ALADAPCIN, A NEW MICROBIAL METABOLITE THAT ENHANCES HOST RESISTANCE AGAINST BACTERIAL INFECTION

PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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We have constructed a new screening system for detecting microbial products that enhance host resistance against bacterial infection. It was found that a new compound with such activity is produced by a soil isolate classified as *Nocardia* sp. SANK 60484. The compound was isolated from the culture filtrate of the organism and named aladapcin after its amino acid composition. Aladapcin was obtained as an amphoteric white amorphous powder with the molecular formula, $C_{13}H_{25}N_5O_5$. It consists of 2 mol of D-alanine and 1 mol of *meso*-diaminopimelic acid. From the analysis of IR, ^{1}H NMR and FAB-MS spectra, the structure was assigned to be a tripeptide. Aladapcin enhanced host resistance against an experimental *Escherichia coli* infection in mice at doses ranging between 1 and 100 μg/kg.

Many antibiotics have been used to protect hosts against invasion by infectious agents. However, if the resistance of the host has deteriorated for some reason such as age or treatment with cytotoxic antitumor agents, antimicrobial agents are not always effective. Therefore, we expect that a compound that is able to stimulate host resistance could be useful as another type of chemotherapeutic agent against infectious diseases. This paper deals with the fermentation, isolation and physico-chemical properties of aladapcin. The characteristics and identification of the producing organism will be published elsewhere.

Screening System

Escherichia coli SANK 73175 was selected as an infectious pathogen to establish a good sensitive assay system in male ICR mice (5-week old, Japan CLEA, Tokyo), a system which positively demonstrated the enhancing activity of isohematinic acid and BM 12,531[1]. *E. coli* SANK 73175 was grown in Trypticase soy broth for 17 hours at 37°C and the cells were harvested by centrifugation. After washing the cells two times with strerilized physiological saline, the cells were resuspended in physiological saline and served for the challenge. Usually when mice were infected with 2×10^8 cells of *E. coli* SANK 73175 intravenously, all of the mice died within 3 days. Therefore, we observed the number of dead and surviving mice for a period of 7 days after the infection. Groups in which the mean survival time was more than twice of that of the control mice were evaluated as positive.

Then we examined the effect of microbial culture media on the assay system. When 0.2-ml portions

of culture media were injected subcutaneously into mice at 24 hours before the infection, the mean survival times of the infected mice were prolonged, and it was difficult to discriminate the effect of an active substance from the effects of freshly prepared media if the activity of the substance was not strong. When the media were passed through Amicon Centriflo CF25 and the injected into mice, the survival times of mice receiving the samples were almost the same as that of the control mice. Therefore, all of the culture filtrates from microorganisms were passed through Amicon Centriflo CF25 and effects of 0.2 ml of the treated filtrate on the protection of mice against infection with *E. coli* SANK 73175 were examined. A soil isolate classified as a *Nocardia* sp. was found to produce an active substance that enhances host resistance.

