STUDIES ON STREPTOMYCETES*

LATERIOMYCINS A AND B, NEW ANTITUMOR AND ANTIBACTERIAL ANTIBIOTICS PRODUCED BY *STREPTOMYCES GRISEORUBER*, STRAIN NO. 71070

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A strain of *Streptomyces* (No. 71070) was isolated from a soil sample collected in Mexico. Taxonomic investigations led to its identity as a strain of *Streptomyces griseoruber* YAMAGUCHI and SABURI, 1955. From the mycelium of *S. griseoruber* strain No. 71070, two antibiotics were obtained in crystalline form and were named lateriomycins A and B, respectively. These antibiotics were especially active against Gram-positive bacteria and YOSHIDA sarcoma. Moreover, they are pH-indicator antibiotics changing from orange yellow in acidic solution to purple in alkaline solution. Comparison of the chemical and biological characteristics of lateriomycins A and B with known antibiotics indicated that they are new antibiotics.

In the course of screening for new antibiotics, it has been found that two new antitumor antibiotics are produced by a streptomycete isolated from a soil sample collected in Aqua Blanca, Mexico. The organism designated as strain No. 71070 was similar to *Streptomyces griseoruber* YAMAGUCHI and SABURI, 1955[1] in morphological and cultural characteristics with few exceptions, and was identified as a strain of that species.

The antibiotic complex produced by *S. griseoruber*, strain No. 71070, is especially active against Gram-positive bacteria and moreover exhibits antitumor activity. The two components of the complex were obtained in crystalline form, and were identified as new antibiotics when compared with known ones. They were named lateriomycin A and lateriomycin B, respectively.

This paper describes the taxonomic and antibacterial characteristics of *S. griseoruber*, strain No. 71070, and the isolation, physicochemical and biological properties of lateriomycins A and B.

Experimental and Results

I. Taxonomic and Antibacterial Characteristics

The aerial mycelium of *S. griseoruber*, strain No. 71070, develops well on glucose-asparagine agar. Its color is white at first and later changes to Pale Vinaceous Pink or Pale Mouse Gray[2]. The chains of spores form loops or coils representative of

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Table 1. Cultural characteristics of *Streptomyces griseoruber*, strain No. 71070

Medium	Characteristics
CZAPEK's agar	Growth (G); abundant, wrinkled, colorless, later Congo Pink (Rdg*. XXVIII, 7″-b). Aerial mycelium (AM); velety, chalk white, later Pallid Mouse Gray (Rdg. LI, 15‴″-f). Soluble pigment (SP); Light Pinkish Cinnamon (Rdg. XXIX, 15″-d).
CZAPEK's agar with glucose	G ; abundant, wrinkled, Light Vinaceous-Cinnamon (Rdg. XXIX, 13″-d) to Fawn Color (Rdg. XL, 13‴). AM ; thin, Pallid Vinaceous-Drab (Rdg. XLV, 5⁗-f). SP ; faint brown or none.
CZAPEK's agar with glycerin	G ; moderate, Pale Vinaceous-Drab (Rdg. XLV, 5⁗-d) to Dark Vinaceous-Drab (Rdg. XLV, 5⁗-i). AM ; thin, Pallid Purple-Drab (Rdg. XLV, 1⁗-f) to Pale Quaker Drab (Rdg. LI, 1‴″-d). SP ; faint brown.
Glucose-asparagine agar	G ; abundant, Buff-Pink (Rdg. XXVIII, 11″-d) to Pale Congo Pink (Rdg. XXVIII, 7″-f). AM ; velvety, Pale Vinaceous-Pink (Rdg. XXVIII, 9″-f) to Pale Mouse Gray (Rdg. LI, 15⁗″-d). SP ; Salmon-Buff (Rdg. XIV, 11′-d).
Nutrient agar	G ; moderate, colorless later dark brown. AM ; poor, powdery, white to Pale Mouse Gray. SP ; dark brown.
Glucose-nutrinet agar	G ; abundant, wrinkled, dark brown. AM ; thin, chalk white to Pallid Quaker Drab (Rdg. LI, 1‴″-f). SP ; dark brown.
Glycerin-nutrient agar	G ; abundant, wrinkled, dark brown. AM ; poor, chalk white. SP ; dark brown.
Glucose-nutrient broth	G ; moderate, faint brown film without sediment. AM ; white to Pallid Mouse Gray. SP ; blackish brown.
Starch agar	G ; abundant, Pallid Vinaceous Drab (Rdg. XLV, 5⁗-f) to Vinaceous-Lavender (Rdg. XLIV, 65‴-f). AM ; abundant, velvety, white to Vinaceous-Lavender. SP ; Pale Persian Lilac (Rdg. XXXVIII, 69″-f) or none.
Egg (37°C)	G ; abundant, spreading, brownish black. AM ; poor, white to Pale Quaker Drab. Color of medium becomes milky white.
Yeast extract agar	G ; abundant, wrinkled, Buff-Pink (Rdg. XXVIII, 11″-d) to brown. AM ; abundant, velvety, chalk white to Pallid Mouse Gray. SP ; Army Brown (Rdg. XL, 13‴-i).
Potato plug	G ; abundant, wrinkled, Pale Purple-Drab (Rdg. XLV, 1⁗-d) to Pale Quaker Drab. AM ; poor, Mouse Gray (Rdg. LI, 15⁗″). SP ; dark brown.
Carrot plug	G ; abundant, wrinkled. AM ; poor, white to Orient Pink (Rdg. II, 9-f) to Light Mouse Gray. SP ; faint brown.
Milk (37°C)	Cream colored ring formed, weak peptonization without coagulation.
Nutrient gelatin (25°C)	G ; poor, lichenoid, dark brown. AM ; poor, white to Mouse Gray. Liquefaction slow.

