BICYCLOMYCIN, A NEW ANTIBIOTIC
I. TAXONOMY, ISOLATION AND CHARACTERIZATION

TOSHIO MIYOSHI, NORIMASA MIYAIRI, HATSUO AOKI,
MASANOBU KOHSAKA, HEI-ICHI SAKAI and HIROSHI IMANAKA

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan

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Bicyclomycin is a new antibiotic obtained from the culture filtrate of *Streptomyces* sp. WS 4545 which was identified as a new species and given the name *Streptomyces sapporonensis*. Its elementary analysis and mass spectroscopic measurement suggest that the molecular formula is $C_{12}H_{18}N_2O_7$. There is no specific ultraviolet absorption. Bicyclomycin is active against Gram-negative bacteria, especially *Escherichia coli*, *Klebsiella*, *Shigella* and *Salmonella* species, and has no cross resistance with the usual antibiotics. It has low toxicity in mice.

In the course of our antibiotics screening program, a *Streptomyces* initially designated WS 4545 was found to produce an antibiotic with activity against Gram-negative bacteria. From its unique chemical and biological properties, it was judged to be a new antibiotic and named bicyclomycin. In this paper, the characteristics of strain WS 4545, fermentation, isolation procedures, chemical and biological properties of bicyclomycin are described.

Characteristics of Strain WS 4545

The bicyclomycin-producing culture is a *Streptomyces* isolated from a soil sample obtained at Sapporo, Hokkaido, Japan.

1. Morphological characteristics

The morphology of the culture was microscopically observed on CZAPEK's agar

Fig. 1. Aerial mycelia of strain WS 4545 (×600×1/1.5)

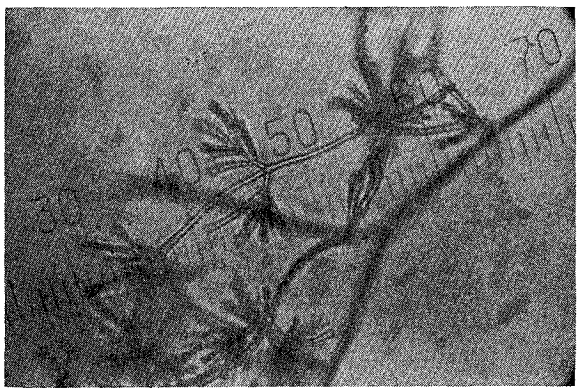


Fig. 2. Electronmicrograph of the spores of strain WS 4545 (×7,000×1/1.5)

and starch ammonium agar, at 30°C for 10~14 days. The aerial mycelium of strain WS 4545 branches and exhibits typical whorl formation (Fig. 1). Spores on BENNETT's agar were observed with an electron microscope (Superscope, Nihondenshi Co., Ltd.). The spores are oval and cylindrical and their surfaces are smooth (Fig. 2).

2. Cultural and physiological characteristics

The cultural characteristics and summarized physiological properties of strain WS 4545 are shown in Tables 1 and 2, respectively. Each of the media used in this study was prepared according to recommendation in WAKSMAN[1] and ISP (International Streptomyces Project)[2]. Matured spores and mycelia on BENNETT's agar were used to inoculate each medium studied. Unless otherwise stated, all cultures were incubated at 30°C for 2 weeks before observation.

On most media, orange vegetative growth develops moderately and the aerial mass is white to white with grayish or yellowish to brownish tinge. No soluble pigment forms on the media tested including tyrosine agar, nutrient agar, yeast malt agar and

Table 1. Cultural characteristics of strain WS 4545.

