ALTEMICIDIN, A NEW ACARICIDAL AND ANTITUMOR SUBSTANCE

I. TAXONOMY, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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Screening of new insecticidal and acaricidal antibiotics was carried out with reference to anti-brine shrimp activity from actinomycete strains isolated from marine environments. Of 200 actinomycete isolates, one isolate was found to produce a new substance, altemicidin. The strain was isolated from sea mud collected at Gamo, Miyagi Prefecture, Japan, and identified as *Streptomyces sioyaensis* SA-1758.

Altemicidin was purified by Diaion CHP-20P and Sephadex LH-20 column chromatographies. The molecular formula was determined as $C_{13}H_{20}N_4O_7S$ by elemental analysis, MS and ^{13}C NMR spectrum.

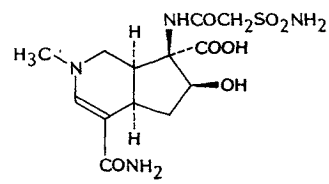
Altemicidin showed not only acaricidal activity but also antitumor activity. The compound showed no antimicrobial activity except the inhibitory activity to *Xanthomonas* strains.

In the screening or monitoring of insecticidal or acaricidal substances of microbial origin, the breeding and rearing of assay organism are problems in the laboratory. It is necessary to maintain and provide the test organism constantly at all times. Brine shrimp, a tiny crustacean, is favorable one. It has been reported that brine shrimp showed high sensitivity against many insecticides and acaricides and has been used for detection of insecticide and acaricide residues.[1,2] The eggs can remain viable for several years when they are stored in a dry condition and the hatching can be obtained in less than 24 hours. They are easily available as feed for tropical fish.

Therefore, we conducted the screening of actinomycete strains isolated from marine environments with reference to anti-shrimp activity which is potential to find insecticidal or acaricidal substances.

As the result of screening of 200 actinomycete isolates, we found that a streptomycete strain SA-1758 produced a new substance which was named altemicidin (Fig. 1). It showed not only acaricidal activity but also antitumor activity.

Fig. 1. Structure of altemicidin.



In this report, we describe the taxonomy of altemicidin-producing organism together with purification, physico-chemical and biological properties of the compound.

Materials and Methods

Isolation of Organism

Strain SA-1758 was isolated from a sea mud collected at Gamo, Miyagi Prefecture, Japan. The isolation medium consisted of ISP No. 4 (Difco) 37 g and corn-steep liquor (Nippon Shokuhin Kako) 1 g in 1 liter of a quarter strength synthetic sea water (Jamarin S, Jamarin Laboratory).

Taxonomical Examination

Morphological, cultural and physiological properties of strain SA-1758 were examined according to the methods described by SHIRLING and GOTTLIEB,[3] and WAKSMAN.[4] Detailed observation of mycelial and spore morphologies was performed with the use of a light microscope (XF-ph-21, Nikon), a transmission electron microscope (EM400, Philips) and a scanning electron microscope (S-570, Hitachi). Chemical analysis of cell wall was performed with the method of BECKER *et al.*[5]

Fermentation

A slant culture of strain SA-1758 on ISP No. 4 medium supplemented with yeast extract 0.1% was inoculated into 100 ml of the medium consisting of surcrose 20 g, corn-steep liquor 10 g and $CaCO_3$ 4 g in 1 liter of a half strength of synthetic sea water (pH 7.4 before autoclaving) and incubated at 27°C for 48 hours on a rotary shaker (180 rpm). For the production of altemicidin, 1 ml of the culture was transferred to 100 ml of the fresh medium and incubated for 96 hours in the same way as above.

Analytical Procedures

HPLC and TLC: Altemicidin contents in fermentation and purification steps were monitored with reversed phase HPLC and silica gel TLC. HPLC was performed with an ODS-PE-1 column (10 × 300 mm, Senshu Science Co., Japan; mobile phase, CH_3CN - 5 mM tetramethylammonium chloride (5 : 15); flow rate, 3 ml/minute; detection, UV 300 nm). It was eluted at 8.6 minutes TLC was performed with Kieselgel 60 F_{254} (Art. No. 5715, Merck) developed with $CHCl_3$ - MeOH - conc NH_4OH (20 : 15 : 8). Spots on TLC were detected by chlorine-tolidine reaction. Rf value of altemicidin was 0.4.

Physico-chemical Characteristics: MP was determined with a Yazawa mp apparatus and was uncorrected. Optical rotation was measured with a Perkin-Elmer model 241 polarimeter. IR and UV spectra were recorded with a Hitachi 260-10 IR spectrophotometer and a Hitachi 220S spectrophotometer, respectively. The ^1H and ^{13}C NMR spectra were measured with a Jeol JNM-GX400 spectrometer. The MS were recorded with a Hitachi M-80H mass spectrometer and a Jeol JMS-AX505 mass spectrometer.