(To be continued)

Table 1 (Continued)

Medium	Characteristics
Nitrate reduction	No reduction.
Cellulose	No growth.
Hydrolysis on starch agar	Hydrolysis, growth zone/enzymatic zone=11~13 mm/15~17 mm.
Calcium malate agar	G ; abundant, Pale Salmon Color (Rdg. XIV, 9′-f) to Flesh Color (Rdg. XIV, 7′-d). AM ; abundant, velvety, white to Mouse Gray. SP ; none or Light Pinkish Cinnamon (Rdg. XXIX, 15″-d).
Tyrosine agar	G ; thin, colorless to Ivory Yellow (Rdg. XXX, 21″-f). AM ; none. SP ; none.
Peptone agar	G ; thin, spreading, dark brown. AM ; thin, powdery, white to Mouse Gray. SP ; dark brown.

* Rdg.: R. RIDGWAY, Color Standard and Color Nomenclature. Published by the author, Washington, D. C., 1912.

Table 2. Carbon source utilization of *Streptomyces griseoruber*, strain No. 71070

Carbon sources	Growth	Carbon sources	Growth
Erithritol	−	Sucrose	+++
D-Sorbitol	+	Lactose	+++
D-Inositol	+++	Raffinose	+++
D-Mannitol	+++	Trehalose	+++
Dulcitol	±	Salicin	−
D-Ribose	++	Inulin	+++
D-Xylose	+++	Cellobiose	+++
L-Arabinose	+++	Glycerin	+++
D-Galactose	+++	Na-acetate	+
D-Glucose	+++	Na-succinate	++
D-Fructose	+++	Na-citrate	+
L-Rhamnose	+++	Control (No carbon)	−
D-Maltose	+++		

Basal medium (g/liter): $(NH_4)_2SO_4$, 2.64; KH_2PO_4, 2.38; K_2HPO_4, 5.65; $MgSO_4 \cdot 7H_2O$, 1.00; $CuSO_4 \cdot 5H_2O$, 0.0064; $FeSO_4 \cdot 7H_2O$, 0.0011; $MnCl_2 \cdot 4H_2O$, 0.0079; $ZnSO_4 \cdot 7H_2O$, 0.0015 and agar, 15.00.

+++; Very good growth, ++; Good growth, +; Growth, ±; Faint growth, −; No growth.

