KOBENOMYCIN, A NEW POLYPEPTIDE ANTIBIOTIC

SABURO OKAMOTO, MIKAO MAYAMA, YU TANAKA,
KATSUYA TAWARA, NOBORU SHIMAOKA, HIROSHI KATO,
HARUO NISHIMURA, MITSUO EBATA and HIDEO OHTSUKA

Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka, Japan

(Received for publication January 21, 1968)

A new colorless crysalline polypeptide antibiotic, kobenomycin, has been isolated from the culture broth of *Streptomyces kobenensis*, nov. sp. The morphological, cultural and physiological characteristics of this organism, and the isolation and properties of the new antibiotic are described. Kobenomycin is highly active only against aerobic sporulating bacilli and moderately active against acid-fast bacilli and protozoa. No activity was observed against the other bacteria and fungi. The antibiotic is toxic to mice and hemolytic to rabbit red cells.

A colorless crystalline antibiotic, active against aerobic spore-forming bacilli, acid-fast bacilli and protozoa, has been isolated from the broth of a strain of *Streptomyces*. This strain, which is indexed M-88 in our culture collection, was isolated from a soil sample collected from Kobe City. The antibiotic, which we have named kobenomycin, is a polypeptide.

Characterization of the *Streptomyces* M-88

Cultures of *Streptomyces* M-88 were incubated on each medium at 28°C for 18 days. The morphological properties of the strain were observed on BENNETT's agar and detailed observations were made with agar-cylinder culture method[1]. The structure of the spore surface was observed with the electron microscope. Aerial mycelium of a cottony type is formed abundantly on BENNETT's agar. The sporophores are straight and branch in biverticillus type. The spores are formed in chains with 5~10 conidia. The spore is cylindrical to ellipsoidal in shape (0.6 μ in width, 1.0~1.5 μ in length) with a smooth surface. Sporangium and flagellated spore are not observed and also fragmentation and sclerotia in substrate mycelium are not observed.

The cultural and physiological characteristics of this strain are listed in Tables 1 and 2. The utilization of carbon sources by this strain is shown in Table 3.

From the results of these studies, the characteristics of *Streptomyces* M-88 can be summarized as follows: Sporophores are straight in biverticillus type (secondary whorl). Structure of spore surface is smooth and shape of spore is cylindrical to ellipsoidal. Production of melanoid pigment and tyrosinase reaction are negative. Growth type on glucose broth is ring type. Acid production from glucose is positive. Good growth is observed at 28°C and 37°C, and fair growth was observed at 45°C. Color of aerial mycelium is white to yellowish gray, substrate mycelium is colorless

Table 1. Cultural characteristics of *Streptomyces* M-88

Medium	Growth	Formation of aerial mycelium	Color		
			Aerial mycelium	Substrate mycelium	Soluble pigment
CZAPEK's agar	Poor	Fair to poor cottony	White	White to yellowish gray	None
Glucose-CZAPEK's agar	Poor	Poor	Yellowish gray	Yellowish gray to pale yellow	None
Cellulose agar	Poor to scant	Poor cottony	White	Colorless	None
Starch agar	Poor	Poor	White	Colorless	None
Ca-malate agar	Fair	Poor	Pale yellow to yellowish gray	Dull yellow	None
Glucose-asparagine agar	Fair	Poor to scant	Pale yellow	Dull yellow to pale yellowish brown	None
Glucose-peptone agar	Good	Fair powdery	Yellowish gray	Dull yellow	None
Nutrient agar	Good	Good	White	Pale yellowish brown to yellowish brown	None
Glucose-bouillon agar	Good	Good	White	Yellowish brown	None
BENNETT's agar	Good	Good, cottony	Yellowish gray	Yellowish brown	None
Glucose-broth	Good	Good, cottony	White	Pale yellowish brown	None
Potato	Fair	Poor	Yellowish gray to pale yellow	Dull yellow	None
Glucose-CZAPEK's solution	Scant		Yellowish gray	Pale yellowish brown	None
Milk	Good	Poor	Yellowish gray	Pale yellowish brown	None
Gelatin	Good	Poor	Brownish white	Grayish brown	Dark brown
Growth response to temperature	Good growth at 28°C and 27°C. Fair growth at 45°C. Fair sporulation at 28°C, 37°C and 45°C.				
Growth type in glucose-broth	Ring type				

