ISOLATION AND CHARACTERIZATION OF A NEW ANTITUMOR ANTIBIOTIC OS-3256-B FROM *STREPTOMYCES CANDIDUS* VAR. *AZATICUS*

KEIKI SATOH, KANKI KOMIYAMA, CHIAKI KITAO, YUZURU IWAI, KIYOO ATSUMI, RUIKO ŌIWA, MICHIKO KATAGIRI, IWAO UMEZAWA, SATOSHI ŌMURA and TOJU HATA*

Kitasato Institute and Kitasato University*
Shirokane, 5-9-1, Minato-ku, Tokyo 108, Japan

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OS-3256-B is a new member of azaamino acid substances obtained from the culture filtrate of *Streptomyces candidus* var. *azaticus* which was reported in a previous paper. This antibiotic is remarkably effective against experimental murine tumors such as S-180 tumor and leukemia L-1210. The antibiotic is also obtainable from alazopeptin by chemical transformation.

Our preceding paper[1] reported that an antitumor antibiotic, alazopeptin, was produced by *Streptomyces candidus* var. *azaticus*. In the following investigation, we found that the same strain produced a new antitumor antibiotic, OS-3256-B, which is a kind of azaamino acid substance containing alanine. This antibiotic has an antibacterial activity against gram-positive bacteria and an antitumor activity. In this paper, isolation, physico-chemical properties and the biological properties of OS-3256-B are described.

I. Production and Isolation of OS-3256-B

A medium containing 2% glycerol, 2% soybean meal, 0.3% meat extract, and 0.3% NaCl, pH 7.0, was used for the production of OS-3256-B. The strain was cultured at 27°C for 30 hours by aeration at 15 liters/min. and stirring at 250 rpm, in a 30-liter jar fermentor. The cultured broth (20 liters) was filtered and passed through a column packed with 1 liter of activated carbon. The column was washed with 5 liters of water, and the new antibiotic followed by alazopeptin were successively eluted with 20% aqueous acetone. The active fraction of OS-3256-B was concentrated *in vacuo* and methanol was added to the residue. After removing the precipitate, the supernatant was concentrated *in vacuo*, and acetone was then added. The precipitate which formed was collected, dissolved in water, and the solution was passed through a column packed with 500 ml of silica gel (Kieselgel 60 Merck) and the activity was eluted with water. To obtain a crude powder, the active fraction was concentrated *in vacuo* and freeze-dried. This crude powder was dissolved in a mixture of ethanol - 20% ammonia water-water (8 : 1 : 1), passed through a column packed with 500 ml of Avicel prepared with the mixed solvent and eluted with the same solvent. The active fraction was concentrated to remove the solvent and the aqueous solution of the crude antibiotic was passed through a column packed with 100 ml Sephadex G-10 and eluted with water. These fractions were checked by paper chromatography using a mixture of 93.8% aqueous *n*-butanol - 44% aqueous propionic acid (1 : 1) for distinguishing OS-3256-B from alazopeptin. The Rf value of the antibiotic was 0.34 on a bioautogram using *Bacillus subtilis* PCI-219 as the test organism.

Table 1. Chromatographic mobility of OS-3256-B

	Solvent system	Rf
Silica gel TLC	*n*-Butanol - methanol - water (2 : 1 : 3)	0.56
	〃 (3 : 1 : 2)	0.22
	n-Butanol - acetic acid - water (3 : 1 : 2)	0.31
Avicel TLC	Ethanol - ammonia water - water (8 : 1 : 1)	0.16
	n-Butanol - acetic acid - water (3 : 1 : 2)	0.46
	n-Butanol - ethanol - water (2 : 2 : 3)	0.55
Paper chromatography	93.8% *n*-Butanol - 44% propionic acid (1 : 1)	0.34
	Ethanol - *t*-butanol - formic acid - water (60 : 20 : 5 : 15)	0.50

After concentration, the aqueous solution of the antibiotic was freeze-dried.

II. Physico-chemical Properties of OS-3256-B

This antibiotic is obtained as a white powder that does not give a clear melting point. It is freely soluble in water and slightly soluble in aqueous methanol. Chromatographic mobility of the antibiotic in a number of solvent systems is shown in Table 1. The Rf value was detected by bioautography against *Bacillus subtilis*. Ninhydrin can also be used for its detection. Elemental analysis gave: C, 46.77; H, 5.99; N, 18.12%. Acid hydrolysis (6 N HCl, 120°C, 2 hours) of the antibiotic yielded alanine, and acid hydrolysis (6 N HCl, 120°C, 6 hours) after HIO_4 oxidation (0.2 M HIO_4, 25°C, 2 hours) yielded glutamic acid. The antibiotic exhibited ultraviolet absorption maxima at 226 nm ($E^{1\%}_{1cm}$ 394) and 276 nm ($E^{1\%}_{1cm}$ 310) in aqueous solution (Fig. 1). The infrared spectrum, run in KBr tablet, is shown in Fig. 2, giving peaks at the following frequencies: 3300, 3050, 2900, 2150, 1660~1550, 1400, 1120 and 600 cm^{-1}.