Table 3. Antibacterial spectrum of *Streptomyces griseoruber*, strain No. 71070, by cross-streak method

Test organisms	Inhibition length (mm) of zone			
	Nutrient agar		Glycerin-nitrient agar	
Escherichia coli	3	3	2	2
Proteus vulgaris	0	0	7	6
Staphylococcus aureus	13	13	6	7
Bacillus subtilis	14	15	8	8
Bacillus cereus	11	11	0	0
Bacillus brevis	13	13	0	0
Sarcina lutea	17	18	7	6
Micrococcus flavus	14	15	7	7
Aerobacter aerogenes	0	0	0	0
Pseudomonas aeruginosa	0	0	3	3
Mycobacterium avium			0	0
Mycobacterium smegmatis			0	0
Mycobacterium phlei			0	0

Sections *Retinaculum-Apertum* or *Spira* of PRIDHAM *et al.*[3] and the spores are ellipsoidal or oval (0.5~0.8 μ × 1.0~1.3 μ) with smooth surfaces. The cultural characteristics of strain No. 71070 are shown in Table 1. On chemically-defined media the vegetative mycelium of strain No. 71070 develops well. It is colorless at first, but changes to pale orange or pale pink. A pale yellowish brown or pale pink soluble pigment is produced. On protein-containing media the color of the vegetative mycelium is pale brown or dark brown, and white or Pallid Mouse Gray aerial mycelium is formed. The soluble pigment is brown or blackish brown. Accordingly, strain No. 71070 is considered to be chromogenic. The utilization of carbon sources of strain No. 71070

was investigated by the method of PRIDHAM and GOTTLIEB[4]. The results are shown in Table 2.

The antibacterial spectrum of strain No. 71070 was determined by the cross-streak method using nutrient agar and glycerin-nutrient agar. It was found that the organism exhibited activity mainly against Gram-positive bacteria (Table 3).

Among the known species of *Streptomyces*, *S. fervens*[5] DEBOER *et al.*, 1960, *S. purpurescens*[6] LINDENBEIN, 1952, and *S. griseoruber* YAMAGUCHI and SABURI, 1955 were considered similar to strain No. 71070 in cultural characteristics. However, some differences were noted in their morphological characteristics. *Streptomyces fervens* is verticillate, and the spores of *S. purpurescens* have echinulate surfaces, our strain has no such morphological characteristics. On the other hand, strain No. 71070 resembled *S. griseoruber* in cultural and morphological characteristics.

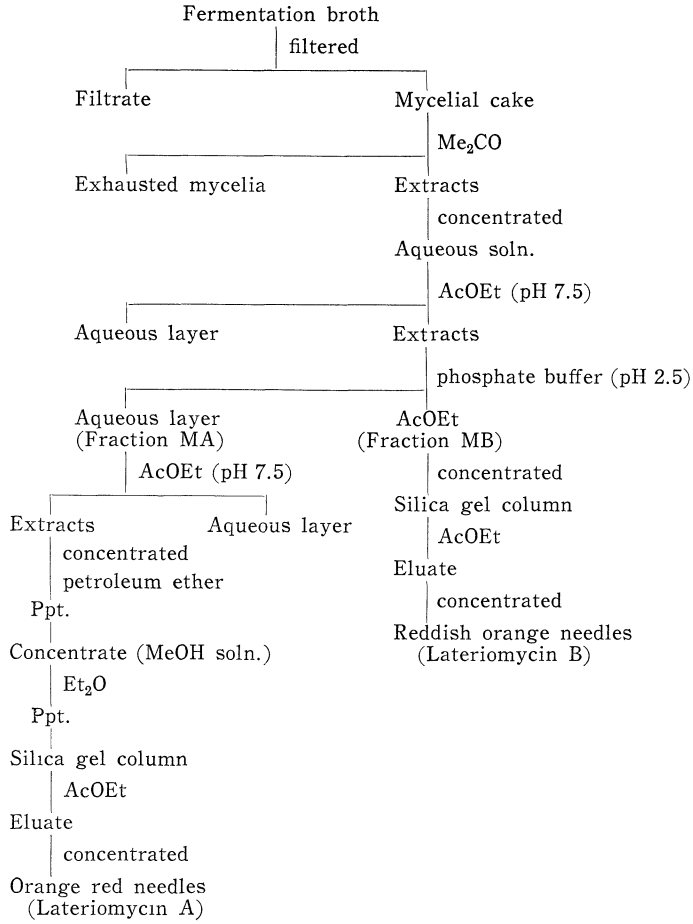
Based on the above-mentioned similarities and differences, strain No. 71070 was identified as *S. griseoruber* YAMAGUCHI and SABURI, 1955.