to yellowish on synthetic media. Color of aerial mycelium is white to yellowish gray, substrate mycelium is yellow to yellowish brown on organic media. Soluble pigment is not produced on synthetic and organic media. Reduction of nitrate is positive.

Table 2. Physiological properties of *Streptomyces* M-88

Property	Results
Formation of melanoid pigment	Negative
Tyrosinase reaction	Negative
Acid formation from glucose	Positive
Starch hydrolysis	Positive
Nitrate reduction	Positive
Gelatin liquefaction	Positive
Milk peptonization	Positive
Cellulose reaction	Negative

Among many species of *Streptomyces* described in BERGEY's Manual of Determinative Bacteriology, 1957, 7 Ed.[2], WAKSMAN's "The Actinomycetes" 1961, Vol. 2[3], and other literature, *Streptomyces* M-88 is closely related to *Streptomyces mashuensis* in morphological properties (straight biverticillus), color of substrate mycelium (yellowish), lack of soluble pigment on synthetic and organic media, growth type on glucose broth and some biochemical properties such as melanoid pigment (negative), tyrosinase reaction (negative), starch hydrolysis, gelatin liquefaction and milk peptonization (positive). This strain was then compared in detail with *Streptomyces mashuensis*, and after actual comparison, it was recognized that *Streptomyces* M-88 differed from

Table 3. Utilization of carbon sources by *Streptomyces* M-88 on the basal medium of PRIDHAM *et al.*

Carbon sources	Result	Carbon sources	Result
Glucose	++ (Good growth)	*d*-Xylose	±
Glycerine	++	*d*-Mannitol	±
d-Mannose	++	Rhamnose	±
d-Fructose	++	Raffinose	±
Inositol	++	Dulcitol	±
Soluble starch	+ (Fair growth)	Inulin	±
Potato starch	+	*l*-Arabinose	− (No growth)
Dextrin	+	Salicin	−
Lactose	+	Sorbose	−
Maltose	+	Sorbitol	−
Galactose	+	*d*-Mannitol	−
Sucrose	± (Faint growth)	Cellulose	−

Table 4. Distinguishable characteristics between *Streptomyces* M-88 and *Streptomyces mashuensis* based on actual comparison

Property	Medium	*Streptomyces* M-88	*Streptomyces mashuensis*
Acid production from glucose		Positive	Negative
Growth	CZAPEK's agar	Poor	Good
	Glucose-CZAPEK's agar	Poor	Good
	Starch agar	Poor	Good
Aerial mycelium formation	CZAPEK's agar	Fair	Scant to none
	Ca-malate agar	Fair	None
	Nutrient agar	Good	None
	Glucose-bouillon agar	Good	Scant
	Glucose-broth	Good	None
Color of aerial mycelium	BENNETT's agar	Yellowish gray	Brownish white
Color of substrate mycelium	CZAPEK's agar	White to yellowish gray	Pale olive
	Glucose-CZAPEK's agar	Yellowish gray	Pale olive
	Starch agar	Colorless	Pale olive to pale yellowish brown
Utilization of carbon sources	Lactose	+ (Fair growth)	− (No growth)
	Galactose	+	−
	Sucrose	± (Faint growth)	++ (Good growth)

S. mashuensis in the following points: acid production from glucose; growth on CZAPEK's agar, glucose-CZAPEK's agar and starch agar; formation of aerial mycelium on CZAPEK's agar, calcium-malate agar, nutrient agar, glucose-bouillon agar and glucose broth; color of aerial mycelium on BENNETT's agar; color of substrate mycelium on CZAPEK's agar, glucose-CZAPEK's agar and starch agar; and utilization of lactose, galactose and sucrose. These results obtained are presented in Table 4.

