ALDECALMYCIN, A NEW ANTIMICROBIAL ANTIBIOTIC FROM *Streptomyces*

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

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A new antibiotic, aldecalmycin, has been discovered in the culture broth of *Streptomyces* sp. MJ147-72F6. Aldecalmycin was purified by solvent extraction, Diaion HP-20 chromatography, silica gel chromatography, Sephadex LH-20 chromatography, HPLC and centrifugal partition chromatography. The ^{1}H and ^{13}C NMR spectra of aldecalmycin showed the presence of keto-enol tautomers. Aldecalmycin is equipotent in inhibiting the growth of sensitive and methicillin-resistant *Staphylococcus aureus* (MRSA).

During our screening of soil microorganisms for new antibiotics, we have isolated aldecalmycin (Fig. 1) which was produced by *Streptomyces* sp. MJ147-72F6. In this paper, the taxonomy, fermentation, isolation, physico-chemical and biological properties of aldecalmycin are described.

Materials and Methods

Microorganism

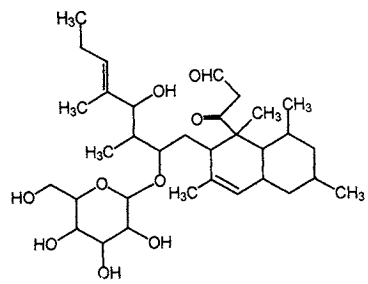
The producing organism, strain MJ147-72F6 was isolated from a soil sample collected at Setagaya-ku, Tokyo, Japan.

Streptomyces aminophilus[1] IMC S-0101 (ISP 5186) and *Streptomyces cacaoi*[2] IMC S-0209 (ISP 5057) were used as reference strains.

Taxonomic Studies

Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[3] and by the methods of WAKSMAN[4]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[5]. The substrate and aerial mass color including soluble pigments were assigned by the Color Harmony Manual, 1958 (Container Corporation of America, Chicago). Characteristics of the spores and mycelia were observed with a scanning electron microscope (Hitachi S-570). 2,6-Diaminopimelic acid in the cell wall was analyzed from the hydrolysate of the grown culture according to the method of BECKER *et al.*[6].

Fig. 1. Structure of aldecalmycin.



Time Course of the Production

The time course of antibiotic production was followed in shaker flasks. A slant culture of the strain MJ147-72F6 was inoculated into a 500-ml Erlen-

meyer flask containing 110 ml of seed medium composed of galactose 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, corn steep liquor (Iwaki) 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and a drop of silicone (Shin-etsu Chemical Industry) (pH 7.4 before sterilization). The flask was shaken on a rotary shaker (180 rpm) at 27°C for 72 hours. Two-ml of this seed culture was transferred to five 500-ml Erlenmeyer flasks containing 110 ml of a production medium consisting of glucose 1.0%, yeast extract 0.5%, potato starch 2.0%, Casamino acid (Difco) 0.5%, $CaCO_3$ 0.4% (pH was not adjusted). The fermentation was continued at 27°C for 168 hours on a rotary shaker (180 rpm). Each day 10 ml samples of the fermentation broth were drawn from each flask. The pH value, packed cell volume, concentration of carbohydrate and production of aldecalmycin were measured with each sample. Quantitative analysis of carbohydrate was done by the anthrone-sulfuric acid method[7]. Aldecalmycin in the broth filtrate was extracted with EtOAc at pH 3.0 and the extract was treated with 2,4-dinitrophenylhydrazine. The amounts of aldecalmycin were determined by quantitative analysis of the 2,4-dinitrophenylhydrazone derivative using HPLC (Shiseido CAPCELL PAK C_{18} UG120A 4.6 i.d. × 250 mm, MeOH - H_2O (95 : 5) as a mobile phase, room temperature).

Physico-chemical Properties

MP was determined on a Yanagimoto micro melting point apparatus. Optical rotation was measured with a Perkin-Elmer 241 polarimeter. IR spectrum was recorded with a Hitachi I-5020 spectrometer. UV spectra were taken on a Hitachi U-3210 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX400 spectrometer. Mass spectra were obtained with a JEOL JMS-SX102 spectrometer.

