STUDIES ON ACULEACIN. II

ISOLATION AND CHARACTERIZATION OF ACULEACINS B, C, D, E, F and G

SHUZO SATOI, AKIRA YAGI, KATSUMI ASANO, KIMIO MIZUNO and TETSUO WATANABE

Research Laboratories, Toyo Jozo Co., Ltd.,
Ohito-cho, Tagata-gun, Shizuoka-ken, Japan

(Received for publication November 8, 1976)

Six new antibiotics were isolated as the minor components related to aculeacin A from the culture broth of *Aspergillus aculeatus* M-4214 and named as aculeacins B, C, D, E, F and G. Their physico-chemical properties were analogous to those of aculeacin A and they showed significant activity against fungi. All of the minor components liberated palmitic acid on alkaline hydrolysis. Amino acid analysis showed that threonine and hydroxyproline are common constituents of aculeacins.

In the previous paper,[1] we reported a new antifungal antibiotic aculeacin A, which is a main product of *Aspergillus aculeatus* M-4214. Further investigation of the bioactive metabolites produced by this strain led to the isolation of six minor components which are named aculeacins B, C, D, E, F and G in the order of their mobility on silica gel TLC. In this paper, we describe the isolation and characterization of these minor components.

Isolation

A crude preparation of aculeacin complex was obtained from fermentation broth by the procedure employed for the isolation of aculeacin A. The mycelial cake of *Aspergillus aculeatus* (450 kg) was extracted with methanol (1,200 liters). The extract was concentrated to remove methanol. Antibiotics in the aqueous solution (150 liters) were extracted with *n*-butanol (150 liters), the butanol layer was decolorized with charcoal and concentrated. The antibiotics were precipitated by the addition of *n*-hexane to the butanol solution, and the precipitate was collected to yield a crude powder (698 g). The crude powder was washed with ethyl acetate and dissolved in methanol, then the antibiotics were re-precipitated by the addition of ethyl acetate, and a crude preparation (342 g) was obtained.

TLC of this crude preparation revealed, in addition to a main spot due to aculeacin A, six spots that were detected by bioautography using *Candida albicans* as test organism. The Rf values of six minor components are shown in Table 1 together with those of aculeacin A.

In order to remove aculeacin A, the main component of the crude mixture, the complex (340 g) was applied to a silica gel column and eluted with a solvent system consisting of *n*-butanol - ethyl acetate - water (10: 2: 1). The eluates were spotted on TLC and the spots detected by exposure to I_2 vapour or by bioautography. The antibiotic principles were separated into three fractions: Fraction II contained only aculeacin A; the other fractions (Fractions I and III) were further purified by silica gel column chromatography. All the minor components were successfully separated by silica gel chromatography, and obtained as colorless amorphous powders.

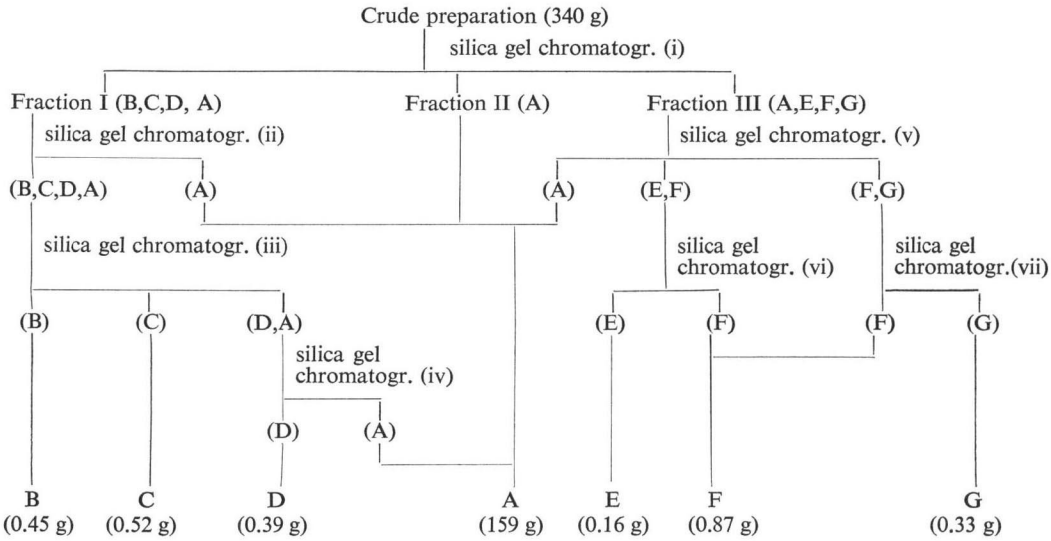
In Chart 1, the separation procedure and the solvent systems for silica gel column chromatography are illustrated.

