented as I. Aspochracin is the sole cyclotriptide which has ever been discovered in the nature.

The mass spectrum of II shown in Fig. 5 scarcely supplies any information for the structural elucidation. However, when it was compared with the spectra of destruxins A and B, an interesting coincidence was observed in their fragmentation patterns. The fragment ions of M-57 (m/e 381), M-85 (m/e 353) and M-142 (m/e 296) in Fig. 5 are also found in the spectra of destruxins A and B, in which the same sequence of two N-methylamino acids, N-methylalanine and N-methylvaline,

TABLE III. ELEMENTAL COMPOSITIONS OF FRAGMENT IONS OBSERVED IN THE HIGH RESOLUTION MASS SPECTRUM OF HEXAHYDROASPOCHRACIN

m/e	Constitution	Observed	Calcd.	Error
381 (M- 57)	$\text{C}_{20}\text{H}_{35}\text{O}_4\text{N}_3$	381.266	381.263	+0.003
353 (M- 85)	$\text{C}_{18}\text{H}_{31}\text{O}_4\text{N}_3$	353.231	353.232	-0.001
296 (M-142)	$\text{C}_{16}\text{H}_{28}\text{O}_3\text{N}_2$	296.210	296.212	-0.002

FIG. 6. Fragmentation Patterns of Hexahydroaspochracin in Mass Spectrometry.

exists in the cyclic depsipeptide structures as in the case of II. From the elemental compositions shown in Table III, M-57 and M-85 are considered to originate in the elimination of the fragments (a) and (b) illustrated in Fig. 6. Another type of cleavage at (b) and (c) is expected to give the fragment ion of M-142. It is noteworthy that these unusual

TABLE IV. INSECTICIDAL ACTIVITY OF ASPOCHRACIN ON THE FINAL INSTAR LARVAE OF FALL WEBWORM BY INJECTION

Amount injected, γ /g	% killing after 24 hr
170	100
85	33
42	0
Control	0

TABLE V. INSECTICIDAL ACTIVITY OF ASPOCHRACIN ON THE FIRST INSTAR LARVAE OF SILKWORM BY DIPPING

Concentration, γ /ml	% killing after 24 hr
3200	93
1600	82
800	52
400	41
200	25
100	0
Control	0

TABLE VI. INSECTICIDAL ACTIVITY OF ASPOCHRACIN ON EGGS OF SILKWORM BY DIPPING

Concentration, γ /ml	% killing after 24 hr
800	100
400	83
200	49
100	15
Control	0

fragmentations were observed in the compounds having the common peptide unit composed of N-methylamino acids.*

Insecticidal activities of aspochracin were examined on larvae of silkworm and fall webworm. Toxicity of aspochracin was found to be rather inferior to those of destruxins, and the minimum amount to cause immediate paralysis followed by death by injection into the final instar larvae of

silkworm was 17 γ /g and that for the final instar larvae of fall webworm was 170 γ /g as shown in Table IV. Contrary to destruxins, aspochracin coated on mulberry leaves at doses of 50~200 γ was easily taken by final instar silkworm larvae resulting in immediate knockdown followed occasionally by death. Besides, as shown in Tables V and VI aspochracin showed contact toxicity to some extent to the first instar larvae as well as eggs of silkworm by the dipping method. Hydrogenation of aspochracin to give hexahydroaspochracin completely diminished the insecticidal effect, suggesting the participation of the conjugated triene in the side chain of the former in the biological action. Extremely low mammalian toxicity is a characteristic of aspochracin, since mice injected intravenously with this compound were never killed even at the dosage of 165 mg/kg. Similar to destruxins, aspochracin did not show any appreciable antimicrobial activity even at the concentration of 100 γ /ml.

EXPERIMENTAL

Infrared spectra were measured on a JASCO IR-S KCl spectrometer and ultraviolet spectra were measured on a Cary 14-PM spectrophotometer. NMR spectra were recorded with a JEOL NMH-100 spectrometer at 100 Mc in around 10% CDCl_3 solutions with tetramethylsilane as an internal standard. Mass spectra were measured with a JMS-OISG double focusing mass spectrometer and a Hitachi RMU-7 mass spectrometer. The energy of the bombarding electrons was 75 eV. Paper chromatography was carried out with Toyo Roshi No. 51 filter papers, which were developed with the solvent systems of (1) *n*-butanol-acetic acid-water (4:1:1, v/v), (2) phenol-water (3:1, w/w) and (3) isopropanol-28% ammonium hydroxide-water (10:1:1, v/v) by the ascending method. For thin layer chromatography, Kieselgel G (Merck) plates of 0.25 mm thickness were used unless otherwise stated.

