STUDIES ON ACULEACIN. I

ISOLATION AND CHARACTERIZATION OF ACULEACIN A

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Aculeacin A, a new antifungal antibiotic was isolated from the mycelial cake of *Aspergillus aculeatus* M-4214. The antibiotic is a white amorphous powder soluble in lower alcohols and hardly soluble in other organic solvents or water. Aculeacin A gave palmitic acid and five ninhydrin-positive products including threonine, hydroxyproline upon acid hydrolysis. The antibiotic showed a potent activity against molds and yeasts, but exhibited no antibacterial activity. Aculeacin A has relatively low toxicity in mice.

In the continuing search for new antifungal antibiotics, a fungal strain originally designated M-4214 was found to produce antifungal principles. The strain was isolated from a soil sample collected at Ehime prefecture and classified as *Aspergillus aculeatus*.

The antibiotics were extracted from the mycelial cake and separated into seven components by means of silica gel chromatography as will be described in a succeeding paper. The major component was named aculeacin A, its isolation and properties are presented in this paper.

Fermentation and Isolation

The producing organism was grown in submerged culture in a 2,500-liter fermentor at 26°C, the medium contained 1.5% dextrin, 2.0% Polypeptone, 0.45% corn steep liquor, 0.2% KH_2PO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.01% $FeSO_4 \cdot 7H_2O$ (pH 6.5). The deep-aerated culture under these conditions contained 100~120 mcg of aculeacin per ml after 100 hours cultivation.

The activity of the antibiotic was determined by the conventional paper disk-agar diffusion assay method using *Candida albicans* or *Trichophyton asteroides* as test organisms. The antibiotics were principally obtained from the mycelial cake, because the activity in the mycelial mass was approximately 10 times higher than that in the filtrate.

The fermented broth (1,900 liters) was filtered and the antibiotics were extracted from the harvested mycelial cake (150 kg) with methanol (400 liters). The extract was concentrated to remove methanol. The antibiotic was extracted with *n*-butanol (50 liters) from the concentrate (50 liters). The butanol layer was decolorized with charcoal and concentrated *in vacuo*. Then the antibiotics were precipitated by the addition of *n*-hexane to the butanol solution, the precipitate was collected by filtration and dried to yield a crude powder (317 g). After washing with ethyl acetate, the crude powder was dissolved in a small amount of methanol and the antibiotics were re-precipitated by the addition of ethyl acetate to yield a yellow powder (112.5 g).

The material thus obtained was dissolved in a small volume of *n*-butanol, applied on a silica gel column, and developed with a mixture of ethyl acetate - *n*-butanol - water (10: 2: 1). The major

bioactive fractions that gave a single spot on thin-layer chromatogram were concentrated to dryness. The antibiotic was dissolved in *n*-butanol, the solution washed with water to remove a trace contaminant of silica gel, and the solvent evaporated to give a pure preparation of aculeacin A (66.7 g).

Physico-chemical Properties

Aculeacin A is a white amorphous powder, m.p. 162~166°C, it is soluble in lower alcohols, dimethyl formamide and dimethyl sulfoxide, but hardly soluble in other organic solvents or water. It is optically active; $[\alpha]_D^{24}$ −54° (*c* 1.0, methanol). Elemental analysis gave the following composition, C, 56.38%; H, 8.01%; N, 9.29%. The ultraviolet absorption spectrum in methanol has a maximum at 278 nm ($E_{1cm}^{1\%}$ 15.8) with shoulders at 226 nm ($E_{1cm}^{1\%}$ 140) and 283 nm ($E_{1cm}^{1\%}$ 12.5). Other maxima are at 247 nm ($E_{1cm}^{1\%}$ 148) and 295.5 nm ($E_{1cm}^{1\%}$ 22.0) in 0.01 N KOH - 90% methanol, as shown in Fig. 1. The infrared spectrum is illustrated in Fig. 2 which shows characteristic bands at 3350, 2910, 2840, 1620, 1510 and 1435 cm^{-1}. The NMR spectrum is shown in Fig. 3. Aculeacin A gives positive reactions to PAULY, FOLIN, IO_4-benzidine and $KMnO_4$ tests, but negative reactions to ninhydrin, SAKAGUCHI, EHRLICH, $FeCl_3$, DRAGENDORFF and BENEDICT tests.

On silica gel thin-layer chromatography (Eastman Kodak No. 6060) carried out using various solvent systems, the antibiotic showed the following Rf values; 0.47 in chloroform - methanol (10: 3), 0.28 in ethyl acetate - methanol - water (20: 4: 1), 0.37 in ethyl acetate - *n*-butanol (3: 1) saturated with water.