Table 1. Rf values of aculeacins on silica gel thin-layer chromatograms

Solvent systems	Aculeacins						
	B	C	D	E	F	G	A
I	0.52	0.46	0.35	0.18	0.15	0.13	0.20
II	0.91	0.87	0.67	0.58	0.38	0.38	0.48

Silica gel plate: Eastman sheet No. 6060
Solvent I: ethyl acetate - isopropanol - water (10: 2: 1)
Solvent II: chloroform - methanol (10: 3)

Chart 1. Separation and purification of aculeacins



Solvent systems for silica gel chromatography
(i) : ethyl acetate - *n*-butanol - water (10: 2: 1)
(ii) : ethyl acetate - isopropanol - water (10: 1: 0.5)
(iii) : chloroform - methanol (10: 1)
(iv) : ethyl acetate - isopropanol - water (10: 1: 0.5)
(v) : ethyl acetate - *n*-butanol - water (10: 2: 1)
(vi) : chloroform - methanol (10: 2)
(vii) : chloroform - methanol (10: 2)

Physico-chemical Properties

The solubilities of aculeacins B, C, D, E, F and G are similar to that of aculeacin A, *i.e.*, they are soluble in lower alcohols, DMF and DMSO, but almost insoluble in other organic solvents or water. Their UV and IR spectra are similar to those of aculeacin A, as illustrated in the previous paper.[1] Their IR spectra showed characteristic bands at 3350, 2910, 2840, 1620, 1510 and 1435 cm^{-1}.

The components gave positive reactions in the PAULY, FOLIN, IO_4-benzidine and $KMnO_4$ tests, but negative reactions to ninhydrin, SAKAGUCHI, EHRLICH, $FeCl_3$, DRAGENDORFF and BENEDICT tests.

The physical and chemical properties of aculeacins B, C, D, E, F and G are summarized in Table 2.

Acid hydrolyzates of aculeacins (6 N HCl, 105°C, 20 hours) were subjected to amino acid analysis and the results are summarized in Table 3. The data show that all components liberated threonine and hydroxyproline and that aculeacin E liberated proline as the usual amino acid. The amounts of

threonine determined on the acid hydrolyzates are shown in Table 4.

On alkaline hydrolysis, all the minor components liberated palmitic acid which was identified by GLC and mass spectrometry.

Biological Properties

Minimum inhibitory concentrations (MIC) of aculeacins against yeasts and yeast-like organisms

Table 2. Physico-chemical properties of aculeacins

Aculeacins	m.p. °C	$[\alpha]_D^{24}$ (c 1.0, MeOH)	UV max in MeOH ($E_{1cm}^{1\%}$)	UV max in 0.01 N KOH 90%MeOH ($E_{1cm}^{1\%}$)	Analysis (%)
B	148~151	−45°	279 nm (18.5)	247 nm (104) 297 nm (21.6)	C: 60.52 H: 8.53 N: 9.46
C	164~168	−47.5°	279 nm (18.3)	247 nm (100) 295.5 nm (20)	C: 59.04 H: 8.27 N: 9.66
D	159~162	−46°	278 nm (17.2)	247 nm (100) 295.5 nm (19.5)	C: 57.95 H: 8.02 N: 8.83
E	186~191	−66°	277 nm (16.0)	247 nm (120) 295.5 nm (21.2)	C: 57.46 H: 8.01 N: 9.11
F	163~167	−55°	277 nm (14.5)	247 nm (125) 295.5 nm (19.6)	C: 54.81 H: 7.59 N: 9.04
G	166~170	−52°	277 nm (17.4)	247 nm (115) 295.5 nm (23)	C: 56.08 H: 7.73 N: 8.68
A	162~166	−54°	278 nm (15.8)	247 nm (148) 295.5 nm (22)	C: 56.38 H: 8.01 N: 9.29

Table 3. Amino acid analysis of aculeacins

	pH 5.28 ⟶⟵ pH 3.25 ⟶⟵ pH 4.25 ⟶											
	short column ⟶⟵ long column ⟶											
Time (min)	16	22	37	118	128	132	138	142	146	158	182	293
Color*	V	V	V	Y	Y	Y	Y	Y	V	V	Y	V
Amino acid**	UK	UK	NH_3	UK	UK	UK	HPr	UK	UK	Thr	Pro	UK
B	+	−	+	−	−	+	+	−	+	+	−	−
C	+	−	+	−	−	+	+	−	−	+	−	+
D	−	+	+	−	−	+	+	+	−	+	−	+
E	−	+	+	−	−	−	+	+	−	+	+	−
F	−	+	+	−	+	−	+	+	−	+	−	−
G	−	+	+	+	−	−	+	+	−	+	−	−
A	−	+	+	−	−	+	+	+	−	+	−	−

* Color : Y, yellow (absorbance at 440 nm > absorbance at 570 nm)
V, violet (absorbance at 570 nm > absorbance at 440 nm)

** Amino acid: UK, unknown amino acid; HPr, hydroxyproline, Thr, threonine; Pro, proline.

+: The peak was detected

−: The peak was not detected

Lys, 17.5 min; His, 24 min; Asp, 148 min; Ileu, 288 min; Leu, 297 min.

were determined by conventional two-fold serial agar dilution assay, and compared to those of aculeacin A which is the main component of aculeacins. As shown in Table 5, their antimicrobial spectra were very similar to that of aculeacin A: among these antibiotics, aculeacin D exhibited the strongest activity. Aculeacins also inhibited the growth of filamentous fungi, but inhibition was not complete. Therefore, the growth inhibition test of aculeacins against *Aspergillus fumigatus* and *Trichophyton asteroides* was carried out according to the method described in the previous paper[1], and the values for 80% inhibition are shown in Table 6.

