A NEW ANTITUMOR ANTIBIOTIC, KAZUSAMYCIN

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A new antibiotic kazusamycin, was isolated from the culture broth of *Streptomyces* sp. No. 81-484, which shows antitumor activity against experimental murine tumors.

This antibiotic did not possess antibacterial activity against Gram-positive and Gram-negative bacteria, but showed strong cytotoxic activity against HeLa cells *in vitro*. The chemical and physico-chemical properties of kazusamycin suggest that the molecular formula of this antibiotic is $C_{88}H_{45}O_7$ (MW 556).

In the course of a continuing search for novel antitumor antibiotics of microbial origin, kazusamycin was isolated from the fermentation culture of *Streptomyces* sp. No. 81-484. This paper describes the taxonomy of the producing organism, fermentation, isolation, and physico-chemical and biological properties of this antibiotic.

Materials and Methods

Taxonomic Studies

For taxonomic studies, most cultures were grown in accordance with methods adopted by the International Streptomyces Project[1]. For experiments on cultural properties, all cultures were incubated at 27°C and were observed for 15~20 days. The color recorded for natural cultures was described according to the "Color Harmony Mannual"[2]. Physiological properties including utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB[3]. Diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[4].

Antimicrobial Activity

The antimicrobial spectrum of kazusamycin was determined by the ordinary paper disk method using nutrient agar medium for bacteria and potato agar for fungi and yeast. The agar concentration in nutrient and potato media was 1.0% for all strains except potato medium for *Schizosaccharomyces pombe* (0.5%). The minimum inhibitory concentrations (MIC) were observed after 24-hour incubation for bacteria or longer incubation for fungi.

Antitumor Activity

For determination of the antitumor activity of kazusamycin, female ICR and CDF_1 mice (5-week old) were purchased from the Shizuoka Agricultural Cooperative Association. Sarcoma 180 ascites and P388 leukemia were kindly supplied by the Sasaki Institute and Cancer Chemotherapy Center (Tokyo), respectively. Antitumor activity was evaluated by the increase in life span (ILS): $(T/C-1)\times 100\%$, where "T" was the median survival days (MSD) of the treated group and "C" the MSD of the control group. Survivors were scored 60 days after tumor incubation, and mice remaining alive after this period of observation were considered cured.

Effect of Kazusamycin on HeLa Cells

HeLa cells were maintained in monolayers in Eagle minimum essential medium (MEM) supplemented with 10% bovine serum and 60 μg/ml of kanamycin at 37°C. To determine the cytotoxicity

of kazusamycin on mammalian cells, HeLa cells (4×10^4) in 2 ml of medium were placed in a 2-cm^2 Petri dish and incubated for 48 hours at 37°C in a 5% CO_2 - 95% air atmosphere. Each culture dish was filled with fresh medium containing a different concentration of kazusamycin and incubated for 72 hours. The HeLa cells were trypsinized to form a single cell suspension, and cells were counted in a hemocytometer.

Media and Fermentation

Stock cultures of the producing organism were inoculated into a 500-ml Sakaguchi flask containing 100 ml of the seed medium consisting of glucose 2.0%, peptone 0.5%, dry yeast 0.3%, meat extract 0.5%, NaCl 0.5% and $CaCO_3$ 0.3% (pH 7.0). The flasks were incubated at 28°C for 4 days on a reciprocal shaker. One liter of the resulting culture was then transferred to a 200-liter fermentor containing 130 liters of the same culture medium described above. The fermentation was carried out at 28°C for 120 hours under aeration of 130 liters per minute and agitation of 240 rpm. The activity against HeLa cells *in vitro* reached a maximum after 4~5 days of cultivation.

Isolation and Purification

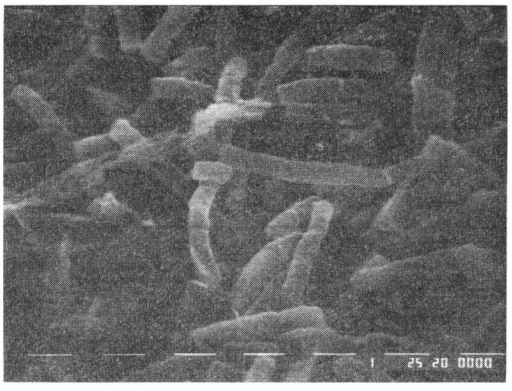
The flow diagram of the isolation and purification method for the preparation of kazusamycin from broth filtrate is shown in Fig. 1. The fermentation broth (118 liters) of *Streptomyces* sp. No. 81-484 was mixed with 3% of Hyflo Super-Cell (Johns-Manville Sales Co., U.S.A.), filtered and washed with 10 liters of water. The broth filtrate was absorbed on a column (4 liters) of Amberlite XAD-7 and the column was washed with water and 20% aqueous methanol, and eluted with 60% aqueous methanol. The active fractions against HeLa cells *in vitro* were collected and concentrated *in vacuo* to 300 ml. The supernatant was separated from the precipitate which contained nybomycin. The supernatant was extracted twice with 300 ml of ethyl acetate. The organic layer was concentrated *in vacuo* and applied to a silica gel column (Wako C-200, 1 liter) and the antibiotic was eluted with a gradient system between *n*-hexane and *n*-hexane - acetone (1: 1). The active fractions were combined and the solvent removed under reduced pressure. The residue was dissolved in a small amount of ethyl acetate and chromatographed on a silica gel column with ethyl acetate. Crude kazusamycin (*ca.* 50 mg) was obtained by evaporation of the active fraction.