Medium	Growth	Aerial mycelium	Soluble pigment	Remarks
CZAPEK's agar	White, faint growth	None	None	
Starch-inorganic salts agar	Brownish orange, flat spreading growth	White, cottony	None	
Starch ammonium agar	Light orange, small colonies	None	None	Diastatic action : weak
Glucose asparagine agar	Brown small colonies	White powdery	None	
Glycerin asparagine agar	Light brownish gray small colonies	Thin, white powdery	None	
Calcium malate agar	White growth	Thin, light brownish white powdery	None	
Tyrosine agar	Brownish small colonies	None	None	
Nutrient agar	Light grayish yellow, wrinkled small colonies	None	None	
Yeast malt extract agar	Light brown wrinkled small colonies	White with grayish tinge, powdery	None	
BENNETT's agar	Brown wrinkled small colonies	Thin, white powdery	None	
Peptone yeast iron agar	No growth			
Oatmeal agar	Brownish yellow colonies	Thin, yellowish white powdery	None	
Glucose CZAPEK's solution	White small colonies at bottom	None	None	
Glucose bouillon	White growth at bottom	None	None	
Milk	Faint growth	None	None	Peptonization: weak Coagulation : negative
Gelatin stab (15°~20°C 20 days)	Faint growth	None	None	Liquefaction : weak
Potato plug	Light brown wrinkled colonies	Light brownish powdery	None	
Cellulose	No growth			

Table 2. Physiological properties of strain WS 4545

Optimum temperature for growth*	25°~37°C
Optimum pH range for growth	6~8
Tyrosinase reaction	negative
Melanoid pigment	negative
Reduction of nitrate	negative
Liquefaction of gelatin	slow
Coagulation of milk	negative
Peptonization of milk	negative
Hydrolysis of starch	weak
Cellulose decomposition	negative
Product	bicyclomycin

* On BENNETT's agar

Table 3. Carbon utilization pattern for strain WS 4545.

Source of carbon	Growth
D-Xylose	±
L-Arabinose	±
D-Fructose	±
D-Glucose	±
L-Rhamnose	−
Sucrose	−
Lactose	−
Trehalose	+
Raffinose	−
D-Mannitol	−
Inositol	+
Salicin	−
Negative control	−

(+) utillization, (±) probable utilization, (−) no utilization.

other proteinous media such as gelatin, milk and potato. Accordingly, strain WS 4545 is considered to be non-chromogenic. The hydrolytic activity on starch, gelatin or milk is weak. Utilization of carbon sources by strain WS 4545 was investigated with the method of PRIDHAM and GOTTLIEB.[3]

The results are shown in Table 3.

D-Trehalose and inositol are easily utilized and L-arabinose, D-xylose, D-fructose and D-glucose are slightly utilized for growth of the organism.

From the observations described above, strain WS 4545 may be characterized as follows:

It forms aerial hyphae with typical whorls. No soluble pigment was produced on either synthetic or organic media, suggesting that the strain is non-chromogenic. The color of vegetative growth on synthetic media is orange to brown. On some media, the aerial mass color is white and in some case late becoming grayish or yellowish to brownish white. The hydrolytic activities on starch, gelatin and milk are weak.

3. Comparison of strain WS 4545 with related *Streptomyces*

After comparing the characteristics of those *Streptomyces* species in the verticillate type and non-chromogenic series described in "The actinomycetes, Vol. 2" by WAKSMAN,[1] the ISP reports by SHIRLING and GOTTLIEB[4,5,6] and other recent literature, some related strains were selected for further detailed comparison. They were *Streptomyces flavopersicus*,[4] *Streptomyces hachijoensis*,[1,4] *Streptoverticillium cinnnamoneum*,[5] *Streptomyces kobenensis*[7] and *Streptomyces triculaminicus*[8]. These five species and strain WS 4545 are similar in the morphology of their sporophores and non-chromogenic character. It was found, however, that these five species were differentiated from strain WS 4545 in the following points.

(1) The aerial mass color of *Streptomyces flavopersicus* is in Red color series on yeast malt agar, oatmeal agar and salts starch agar, whereas that of WS 4545 is white with grayish or yellowish tinge. The color of vegetative growth of the former does not show distinct pigment on the same media. The growth of WS 4545 is orange to brown.

(2) The aerial mass color of *Streptomyces hachijoensis* is in Red color series on yeast malt agar, oatmeal agar, salts starch agar and glycerol asparagine agar, whereas that of strain WS 4545 does not exhibit reddish color on the same media. In addition, the hydrolytic activities of the former on gelatin and milk are moderately strong, whereas those of the latter are weak.