On chromatograms, the antibiotic was visualized as a brown spot after exposure to I_2 vapour. Aculeacin A is stable in acidic and neutral solutions but labile in alkaline solution.

Acid hydrolysis of aculeacin A with 6 N HCl at 110°C for 24 hours in a sealed tube resulted in

Fig. 1. UV spectra of aculeacin A

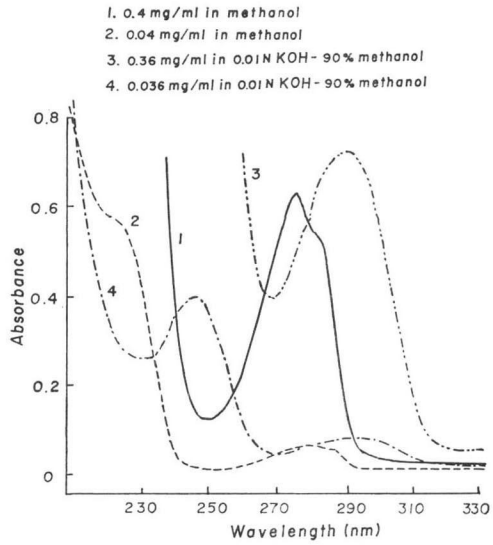


Fig. 2. IR spectrum of aculeacin A in KBr disk

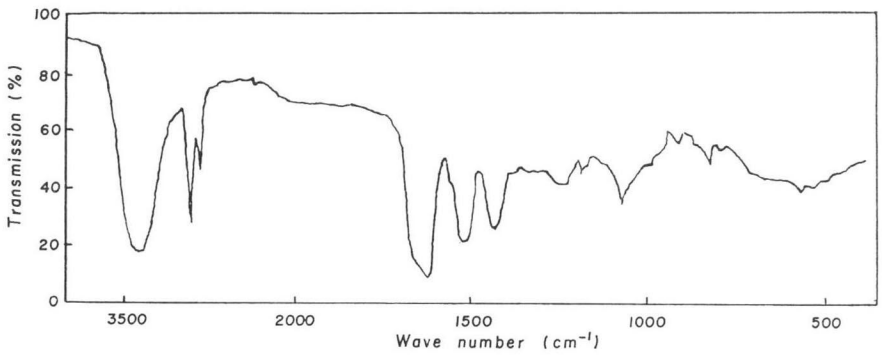


Fig. 3. NMR spectrum of aculeacin A (100 MHz, DMSO-d_6)

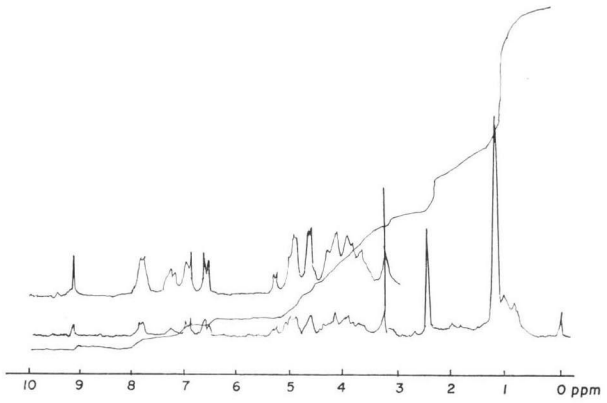
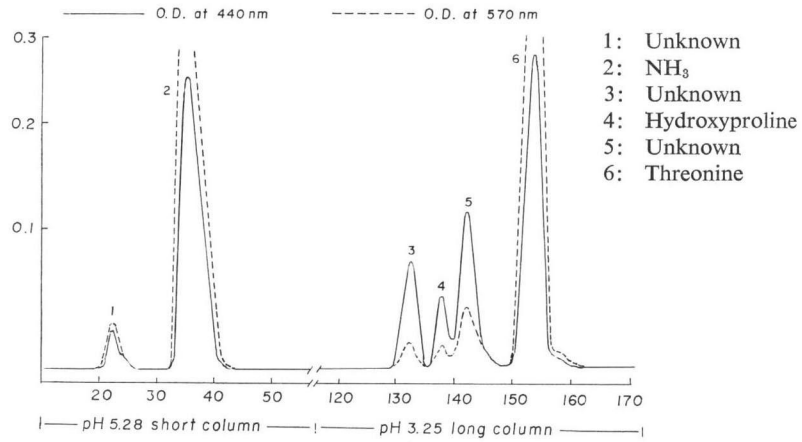


Fig. 4. Amino acid analysis of aculeacin A



liberation of five kinds of ninhydrin-positive fragments. As shown in Fig. 4, threonine, hydroxyproline and three unknown fragments were detected by amino acid autoanalyzer (JEOL, JLC-5AH). In the above conditions, 1.73 μmole of threonine was recovered from 1 mg of aculeacin A.