Therfore, it was decided that *Streptomyces* M-88 is a new species of *Streptomyces* and it was named *Streptomyces kobenensis*, nov. sp.

Production and Isolation of Kobenomycin

Shake-flask fermentation of the kobenomycin-producing cultures was carried out according to the following procedure. An inoculum was prepared by growing a vegetative suspension of the culture on a reciprocal shaker for 48 hours at 28°C. The medium used contained 2 % glucose, 0.5 % peptone, 0.5 % meat extract, 0.3 % NaCl, and 0.35 % $CaCO_3$ (pH 7.0).

A 3 % inoculum was used to seed a production medium containing 2 % potato starch, 3 % soybean meal, 0.3 % NaCl and 0.35 % $CaCO_3$ (pH 7.2). The fermentations were carried out on a reciprocal shaker at 28°C for 7 days. The seed and production media described above were also used for antibiotic production in stirred-jar fermentors. Kobenomycin produced was determined by a paper disc or cup method using *Bacillus subtilis* PCI-219 as the test organism. Activity was found mainly in the culture filtrate. A typical fermentation in the above medium assayed about 200~400 mcg per ml.

Kobenomycin could be removed from the fermentation broth by adsorption on active carbon or cation-exchange resin such as Amberlite IRC-50 and then by elution with aqueous acetone. A procedure for the preparation of kobenomycin is as follows: 20 liters of filtered fermentation broth (pH 5.6) were adjusted to pH 7.0 with 10 % sodium hydroxide and filtered. The filtrate was treated with active carbon (0.5 g per 100 ml) which was removed by filtration. The filtrate was discarded and the carbon was eluted two times by stirring for one hour with 5-liter portions of 80 % aqueous acetone. Each extract was collected by filtration. The extracts were combined and concentrated *in vacuo* to an aqueous solution (500 ml). The precipitate which occurred during the concentration was collected by filtration. Further purification of both precipitate and filtered solution were separately effected by chromatography on carbon. An equal weight of active carbon (15 g) and Hyflo Supercel (15 g) was mixed and packed into a glass column (3.4×14 cm). The precipitate (5 g) suspended in water (about 50 ml) or the filtered solution was placed on the column and washed with water. Stepwise elution was carried out with aqueous acetone of 20 %, 40 % and 80 %. Kobenomycin was eluted with 80 % aqueous acetone. The active fractions of 80 % aqueous acetone were combined and concentrated *in vacuo* to precipitate the crude kobenomycin which was collected and dried *in vacuo* to give 2 g of colorless crude kobenomycin powder.

For crystallization, 2 g of the crude powder was suspended in 50 ml of 80 % aqueous methanol at 60°C and filtered. The residue was dissolved in 50 ml of 50 % aqueous methanol at 60°C and filtered. The filtrate was concentrated *in vacuo* to dryness. Crystalline kobenomycin (420 mg) was obtained by dissolving 640 mg of the dried material in 50 ml of 80 % aqueous methanol at 60°C and allowing it to stand at room temperature. The crystalline kobenomycin was then recrystallized from the same solvent.

Chemical and Physical Properties of Kobenomycin

Kobenomycin is a colorless crystalline polypeptide antibiotic which contains sulfur.

It is soluble in acetic acid, dimethylsulfoxide and pyridine, slightly soluble in water, methanol and ethanol, but insoluble in acetone, ether, petroleum ether and ethyl acetate. It melts at 285~288°C under decomposition.

