STUDIES ON THE ANTIBIOTICS FROM *STREPTOMYCES SPINICHROMOGENES* VAR. *KUJIMYCETICUS*. II

ISOLATION AND CHARACTERIZATION OF KUJIMYCINS A AND B

SADAFUMI OMURA, SHINJURO NAMIKI, MICHINORI SHIBATA, TOSHIO MURO, HIROSHI NAKAYOSHI and JIRO SAWADA

Research Division, Taisho Pharmaceutical Co., Ltd.,
No. 34-1, Takata 3-chome, Toshimaku, Tokyo, Japan

(Received for publication August 11, 1969)

Two new antibiotics, kujimycins A and B, were isolated from the fermentation broth of *Streptomyces* sp. TPR-885 and purified by the extraction with ethyl acetate, gel filtration on Sephadex LH-20 and silica gel column chromatography. Kujimycin A was obtained as colorless powder, m. p. 114~115°C, $C_{40}H_{70}O_{15}$. Kujimycin B was obtained as colorless prisms, m. p. 178~180°C, $C_{42}H_{72}O_{16}$. They were suggested to be neutral macrolides which have antimicrobial activities against Gram-positive bacteria.

Kujimycins are new antibiotics produced by a new strain, *Streptomyces* sp. TPR-885. The microbiological characteristics of the new strain, and the fermentation procedure were described in the previous paper[1].

The kujimycins are composed of several components. Among them two main components, kujimycins A and B were purified and characterized as neutral macrolides which inhibited mainly the growth of Gram-positive bacteria. In this paper are described the isolation of the antibiotics from the fermentation broth and some chemical, physical and biological properties.

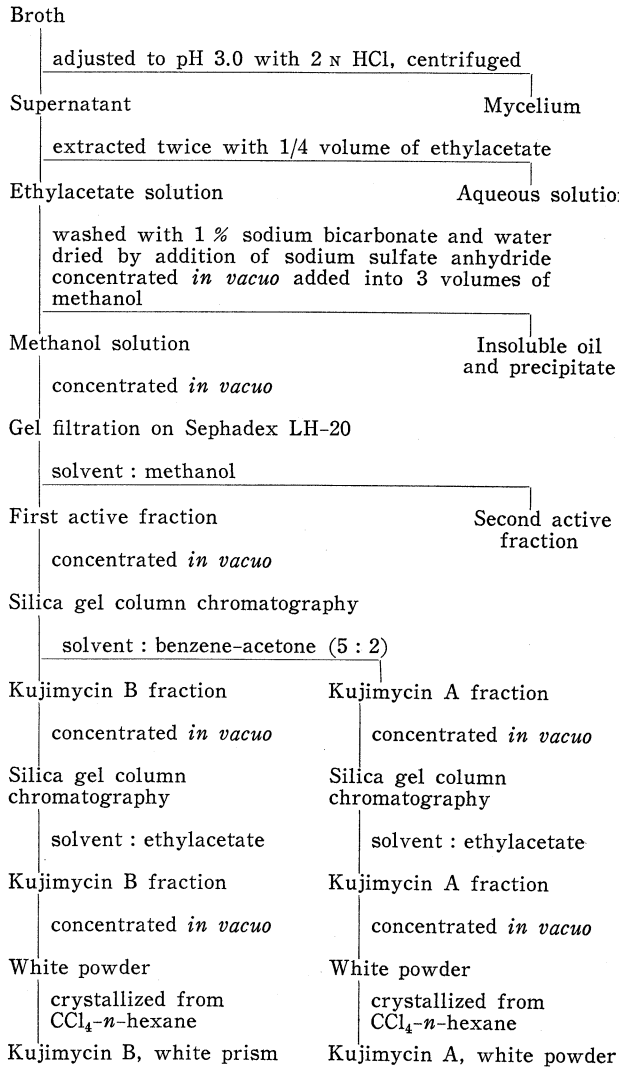
Isolation of Kujimycins A and B

Sixty liters of culture broth were adjusted to pH 3.0 with 2 N hydrochloric acid and centrifuged to separate the mycelium. The beer was extracted twice with 15 liters of ethyl acetate. The combined extracts were washed with 4 liters of 1% aqueous sodium bicarbonate solution and then with 4 liters of water. Ethyl acetate solution was dried by the addition of anhydrous sodium sulfate and concentrated to 50 g of brown syrup *in vacuo*. The syrup was dissolved in 150 ml of methanol to remove the insoluble oil and the precipitate.