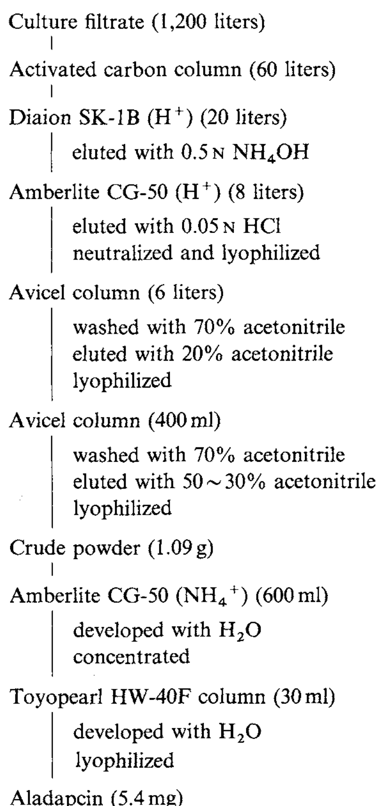
Fermentation

Stock cultures of *Nocarida* sp. SANK 60484 were inculated into 2-liter Erlenmeyer flasks containing 500 ml of seed medium consisting of glucose 1.0% glycerol 1.0%, sucrose 1.0%, pressed yeast 1.0% soybean meal 2.0%, oatmeal 0.5%, Casamino acid 0.5%, $CaCO_3$ 0.1% and CB-442 0.01% (pH 7.0 before sterilization) and incubated at 28°C for 4 days. The second seed culture was carried out at 28°C for 2 days in a 100-liter fermenter containing 50 liters of the medium described above inoculated with 2.5 liters of seed culture. A 15-liter portion of the second culture was transferred to a 600-liter fermenter containing 300 liters of fermentation medium consisting of glucose 2.0%, dextrin 1.1%, pressed yeast 0.9%, meat extract 0.5%, Polypepton 0.5%, $CaCO_3$ 0.3%, NaCl 0.5% and CB-442 0.01% (pH 7.0) and incubated for 4 days at 28°C with an air-flow of 150 liters/minute and an agitation rate of 100 rpm.

Isolation

The isolation method for aladapcin is shown in Fig. 1. The culture filtrate (1,200 liters) was passed through an activated carbon column (60 liters), and then the effluent was applied to a Diaion SK-1B (H^+) column (20 liters). After washing the column with distilled water, the fraction having the enhancing activity for host resistance was eluted with 0.5 N NH_4OH and concentrated *in vacuo*. The concentrate was applied to an Amberlite CG-50 (H^+) column (8 liters) and the column was washed with distilled water and then developed with 0.05 N HCl. The active eluate was concentrated after neutralization and lyophilized. The powder thus obtained was applied to an Avicel column (6 liters) and the column was washed with acetonitrile - water (70 : 30). The active fraction was then obtained by elution with a mixture of acetonitrile - water (20 : 80) and concentrated. Following lyophilization, the resulting powder was applied to an Avicel column (400 ml). After washing with acetonitrile - water (70 : 30), the active substance was eluted with acetonitrile - water (50 : 50

Fig. 1. Purification procedure for aladapcin.



and 30 : 70) and lyophilized. The crude powder thus obtained was dissolved in a small amount of distilled water, applied to an Amberlite CG-50 (NH_4^+) column (600 ml), and the column was developed with distilled water. The concentrated eluate was applied to a column of Toyopearl HW-40F (30 ml) and the active fraction was eluted with distilled water. After concentration and lyophilization, 5.4 mg of purified aladapcin was obtained as a white amorphous powder.

Physico-chemical Properties and Total Synthesis of Aladapcin

Physico-chemical properties of aladapcin are listed in Table 1. Aladapcin was obtained as an amphoteric white powder. It is soluble in water and methanol and insoluble in acetone and chloroform. The FAB-MS showed a protenated molecular ion at *m/z* 332 and the molecular formula was deduced to be $C_{13}H_{25}N_5O_5$ from the HR mass spectrum. After hydrolysis by 6 N HCl at 105°C for 20 hours, 2 mol of alanine and 1 mol of 2,2′-diaminopimelic acid were detected. Alanine was assigned the D-configuration using D-amino acid oxidase. Diaminopimelic acid was identified as the *meso* form by the method of RHULAND *et al.*[2]. The UV spectrum showed only end absorption. Aladapcin gave positive ninhydrin and Rydon-Smith color reactions. The IR and ^1H NMR spectra are shown in Figs. 2 and 3, respectively. The IR spectrum showed a strong absorption band at around 1580 cm^{-1} indicating the presence of amide bond. The ^1H NMR spectrum showed signals for the methyl protons of alanine (1.15 and 1.23 ppm), the methylene protons of diaminopimelic acid (1.3 and 1.7 ppm) and methine protons of alanine (3.95 and 4.18 ppm) and also

Table 1. Physical and chemical properties of aladapcin.