Analytical data of kobenomycin dried at 60°C *in vacuo* to constant weight are as follows: C 51.93 %, H 6.53 %, N 15.75 %, S 4.96 %. The specific rotation of kobenomycin is $[\alpha]_D^{21.5}$ −87.5 ±2 (c 1.0, glacial acetic acid). The ultraviolet absorption spectrum (Fig. 1) is characterized by a maximum in water at 280 mμ ($E_{1cm}^{1\%}$ 22.1) and shoulders at 220 mμ, 275 mμ and 285 μm; in 0.1 N HCl at 280 mμ ($E_{1cm}^{1\%}$ 260) and shoulders at 220 mμ, 257 mμ and 285 mμ; in 0.1 N NaOH at 222 mμ ($E_{1cm}^{1\%}$ 253) and shoulder at 270~280 mμ. The infrared absorption spectrum of kobenomycin in KBr shows the following frequencies (S=strong, B=broad, Sh=shoulder): 3300 (S), 3072 (S), 2959 (S), 1664 (S), 1525 (B), 1452 (B), 1390 (Sh), 1335 (B), 1270 (Sh) and 750 (S) cm^{-1} (Fig. 2).

Fig. 1. Ultraviolet absorption spectra of kobenomycin

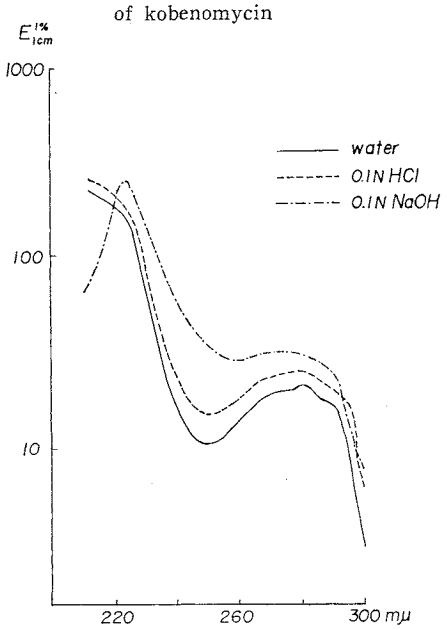
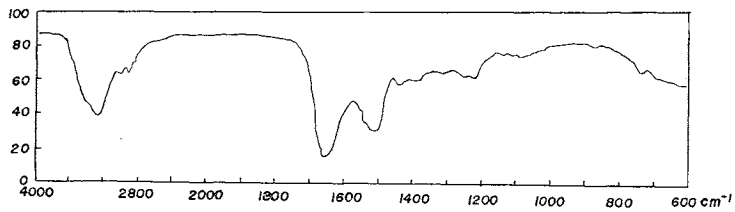


Fig. 2. Infrared absorption spectrum of kobenomycin (KBr)



It gives positive FOLIN-phenol, ninhydrin, biuret, xanthoprotein and EHRLICH reactions. Anthrone, FEHLING, PAULY, SAKAGUCHI and MOLISCH reactions are negative.

Rf values by ascending paper chromatography at room temperature using Toyo filter paper No. 51 are as follows: 0.60 with *t*-butanol-acetic acid-water (2 : 1 : 1), 0.15 with *n*-butanol-acetic acid-water (4 : 1 : 5, upper phase), and 0.83 with acetone-water (1 : 1).

On paper electrophoresis using Toyo filter peper No. 51 at 300 volt for 3 hours, kobenomycin moves slightly to cathode in the pH 2.0, pH 5.0, pH 6.0, pH 7.0 and pH 8.0 buffer solutions. In pH 11.4 buffer solution, it moves slightly to anode.