This residue was then subjected to reverse phase silica gel preparative HPLC (Fine SIL C_{18}-10, Jasco) with a solvent system of methanol and water (70: 30), and about 30 mg of purified kazusamycin was obtained.

Fig. 1. Flow diagram of the isolation and purification of kazusamycin.

Broth filtrate (120 liters)
↓
Amberlite XAD-7 column (4 liters)
→ washed with H_2O and then with 20% aq MeOH
→ eluted with 60% aq MeOH
Active fraction
→ concd *in vacuo*
→ filtered
↓
Supernatant → extracted with EtOAc
Precipitate → recrystallized → Nybomycin
Organic layer
→ concd *in vacuo*
Oily residue
↓
Silica gel column
→ eluted with a gradient system between *n*-hexane and *n*-hexane - acetone (1: 1)
Active fraction
↓
Silica gel column
→ eluted with EtOAc
Reverse phase silica gel preparative HPLC (Fine SIL C_{18}-10)
→ eluted with MeOH - H_2O (70: 30)
Kazusamycin (30 mg)

Fig. 2. Electron micrograph of the spores of strain No. 81-484.
Bars represent 1 μm.



Results

Taxonomic Studies

The chain of the mature spores consisted of more than six spores and was usually straight. The spores were cylindrical, and $0.56 \sim 0.7 \times 0.8 \sim 1.6$ μm in size with smooth surfaces (Fig. 2). Culture characteristics in various media are shown in Table 1. The color of aerial mycelium was relatively close to the yellow series on four different media.

The physiological properties and utilization of carbon sources of strain No. 81-484 are shown in Tables 2 and 3, respectively. Cell wall analysis showed the presence of LL-diaminopimelic acid and was classified as type I. Microscopic studies and cell wall type indicated that strain No. 81-484 belongs to the genus *Streptomyces*.

Physico-chemical Properties of Kazusamycin

The physico-chemical properties of kazusamycin are summarized in Table 4. Kazusamycin was soluble in methanol, ethanol, ethyl acetate, acetone, benzene, chloroform and ethyl ether but insoluble in *n*-hexane and water. The UV spectrum in methanol showed a maximum absorption at 232 nm and shoulder absorption at 245 nm (Fig. 3). In the IR spectrum, alcohol and carbonyl groups were observed at $3200 \sim 3500$ cm^{-1} and 1700 cm^{-1}, respectively (Fig. 4). The formula and molecular weight of

Table 1. Cultural properties of strain No. 81-484.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Glycerol - asparagine agar	Good	Brownish white	Yellowish gray	Brownish white
Inorganic salts - starch agar	Poor	Ivory	Ivory	Ivory
Tyrosine agar	Good	Pale yellow orange	Grayish yellow brown	Light brownish gray
Yeast extract - malt extract agar	Good	Ivory	Pastel yellow	Buff
Oat meal agar	Poor	Pale yellow	Pale yellow	Brownish white
Peptone - yeast extract - iron agar	Poor	Pale yellow orange	Pale yellow orange	Maize

Table 2. Physiological characteristics.

Nitrate reduction	—
Liquefaction of gelatin	—
Coagulation of milk	—
Peptonization of milk	±
Melanin formation	—
Production of H_2S	—
Starch hydrolysis	—
Tyrosinase reaction	—

± Doubtful, — negative.

Table 3. Utilization of carbon sources.

Responses	Carbon source
Positive	Inositol, L-rhamnose
Negative	L-Arabinose, sucrose, D-xylose, D-mannitol, D-fructose, raffinose

Table 4. Physico-chemical properties of kazusamycin.

