A NEW ANTIBIOTIC K-82 A AND MINOR COMPONENTS, PRODUCED BY *STREPTOMYCES LAVENDULAE*, STRAIN NO. K-82

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From the results of taxonomic studies, *Streptomyces* sp. strain No. K-82 isolated from a soil sample collected in Kumamoto city, was identified as a strain belonging to *Streptomyces lavendulae* WAKSMAN & HENRICI 1948. The strain produced an active new antibiotic called K-82 A and minor components named the B complex. Antibiotic K-82 A was isolated as dark reddish needles by silica gel column chromatography and found to have both antibacterial activity and high phage induction activity. The K-82 B complex was found to consist of at least five components, among which K-82 B_2 and B_3 were isolated as crystals. Substance K-82 B_2 was identified as benzoic acid from its physicochemical properties. Substance B_3 like B_2 had only marginal antibiotic activity.

In the course of our screening for new antibiotic, *Streptomyces* sp., strain No. K-82 was found to be highly active against Gram-positive and Gram-negative bacteria. Several active components were isolated in crystalline form and their biological and physicochemical properties were clarified. The present paper deals with the taxonomic studies of *Streptomyces* sp., strain No. K-82, fermentation, isolation, and physicochemical properties of the antibiotics.

Materials and Methods

Mycological properties

The organism was examined by a light microscope (L type, Nihon Kogaku K. K.) and an electron microscope (JEM-50 B, Japan Electron Optics Laboratory, Co., Ltd.) every 7 days after incubation on modified glucose asparagine agar at 28°C. The methods and media for examination of cultural and physiological properties were made according to the recommendation of the International Streptomyces Project.[1] Color determinations and examinations of carbon utilization of the culture were made according to RAYNER's description[2] and PRIDHAM's method[3], respectively.

Fermentation

Shaking culture fermentation were carried out with 50 ml of a medium in 200-ml Erlenmeyer flasks. Spores from a slant culture were inoculated into a seed culture medium containing 5% starch, 0.5% peptone, 1% soybean flour, 1% corn steep liquor, 0.5% NaCl and 0.3% $CaCO_3$. After incubation for 24 hours at 34°C, the resultant culture was inoculated into the same main culture medium as described above, with the inoculum size of 4%. Fermentations were carried out for 96 hours at 34°C on a rotary shaker. Jar fermentations were carried out for 48 hours at 34°C with stirring (350 rpm) and under aeration (20 liters/min). Six hundred ml of the seed culture, prepared as described above, were inoculated in a 30-liter jar-fermentor containing 14 liters of the medium.

Antibiotic Assay

(1) Antibacterial activity: The conventional serial agar dilution method and cup or paper disk method were applied in this study using *Staphylococcus aureus* or *Sarcina lutea* as a test organism.

(2) Phage induction activity: The serial phage induction-agar dilution method[4] was applied in this study, using *E. coli* K-12 λ as a lysogenic strain. The assay medium consisted of 1% peptone, 0.2% casamino acid, 0.2% NaCl and 0.6 or 1.2% agar.

Thin-layer and paper chromatography

Thin-layer plates were prepared with a Desaga applicator from silica gel G (Type 60, Merck). The detection of the antibiotic was made by bioautography on bouillon agar seeded with *S. aureus*.

Results and Discussion

Taxonomic Characteristics of *Streptomyces* sp., Strain No. K-82

The spore chains terminate in loops and in spirals with a few turns. Spores are oval with smooth surface. In general, the aerial mass color is pink to vinaceous and melanoid pigments are observed on proteinaceous media. In carbon-source utilization studies, good growth or moderate growth was observed with D-glucose, D-fructose, L-arabinose. No growth was observed with D-xylose, *i*-inositol, D-mannitol, raffinose, sucrose and rhamnose. The growth of the strain occurred between 20° and 43°C and its optimum temperature was 37°C. The growth and sporulation occur at pH's ranging from 5.0 to 8.0 and its optimum pH was 7.0.