II. Production and Isolation of Lateriomycins A and B

(1) Production

The antibiotic complex was produced in a medium (adjusted to pH 7.0) containing 3.0 % soluble starch, 2.0 % soy bean flour, 1.0 % meat extract and 0.5 % sodium chloride. For a typical run, 500 liters of the medium in a 1,000-liter stainless steel fermentor was inoculated with 30 liters of a seed culture. The fermentation was carried out at 28°C for four days with aeration (500 liters/min.) and agitation (160 r.p.m.). When the fermentation was completed, as determined by bioassay of mycelial activity and culture filtrate using *Bacillus subtilis* in a paper disc assay, the mycelium was harvested and washed. Preliminary experiment showed that two or three times as much antibiotic was present in the mycelium as in the cultural filtrate.

Chart 1. Isolation of lateriomycins



(2) Isolation

The extraction and purification procedure for lateriomycins A and B is summarized in Chart 1.

The mycelial cake (70 kg) obtained by filtration of the culture broth (480 liters) was extracted with acetone (150 liters) with stirring for one hour. The extract was concentrated *in vacuo* to about one-thirtieth of the original volume. The concentrated solution was adjusted to pH 7.5 and extracted three times with ethyl acetate (1/3 vol.). The extract was reextracted thrice with 0.1 M phosphate buffer (adjusted to pH 2.5 with dil. HCl) (1/3 vol.). The active material was divided into a basic fraction (MA) and a neutral fraction (MB). After MA was adjusted to pH 7.5, it was reextracted with ethyl acetate. The extract was washed twice with distilled water (1/10 vol.) and concentrated *in vacuo*, and a large amount of petroleum ether was added to this concentrated solution, yielding an orange yellow precipitate. The precipitate was dissolved in a small amount of methanol and reprecipitated by the addition of diethyl ether to the solution with vigorous stirring. This second precipitate was recovered by filtration and air-dried. Fraction MA thus obtained is an orange yellow powder (*ca.* 2.5 g). Crude fraction MA powder (1 g) was purified by column chromatography on silica gel (Merck) by development with benzene and elution with ethyl acetate. The active effluents were collected and concentrated *in vacuo* to yield orange red needles. The crystalline product was recrystallized from a mixture of ethyl acetate and methanol (2 : 1, v/v) giving pure lateriomycin A (*ca.* 200 mg) as orange red needles (Plate 1).

Plate 1. Crystals of lateriomycin A

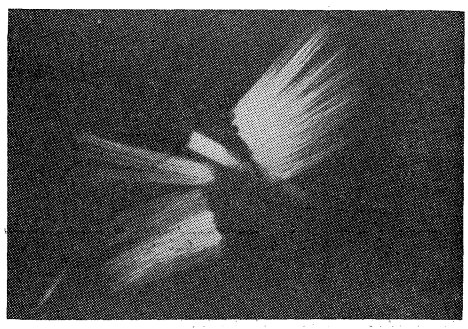
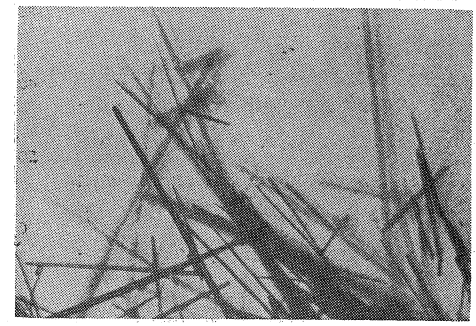


Plate 2. Crystals of lateriomycin B



Fraction MB was washed with distilled water and concentrated *in vacuo*, giving a dark reddish oily substance (300 ml). The oily substance was dissolved in a small amount of methanol and purified by chromatography on silica gel. After impurities were removed by elution with petroleum ether and diethyl ether, the main active fraction was eluted with ethyl acetate. Reddish orange needles were obtained by concentration of the active eluate under reduced pressure. Pure lateriomycin B (200 mg) was obtained as orange needles by recrystallization from a mixture of methanol and ethyl acetate (1 : 1) (Plate 2).

III. Physicochemical and Biological Properties of Lateriomycins A and B

(1) Lateriomycin A

Lateriomycin A occurs as orange red needles and melts with decomposition at 202~206°C. It is soluble in methanol, ethanol, 1-butanol, acetone, ethyl acetate,

chloroform and benzene; is slightly soluble in diethyl ether; but is insoluble in petroleum ether and water.