Alkaline hydrolysis of aculeacin A with 0.5 N ethanolic potassium hydroxide at 90°C for 30 minutes gave rise to the liberation of a fatty acid, which was identified as palmitic acid by GLC and mass spectrometry.

The molecular ion of aculeacin A or its acyl derivative could not be detected by mass spectrometry. The molecular weight was estimated to be about 1,000 by RAST's method and gel filtration using Sephadex LH-20. On amino acid analysis, 1.73 μmole of threonine was recovered from 1 mg of aculeacin A; therefore, it was assumed that aculeacin A contains two moles of threonine, suggesting that the molecular weight lies between 1,150 and 1,250. These results and repeated elemental analysis yielded a tentative formula of $C_{57\sim60}H_{94\sim102}N_8O_{20\sim21}$ (M.W. 1,211~1,271).

This formula was not inconsistent with the number of protons observed in proton NMR spectrum.

Biological Properties

The antimicrobial spectrum of aculeacin A was determined by the conventional two-fold serial agar

dilution method. The result is given in Table 1. Aculeacin A showed strong activity against yeasts and yeast-like organisms, except *C. tropicalis,* but no activity against bacteria, except *Mycobacterium* ATCC 607 which was only slightly affected.

Aculeacin A also strongly inhibited the growth of filamentous fungi, including dermatophytes, but the activity was not fungicidal; the inhibitory effect of aculeacin A on the mycelial growth of typical dermatophytes cultivated on SABOURAUD glucose agar plates is shown in Fig. 5. Aculeacin A slowed down the growth of filamentous fungi at very low concentrations but did not completely inhibit even at 25 mcg/ml.

Similar growth inhibition test was carried

Table 1. Antimicrobial spectrum of aculeacin A by agar dilution method

Test organisms	Medium and conditions[a)]	MIC[b)] mcg/ml
Staphylococcus aureus	1	>500
Bacillus subtilis	1	>500
Sarcina lutea	1	>500
Escherichia coli NIHJ	1	>500
Klebsiella pneumoniae	1	>500
Pseudomonas aeruginosa	1	>500
Mycobacterium sp 607	1	500~62.5
Candida albicans 1	2	0.2
〃 〃 2	2	0.1
〃 〃 3	2	0.1
〃 〃 4	2	0.2
〃 〃 5	2	1.6
〃 〃 6	2	0.1
〃 〃 7	2	3.2
〃 〃 8	2	0.1
Candida krusei	2	0.8
〃 〃	2	0.2
Candida parakursei	2	3.2
Candida tropicalis	2	>100
Candida pseudotropicalis	2	0.8
Candida guilliermondii	2	0.8
Candida pulcherrima	2	0.2
Saccharomyces cerevisiae	2	1.6
Saccharomyces sake	2	1.6
Mycotorula japonica 1	2	0.1
〃 〃 2	2	0.1
Torula utilis 1	2	0.1
〃 〃 2	2	0.1

a): Medium 1: nutrient agar (37°C, 18 hours)
Medium 2: SABOURAUD medium (30°C, 48 hours)

b): minimum inhibitory concentration

Fig. 5. Inhibition of mycelial growth of some dermatophytes by aculeacin A

A loopful of the test organism was inoculated in the center of agar plates containing the drug in varying concentrations and then cultured for two or three weeks until the diameter of mycelial growth of the control group without the drug had reached 70~80 mm. The growth rate was determined by measuring the diameter of the giant colony and calculating growth area.

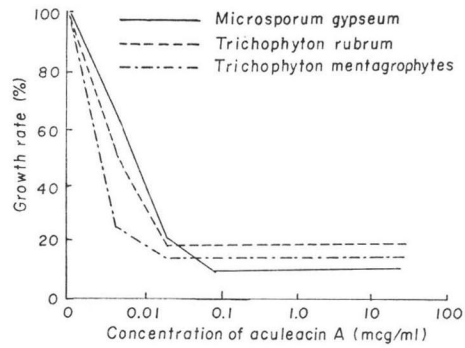


Table 2. Growth inhibition of filamentous fungi by aculeacin A[a)].

Organism	Concn. for 80% inhibition (mcg/ml)
Aspergillus fumigatus	0.5
Aspergillus niger	>100
Microsporum gypseum	0.008
Trichophyton mentagrophytes	0.007
Trichophyton rubrum	0.006
Ascochyta sojaecola	0.08
Cercospora kikuchii	4.0
Colletotrichum linicolum	0.4
Diaporthe phaseolorum	0.01
Corynespora cassiicola	2.0
Fusarium oxysporum f. *lini*	2.0
Glomerella cingulata	0.06
Helminthosporium oryzae	0.008
Sclerotium bataticola	0.08

a): Incubation at 30°C for 7 days on SABOURAUD-dextrose agar.