Fig. 1. Ultraviolet absorption spectrum (25 mcg/ml water)

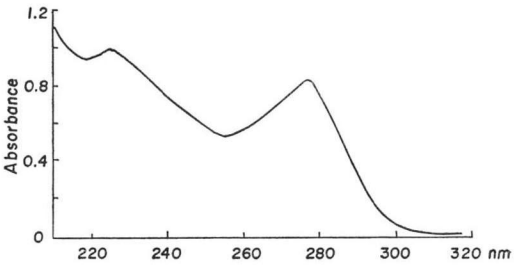
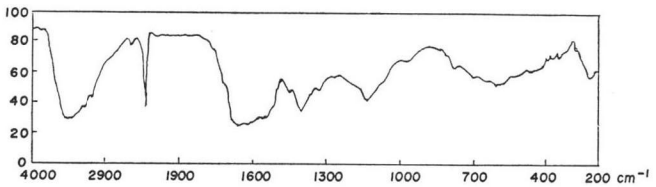


Fig. 2. Infrared absorption spectrum (KBr)



III. Chemical Transformation from Alazopeptin to OS-3256-B

A solution of 50 mg of alazopeptin dissolved in 10 ml of water was adjusted to pH 4.0 with 0.01 N hydrochloric acid. The solution was incubated for 20 hours at 37°C and then neutralized with 0.01 N sodium hydroxide. This solution was passed through a column of activated carbon. The column was washed with water, and the antibiotic transformed from alazopeptin was eluted with 20% aqueous acetone. The eluate containing the antibiotic was

concentrated, passed through a column of Sephadex G-10 and the column was eluted with water. The active eluate was concentrated and lyophilized to give a white powder; yield, 8.5 mg. This compound was identified as OS-3256-B by paper chromatography, thin-layer chromatography, UV and IR spectra.

IV. Antimicrobial Activity of OS-3256-B

Antimicrobial activities of the antibiotic by the agar dilution method are listed in Table 2. It was active against *Staphylococcus aureus*, *Sarcina lutea* and *Escherichia coli* NIHJ in the concentration of 25~50 mcg/ml, but less active against most of the gram-negative bacteria and fungi. When antimicrobial activities of the antibiotic were assayed on DAVIS' agar medium, the minimum inhibitory concentrations were 1.56 mcg/ml against *Bacillus subtilis* PCI 219 and 3.12 mcg/ml against *Escherichia coli* JC-2.

Table 2. Antimicrobial spectrum of OS-3256-B

Test organism	Minimum inhibitory concentration (mcg/ml)
Bacillus subtilis PCI 219	>100
Staphylococcus aureus FDA 209P	25
〃 JC-1	25
Sarcina lutea PCI 1001	25
Mycobacterium smegmatis ATCC 607	>100
Escherichia coli NIHJ	50
〃 JC-2	>100
Klebsiella pneumoniae	>100
Salmonella typhimurium	>100
Xanthomonas oryzae	>100
Candida albicans	>100
Saccharomyces sake	100
Aspergillus niger	>100
Trichophyton interdigitale	100

V. Effect of OS-3256-B on Murine Malignant Tumors

1. Effect on leukemia L-1210: Leukemia L-1210 cells were carried intraperitoneally in strain CDF_1 mice. Ascites tumor cells were counted in a hemocytometer and diluted in HANK's balanced salt solution to 1×10^6 cells/ml. CDF_1 mice weighing 19~22 g received 0.1 ml injections of tumor cell suspension intraperitoneally. The antibiotic was dissolved in sterilized distilled water. Treatments were begun 24 hours after tumor inoculation.

The effects of the antibiotic on the survival time of mice are presented in Table 3. When 9 mg/kg/day of the antibiotic was injected once daily for 10 days, the highest survival ratio of leukemic mice was obtained, and this was followed closely by 4.5 mg/kg/day. When large doses of the antibiotic, 28.6 or 18 mg/kg/day, were injected for 4 days, the survival effect was reduced as compared with the daily treatment with small doses for 10 days.

Table 3. Effect of OS-3256-B on Leukemia L-1210

Dose (mg/kg/day)	Route and regimen	Survival days		Survival ratio (T/C %)
		Median	Range	
None	—	7.6	7~ 8	100
9.0	ip, day 1~10	14.6	9~16	192.1
4.5	〃	14.4	14~15	189.5
2.3	〃	11.8	11~13	155.3
1.2	〃	9.8	9~10	128.9
0.6	〃	8.4	8~ 9	110.5
28.6	ip, day 1~4	13.2	13~14	173.7
18.0	〃	13.0	12~14	171.1

Groups of 5 mice were inoculated with 10^5 L-1210 cells ip on day 0. The LD_{50} of OS-3256-B is more than 150 mg/kg (ip).