Antimicrobial Activity

The antibiotic activities were monitored by cup or paper-disk agar diffusion assay against *Bacillus stearothermophilus*.

The MICs of aldecalmycin were determined by the agar dilution method in Mueller-Hinton agar against Gram-positive, Gram-negative bacteria and yeast.

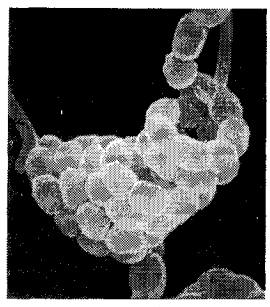
Results and Discussion

Taxonomy of the Producing Microorganism

Strain MJ147-72F6 had branched substrate mycelia, from which aerial hyphae developed in the form of 6~8 spirals. The whirl-formation or sporangia were not observed. Matured spore-chains had more than 50 spores, which were 0.6~0.7 × 0.7~1.2 μm in size and smooth surface as shown in Fig. 2. The cultural characteristics of the strain MJ147-72F6 are summarized in Table 1. Aerial mycelia with poor sporulation but good growth were observed in inorganic salts - glycerol - yeast extract - aloe (*Aloe arborescens*) extract agar consisting of homogenized aloe extract 0.25%, glycerol 0.075%, yeast extract 0.025%, K_2HPO_4 0.025%, $MgSO_4 \cdot H_2O$ 0.025%, NaCl 0.025%, $(NH_4)_2SO_4$, 0.05%, $CaCO_3$ 0.05%, trace salts solution ($FeSO_4 \cdot 7H_2O$ 0.01%, $MnCl_2 \cdot 4H_2O$ 0.01%, $ZnSO_4 \cdot 7H_2O$ 0.01%) 0.025%, and agar 1.8% (pH 7.0). The vegetative growth color was colorless to pale yellowish brown on some media tested. Soluble pigments and melanoid pigments were not produced. The physiological properties of the strain MJ147-72F6 were summarized in Table 2 and utilization of carbon sources of this strain are represented in Table 3.

Fig. 2. Scanning electron micrograph of strain MJ147-72F6.

Bar represents 1.76 μm.



Analysis of the whole-cell hydrolysate of the

Table 1. Cultural characteristics of strain MJ147-72F6.

Medium	Aerial mycelium	Growth	Soluble pigment
Sucrose nitrate agar	Thin, yellowish gray [1½ec, Putty]	Colorless	None
Glycerol-nitrate agar	None	Colorless	None
Glucose-asparagine agar	None	Poor, pale yellow	None
Starch agar	Scant, white	Pale yellowish brown [2ie, Lt Mustard Tan] to grayish yellow brown [2pl, Mustard Brown]	None
Calcium malate agar	None	Colorless	None
Nutrient agar	None	Poor, yellowish brown [3ni, Clove Brown]	None
Yeast extract-malt extract agar (ISP No. 2)	None	Pale yellowish brown [2ng, Dull Gold to 2pi, Mustard Brown]	Faint, brownish
Oatmeal agar (ISP No. 3)	Scant, yellowish gray [2ba, Pearl]	Colorless	None
Inorganic salts-starch agar (ISP No. 4)	Yellowish gray [1ba, Yellow Tint]	Pale olive [1½ie, Lt Olive] to pale yellowish brown [2ie, Lt Mustard Tan to 2le, Mustard]	None
Glycerol asparagine agar (ISP No. 5)	None	Light brownish gray [2ge, Covert Tan to 2ig, Slate Tan]	Faint, brownish gray
Tyrosine agar (ISP No. 7)	None	Pale yellowish brown [2ie, Lt Mustard Tan] to olive gray [1li, Lt Olive Drab]	None
Inorganic salts-glycerol-yeast extract-aloe extract agar	Yellowish gray	Pale yellow to pale olive	None

Observation after incubation at 27°C for 21 days.

Table 2. Physiological properties of strain MJ147-72F6.

Temperature range for growth (°C)	6~37
Optimum temperature (°C)	24
Formation of melanoid pigment	−
Liquefaction of gelatin (20°C)	+
Liquefaction of glucose peptone gelatin (27°C)	−
Coagulation of milk (37°C)	−
Peptonization of milk (37°C)	−
Hydrolysis of starch	+
Reduction of nitrate	−
Decomposition of cellulose	−

Table 3. Utilization of carbon sources of strain MJ147-72F6.