The results of elementary analyses were as follows: C, 60.13, 60.15, 60.18; H, 5.84, 6.06, 6.02; N, 1.90, 1.92, 2.01.

The molecular weight was found to be 1,151 or 1,330 by osmometry (in $CHCl_3$).

The optical rotation was found to be $[\alpha]_D^{21}$ +186° (*c* 0.3, in $CHCl_3$). The antibiotic gave positive MOLISCH and ferric chloride tests, and negative ninhydrin, FEHLING, biuret, EHRLICH and PAULY tests. The ultraviolet and visible absorption spectra are shown in Figs. 1 and 2. Maximum absorptions were shown at 234 mμ ($E_{1cm}^{1\%}$ 581), 253 mμ ($E_{1cm}^{1\%}$ 403), 290 mμ ($E_{1cm}^{1\%}$ 131), 480 mμ ($E_{1cm}^{1\%}$ 176) with a shoulder at 530 mμ in methanol. The infrared absorption spectrum is shown in Fig. 3. Lateriomycin A is orange yellow in acidic methanol solution and purple in alkaline solution.

The antibacterial spectra of lateriomycins A and B were studied by using nutrient agar with Gram-positive and Gram-negative bacteria as the test organisms and incubation for 24 hours at 37°C. For acid-fast bacteria, glycerin-

Fig. 1. Ultraviolet absorption spectrum of lateriomycin A (MeOH)

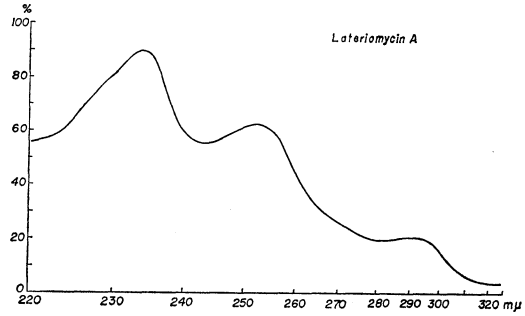


Fig. 2. Visible absorption spectrum of lateriomycin A (MeOH)

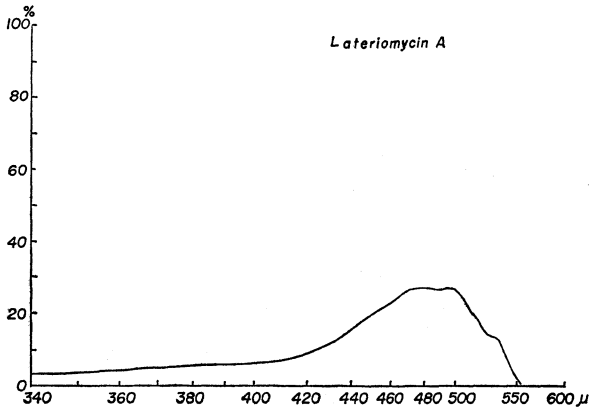
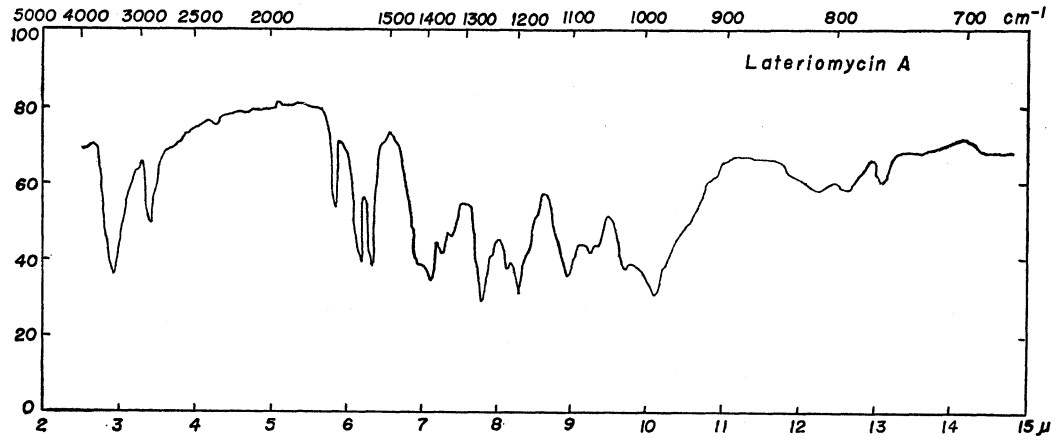