These properties were compared with the species descriptions in International Streptomyces Project and BERGEY's Manual of Determinative Bacteriology, 8th ed.[5] From the melanoid pigment formation, spore surface and spore chain morphology, the strain was identified as a strain belonging to *Streptomyces lavendulae* WAKSMAN & HENRICI 1948.

Production and Isolation of Antibiotic K-82 A and the K-82 B Complex

The culture filtrate (10 liters) was adjusted to pH 2.0 with 6 N HCl and extracted two times with ethylacetate at one fourth volume of the filtrate. After washing and dehydration with Na_2SO_4, the extract were concentrated *in vacuo* and fractionated with ether into ether-soluble and ether-insoluble fractions. The insoluble fraction was dissolved in methanol and reprecipitated with ether. The precipitate was filtered off, followed by vacuum drying to give 1 g of crude K-82 A and the filtrate plus ether-soluble fractions were combined and concentrated *in vacuo* to give crude K-82 B. The antibiotic K-82 A was then subjected to column chromatography on silica gel. The column was eluted with solvent mixture I ($CHCl_3$ - MeOH - AcOH, 30: 1: 1). The active fractions were concentrated *in vacuo* to give a dark reddish powder of partially purified K-82 A. The partially purified K-82 A was then dissolved in a small amount of 1 N ammonium hydroxide and after addition of methanol, acidification with acetic acid gave dark reddish needles of purified K-82 A.

On the other hand, the crude K-82 B complex was found to contain, at least, five components by thin-layer chromatography and these were named K-82 B_1, B_2, B_3, B_4 and B_5 in the order of increasing Rf values. Purification of these components was also made by silica gel column chromatography. Using solvent mixture II (C_6H_6 - AcOEt, 3: 1), the K-82 B_2 and B_3 fractions were separated from the K-82 B_4 and B_5 fractions. Further purifications of the former two fractions were made by silica gel column chromatography using solvent mixture III (C_6H_6 - AcOEt, 9: 1). The active fractions were concentrated *in vacuo* to give light yellow oils of partially purified K-82 B_2 and B_3. The partially purified K-82 B_2 and B_3 were rechromatographed on another silica gel column. Elution with solvent mixture IV (C_6H_6 - Et_2O, 9: 1), followed by evaporation gave pure colorless prisms of K-82 B_2 and yellow needles of K-82 B_3.

Crude K-82 B_4 and B_5 were similarly purified by column chromatography on silica gel. K-82 B_4

and B_5 were eluted with solvent mixture II and V (C_6H_6 - AcOEt, 1: 1), respectively. The B_5 fraction was concentrated *in vacuo* and rechromatographed on active carbon column and elution with solvent mixture VI (AcOEt - MeOH, 1: 1) and gave a light yellow oil (K-82 B_5).

Physicochemical Properties

Physicochemical properties of K-82 A, B_2 and B_3 are shown in Table 1. K-82 A gave negative MOLISCH, anthrone, FEHLING, BENEDICT, ninhydrin, ferric chloride, ELSON-MORGAN, biuret, xanthoprotein, PAULI, SAKAGUCHI and EHRLICH reactions. No conclusion about a molecular weight determination of K-82 A could be drawn from mass-spectrometry, although ion peaks at *m/z* 398 and *m/z* 354 were observed in the higher mass units region. Further investigation is necessary. The ultraviolet absorption and infrared absorption spectra of K-82 A, B_2 and B_3 are shown in Figs. 1 and 2, and Figs. 3 and 4, respectively. K-82 B_2 was identified as benzoic acid, $C_7H_6O_2$ from the elementary analysis and infrared spectrum. The Rf values of these antibiotics on TLC are given in Table 2.

Table 1. Physicochemical properties of antibiotics K-82 A, B_2 and B_3.