2. Effect on sarcoma-180: A small piece (about 2 mm^3) of sarcoma-180 solid tumor was inoculated subcutaneously into the axillar region of *dd* mice weighing 20~22 g. Treatments once daily for 10 days, were begun 1 or 5 days after tumor inoculation.

As shown in Fig. 3 a~c, when 3.1~12.5 mg/kg/day of the antibiotic was given from one

Fig. 3. Effect of OS-3256-B on sarcoma-180 (Injected ip once daily for 10 days from 1 day after tumor inoculation)

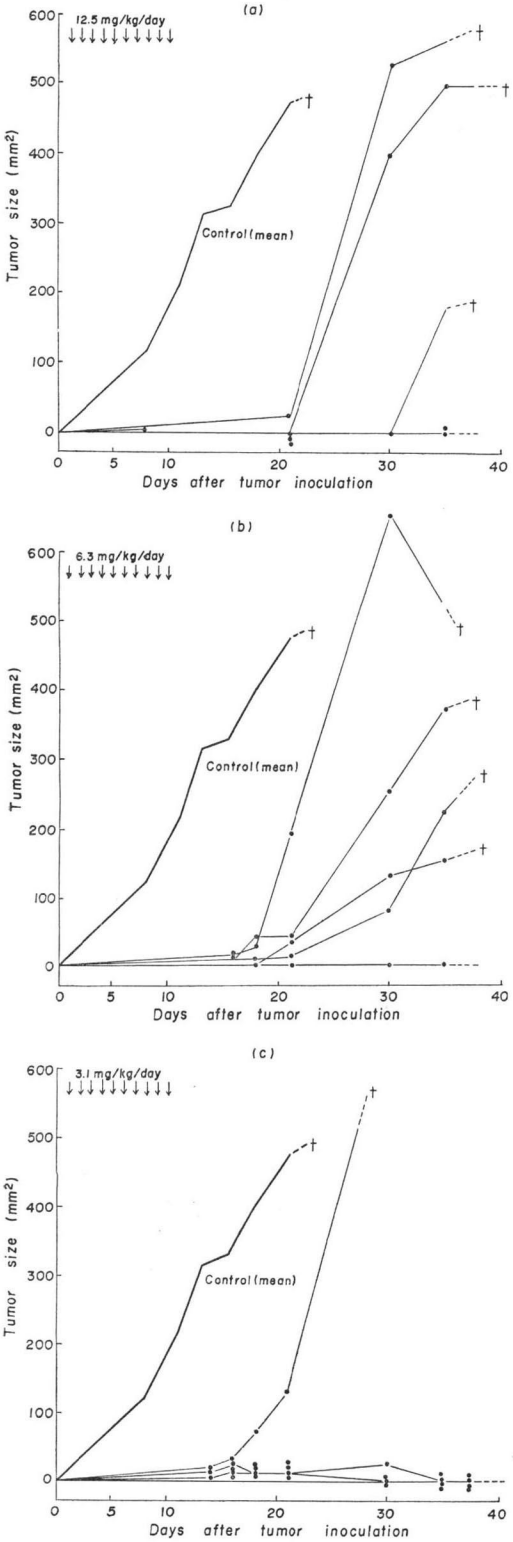
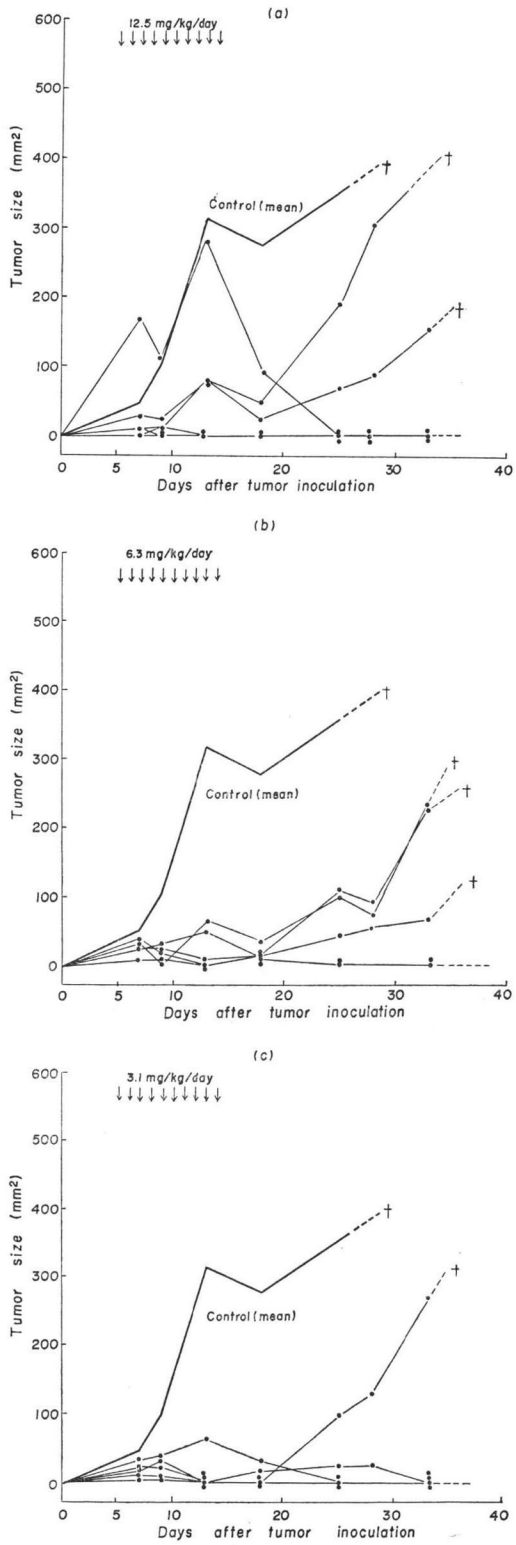