Molecular formula	$C_{33}H_{45}O_7$
Molecular weight (FD-MS)	556
$[\alpha]_D^{20}$ (c 0.1, MeOH)	$-83.5°$
UV absorption in MeOH (ε)	λ_{max} 232 nm (33,000) (shoulder) 245 nm (30,400)
IR ν_{max} (KBr, cm^{-1})	3450, 2980, 2950, 2900, 1705, 1640, 1458, 1380, 1285, 1258, 1160, 1100, 1043, 965, 820
Rf value (Kieselgel 60 F_{254})	0.33 (EtOAc - MeOH, 40: 1) 0.28 ($CHCl_3$ - MeOH, 10: 1)

Fig. 3. UV spectrum of kazusamycin (MeOH).

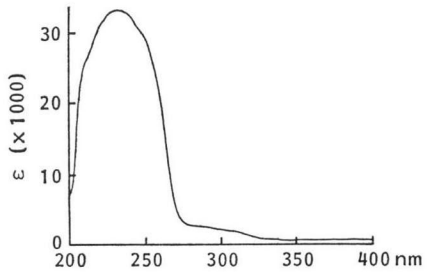


Table 5. Antimicrobial spectrum of kazusamycin.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	>100
Bacillus subtilis PCI 219	>100
Micrococcus luteus ATCC 9341	>100
Escherichia coli NIHJ	>100
Shigella sonnei E-33	>100
Trichophyton rubrum KF 53	>100
Candida albicans KF 1	>100
Saccharomyces sake KF 26	>100
Aspergillus niger ATCC 6275	>100
Schizosaccharomyces pombe IAM 4863	0.1
Rhizopus javanicus IAM 6241	2.5

kazusamycin were proposed from high-resolution mass analysis and field desorption mass spectrometry. Field desorption mass spectrometry of this antibiotic gave peaks at m/z 557 ($[M+H]^+$) and at m/z 579 ($[M+Na]^+$). HR-MS: found 538.33047, calcd for $C_{33}H_{46}O_6$ (M^+-H_2O) 538.32918. The ^1H NMR spectrum of kazusamycin (in $CDCl_3$, 400 MHz) is shown in Fig. 5.

Biological Properties

The antimicrobial spectrum of kazusamycin is shown in Table 5. The antibiotic was active on *Schizosaccharomyces pombe* IAM 4863 and *Rhizopus javanicus* IAM 6241, but inactive against Gram-positive and Gram-negative bacteria, and other microorganisms as follows, *Candida albicans, Trichophyton rubrum* and *Saccharomyces sake*.

The effect of adding kazusamycin to asynchronous exponentially growing cultures of HeLa cells was determined. Fig. 6 shows that a concentration of 3.3 ng/ml of the antibiotic was effective in completely preventing cell growth.

The antitumor activities of kazusamycin on P388 leukemia and sarcoma 180 tumor are shown in Tables 6 and 7, respectively. The intraperitoneal injection of kazusamycin caused a prolongation of the life span of the treated mice with both tumors.

Fig. 4. IR spectrum of kazusamycin (KBr).

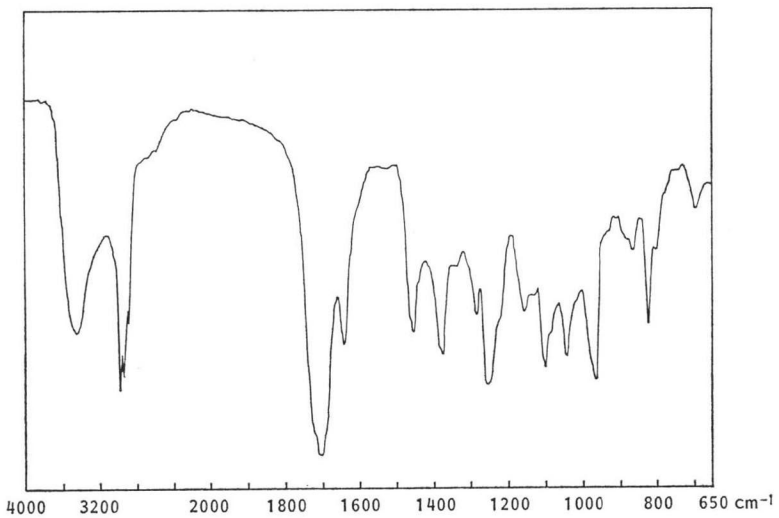


Fig. 5. ^{1}H NMR spectrum of kazusamycin (400 MHz, in $CDCl_3$).