	A	B_2	B_3
Appearance	dark reddish needle	colorless prism	yellow needle
Melting point (°C)	210~230 (dec.)	106~109	150~153
$[\alpha]_D^{25}$	+20 (*c* 0.1, MeOH)	—	—
pKa′	6.45 (MeOH)	—	—
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	230 (790)	228 (728)	287 (64.0)
	250 (787.5)	273 (93.2)	
	280 (525)	300 (46.8)	
	390 (306)		
Elemental analysis			
Found (%)	C: 62.65	C: 68.57	C: 56.58
	H: 3.65	H: 5.31	H: 5.37
	N: 11.85	N: 0.00	N: 0.00
Molecular weight	—	122*	—
Solubility			
Soluble in	alkaline water N,N-dimethylformamide	most organic solvents	most organic solvents
Slightly soluble in	lower alcohols	*n*-hexane	*n*-hexane
Insoluble in	acidic water, ether, *n*-hexane	water	water

* Mass-spectrometry.

Fig. 1. UV absorption spectra of antibiotic K-82 A.

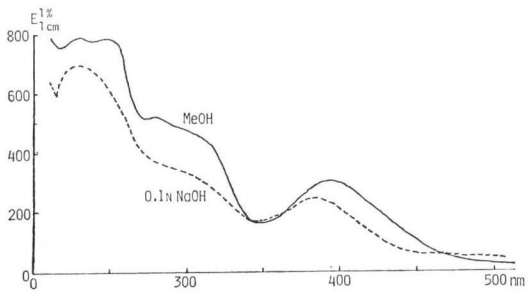

Fig. 2. UV absorption spectra of antibiotics K-82 B_2 and B_3 (MeOH).

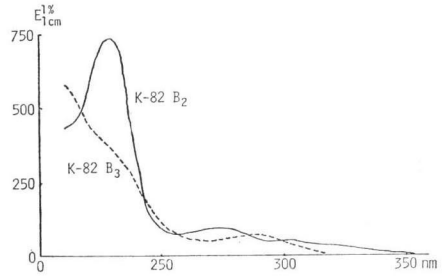

Fig. 3. Infrared absorption spectra of antibiotic K-82 A and rufochromomycin (KBr).

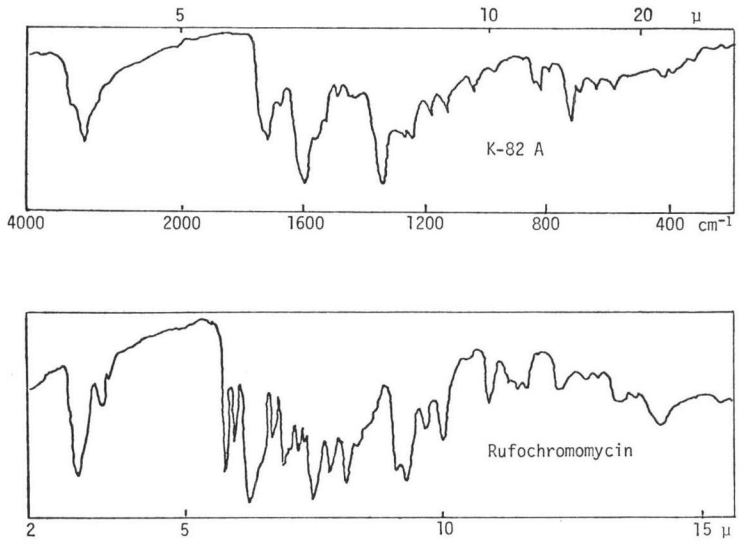

Fig. 4. Infrared absorption spectra of antibiotics K-82 B_2 and B_3 (KBr).