L-Arabinose	+
D-Xylose	+
D-Glucose	+
D-Fructose	(+)
Rhamnose	+
Sucrose	−
Raffinose	−
Inositol	−
D-Mannitol	(+)

−: not utilized; +: utilized; (+): probably utilized.

strain showed the presence of LL-diaminopimelic acid. On the basis of these characteristics, the strain MJ147-72F6 was found to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *S. aminophilus*[1] and *S. cacaoi*[2] were selected as similar to strain MJ147-72F6. The comparisons of these strains are summarized in Table 4. The physiological properties of *S. aminophilus* and *S. cacaoi* were similar to the strain MJ147-72F6 except for liquefaction of gelatin and peptonization of milk. Although aerial mycelium formations of these strains were good in various media, which was different from that of the strain MJ147-72F6. The strain MJ147-72F6 was considered to be different from these strains on the basis of the results described above. Therefore, the strain MJ147-72F6 was designed as *Streptomyces* sp. MJ147-72F6. This strain was deposited in the National Institute of Bioscience and Human-Technology,

Table 4. Comparison of taxonomic characterization of strain MJ147-72F6 with *Streptomyces aminophilus* and *Streptomyces cacaoi*.

	MJ147-72F6	*Streptomyces aminophilus* IMC S-0101 (ISP 5186)	*Streptomyces cacaoi* IMC S-0209 (ISP 5057)
Spore chain morphology	Spirals	Spirals	Spirals, loops and hooks
Spore surface	Smooth	Smooth	Smooth
Number of Spore chain	10~50	3~10	10~50
Aerial mass	None~Yellowish gray	White~Yellowish gray	White~Yellowish gray
Color of growth	Colorless~Pale yellowish brown	Pale yellow	Pale yellow
Soluble pigment	−	−	−
Formation of melanoid pigment	−	−	−
Liquefaction of gelatin	+	−	−
Liquefaction of glucose peptone gelatin	−	−	−
Coagulation of milk	−	+	+
Peptonization of milk	−	+	+
Reduction of nitrate	−	+	+
Hydrolysis of starch	+	+	+
Utilization of[a]			
L-Arabinose	+	+	+
D-Fructose	+	+	(+)
D-Glucose	+	+	+
Inositol	−	−	−
D-Mannitol	(+)	+	(+)
Raffinose	±	±	−
Rhamnose	(+)	−	−
Sucrose	−	−	−
D-Xylose	+	+	+

[a] +: utilized, ±: doubtful, (+): probably utilized, −: not utilized.

Agency of Industrial Science and Technology, Ibaragi, Japan, with an accession number of FERM P-12174.

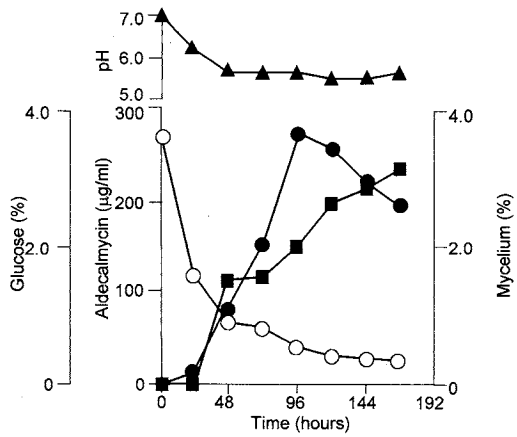
Fermentation

The production of aldecalmycin is shown in Fig. 3. The maximum production of aldecalmycin in shaker flasks was obtained after 96 hours.

A well-grown culture of the strain MJ147-72F6 was inoculated into sixteen 500-ml Erlenmeyer flasks containing 110 ml of seed medium as described in the experimental paragraph. The culture was incubated at 27°C for 72 hours on a rotary shaker (180 rpm). Four hundred-ml of the seed culture was transferred to four 20-liter jar fermentors containing 12 liters of modified production medium consisting of glucose 1.0%, yeast extract 0.5%, potato starch 2.0%, Casamino acid (Difco) 0.5%, $CaCO_3$ 0.4% and Pronal 502 (Toho Chemical Industry) as an antifoaming agent. The fermentation was carried out at 27°C for 72 hours with aeration of 12 liters per minute and agitation of 200 rpm.