Biological Procedures

Brine Shrimp: The 100-mg of brine shrimp eggs (Warner Lambert, U.S.A.) were hatched in 200 ml of synthetic sea water at 27°C with forced aeration. After 24 hours, the hatched nauplii suspension was stood for 1 hour without aeration. And then the nauplii were collected by pippete from middle layer of the salts solution, in which much of nauplii were swimming, for increase of the density of shrimp and exclusion of unhatched or dead eggs which were submerged in bottom or floated on the salts solution. By this procedure 40 ~ 60 larvae per ml can be obtained with this method. 0.5 ml of nauplii suspension (20 ~ 30 larvae) was pippeted into each well of 24-hole titer plate (Corning Laboratory, U.S.A.) and incubated with or without diluted altemicidin or diluted altemicidin-containing broth filtrate for 48 hours at 27°C. The activity were expressed as follows:

% Mortality = (number of dead or stopped swimming shrimps/total number of shrimps) × 100

Mite: The acaricidal effect of altemicidin against the two spotted spider mite (*Tetranychus urticae*) was examined by a pot test in green house. Twenty adult mites were inoculated on leaves of kidney bean in the first stage. One day after the inoculation, they were sufficiently sprayed with the diluted altemicidin preparation. The numbers of adult mites surviving on the leaves were determined 14 days after the treatment. The prevention value (%) was calculated from the following formula:

$100 \times (1 - (\text{number of the mites on treated leaves})/(\text{number of the mites on nontreated leaves}))$

Tumor Cells: L1210 was cultured with EAGLE's minimum essential medium supplemented with 10% fetal bovine serum (FBS). IMC carcinoma was cultured with RPMI-1640 medium supplemented with 10% FBS. Exponentially growing tumor cells (2×10^4 cells/microtiter well) were cultured for 48 hours with altemicidin. Cell growth was measured by a Coulter counter after dilution with saline.

Microorganisms: To determine the MIC of altemicidin, bacteria were incubated at 37°C for 18 hours in Mueller-Hinton agar (Difco), and yeasts and molds were incubated at 27°C for 42 hours in Nutrient Agar (Difco) supplemented with glucose 1.0%.

Results

Taxonomic Features of Strain SA-1758

Cultural and physiological features of strain SA-1758 were summarized in Table 1. Aerial mass color of the strain was white to light gray or grayish white depending on the medium. Mature spores occurred in chains of more than 20 spores forming spirals. The spore was oval to globose with smooth surfaces and $0.61 \sim 0.64 \times 0.80 \sim 0.84\,\mu m$ in size. The reverse side color of colonies was pale yellow to pale yellowish brown. Soluble pigment and melanoid pigment were not formed. Milk was peptonized. The whole cell-acid hydrolysate contained LL-diaminopimelic acid. The strain was tolerant to 3% of sodium chloride.

From these taxonomic properties, strain SA-1758 was considered to belong to the genus *Streptomyces*. Compared with the published description of *Streptomyces* species and the type cultures, *Streptomyces sioyaensis*[6] seemed to be the closest to the strain except small differences in soluble pigment formation and milk peptonization.

Therefore, strain SA-1758 was classified and designated as *S. sioyaensis* SA-1758.

Isolation

Altemicidin production began after 48 hours cultivation and reached maximum (47.6 μg/ml) after 4 day's incubation.

The culture broth (10 liters) was filtered by using Hyflo Super-Cel (John-Manville Co., U.S.A.) and then charged to a column of activated charcoal (500 ml). After the column was washed with water (2 liters), the active principle was eluted with 0.05 N HCl-50% aq acetone (16 g fractions). Active fractions (Nos. 31 ~ 80) were combined and concentrated to 20 ml.

Table 1. Taxonomic features of strain SA-1758.

Cultural characteristics[a]	
Growth	Good ~ abundant; ISP Nos. 2, 3, 4, 5, 7
	Moderate; W1, W2, ISP No. 6, NA
Reverse side color	Pale yellow; W1, W2, ISP Nos. 3, 4, 5, 6, NA
	Pale yellowish brown; ISP Nos. 2, 7
Aerial mass color	White; W2, ISP No. 7, NA
	White ~ grayish white; ISP Nos. 2, 4, 5
	Light gray; ISP No. 3
	No aerial mycelium; W1, ISP No. 6
Physiological characteristics	
Carbon utilization:	
Positive;	Glucose, sucrose, inositol, mannitol, fructose, raffinose
Negative;	Arabinose, rhamnose
Doubtful;	Xylose
Melanoid pigment	Negative
Soluble pigment	Negative
Gelatin liquefaction	Negative
Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Positive (weak)
Nitrate reduction	Positive
NaCl tolerance	1 ~ 3%

[a] W1: Sucrose-nitrate agar (Waksman No. 1 medium), W2: glucose-asparagine agar (Waksman No. 2 medium), ISP No. 2: yeast extract-malt extract agar, ISP No. 3: oatmeal agar, ISP No. 4: inorganic salts-starch agar, ISP No. 5: glycerol-asparagine agar, ISP No. 6: peptone-yeast extract-iron agar, ISP No. 7: tyrosine agar, NA: nutrient agar.