Table 3. Acute intravenous lethalities of amphotericin B and aculeacin A in mice

Drug	LD_0 (mg/kg)	LD_{50} (mg/kg)	LD_{100} (mg/kg)	Slope function
Amphotericin B	3.47	5.50 (4.0)*	8.64	1.2 (1.45)*
Aculeacin A	273	350	440	1.2

( )*: cited from G. R. KEIM, Jr. *et al.*[1]

Amphotericin B was supplied by Squibb Institute for Medical Research. Amphotericin B and aculeacin A were solubilized in sodium deoxycholate (amphotericin B - sodium deoxycholate=1: 1, aculeacin A - sodium deoxycholate=1: 0.25) and administered intravenously.

Table 4. Biochemical findings in rats injected intraperitoneally amphotericin B or aculeacin A

Dose mg/kg		Animal No.	Cholesterol (mg/dl)[a]	Albumin (g/dl)[b]	Na (mEq/dl)[c]	K (mEq/l)[c]	Blood urea-N (mg/dl)[d]
Control		7	43.3±4.4	4.25±0.34	148.0±3.4	5.00±0.42	15.2± 2.0
Amp-B[e]	1	7	39.7±5.6	4.01±0.32	146.4±3.0	5.26±0.63	19.8± 2.9
	5	7	51.9±6.7	3.76±0.28	144.6±1.7	4.83±0.26	24.6± 3.7
	10	6	59.4±4.1	3.45±0.28	142.5±1.9	6.55±0.87	40.7±16.5
Acu-A[f]	5	7	34.3±2.7	4.03±0.31	147.6±3.3	5.10±0.48	13.2± 1.1
	10	7	38.1±4.0	4.19±0.17	146.6±2.4	4.81±0.36	14.8± 1.0
	50	7	67.9±8.0	4.26±0.18	143.7±2.6	5.41±0.35	17.3± 1.3

a) ZURKOWSKI method, b) HABCA method, c) Flame photometric method, d) Diacetyl monoxyme method using thiosemicarbazide, e) Amphotericin B, f) Aculeacin A.

out using other filamentous fungi and the values for 80% inhibition were determined by graphic analysis; the results are shown in Table 2.

Acute and subacute toxicities of aculeacin A were determined in comparison to that of amphotericin B[1] which is a clinically useful polyene antibiotic. As shown in Table 3, acute lethality values in male mice showed that aculeacin A is less toxic than amphotericin B in intravenous injection. Some biological results of subacute toxicity test in male Wister rats are presented in Table 4, which shows that the nephrotoxicity of aculeacin A is much lower than that of amphotericin B.

Discussion

Aculeacin A is a peptide antibiotic containing palmitic acid. It shows a UV maximum at 278 nm in methanol. Among the peptide antibiotics with a weak UV absorption maximum at about 280 nm, athlestatin from *Aspergillus niger*[2] and echinocandin B from *A. nidulans*[3,4] are very similar to aculeacin A in chemical and biological properties, but they differ from it in the following points.

Athlestatin shows a distinct UV maximum at 225 nm in addition to a maximum at 278 nm; also, it can be differentiated from aculeacin A by the data in optical rotation or elemental analysis. On the other hand, echinocandin B liberates linoleic acid on hydrolysis. Therefore, aculeacin A is concluded to be a new antibiotic.

The amino acid composition of aculeacin A has not fully been elucidated; in addition to threonine and hydroxyproline, two imino acids and a basic amino acid, which was positive for the PAULY reaction and different from histidine were detected. The UV spectrum of aculeacin A in methanol showed bathochromic shift in alkaline solution. This suggested that the antibiotic molecule includes an enol or a phenol function.

Aculeacin A showed strong antibiotic activities against yeasts and yeast-like organisms, dermato-

phytes and phytopathogenic fungi. Filamentous fungi were strongly inhibited at very low concentrations, but not killed even at the concentration of 25 mcg/ml. It is not presently known if the antifungal mechanism in yeasts and filamentous fungi is the same.

Aculeacin A is much less toxic than amphotericin B; it has no influence on BUN (blood urea nitrogen) value even at a dose of 50 mg/kg.

The complete toxicity studies will be reported in detail later. *In vitro* anti-*Candida* activity was verified by the successful therapy of experimental mouse infections (TORIYA *et al.*, 197th Meeting of Japan Antibiotics Research Association).

References

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