Rf values by thin-layer chromatography using silica gel G (250 μ) are as follows: 0.30 with *n*-butanol-acetic acid-water (4 : 1 : 2), 0.50 with *n*-

Table 5. Amino acid composition of kobenomycin

Amino acid	Molar ratio
Aspartic acid	0.95 (1)
Serine	0.90 (1)
Proline	0.76 (1)
Leucine	1.00 (1)
Valine	0.90 (1)
Phenylalanine	0.93 (1)
Glycine	1.83 (2)
Glutamic acid	3.87 (4)
Tryptophan	0.68 (1)
Unknown (1)	(1)*
Unknown (2)	(1)*
Unknown (3)	(1~2)*
Unknown (4)	(2)*

* Presumptive value

butanol-ethanol-0.1 N HCl (1 : 1 : 1), 0.75 with *n*-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 10), 1.0 with $CHCl_3$-methanol-17 % NH_4OH (2 : 1 : 1, upper phase) and 0.57 with ethanol-water (4 : 1).

The presence of amino acids in kobenomycin was confirmed by paper chromatography of its acid and alkali hydrolysates. The amino acid composition of kobenomycin acid hydrolysate [6 N hydrochloric acid at 110°C for 24 hours] and alkali hydrolysate [barium hydroxide at 110°C for 24 hours] was determined quantitatively by an automatic amino acid analyzer. Tryptophane content was also determined by UV absorption method. The results were shown in Table 5. The molecular weight of kobenomycin was suggested to be 2,150~2,200, from its amino acid composition.

Biological Properties

Antimicrobial activity: To determine the minimal inhibitory concentration of kobenomycin, the antibiotic was examined by the streak plate dilution method or by the tube dilution method. The antimicrobial spectrum of kobenomycin is fairly specific in its activity, aerobic sporulating bacilli are inhibited strongly, and also it is effective against acid-fast bacilli and some protozoa, such as *Trichomonas vaginalis* and *Tetrahymena geleii*. The other bacteria, yeast and fungi are not affected even at a concentration of 200 mcg per ml. Table 6 shows the minimal inhibitory concentration of kobenomycin for aerobic sporulating bacilli, acid-fast bacilli and protozoa.

Table 6. Minimal inhibitory concentration of kobenomycin for aerobic spore-forming bacilli, acid-fast bacilli and protozoa

Test organisms	Minimal inhibitory concentration mcg/ml
1. *Bacillus subtilius*, PCI-219	5.0
2. *Bacillus anthracis*	1.0
3. *Bacillus megatherium*	5.0
4. *Bacillus cereus*	0.5
5. *Bacillus agri*	1.0
6. *Mycobacterium phlei*	5.0
7. *Mycobacterium smegma*	20.0
8. *Mycobacterium* 607	20.0
9. *Mycobacterium avium*	20.0
10. *Mycobacterium tuberculosis*, H37Rv	200.0
11. *Trichomonas vaginalis*, 4F	12.5
12. *Tetrahymena geleii*, W	50.0

Culture medium : 1~5, peptone-meat extract agar ; 6~9, peptone-meat extract-glycerol agar ; 10, KIRCHNER+10% human plasma ; 11, glucose-peptone-yeast extract+0.2% cystein and 10% human plasma ; 12, peptone-yeast extract.
End point observed : 1~9, 24 hours at 37°C ; 10, 3 weeks at 37°C ; 11, 2 days at 37°C ; 12, 4 days at 28°C.

Stability: Kobenomycin was dissolved in dimethylsulfoxide at 10 mg/ml and was diluted with sterile distilled water to give test solutions containing 1,000 mcg/ml. The test solutions were adjusted to various pH levels ranging from 2.0 to 11.4 with glycine-HCl, phosphate and citrate buffers, and the solutions were heated at 100°C for various time lengths. Each sample was diluted with phosphate buffer at pH 6.0 to provide a suitable dilution for assay. The potency of kobenomycin was estimated by the agar cup diffusion method using *B. subtilis* PCI-219 as the test organism. The results of

Table 7. Stability of kobenomycin solution (1,000 mcg/ml) at various pH levels at 100°C

pH	Percent activity after heating	
	5 minutes	30 minutes
2.0	95.0	94.0
5.0	95.0	90.0
7.0	95.0	67.5
8.0	87.5	55.0
11.4	50.0	16.3

thermostability at various pH levels are shown in Table 7. It will be seen that greater loss of activity was observed in alkaline solution than in acid.