The solution was chromatographed on a column of Diaion CHP-20P (250 ml) by elution with water (5 ml fractions). Active fractions (Nos. 64~84) were collected and concentrated to give a hygroscopic solid (678 mg).

The crude solid was dissolved in 50% aq MeOH (4 ml) and applied to a column of Sephadex LH-20 (250 ml). The column was developed with 50% aq MeOH (2.5 ml fractions). Active eluate (fraction Nos. 53~60) were concentrated and lyophilized. Thus, 232 mg of white powder of altemicidin was obtained (recovery 50.7%).

Physico-chemical Properties

Altemicidin was obtained as white powder: MP 195~199°C (dec), $[\alpha]_D^{27}$ −7.6° (*c* 1.0, water). It was soluble in water, slightly soluble in lower alcohol and hardly soluble or insoluble in other organic solvents. The UV spectra of altemicidin are as follows: UV λ_{max} (1/15 M phosphate buffer, pH 6.8) nm (ε) 300 (20,300); λ_{max} (0.1 M HCl) nm (ε) 305 (11,300); λ_{max} (0.1 M NaOH) nm (ε) 300 (20,800). Altemicidin was positive to chlorine-tolidine reaction. The molecular formula of altemicidin was established as $C_{13}H_{20}N_4O_7S$ (MW 376) by elemental analysis, secondary ion mass spectrometry (SI-MS), high-resolution fast atom bombardment mass spectrometry (HRFAB-MS) and ^{13}C NMR spectrum, *Anal* calcd for $C_{13}H_{20}N_4O_7S \cdot H_2O$: C 39.20, H 5.58, N 14.07, S 8.04, found: C 39.23, H 5.29, N 12.73, S 6.51, SI-MS *m/z* 377 $(M+H)^+$; HRFAB-MS $(M-H)^-$ *m/z* 375.1009 (calcd for $C_{13}H_{19}N_4O_7S$ 375.0975). The IR spectrum is shown in Fig. 2. The ^{13}C NMR spectrum (D_2O, 100 MHz) of altemicidin showed thirteen carbon signals at δ 179.7, 174.2, 164.4, 147.3, 97.1, 76.0, 69.1, 60.3, 45.5, 43.2, 41.4, 40.8 and 31.7. The ^1H NMR spectrum is shown in Fig. 3.

These physico-chemical properties indicate that altemicidin is a new compound different from any compound ever reported. Structure determination of altemicidin will be described in the accompanying paper.[7]

Biological Properties

The 50% lethal concentration (LC_{50}) value of altemicidin against brine shrimp was 3.0 μg/ml, while

Fig. 2. IR spectrum of altemicidin (KBr).