Nature	Amphoteric, white powder
Molecular formula	$C_{13}H_{25}N_5O_5$
MW (FAB-MS)($(M+H)^+$)	331 (332)
Amino acid analysis	*meso*-Diaminopimelic acid (1 mol), D-alanine (2 mol)
Solubility:	
Soluble	H_2O, MeOH
Insoluble	Acetone, $CHCl_3$
Rf (Eastman cellulose sheet)	0.42 (BuOH - AcOH - H_2O, 4 : 1 : 2)
Color reaction	Ninhydrin, Rydon-Smith (positive)
UV (H_2O)	End absorption

Fig. 2. IR spectrum of aladapcin in KBr.

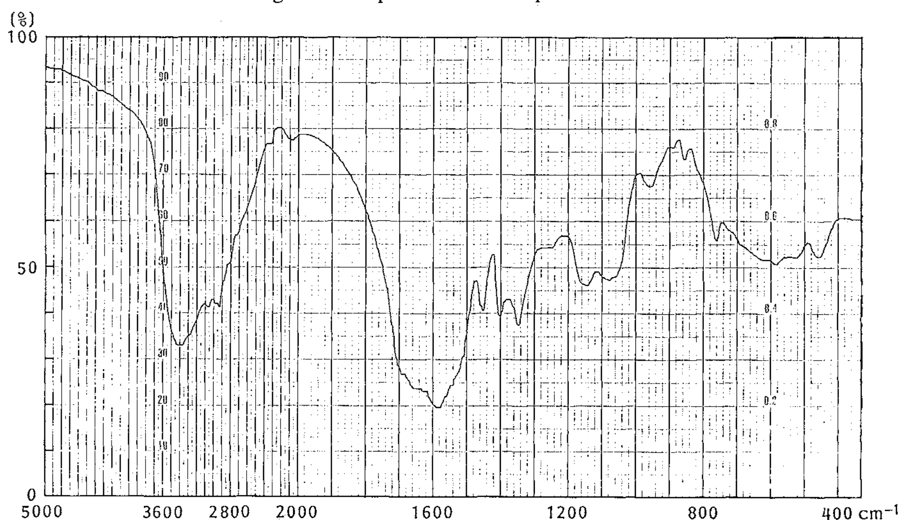


Fig. 3. ^1H NMR spectrum of aladapcin in D_2O (400 MHz).

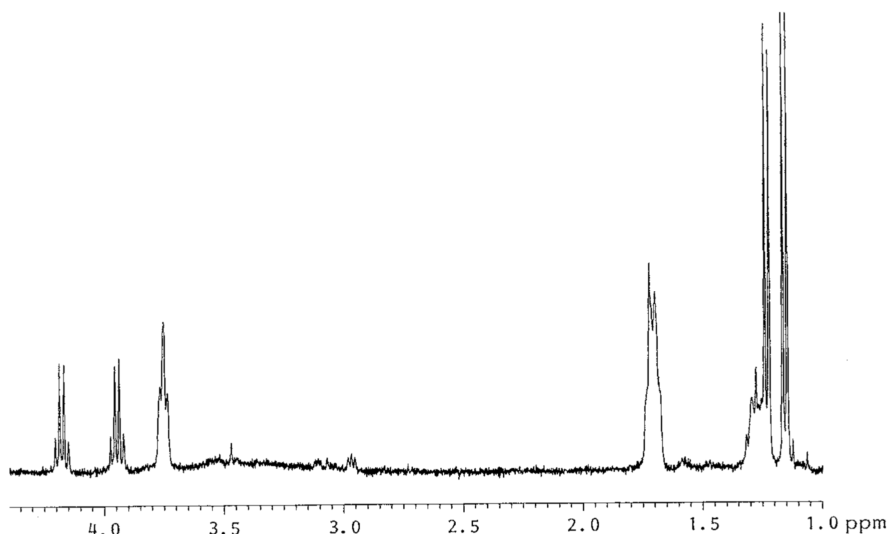


Table 2. Dose dependence of the effect of aladapcin on the resistance to bacterial infection.