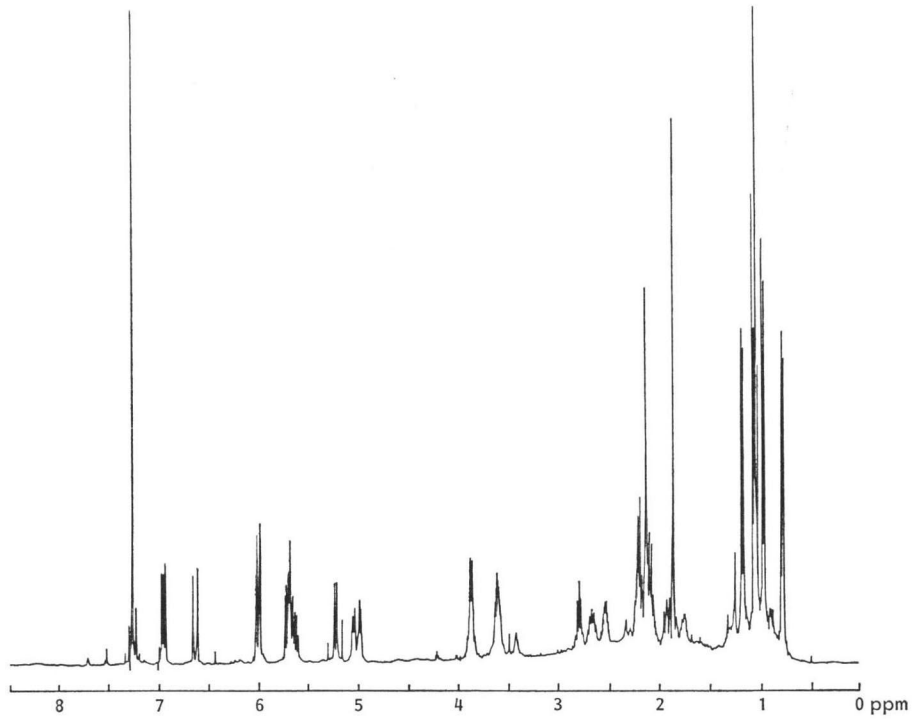


Table 6. Antitumor activity of kazusamycin on P388 leukemia.

Dose (mg/kg/day)	MSD	ILS (%)
0.016×5	19	58
0.008×5	16	33
Control	12	0

P388 leukemia cells (1×10^5) were ip inoculated into CDF_1 mice (5-week old, female).

Mice were given ip injections of the antibiotic on days 1~5.

Table 7. Antitumor activity of kazusamycin on sarcoma 180.

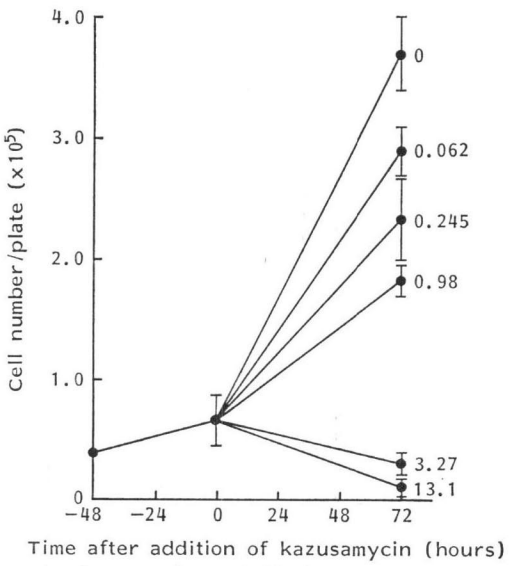
Dose (mg/kg/day)	MSD	ILS (%)
0.031×5	20	67
0.016×5	28	133
0.008×5	22	83
Control	12	0

Sarcoma 180 cells (1×10^6) were inoculated ip into ICR mice (5-week old, female).

Mice were given ip injections of the antibiotic on days 1~5.

Fig. 6. Cytocidal activity of kazusamycin on HeLa cells.

Numbers in figure indicate concentration (ng/ml) of the antibiotic.



Discussion

A new antibiotic, kazusamycin, was isolated from the culture filtrate of *Streptomyces* sp. No. 81-484. Among known antibiotics, kazusamycin was similar to leptomycins A and B[5,6] in physico-chemical and biological characteristics, *i.e.*, IR and UV absorption spectra, ^{1}H NMR spectra, antibacterial spectra, and cytocidal activity on mammalian cells. However, the molecular weights of kazusamycin, leptomycins A and B are 556, 526 and 540, respectively. Besides kazusamycin, *Streptomyces* sp. No. 81-484 produced an antibiotic identical to nybomycin[7]. Because kazusamycin showed significant cytotoxic activity on HeLa cells *in vitro*, it seems that the antitumor effect *in vivo* is due to direct cytotoxic activity.

Acknowledgments

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