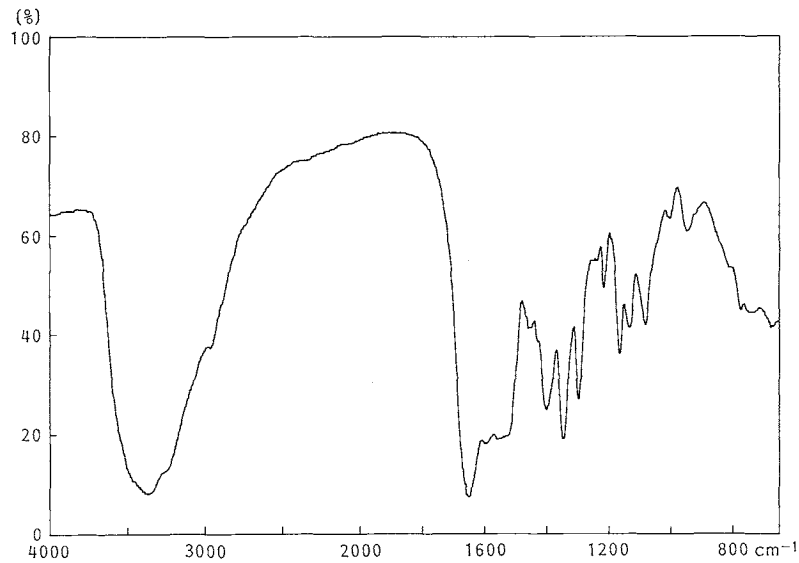
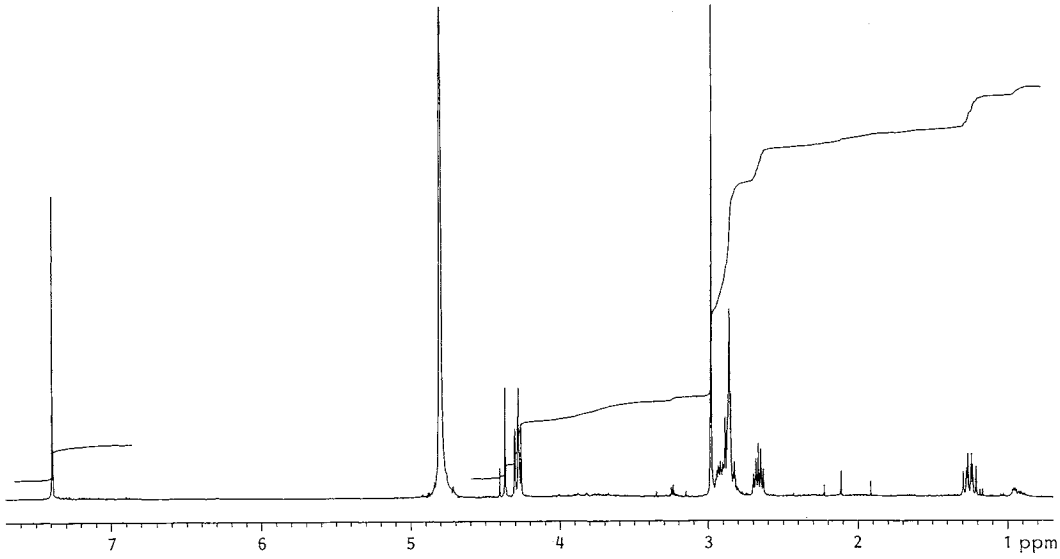


Fig. 3. ^1H NMR spectrum of altemicidin in D_2O.



that of polynactin tested as control agent was 4.0 μg/ml. The activity of altemicidin against the two spotted spider mite on kidney bean leaves is shown in Table 2. The 50% prevention value of altemicidin was about 10 ppm but the anti-mite activity was less than that of polynactin in spite of these two compounds having about the same activity against the shrimp.

Table 2. Acaricidal effect of altemicidin in pot test.

Concentration (ppm)	Preventive value (%)[a]	
	Altemicidin	Polynactin
100	100	—
10	47	99
1	12	32

[a] Average of triplicates.

Altemicidin also strongly inhibited the growth of tumor cells. Fifty % inhibitory concentration (IC_{50}) values (μg/ml) were as follows: L1210, 0.84; IMC carcinoma, 0.82.

Altemicidin showed no inhibitory activity at 100 μg/ml against Gram-positive and Gram-negative bacteria, mycobacteria, yeasts and molds except *Xanthomonas* species: (MIC) *Xanthomonas oryzae*, 6.25 μg/ml; *Xanthomonas citri*, 100 μg/ml. Acute toxicity (LD_{50}, iv) in mice was about 0.3 mg/kg.

Discussion

Screening for anti-invertebrate antibiotics utilizing antimicrobial activity as the primary detection method is often plagued by rediscovery of antibiotics such as valinomycin, antimycin, cycloheximide, gougerotin, destomycin, streptothricin *etc.* Therefore, we selected anti-shrimp substances without or with weak antimicrobial activity. As the result of selection from 200 actinomycete isolates after excluding antimicrobial antibiotic producers, one strain was found to produce a new compound. Accordingly, the combination screening of anti-shrimp assay and antimicrobial assay was considered to be a useful and rapid screening method to discover new anti-invertebrate substances.

Altemicidin also inhibited the growth of tumor cells, although it showed high acute toxicity in mice. Brine shrimp have been utilized in other various bioassay systems in addition to the detect of pesticides residues; for example, the analysis of mycotoxins[8] and active plant constituents including podophyllotoxin, strychnine, digitalin *etc.*[9] Therefore, it should be noted that anti-shrimp assay detects not only specific insecticides or acaricides but also mammalian toxicants.

Altemicidin with monoterpene-alkaloid skeleton (Fig. 1), whose structure elucidation will be detailed in the accompanying paper,[7] was produced by a streptomycete strain inhabiting marine environment. Many alkaloids, most of which show a variety of biological activities including high cytotoxicity, have been isolated from marine plants and animals.[10] But it is rare that actinomycetes produce the cytotoxic alkaloids, and it is the first finding that actinomycete of marine origin produced a cytotoxic alkaloid.

Acknowledgments

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