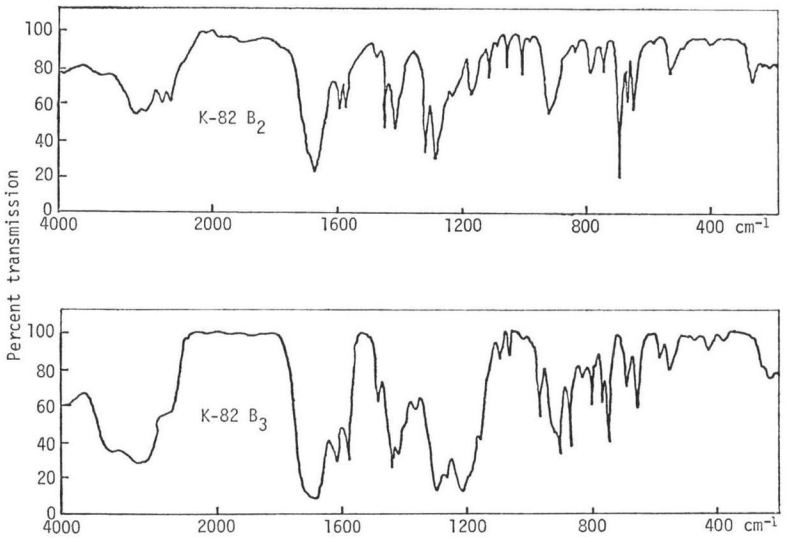

Table 2. Rf values of antibiotics K-82 A, B_2 and B_3.

Solvent system	Rf		
	A	B_2	B_3
$CHCl_3$-MeOH-AcOH (20 : 1 : 1)	0.34	—	—
AcOEt-BuOH-AcOH (10 : 1 : 1)	0.48	—	—
C_6H_6-AcOEt (3 : 1)	0.00	0.43	0.21
C_6H_6-AcOEt (9 : 1)	0.00	0.30	0.05
C_6H_6-AcOEt-MeOH (30 : 1 : 1)	0.00	0.66	0.41

Biological Properties

(1) Antimicrobial activity

The antimicrobial spectra of K-82 A, B_2 and B_3 determined by dilution method on bouillon agar are shown in Table 3. K-82 A was active against Gram-positive and Gram-negative bacteria and in particular, highly active against *B. brevis* and *Sarcina lutea*, whereas substance K-82 B_2 and B_3 showed only weak activities.

Table 3. Antibacterial spectra of antibiotics K-82 A, B_2 and B_3.

Test organism	MIC (mcg/ml)		
	A	B_2	B_3
Escherichia coli IFO 3301	2.0	500	500
Proteus vulgaris IFO 3167	2.0	500	500
Pseudomonas aeruginosa IFO 3923	5.0	500	500
Bacillus brevis IFO 3331	0.2	250	250
Bacillus cereus IFO 3466	1.0	500	500
Bacillus subtilis PCI 219	0.5	500	500
Sarcina lutea IFO 3232	0.5	500	500
Staphylococcus aureus FDA 209 P	0.5	500	500
Mycobacterium avium IFO 3082	2.0	500	500

Table 4. Phage induction activities of K-82 A, bleomycin, mitomycin C and xanthomycin.

	Induction activity (unit/mg)	Antibacterial activity
K-82 A	2.0×10^8	Gram (+), (−) *Mycobacteria*
Bleomycin	3.5×10^5	Gram (+), (−) *Mycobacteria* Fungi
Mitomycin	1.0×10^7	Gram (+), (−)
Xanthomycin	2.0×10^4	Gram (+), (−) *Mycobacteria*

(2) Phage induction activity

The phage induction activity was measured by phage induction-agar dilution method[4]. The activity was expressed as the highest dilution for two times spontaneous phage count. K-82 A showed strong phage induction activity, that is, 2×10^6 units/mg but K-82 B_2 and B_3 showed no activity (Table 4).

Comparison of Antibiotic K-82 A with Known Antibiotics

From the nature, elementary analysis and antimicrobial activity of K-82 A, the antibiotic seemed to resemble rufochromomycin[6]. K-82 A showed, however, absorption maxima [λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)] at 230 (790), 250 (787.5), 280 (525) and 390 (306) in its ultraviolet absorption spectrum. Rufochromomycin showed absorption maxima at 247 (750) and 382 (322). Furthermore, K-82 A was differentiated from rufochromomycin by its infrared spectra as shown in Fig. 3. From these findings K-82 A is believed to be a new antibiotic.

Acknowledgements

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