Fig. 3. Production of aldecalmycin.

● Aldecalmycin, ▲ pH, ■ mycelium, ○ glucose.



Isolation

The culture broth was filtered and the filtrate (40 liters) was absorbed on Diaion HP-20 (4 liters). The column was washed with water (8 liters) and then with 50% acetone (4 liters). The active fractions were eluted with acetone (7 liters) and concentrated under reduced pressure. The concentrate at pH 3.0 was extracted with ethyl acetate. Additionally, the mycelium cake was extracted with methanol (3 liters). The methanol extract was concentrated *in vacuo* and extracted at pH 3.0 with ethyl acetate. Both extracts were combined and evaporated to dryness to give brown oil (*ca.* 100 g). This oily substance (50.4 g) was washed with *n*-hexane (600 ml) and then the residue was dissolved in methanol (500 ml). The methanol solution was

Table 5. Physico-chemical properties of aldecalmycin.

Appearance	White powder
MP (°C)	125~128
Optical rotation $[\alpha]_D^{26}$	−78.7° (*c* 1.0, MeOH)
Molecular formula	$C_{33}H_{54}O_9$
Elemental analysis	
Calcd:	C 65.64, H 9.18, O 25.18 (as $C_{33}H_{54}O_9 \cdot \frac{1}{2}H_2O$)
Found:	C 65.48, H 9.29, O 25.47
FAB-MS (*m/z*)	593 $(M-H)^-$
HRFAB-MS (*m/z*)	
Calcd:	593.3689 (as $C_{33}H_{53}O_9$)
Found:	593.3687 $(M-H)^-$
UV λ_{max} nm ($E^{1\%}_{1cm}$)	
in MeOH	272 (30), 299 (31)
in HCl-MeOH	271 (24), 303 (sh, 13)
in NaOH-MeOH	304 (395)
IR ν_{max} (cm^{-1}) KBr	3430, 2960, 2910, 1694, 1626, 1456, 1379, 1074, 1038, 995
TLC (Rf value)[a]	0.39

[a] Silica gel TLC (Merck Art. 5715): $CHCl_3$-MeOH (20:3).

Fig. 4. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra of aldecalmycin in dioxane-d_8.