Isolation of aspochracin

(A) *Assay method.* Culture filtrate was diluted with water in serial concentrations and injected into

* The mass spectra of destruxins have been reported by A. Suzuki *et al.* of this laboratory at the IVth Symposium of Mass Spectrometry on Organic Compounds held at Meijo University, November, 1968. We are much obliged to Dr. A. Suzuki for his useful advice and discussion on the examination of the spectrum of aspochracin.

4~5th instar silkworm larvae at a dose of 0.05 ml/larva. During the isolation process, the activity of each fraction was also detected by injecting aqueous solutions containing 5% methanol into larvae.

(B) *Culture of Aspergillus ochraceus*. *Asp. ochraceus* on an agar plate was inoculated into two 5 liter-Fernbach flasks containing 1 liter each of the medium composed of sucrose 25, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.5, $NaNO_3$ 2, KCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.02 and yeast extract powder 5 g/liter and incubated for three days at 27°C with shaking. The seed culture broth thus obtained was transferred into a 100 liter-fermentation tank containing 50 liters of the same medium. Incubation was continued for 64~88 hr at 27°C with aeration and agitation. The main activity was observed in the culture filtrate.

(C) *Isolation of aspochracin*. The procedure of isolation is illustrated in Fig. 1. Thirty four liters of culture filtrate (pH 6.5) was adjusted to pH 7.0 and extracted with two 17 liter-portions of ethyl acetate with agitation. The combined extracts were concentrated to 750 ml under reduced pressure, washed with three 500 ml-portions of 10% aqueous sodium bicarbonate and dried over anhyd. sodium sulfate. Evaporation of the solvent under reduced pressure yielded 34 g of a brown powder.

Fourteen grams of the brown powder thus obtained was dissolved in 40 ml of benzene-ethyl acetate-acetone (60:30:10, v/v) and applied to a column (4×30 cm) prepared with 200 g of silicic acid (Mallinckrodt). The column was eluted with 2.6 liters of the same solvent and subsequently with 1.6 liters of a mixture of benzene-ethyl acetate-acetone (45:40:15, v/v). The eluate with the latter solvent showed toxicity to silkworms by injection and was evaporated to dryness under reduced pressure to give 2.1 g of yellow powder. The powder was dissolved in 10 ml of chloroform and applied to a column (2×40 cm) packed with silicic acid, which was eluted with 450 ml of chloroform. The eluant was then changed to chloroform containing 2% acetone. Approximately 300 ml of eluate was collected and evaporated under reduced pressure to give 0.9 g of aspochracin as a pale yellow powder. The yield was 64 mg per liter of the culture filtrate. All the above-mentioned chromatographic procedures were carried out at 5°C.

I thus obtained gave a single spot on silicic acid t.l.c. with various solvent systems illustrated in Table VII.

TABLE VII. R_f VALUES OF ASPOCHRACIN IN SILICIC ACID THIN LAYER CHROMATOGRAPHY

Solvent system	R_f
Benzene-ethyl acetate-methanol-acetone (20:50:15:15, v/v)	0.6
Ethyl acetate	0.3
Chloroform-acetone (3:2, v/v)	0.5
Chloroform-methanol (10:1, v/v)	0.8
Benzene-methanol (10:1, v/v)	0.6

I showed blue-black coloration with the chlorine-*o*-tolidine-potassium iodide reagent and reduced potassium permanganate, but it was negative to ninhydrin. UV absorption maximum at 297 m μ (ϵ 29,500) did not change in either acidic or alkaline methanol. Elemental analyses gave variable values of C 57.9~65.5, H 7.5~9.2 and N 10.7~12.1%. On storage at room temperature under nitrogen atmosphere for 10 days, I caused around 35% decomposition, which was shown by UV absorption measurement.