Fig. 3. Infrared absorption spectrum of lateriomycin A (KBr)



nutrient agar was used with incubation for 48 hours, at 37°C. For molds and yeasts, glucose-nutrient agar was used with incubation for 48 hours at 28°C. The results are presented in Table 4. Lateriomycin A exhibited strong activity against Gram-positive bacteria (MIC; 0.05~0.5 mcg/ml), weak activity against mycobacteria (MIC; 10~20 mcg/ml) and no activity against Gram-negative bacteria, molds and yeast (MIC; >100 mcg/ml). Moreover, it exhibits inhibitory activity against YOSHIDA sarcoma and sarcoma 180.

Acute toxicity studies with mice showed that lethal doses were 0.4~0.8 mg/kg (intra-peritoneal administration).

(2) Lateriomycin B

Lateriomycin B occurs as reddish orange needles and melts with decomposition at 234~236°C. The antibiotic is soluble in methanol, ethanol, 1-butanol, acetone, chloroform and benzene; but is insoluble in petroleum ether and water.

The results of elementary analyses were as follows: C, 65.21; H, 5.22; O, 30.09. The molecular weight was found to be 410 by osmometry (in $CHCl_3$). The optical rotation was $[\alpha]_D^{22}$ $-68°\pm4$ (c 0.06, in $CHCl_3$). The antibiotic gave positive MOLISCH and ferric chloride tests, and negative biuret and FEHLING tests. The ultraviolet and visible absorption spectra

Fig. 4. Ultraviolet absorption spectrum of lateriomycin B (MeOH)

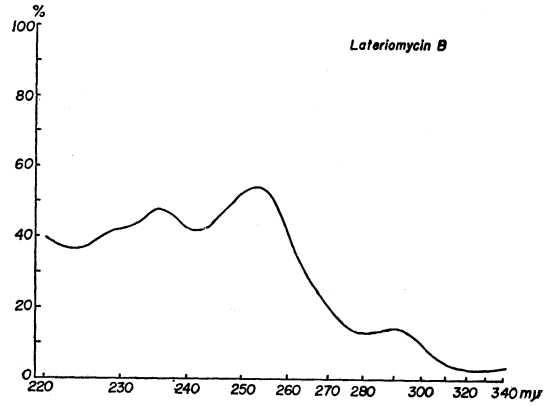


Fig. 5. Visible absorption spectrum of lateriomycin B (MeOH)

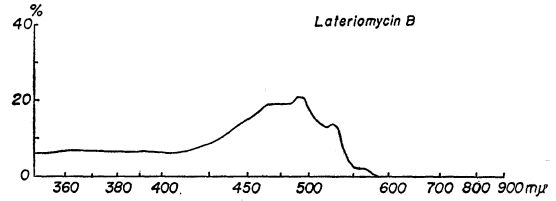
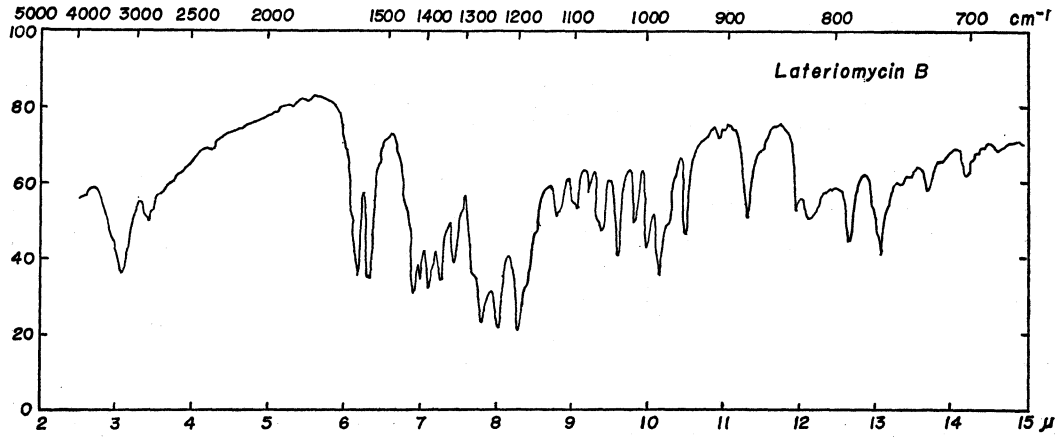