Further purification of the antibiotics was carried out by gel filtration on Sephadex LH-20. The methanol solution from which the insoluble impurity was removed by filtration was concentrated to 50 ml *in vacuo* and charged on a column (35×550 mm) packed with 500 ml of Sephadex LH-20 using methanol. The active components were developed with methanol, and two active peaks were obtained by fractionation. The first active fraction contained two main components, kujimycins A and B, which

could be separated from each other by silica gel thin layer chromatography using the solvent system of benzene-acetone (1 : 1). The second active fraction contained other antibiotics. Two grams of yellowish white powder were obtained from the first active fraction by concentration *in vacuo*. This crude powder was dissolved in a small volume of benzene and charged on a silica gel column (120 g, 20×500 mm). After the column was washed with 200 ml of benzene, the active components were developed with benzene - acetone (5 : 2). Kujimycin B was eluted first, followed by kujimycin A. After each fraction of the effluent was confirmed to contain kujimycin A or kujimycin B by silica gel thin-layer chromatography, as developed with the solvent mixture of benzene - acetone (1 : 1), each fraction of kujimycins A and B was concentrated *in vacuo*. Each of the crude kujimycin A (450 mg) and kujimycin B (600 mg) was dissolved in ethyl acetate and charged on a silica gel column (90 g, 20×300 mm). Each of them was developed with ethyl acetate. The active fraction was concentrated *in vacuo*.

Fig. 1. Purification procedure of kujimycins A and B



Each of the dried materials (kujimycin A 330 mg, kujimycin B 430 mg) was crystallized from carbon tetrachloride - *n*-hexane or ether - *n*-hexane mixture. Two hundred and forty milligrams of kujimycin A was obtained as a white amorphous powder. Also, 350 mg of kujimycin B was obtained as white prisms (Fig. 1).

Physico-chemical Properties of Kujimycins A and B

Kujimycins A and B are both easily soluble in methanol, ethanol, butanol, acetone, ethyl acetate, butyl acetate, diethylether, benzene and chloroform, slightly soluble in hot water and carbon tetrachloride but insoluble in cold water, *n*-hexane and petroleum ether.

Kujimycin A was obtained as a white amorphous powder which melted at 114~

Fig. 2. Ultraviolet absorption spectra of kujimycins A and B.

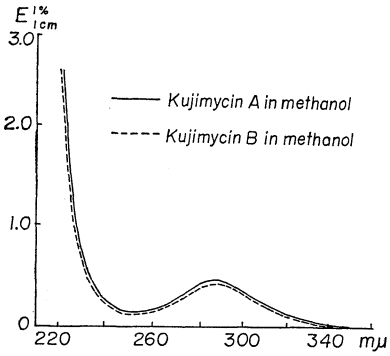


Fig. 3. Infrared absorption spectra of kujimycins A and B (KBr)

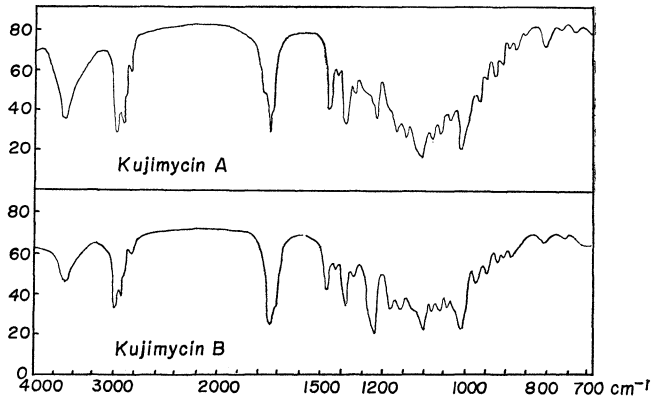


Table 1. The properties of kujimycins A and B on thin-layer chromatogram

Plate		Silica gel (0.25 mm)	Alumina (0.25 mm)	
Solvent system		Benzene-acetone (1 : 1)	Ethylacetate	Benzene-acetone (1 : 1)
Kujimycin A	Rf value	0.46	0.12	0.38
	Color of spot 1	Bluish green	Yellowish green	Yellowish green
	2	Violet	Bluish violet	Bluish violet
Kujimycin B	Rf value	0.62	0.55	0.65
	Color of spot 1	Brown	Dark violet	Dark violet
	2	Purple	Dark blue	Dark blue

Color 1: Colorization with concentrated sulfuric acid at 100°C for 5 minutes
Color 2: Colorization with anisaldehyde reagent at 100°C for 2 minutes

115°C. The vapor pressure determination of the molecular weight gave 780±20, and the data of elemental analysis favored a molecular formula of $C_{40}H_{70}O_{15}$ (Mol. wt. 791) for kujimycin A.