Amino acid composition of aspochracin

(A) *Acid hydrolysis*. I (100 mg) was treated with 5 ml of 6 N hydrochloric acid at 105°C for 16 hr in a sealed tube. The dark hydrolysate thus obtained was evaporated to dryness, and the residue was subjected to p.p.c. using the solvent systems (1) and (2). The dried chromatograms were treated with ninhydrin (1% in acetone containing 5% pyridine) to reveal a violet spot at room temperature together with two weakly colored spots. The latter two gave transient orange-red coloration, characteristic of N-methylamino acids, with the *p*-nitrobenzoyl chloride-pyridine reagent.⁵⁾ As shown in Table VIII, the R_f values were identical with authentic samples of

TABLE VIII. R_f VALUES OF AMINO ACIDS CONSTITUTING ASPOCHRACIN IN PAPER CHROMATOGRAPHY

Amino acid	R_f	
	Solvent system (1)	Solvent system (2)
Ornithine	0.06	0.18
N-Methylalanine	0.36	0.75
N-Methylvaline	0.54	0.85

5) P. A. Plattner and U. Nager, *Helv. Chim. Acta*, **31**, 2203 (1948).

ornithine, N-methylalanine and N-methylvaline, respectively. Moreover, high-voltage paper electrophoresis was applied to the hydrolysate at 3500 V (130 V/cm) and 2.4 mA/cm for 30 min at pH 1.9. Two weakly ninhydrin-positive spots corresponding to the N-methylamino acids were observed at 7 and 9 cm toward the cathode, and a distinctly colored spot corresponding to ornithine at 17 cm. However, no amino acids other than these were detected.

(B) *Isolation and identification of amino acids.* I (300 mg) was hydrolyzed with 15 ml of 6 N hydrochloric acid at 105°C for 16 hr. After evaporation of hydrochloric acid, the hydrolysate was dissolved in 1 ml of water and applied to four sheets of filter paper (40×40 cm) which were developed with the solvent system (1). The zones at *R_f* values 0.03~0.10, 0.30~0.45 and 0.50~0.60 were cut into strips which were respectively extracted with 50% aqueous methanol. Each extract was concentrated to dryness under reduced pressure, and the residue was recrystallized twice from aqueous ethanol. From the first zone 31.6 mg of ornithine was obtained as monohydrochloride, from the second 22.8 mg of N-methylalanine hydrochloride and from the third 27.3 mg of N-methylvaline hydrochloride. Their infrared spectra were identical in every respects with those of corresponding authentic samples. Their optical rotations were measured from their ORD curves in aqueous solution: ornithine, $[\alpha]_D + 10.6^\circ$ ($c=0.8$); N-methylalanine, $[\alpha]_D + 4.1^\circ$ ($c=0.7$); N-methylvaline, $[\alpha]_D + 12.7^\circ$ ($c=0.8$).

Hydrogenation of aspochracin to hexahydroaspochracin

I (1.5 g) in 20 ml of ethanol was hydrogenated under ordinary pressure and at room temperature over 250 mg of Adams' catalyst. When approximately three moles were consumed uptake of hydrogen ceased. After removal of the catalyst, the solvent was evaporated under reduced pressure. The residue was dissolved in 10 ml of ethyl acetate which was washed with 5% aqueous sodium bicarbonate. After evaporation of the solvent under reduced pressure, the residual syrup was dissolved in 3 ml of benzene-ethyl acetate-acetone (60:30:10, v/v) and applied to a silicic acid column (1×10 cm), which was eluted with the same solvent. Orange-yellow bands of impurities were removed at first, and the succeeding colorless fraction was collected. The solvent was evaporated from the fraction under reduced pressure, and the residue was recrystallized twice from ethyl acetate-

n-hexane to give 130 mg of hexahydroaspochracin (II), mp 137°C. *Anal.* Found: C, 62.61; H, 9.72; N, 12.67. Calcd. for $C_{23}H_{42}O_4N_4$: C, 62.98; H, 9.65; N, 12.78%. The molecular weight measured by the vapor pressure osmometer method (solvent; $CHCl_3$) was 434.8. The high resolution mass spectrum of II showed a molecular ion peak at 438.315 (Calcd. for $C_{23}H_{42}O_4N_4$; 438.321). $[\alpha]_D^{25} - 101^\circ$ ($c=2.5$, MeOH).