Acute toxicity in mice: Because kobenomycin is insoluble in water, aqueous solutions of the antibiotic were prepared by the following method: the antibiotic is dissolved in a small amount of dimethylsulfoxide and diluted to the desired concentration with sterile distilled water. Acute toxicity studies were carried out in DS mice weighing 15~16 g following 10-day observation period. The antibiotic was found to be toxic to mice by parenteral administration: the LD_{50} for mice was 15 mg/kg given intravenously and 25 mg/kg given intraperitoneally. Kobenomycin was well tolerated subcutaneously by mice: the LD_{50} was >100 mg/kg. It may be that the antibiotic is not well absorbed after subcutaneous injection.

Hemolysis of rabbit erythrocytes: Kobenomycin was serially diluted with dimethylsulfoxide. An equal volume of pH 6.0 phosphate buffer solution was added to each concentration of the antibiotic solvent solution. Hemolytic activity for rabbit erythrocytes was examined by adding 0.5 ml of 3 % cells in saline solution to 0.5 ml of the diluted kobenomycin solvent-phosphate buffer solution. After incubation overnight at 37°C, the highest dilution producing complete hemolysis was determined. Rabbit red cells were completely lysed by kobenomycin at a concentration of 30 mcg/ml.

Discussion

On the basis of its physicochemical properties, kobenomycin is clearly a new sulfur-containing polypeptide antibiotic. It is readily differentiated from the other sulfur-containing polypeptide antibiotics by amino acid composition. Arsimycin[4] has no aspartic acid, leucine and serine. Leucopeptin[5] has no leucine and phenylalanine. Cinnamycin[6] has no leucine, serine and glycine. Duramycin[7] has no leucine and serine, and leucinamycin[8] has no serine.

A comparison of the biological properties of kobenomycin and leucinamycin indicates a close similarity between the two. Their antimicrobial spectra are similar, except that kobenomycin has no antifungal activity. The only greater susceptibility of aerobic spore-forming bacilli to kobenomycin and leucinamycin suggests that both new antibiotics might be useful as tools to study spore germination.

References

1) Nishimura, H. & K. Tawara : A method for the microscopical observation of *Streptomyces* using agar-cylinder culture. J. Antibiotics, Ser. A 10 : 227, 1957
2) Breed, R. S.; E. G. D. Murray, N. R. Smith & 94 contributors : Bergey's Manual Determinative Bacteriology. 7 Ed. 1957, The Williams & Wilkins Company
3) Waksman, S. A. : The Actinomycetes. Vol. 2, 1961, The Williams & Wilkins Company
4) Reitstötter, J. : Herstellung und Gewinnung des Antibiotikums Arsimycin. German Pat. 1,090,380, Oct. 6, 1960
5) Kondo, S.; M. Sezaki, M. Shimura, K. Sato & T. Hara : Leucopeptin, a new peptide antibiotic. J. Antibiotics, Ser. A 17 : 262~263, 1964
6) Dvonch, W.; O. L. Shotwell, R. G. Benedict, T. G. Pridham & L. A. Lindenfelser : Further studies on cinnamycin, a polypeptide antibiotic. Antibiot. & Chemoth. 4 : 1135~1142, 1954
7) Shotwell, O. L.; F. H. Stodola, W. R. Michael, L. A. Lindenfelser, R. G. Dworschack & T. G. Pridham : Antibiotics against plant disease. III. Duramycin, a new antibiotic from *Streptomyces cinnamomeus* forma *azacoluta*. J. Am. Chem. Soc. 80 : 3912~3915, 1958
8) Mizuno, K.; Y. Ohkubo, S. Yokoyama, M. Hamada, K. Maeda & H. Umezawa : A new antibiotic, leucinamycin. J. Antibiotics, Ser. A 20 : 194~199, 1967.