Fig. 6. Infrared absorption spectrum of lateriomycin B (KBr)



are shown in Figs. 4 and 5. Maximum absorptions were shown at 236 mμ ($E^{1\%}_{1cm}$ 716), 253 mμ ($E^{1\%}_{1cm}$ 864), 290 mμ ($E^{1\%}_{1cm}$ 227), 490 mμ ($E^{1\%}_{1cm}$ 348) and 529 mμ ($E^{1\%}_{1cm}$ 226) in methanol. The infrared absorption spectrum is shown in Fig. 6. Lateriomycin B, like lateriomycin A, is orange yellow in acidic methanol solution and purple in alkaline solution. As shown in Table 4, lateriomycin B exhibited antibiotic activity against Gram-positive bacteria, but its activity was one-twentieth to one-fiftieth that of lateriomycin A. It exhibited considerable tumor inhibition with YOSHIDA sarcoma tests. The LD_{50} of lateriomycin B in mice was 6.25 mg/kg (intraperitoneal administration).

Table 4. Antibacterial spectra of lateriomycins A and B

Test organisms	Minimum inhibitory concentration mcg/ml	
	Lateriomycin A	Lateriomycin B
Escherichia coli	>100	>100
Proteus vulgaris	>100	>100
Pseudomonas aeruginosa	>100	>100
Bacillus subtilis	0.1	2~5
Bacillus cereus	0.2	5
Bacillus brevis	0.2	2~5
Staphylococcus aureus	0.5	5~10
Sarcina lutea	0.05	5
Micrococcus flavus	0.05	2
Mycobacterium avium	20	20
Mycobacterium sp. ATCC 607	20	>100
Mycobacterium smegmatis	10	10
Mycobacterium phlei	20	>100
Saccharomyces cerevisiae	>100	>100
Candida albicans	>100	>100
Penicillium chrysogenum	>100	>100
Aspergillus niger	>100	>100

Lateriomycins A and B exhibited stronger activity on basic assay media than on neutral or acidic media. Therefore, they would be classified as physiologically basic substances[7]. The antitumor activities of lateriomycins A and B will be reported in detail elsewhere.

Discussion

Lateriomycins A and B turn orange yellow in acidic solution and purple in alkaline solution. Therefore they are classified as pH-indicator antibiotics. Also, they are especially active against Gram-positive bacteria and exhibit antitumor activity. Lateriomycin A contains nitrogen in its molecule. Lateriomycin B does not.

Based on its characteristics, lateriomycin A was found to resemble the following antibiotics; antibiotic 289[8], antibiotic SKCC 1377[9], danubomycin[10], daunomycin[11], luteomycin[12], mezzanomycin (antibiotic P-575[13]), miromycin (antibiotic P-285[14]), pluramycin[15], ractinomycin A[16], rubidomycin[17], rubomycin[18] and vinacetin[19]. Antibiotic 289 and luteomycin differ from lateriomycin A with respect to ultraviolet spectra and result of the Molisch test. Antibiotic SKCC 1377 is water soluble. Differences between lateriomycin A and danubomycin exist in regard to elementary analyses. Lateriomycin A clearly differs from mezzanomycin and miromycin in its color reactions, antibacterial spectrum and ultraviolet absorption spectrum. Differences between lateriomycin A and pluramycin exist in regard to ultraviolet absorption spectra, visible light absorption spectra and result of the ferric chloride test. Ractinomycin A definitely differs from lateriomycin A in its antibacterial and infrared spectra. Daunomycin, rubidomycin and rubomycin differ from lateriomycin A in elementary analyses and antibacterial spectra. Also, vinacetin is active against mycobacterium and is more active in acid state than in alkaline, thus differing from lateriomycin A.

Based on its characteristics, lateriomycin B resembles ayamycin A_2[20], collinomycin[21], minomycin[22] and resistomycin[23] because of pH-indicator characteristics. Lateriomycin B

obviously differs from these antibiotics in its elememtary analysis, ultraviolet spectrum, infrared spectrum, color reactions and antimicrobial spectrum.

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