Constitution of hexahydroaspochracin

Hexahydroaspochracin (100 mg) was treated with 5 ml of 6 N hydrochloric acid at 105°C for 16 hr in a sealed tube. The hydrolysate was diluted with 70 ml of water and extracted with ether. The aqueous phase was concentrated to dryness under reduced pressure, and the residue was subjected to p.p.c. with the solvent systems (1) and (2). The dried chromatograms were treated with ninhydrin and the *p*-nitrobenzoyl chloride-pyridine reagent to reveal the presence of ornithine, N-methylalanine and N-methylvaline.

On the other hand, the ether phase was washed with a small amount of water and dried over anhyd. sodium sulfate. After evaporation of the solvent, the residue was applied to a sheet of filter paper (40×40 cm) which was developed with the solvent system (3). The zone at *R_f* 0.6~0.8 which shows the presence of an acid with bromphenol blue was extracted with 80% methanol. The extract was concentrated under reduced pressure, and the residue was dissolved in dilute aqueous sodium hydroxide and shaken with ether. The aqueous phase was acidified and reextracted with ether. The ether extract was dried over anhyd. sodium sulfate and evaporated to give 17 mg of a colorless oil. The oil was treated with ethereal diazomethane and resulting methyl ester was subjected to gas chromatography. Retention times were determined on a Shimadzu GC-1B equipped with a hydrogen flame detector in the following conditions: column packing, diethylene glycol succinate in a stainless steel column (225 cm×4 mm i.d.); column temperature, 125°C; carrier gas, nitrogen; flow rate, 20 ml/min. The sample as well as the mixture with authentic methyl caprylate gave a single peak at a retention time of 8 min, whereas methyl caproate and methyl caprate as reference compounds showed peaks at retention times of 3.7 and 19.0 min, respectively.

Amino acid sequence in hexahydroaspochracin

(A) *Alkaline hydrolysis of hexahydroaspochracin.* II

(200 mg) was treated with 8 ml of 4 N barium hydroxide at 105°C for 16 hr in a sealed tube. The hydrolysate was diluted with 50 ml of water, neutralized with dilute sulfuric acid and filtered. The filtrate was concentrated under reduced pressure, and the residue was subjected to p.p.c. with the solvent system (1). Two peptide spots (peptides A and B) at R_f 0.86 and 0.42 were detected with both ninhydrin and chlorine-*o*-tolidine-potassium iodide. Then, the peptides were isolated from the residue in the following manner by using four sheets of filter paper (40×40 cm) and the solvent system (1).

The zone at R_f 0.7~0.9 was extracted with 50% aqueous methanol. The extract was concentrated under reduced pressure, and the residue was further applied to preparative p.p.c. with the solvent system (3). The zone at R_f 0.55~0.83 was reextracted with 50% methanol and the extract was concentrated under reduced pressure to yield 45.0 mg of pure peptide A.

On the other hand, the zone at R_f 0.4~0.6 was extracted with 50% methanol. The extract contaminated with N-methylalanine was further purified by preparative t.l.c. on a silicic acid plate (20×20 cm; 2.5 mm in thickness) with a solvent system of isopropanol-water (3:2, v/v). The fraction at R_f 0.2~0.4 was again extracted with 50% methanol and, after evaporation of the solvent, the residue was applied again to a sheet of filter paper (40×40 cm) with the solvent system (3). The zone at R_f 0.2~0.5 which was positive to ninhydrin was extracted with 50% methanol, and the extract was concentrated to yield 9.2 mg of pure peptide B.

(B) *Acid hydrolysis of peptides A and B.* In a typical experiment, 3 mg of the peptide was dissolved in 0.3 ml of water and treated with 0.6 ml of conc. hydrochloric acid at 105°C for 16 hr in a sealed tube. The hydrolysate was diluted with 5 ml of water and extracted with ether. The ether phase was washed with a small amount of water and dried over anhyd. sodium sulfate. After evaporation of the solvent, the residue was dissolved in 0.1 ml of methanol and applied to p.p.c. with the solvent system (3) to detect caprylic acid by bromphenol blue. On the other hand, the aqueous phase was evaporated to dryness under reduced pressure and the residue was subjected to p.p.c. in the solvent system (1) to determine the amino acid compositions. As the results, the constitutions of peptides A and B were determined as follows: Peptide A; caprylic acid, ornithine, N-methylalanine and N-methylvaline. Peptide B;

ornithine, N-methylalanine and N-methylvaline.