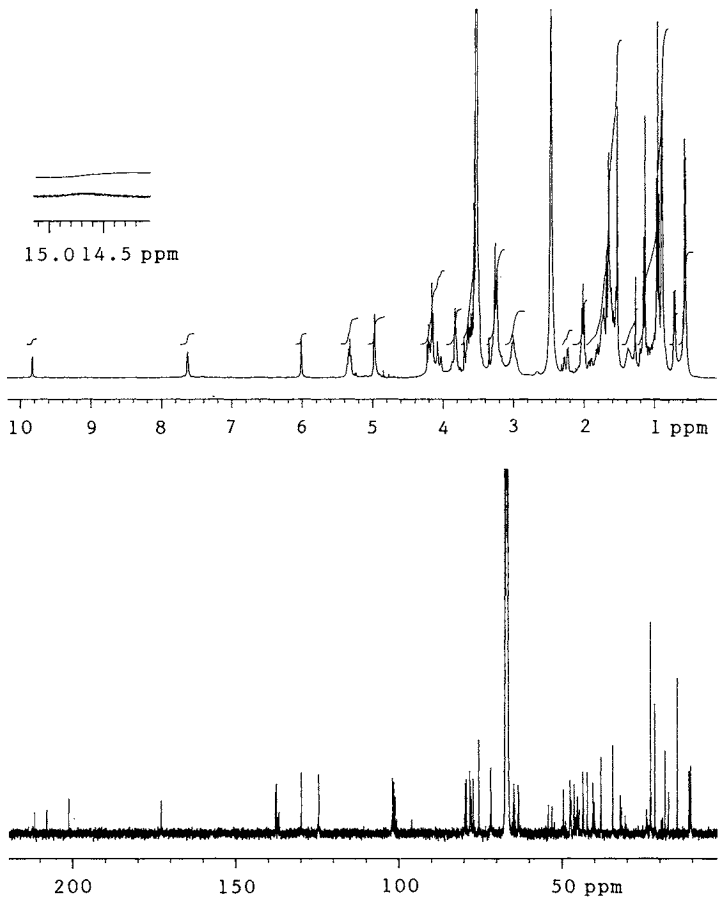


Table 6. The antimicrobial activities of aldecalmycin.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	6.25	*Corynebacterium bovis* 1810	12.5
S. aureus Smith	12.5	*Escherichia coli* NIHJ	>100
S. aureus MS9610	6.25	*E. coli* K-12	>100
S. aureus No. 5 (MRSA)	12.5	*Shigella dysenteriae* JS11910	>100
S. aureus No. 17 (MRSA)	12.5	*Salmonella typhi* T-63	>100
Micrococcus luteus FDA 16	12.5	*Proteus vulgaris* OX19	>100
M. luteus IFO 3333	12.5	*Serratia marcescens*	>100
M. luteus PCI 1001	50	*Pseudomonas aeruginosa* A3	>50
Bacillus anthracis	6.25	*P. aeruginosa* GN315	100
B. subtilis NRRL B-558	6.25	*Klebsiella pneumoniae* PCI 602	>100
B. subtilis PCI 219	6.25	*Mycobacterium smegmatis* ATCC 607*	100
B. cereus ATCC 10702	6.25	*Candida albicans* 3147	100

Mueller Hinton agar 37°C 18 hours.
* 37°C 42 hours.

dried under reduced pressure to give a brown solid (39.8 g). The brown solid was suspended in chloroform and was charged on a silica gel column (Wakogel C-200, 1 kg), washed with chloroform - methanol (15 : 1, 3 liters), and developed with chloroform - methanol (8 : 1, 4.5 liters). The active fractions were concentrated *in vacuo* to dryness (4.9 g). The crude compound was applied on a Sephadex LH-20 column (3.7 liters) and eluted with methanol. The fractions containing aldecalmycin were concentrated under reduced pressure to give pale yellow powder (3.2 g).

For further purification, 1 g of the crude powder was purified in ten portions by reverse phase HPLC (Senshu Pak ODS-6251 30 i.d. × 250 mm) with acetonitrile - water (3 : 1) at a flow rate of 10 ml/minute. The fractions including aldecalmycin were collected and evaporated to dryness to yield white powder (526 mg). Finally, the white powder was subjected to centrifugal partition chromatography. The chromatography was performed using CPC-L.L.N model NMF (Sanki Engineering Limited) with a solvent system of chloroform - methanol - water (5 : 6 : 4). The upper phase of the solvent (360 ml) was pumped by the ascending method. By a change of the flow direction (descending method), the fractions containing aldecalmycin were eluted with the lower phase of the solvent system (125 ml) to give pure aldecalmycin (290 mg).

Physico-chemical Properties of Aldecalmycin

Physico-chemical properties of aldecalmycin are shown in Table 5. The molecular formula of aldecalmycin was established as $C_{33}H_{54}O_9$ by HRFAB-MS and elemental analysis. The UV spectra showed the characteristic absorption maximum at 304 nm in alkaline methanol. Aldecalmycin gave positive color reactions to 2,4-dinitrophenylhydrazine and molybdophosphoric acid-sulfuric acid reagents, negative to ninhydrin and Rydon-Smith reagents. The ^1H and ^{13}C NMR spectra of aldecalmycin in dioxane-d_8 are shown in Fig. 4. The spectra showed the presence of a *ca.* 1 : 2 mixture of keto-enol tautomers. We could not get assignable NMR spectra in another solvents such as methanol, chloroform or pyridine.

Biological Activities

Antimicrobial activities of aldecalmycin are shown in Table 6. Aldecalmycin showed the antimicrobial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) as same extent as against sensitive ones or other Gram-positive bacteria and its MICs were 6.25 ~ 12.5 μg/ml. Aldecalmycin did not show the antimicrobial activities against Gram-negative bacteria or yeast. Aldecalmycin did not show any toxicity (LD_{50} in mice) at 100 mg/kg with iv administration.

References

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