Fig. 4. Effect of OS-3256-B on sarcoma-180 (Injected ip once daily for 10 days from 5 days after tumor inoculation)



day after tumor inoculation, almost complete inhibition of tumor growth was observed until 1~2 weeks after the final injection. After that, a remarkable tumor growth began in most of the animals treated with 6.3 or 12.5 mg/kg/day, whereas a complete inhibition and longer survival were observed in 4 out of 5 animals treated with 3.1 mg/kg/day. When the treatment was started five days after tumor inoculation, a strong inhibitory effect on tumor growth was also observed with 3.1~12.5 mg/kg/day (Fig. 4 a, b, c). At a dose of 3.1 mg/kg/day, 4 out of 5 animals were alive for at least 70 days without tumor, and this was followed by 12.5 mg/kg/day (3 out 5 animals).

VI. Effect of OS-3256-B on HeLa Cells in Cell Culture

A stock of HeLa cells was grown in EAGLE's minimum essential medium supplemented with 10% calf serum. After 48 hours of cultivation of HeLa cells (1×10^5 ml) in Leighton tubes each inserted with a coverslip, the antibiotic dissolved in the growth medium was added to the tube. Morphological changes of HeLa cells were observed under a light microscope after 72 hours cultivation from the addition of the antibiotic.

With this result, shrinkage and decreasing numbers of nucleoli were observed, but no remarkable morphological change of nuclei was observed at concentrations of 500~62.5 mcg/ml. An inhibition of mitosis was observed at a concentration of 31.5 mcg/ml. These findings indicated that OS-3256-B mainly affect nucleoli of HeLa cells.

Discussion

The properties of OS-3256-B are very similar to those of the azaamino acid group of antibiotics, for example, DON[2], duazomycin A, B, and C,[3,4,5] azaserine[6,7] and alazopeptin[8]. These compounds have a diazo base but only alazopeptin contains alanine. OS-3256-B resembles alazopeptin, but its analytical data and ultraviolet absorption spectrum are different from those of alazopeptin. On paper chromatograms, the Rf values of OS-3256-B differ from those of alazopeptin (Table 4).

Table 4. Paper chromatography of OS-3256-B and azaamino acid group of antibiotics

Solvent system	I	II
Duazomycin A	0.76	0.84
Alazopeptin	0.55	0.70
DON	0.40	0.40
OS-3256-B	0.34	0.50

I; 93.8% *n*-BuOH-44% propionic acid=1 : 1.
II; EtOH-*t*-BuOH-HCOOH-H_2O=60 : 20 : 5 : 15.

From the results obtained by treating tumor-bearing mice, OS-3256-B was quite effective on both leukemia L-1210 and sarcoma-180 solid tumor. The best survival effect on mice bearing leukemia L-1210 was obtained by daily injection. Only two treatment schedules were designed in this experiment, so other treatment schedules will be designed in the future.

It was of interest that, in the treatment of sarcoma-180 tumor started 24 hours after tumor inoculation, dosages of 12.5 and 6.3 mg/kg/day inhibited the tumor growth within 2~3 weeks, but a marked tumor growth was observed afterward in most of the treated animals. On the other hand, complete inhibition of tumor growth was observed for more than 9 weeks at a dose of 3.1 mg/kg/day.

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