(C) *N-Terminal amino acids of peptides A and B.* The peptide (1 mg) was dissolved in 1 ml of 1% aqueous triethylamine and added to 2 ml of ethanol containing 0.1 ml of 2,4-dinitrofluorobenzene (DNFB). After standing for 2 hr at room temperature, an additional 2 ml of aqueous triethylamine was added. Excess DNFB was removed by extraction with ether. The ether extract was washed with a small amount of water, and the washing was combined with the aqueous phase. After evaporation of the water under reduced pressure, the residue was again dissolved in a mixture of 0.2 ml of water and 0.3 ml of conc. hydrochloric acid and heated at 110°C for 16 hr in a sealed tube. The hydrolysate was diluted to 5 ml with water and extracted with three 5 ml-portions of ethyl acetate. Both ethyl acetate extract and aqueous phase were respectively concentrated, and subjected to t.l.c. in the following way. Silicic acid plates were saturated overnight in the vapor of the lower phase of toluene-pyridine-ethylene chlorohydrin-0.8 N ammonium hydroxide (10:3:6:6, v/v).⁶⁾ Each sample was applied to the plate and developed with the upper phase. The spot on the chromatogram was identified with corresponding synthetic DNP-amino acids. Peptide A showed the presence of δ -DNP-ornithine at R_f 0.20 in the aqueous phase of the hydrolysate, whereas peptide B revealed the presence of di-DNP-ornithine at R_f 0.73 in the ethyl acetate extract.

(D) *C-Terminal amino acid of peptides A and B.* The peptide (5 mg) was added to 0.3 ml of anhyd. hydrazine and heated at 110°C for 11 hr in a sealed tube. Excess hydrazine was removed in a vacuum desiccator over conc. sulfuric acid, and the residue was dissolved in 1 ml of water. The aqueous solution thus obtained was shaken for 3 hr with 0.2 ml of benzaldehyde at room temperature and subsequently shaken with ether. The aqueous layer was separated and evaporated to dryness under reduced pressure. The residue was dissolved in 0.05 ml of water and subjected to p.p.c. with the solvent system (1) to detect free amino acid liberated. Only a spot corresponding to N-methylalanine appeared in the hydrolysate from the both peptides.

6) G. Biserte and R. Osteux, *Bull. soc. chim. biol.*, **33**, 50 (1951).

Biological activities of aspochracin

(A) *Insecticidal activity.* For silkworm by injection: I was dissolved in a small amount of methanol and then diluted in series with water, the methanol content being restricted under 5%. Each solution was injected into the final instar larvae of silkworm (*Bombyx mori* L.) at a dose of 0.05 ml/larva.

For fall webworm by injection: 0.6 μ l of acetone solution containing a definite amount of I was injected into the final instar larvae of fall webworm (*Hyphantria cunea* Drury).

For silkworm by oral application: 50~200 γ of I dissolved in methanol was applied to mulberry leaves cut off in 4 $\times$ 4 cm size. After evaporation of the solvent, the leaves were fed to the final instar larvae of silkworm.

For silkworm by dipping: I was dissolved in a small amount of acetone and then diluted in series with water (maximum acetone content, 12.5%). The first instar larvae and eggs were dipped for 15 sec in test solutions. As for eggs, they were tested one day before hatching.

Mortality counts were made in every experiments 24 hr after the treatment.

(B) *Antimicrobial activity.* Antimicrobial activity of I was examined at the concentration of 100 γ /ml against the following microorganisms by the agar dilution method, the paper disc method and the dilution method in liquid medium: *Escherichia coli*

NIHJ, *Staphylococcus aureus* 209P, *Staph. aureus* (Resistant), *Shigella flexneri* 2a, *Sh. flexneri* 2a (resistant), *Pseudomonas aeruginosa*, *Candida albicans*, *Trichophyton asteroides*, *Tri. interdigitale*, *Tri. rubrum*, *Penicillium chrysogenum*, *Aspergillus niger*, *Xanthomonas oryzae*, *X. citri*, *X. pruni*, *Erwinia aroideae*, *Piricularia oryzae*, *Alternaria kikuchiana*, *Glomerella cingulata*, *Fusarium moniliforme* and *F. lycopersici*.

(C) *Mammalian toxicity.* I dissolved in aqueous ethanol (maximum ethanol content, 15%) in series was injected intravenously into mice.

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