Calc'd : C 60.74, H 8.92 %
Found : C 60.66, H 8.87 %

The optical rotation is $[\alpha]_D^{20}$ −83° (*c* 1.0 in methanol), and the ultraviolet absorption spectrum shows a maximum at 289 mμ ($E_{1cm}^{1\%}$ 0.5) in methanol as shown in Fig. 2. The infrared absorption spectrum of kujimycin A in potassium bromide pellet shows the following frequencies: 3470 (B), 2980 (S), 2890 (Sh), 2830 (W), 1760 (Sh), 1735 (S), 1720 (Sh), 1460 (S), 1425 (W), 1380 (S), 1335 (W), 1230 (S), 1165 (B), 1145 (W), 1105 (B), 1075 (W), 1060 (W), 1035 (W), 1002 (S), 970 (W), 915 (S), 903 (Sh), 883 (W), 867 (W), 795 (S), 750 (W), 700 (W) cm^{-1} (S: strong, W: weak, B: broad, Sh: shoulder) (Fig. 3).

Kujimycin B was obtained as white prisms. It has a melting point at 178~180°C. The vapor pressure determination of the molecular weight gave 845±20, and the data of elemental analysis favored a molecular formula of $C_{42}H_{72}O_{16}$ (Mol. wt. 833) for kujimycin B.

Calc'd : C 60.56, H 8.71 %
Found : C 60.77, H 8.73 %

The optical rotation is $[\alpha]_D^{20}$ −78° (*c* 1.0 in methanol) and the ultraviolet absorption spectrum shows a maximum at 289 mμ ($E_{1cm}^{1\%}$ 0.42) in methanol as shown in Fig. 2. The infrared absorption spectrum of kujimycin B in potassium bromide pellet shows the following frequencies: 3470 (B), 2980 (S), 2940 (S), 2890 (Sh), 2830 (W), 1742 (S), 1720 (Sh), 1460 (S), 1420 (W), 1370 (S), 1335 (W), 1230 (S), 1165 (B), 1145 (W), 1120 (W), 1100 (S), 1075 (W), 1060 (W), 1042 (W), 1030 (W), 1005 (S), 970 (W), 945 (W), 920 (W), 903 (W), 880 (W), 805 (W) cm^{-1} (Fig. 3). Kujimycin A differs from kujimycin B in the absorption bands of 1760, 1120, 1042, 945, 880, 805 cm^{-1}. Both kujimycins give a positive reaction with anisaldehyde reagent (ethanol 18 ml, conc. H_2SO_4 1 ml, *p*-anisaldehyde 1 ml, as a spray on thin-layer plate and colorization at 100°C for 2 minutes) but negative reactions with FEHLING, biuret, ninhydrin, SAKAGUCHI and ferric chloride reactions. TOLLENS, MOLISCH, anthrone and SELIWANOFF reactions were positive but the existence of a sugar could not be presumed from the results because only the use of sulfuric acid or hydrochloric acid gave remarkable color. FISCHBACH-LEVINE reactions[2] were positive. Kujimycins A and B give a purple reaction in hydrochloric acid-acetone but violet in chloroform extract for the erythromycin test and give a yellow reaction in hydrochloric acid but pale yellowish green