Table 4. Threonine content of aculeacin minor components

Aculeacin	Amount of threonine μmole/mg of aculeacin*
B	1.87
C	1.87
D	1.80
E	1.57
F	1.77
G	1.42
A	1.73

* The data were estimated from amino acid analysis of the acid hydrolyzates of aculeacins

Discussion

The aculeacin complex consists of one major component and six minor components: component A constitutes over 90% of the complex. The minor components showed very similar properties to the A component, namely, they contain threonine, hydroxyproline and palmitic acid, as well as the chromophore moiety which absorbs UV at about 278 nm. Among the peptide antibiotics, athlestatin[2] and echinocandin B[3,4], are recognized as the analogous antibiotics, but they differ from aculeacin minor components in the following points.

Athlestatin shows UV maxima at 225 and 278 nm, and the data in optical rotation, elemental analysis or melting point are not consistent with aculeacins. On the other hand, echinocandin B liberates linoleic acid on hydrolysis. Therefore, aculeacin minor components are decided to be new antibiotics.

Table 5. Minimum inhibitory concentration (mcg/ml) of aculeacin minor component by the agar dilution method against yeasts and yeast like fungi

Test organism	MIC (mcg/ml) of aculeacins						
	B	C	D	E	F	G	A
Candida albicans 1	0.8	0.2	0.05	6.3	0.4	0.4	0.2
〃 〃 2	0.4	0.1	0.025	3.2	0.2	0.2	0.1
〃 〃 3	0.4	0.1	0.025	3.2	0.2	0.2	0.1
〃 〃 4	0.4	0.1	0.05	3.2	0.4	0.2	0.1
〃 〃 5	0.4	0.1	0.05	3.2	0.2	0.1	0.1
〃 〃 6	0.4	0.1	0.05	3.2	0.2	0.1	0.1
〃 *krusei*	1.6	0.4	0.1	3.2	0.8	0.4	0.4
〃 *parakrusei*	6.3	1.6	0.4	50	3.2	3.2	1.6
〃 *tropicalis*	>100	>100	>100	>100	>100	>100	>100
〃 *pseudotropicalis*	6.3	3.2	0.4	50	6.3	6.3	3.2
Saccharomyces cerevisiae	3.2	1.6	0.2	25	3.2	3.2	0.8
Saccharomyces sake	6.3	3.2	0.4	100	6.3	6.3	1.6
Mycotorula japonica	0.2	0.05	0.025	0.8	0.1	0.1	0.05
Torula utilis	0.05	0.0125	0.0063	0.2	0.05	0.05	0.025

Medium: SABOURAUD medium (30°C for 48 hours)

All aculeacins including the A component were active against yeasts, except *C. tropicalis* and filamentous fungi and they did not affect the growth of bacteria.

Aculeacin D was 2~4 times more active than component A against yeasts and yeast-like organisms, while the MIC values of aculeacin E were the highest. It is presently not known if the difference between aculeacin D and E activities correlates with their amino acid composition.

Table 6. Inhibition of growth of filamentous fungi by aculeacins

Aculeacins	Concentration for 80% inhibition (mcg/ml)	
	Asp. fumigatus	*Trichophyton asteroides*
B	7.0	0.04
C	0.7	0.01
D	0.4	0.005
E	2.0	0.003
F	0.6	0.003
G	0.7	0.005
A	0.5	0.004

The fungi were cultured in SABOURAUD medium at 26°C for 12 days.

References

1) MIZUNO, K.; A. YAGI, S. SATOI, M. TAKADA, M. HAYASHI, K. ASANO & T. MATSUDA: Studies on aculeacin. I. Isolation and characterization of aculeacin A. J. Antibiotics 30: 297~302, 1977
2) FUJISAWA, T.; H. SAKAI, H. MIYAIRI, M. TAKASHIMA & K. SHIMIZU: Process for producing athlestatin. (in Japanese) Japanese Patent 41-12668, 1966
3) BENZ, F.; F. KNÜSEL, J. NÜESCH, H. TREICHLER & W. VOSER: Echinocandin B, a new polypeptide antibiotic from *Aspergillus nidulans* var. *echinulatus*. Helv. Chim. Acta 57: 2459~2477, 1974
4) NUESCH J.; H. TREICHER, F. BENZ, H. BICKEL, W. VOSER & W. KELLER: Process for producing new antibiotic A 32204. Switzerland Patent 568386, 1975