Expt	Group of mice (μg/kg)	Mean survival (days)	Number of dead mice	Expt	Group of mice (μg/kg)	Mean survival (days)	Number of dead mice
I	Control	1.4	0/10	II	Control	1.7	0/10
	1	3.1	3/10		0.3	2.2	1/10
	10	4.7	6/10[a]		3	3.6	3/10
	30	3.6	4/10[a]		10	3.7	4/10[a]
	100	4.3	5/10[a]		30	3.7	4/10[a]
					100	2.5	2/10

Mice were infected with *Escherichia coli* SANK 73175 intravenously.
[a] Significant by the FISHER's test.

diaminopimelic acid (3.75 ppm). The FAB-MS spectrum of aladapcin contained fragment ion peaks (at *m/z* 127, 161, 172, 215, 243, 286 and 287) in addition to the protonated molecular ion.

Table 3. Time dependence of the effect of aladapcin on the resistance to bacterial infection.

Group of mice	Mean survial (days)	Number of survivors
Control	1.2	0/10
−48, −24 hours	3.4	3/10
−24 hours	3.0	3/10

Mice were treated with 10 μg/kg of aladapcin and then infected with *Escherichia coli* SANK 73175 intravenously.

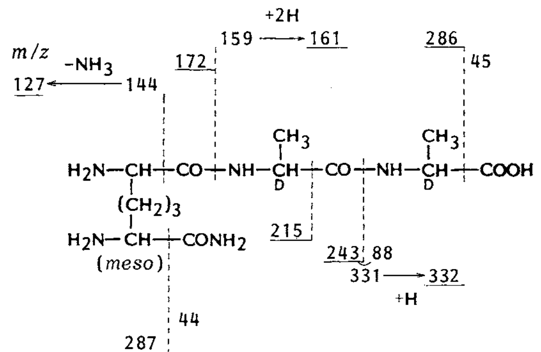
Biological Activities of Aladapcin

Protective effects of purified aladapcin against infections were examined in systems with *E. coli* SANK 73175, as discussed above. To determine the optimal dose, mice were administered 0.3 to 100 μg of aladapcin per kg subcutaneously, and 24 hours later they were challenged with *E. coli* SANK 73175 intravenously. As shown in Table 2, treatment with aladapcin prolonged the survival times of mice at doses ranging from 1 to 100 μg/kg. Therefore, a dose of 10 μg/kg was chosen to examined the effect of a two-shot treatment with aladapcin as follows. Mice received aladapcin subcutaneously at 48 and 24 hours before the infection, and the survival times were compared with those of mice only treated at 24 hours befoer the challenge. As shown in Table 3, the two-shot treatment was equally effective to the one-shot treatment.

Discussion

Aladapcin, a new microbial metabolite that enhances host resistance against bacterial infection was isolated from the culture broth of a *Nocardia* sp. SANK 60484. The physico-chemical properties suggested that this compound is a peptide, and from the fragmentation pattern in the FAB-MS, the chemical structure of aladapcin was deduced as shown in Fig. 4. GOTOH *et al.*[3] isolated FK-156 which has activity similar to that of aladapcin. The chemical structure of FK-156 also resembles that of aladapcin.

Fig. 4. Structure and mass fragmentation patterns of aladapcin.



One of the characteristic properties of aladapcin is its biological activity at extremely low doses (from 1 to 100 μg/kg). When mice were treated with aladapcin two times, we failed to detect any augumentation of the activity (Table 3). Therefore, we can speculate that this compound might act effectively in a short term. Preliminary experiments showed that aladapcin enhanced the superoxide anion releasing activity of polymorphonuclear leucocytes (PMN) after stimulation with concanavalin A and cytochalacin D, suggesting that aladapcin, like other immunomodulators of microbial origin (for example muramyl dipeptide[4,5] and isohematinic acid[1]), enhances host resistance through stimulating the activities of PMN.

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