Table 2. Antimicrobial spectra of kujimycins A and B

Test organism	Minimum inhibitory concentration (mcg/ml)		Test organism	Minimum inhibitory concentration (mcg/ml)	
	Kujimycin A	Kujimycin B		Kujimycin A	Kujimycin B
Staphylococcus aureus FDA 209P	3.13	6.25	*Sarcina lutea* NIHJ	0.4	0.4
Staphylococcus aureus Smith	3.13	6.25	*Bacillus subtilis* PCI 219	0.8	0.8
Staphylococcus aureus Komiya	6.25	12.5	*Bacillus megaterium* IAM 1030	0.4	1.6
Staphylococcus TPR-5 (SA, PC-R)	3.13	6.25	*Corynebacterium xerosis*	1.6	3.2
Staphylococcus TPR-14 (SA, PC, CP-R)	12.5	12.5	*Escherichia coli* B	>100	>100
Staphylococcus TPR-7 (SA, TC-R)	6.25	12.5	*Escherichia coli* K 12	>100	>100
Staphylococcus TPR-1 (SA, PC, TC-R)	3.13	6.25	*Klebsiella pneumonaiae*	>100	>100
Staphylococcus TPR-16 (SA, PC, TC, CP-R)	6.25	6.25	*Proteus vulgaris* HX 19	>100	>100
Staphylococcus TPR-3 (SA, PC, TC, SM-R)	6.25	12.5	*Pseudomonas aeruginosa*	>100	>100
Staphylococcus TPR-13 (SA, PC, CP, EM, OM-R)	6.25	12.5	*Salmonella enteritidis*	>100	>100
Staphylococcus TPR-21 (SA, PC, TC, SM, EM, OM, LM, SPM, LCM-R)	>100	>100	*Salmonella paratyphi* B	>100	>100
Staphylococcus TPR-20 (SA, PC, TC, SM, KM, em, om, lm, spm, lcm-R)	100	12.5	*Shigella sonnei* EW 33	>100	>100
Micrococcus flavus	1.6	0.8	*Mycobacterium phlei*	3.2	3.2
			Mycobacterium sp. 607	25	25
			Saccharomyces cerevisiae	>100	>100
			Penicillium chrysogenum	>100	>100
			Aspergillus niger	>100	>100

Asssay: Two-fold dilution method of glycerin bouillon agar

Abbreviations: SA: Sulfonamide, PC: Penicillin G, TC: Tetracycline, SM: Streptomycin, KM: Kanamycin, CP: Chloramphenicol, EM: Erythromycin, OM: Oleandomycin, LM: Leucomycin, SPM: Spiramycin, LCM: Lincomycin, R: Resistant, em, om, lm, spm, lcm: inducible resistant

in butanol extract for the carbomycin test respectively.

When Kujimycins A and B were examined by thin-layer chromatography using several solvent systems, a single spot on bioautograms with *Sarcina lutea* and on thin-layer plates colorized by concentrated sulfuric acid or anisaldehyde reagent was observed. Rf values and the color of spots of kujimycins A and B are shown in Table 1.

Biological Properties

Kujimycins A and B show inhibitory activities against Gram-positive bacteria. The sensitivity of some microorganisms to kujimycins A and B were determined by two-fold agar dilution method using the glycerin bouillon agar. The results of the antimicrobial test are shown in Table 2.

Though the acute toxicities of kujimycins A and B were examined by the maximum administration of 300 mg/kg intravenously in *ddY* strains of albino mice, all of 10 mice as a group tolerated the drug well, and none of the toxicities were observed at these doses.

Discussion

Kujimycins A and B were suggested to be members of the neutral macrolides by the following results.

1. The data of the molecular weight and the elemental analysis give 780 ± 20, $C_{40}H_{70}O_{15}$ for kujimycin A and 845 ± 20, $C_{42}H_{72}O_{16}$ for kujimycin B respectively.
2. The infrared spectra showing strong peaks at 1720~1760 cm^{-1} and 1230 cm^{-1} suggest the presence of a lactone and an acetyl group.
3. The FISCHBACH-LEVINE's tests are positive.
4. The antibiotics in some solvents do not transfer to the aqueous layer under acidic conditions.
5. The antibiotics mainly inhibit Gram-positive bacteria.

According to the above results, kujimycins A and B were compared with the descriptions of azalomycin B[3], aldgamycin E[4], aldgamycin E-like antibiotic[5], bandamycin A[6], bandamycin B[7], chalcomycin[8], elaiophylin[9], lankamycin[10], megacidin[11], mikonomycin[12], and neutramycin[13]. Kujimycins A and B can be differentiated from these known neutral macrolide antibiotics as listed above on the basis of physical, chemical and biological properties. Lankamycin comparatively resembled kujimycins A and B but gives irregular thick crystals[14] which show double melting points at 147~150°C and 181~182°C and such weak antimicrobial activity with minimum inhibitory concentrations above 100 mcg/ml against Gram-positive bacteria. As a contrast, kujimycin A is obtained as an amorphous powder and kujimycin B is obtained as prisms having a single melting point at 114~115°C and 178~180°C, respectively. Also their minimum inhibitory concentrations show levels of 0.4~12.5 mcg/ml against some Gram-positive bacteria. The infrared absorption spectrum of lankamycin is different from those of kujimycins A and B at 1634, 1497, 752 and 692 cm^{-1} [14]. The carbomycin test for lankamycin is reported to be colorless[15]. However kujimycins A and B give yellow reaction in hydrochloric acid and pale yellowish green reaction in butanol extract by the carbomycin test.

Acknowledgement

The authors wish to thank Mr. S. UEHARA, Excutive Vice President, Dr. S. IKAWA, Director and Dr. I. TANAKA, Manager of Research Department of Taisho Pharmaceutical Co., Ltd., for their encouragement.

References

1) NAMIKI, S.; S. OMURA, H. NAKAYOSHI & J. SAWADA: Studies on the antibiotics from *Streptomyces spinichromogenes* var. *kujimyceticus*. I. Taxonomic and fermentation studies with *Streptomyces spinichromogenes* var. *kujimyceticus*. J. Antibiotics 22 : ～ , 1969.
2) FISCHBACH, H. & J. LEVINE: The identification of the antibiotics. Antibiot. & Chemoth. 3 : 1159～1169. 1953.
3) ARAI, M.: Azalomycins B and F, two new antibiotics. II. Properties of azalomycins B and F. J. Antibiotics, Ser. A 13 : 51～56, 1960.
4) KUNSTMANN, M. P.; L. A. MITSCHER & E. L. PATTERSON: Aldgamycin E, a new neutral macrolide antibiotic. Antimicr. Agents & Chemoth. -1964 : 87～90, 1965.
5) HASEGAWA, T.; E. HIGASHIDE, M. SHIBATA, Y. KAMEDA & K. MIZUNO: Aldgamycin E-like antibiotic by *Streptomyces* sp. No. B-10000. Ann. Rept. Takeda Res. Lab. 25 : 15～23, 1966.
6) KONDO, S.; J. MARIE, J. SAKAMOTO & H. YUMOTO: Bandamycin, a new antibiotic. J. Antibiotics, Ser. A 14 : 365～366, 1961.
7) KONDO, S.; T. MIYAKAWA, H. YUMOTO, M. SEZAKI, M. SHIMURA, K. SATO & T. HARA: Isolation and characterization of a new antibiotic, bandamycin B. J. Antibiotics, Ser. A 15 : 157～160, 1962.
8) FROHARDT, R. P.; R. F. POTTILLO & J. EHRLICH: Chalcomycin and its fermentative production. U. S. Patent 3,065,137, Nov. 20, 1962.
9) ARCAMONE, F. M.; C. BERTAZZOLI, M. CHIONE & T. SCOTTI: Melanosporin and elaiophylin, new antibiotics from *Streptomyces melanosporus* (sive melanosporofaciens) n. sp. Giorn. Microbiol. 7 : 207～216, 1959.
10) GÄUMANN, E.; R. HÜTTER, W. KELLER-SCHIERLEIN, L. NEIPP, V. PRELOG & H. ZAHNER: Stoffwechselprodukte von Actinomyceten. 21 Mitteilung: Lankamycin und Lankacidin. Helv. Chim. Acta 43 : 601～606, 1960.
11) ETTLINGER, L.; E. GÄUMANN, R. HÜTTER, W. KELLER-SCHIERLEIN, F. KRADOLFER, L. NEIPP, V. PRELOG, P. REUSSER & H. ZAHNER: Stoffwechselprodukte von Actinomyceten. 11 Mitteilung: Megacidin. Monatsh. Chem. 88 : 989～995, 1957.
12) GÄUMANN, E.; V. PRELOG & E. VISCHLER: Mikonomycin. South African Patent 60/1853, 1960.
13) LEFEMINE, D. V.; F. BARBATSCHI, M. DANN, S. O. THOMAS, M. P. KUNSTMANN, L. A. MITSCHER & N. BOHONOS: Neutramycin, a new neutral macrolide antibiotic. Antimicr. Agents & Chemoth. -1963 : 41～44, 1964.
14) GÄUMANN, E.; V. PRELOG & E. VISCHLER: Lankamycin. Japanese Patent 16,700, Oct. 17, 1962.
15) HÜTTER, R.; W. KELLER-SCHIERLEIN & H. ZAHNER: Zur Systematik der Actonomyceten. 6. Die Produzenten von Makrolid-Antibiotica. Archiv für Mikrobiol. 39 : 158～194, 1961.