(3) The aerial mass color of *Streptoverticillium cinnamoneum* is grayish yellowish pink on yeast malt agar, oatmeal agar, salts starch agar and glycerol asparagine agar, whereas that of strain WS 4545 is white with grayish or yellowish tinge and does not show pinkish color on the same media. The former's activities of hydrolysis on starch and liquefaction of gelatin are positive and those of the latter are negative.

(4) The aerial mass color of *Streptomyces kobenensis* is white to yellowish gray and the vegetative growth is yellow to yellowish brown, similar to strain WS 4545. However, the former produces dark brownish melanoid pigment on gelatin medium, whereas the latter does not produce any melanoid pigment on the proteinous media. Furthermore, the hydrolytic activities of *S. kobenesis* on starch, gelatin and milk are positive, whereas those of WS 4545 are negative.

(5) *Streptomyces triculaminicus* is also a species which belongs to whorl forming, non-chromogenic group. However, the aerial mass color is white to pink on ordinary media. In addition, gelatin liquefaction is strongly positive. These characters apparently differentiate *S. triculaminicus* from strain WS 4545.

As a result of the above comparisons, strain WS 4545 is considered a new species, and the name *Streptomyces sapporonensis* nov. sp. SAKAI and MIYOSHI is proposed. A culture of the new species has been deposited in the American Type Culture Collection, with accession number ATCC 21532.

Production of Bicyclomycin

For production of the antibiotic, a 48-hour shake flask culture was used as a seed culture. For a typical run, the culture was then transferred to a 30-liter jar fermentor containing 20 liters of the medium composed of 5% potato-starch, 3% Pharmamedia (Trader Oil Mill Co.), 1% $MgSO_4 \cdot 7H_2O$, 1.09% KH_2PO_4 and 0.74% $Na_2HPO_4 \cdot 12H_2O$. Fermentation was carried out at 30°C for 5 days under aeration of 20 liters per minute and agitation of 300 rpm.

Antibiotic activity was found mainly in the broth filtrate and was determined by an agar diffusion method with *Escherichia coli* IAM 1159 as the test organism using crystalline bicyclomycin as the assay standard.

Isolation Procedure

The culture broth was filtered with the aid of filter aid (Radiolite). The filtrate was concentrated *in vacuo* to about one-tenth volume to which 4 volumes of acetone was added to precipitate impurities. The supernatant collected by suction was concentrated to evaporate the acetone and then was treated with one-third volume of *n*-butanol and agitated vigorously to extract impurities having activity against *E. coli*. The aqueous layer was concentrated again to 1/100 or 1/200 volume of the original filtrate, solidified with Radiolite and then lyophilized. The dry powder obtained, was washed with a small volume of ethyl acetate and then extracted with a solvent mixture of ethyl acetate and ethanol (2:1, v/v). The extracts active against *E. coli* were collected and concentrated under reduced pressure. When this concentrate was kept in the cold, the antibiotic crystallized as a white powder. After treatment with charcoal, the crude crystals were recrystallized from a mixture of methanol and acetone as the crystal of type A (rhombic). The crystal of type B (monoclinic) was obtained from ethanol and that of type C (monohydrate) was crystallized from hot

water.

Characterization of Bicyclomycin

Bicyclomycin is weakly basic, freely soluble in water (1 g in 5.2 ml), soluble in methanol (1 g in 19 ml), sparingly soluble in ethanol (1 g in 127 ml), slightly soluble in acetone (1 g in 760 ml), and insoluble in chloroform, ethyl acetate, benzene and *n*-hexane. The optical rotation is $[\alpha]_D^{23}+63.5$ (*c* 1, methanol). As shown in Fig. 3, the ultraviolet absorption spectrum showed only end absorption. It gives positive FEHLING, MOLISCH, permanganate, and negative nihydrin and EHRLICH reactions.

Elementary analysis gave: C 47.44, H 6.16, N 9.36.
Calcd. for $C_{12}H_{18}N_2O_7$: C 47.68, H 6.00, N 9.27.

The molecular formula of bicyclomycin is confirmed to be $C_{12}H_{18}N_2O_7$ (MW 302.29) by the parent peak in the mass spectrum at *m/e* 302. The compound crystallized from water was found to be bicyclomycin monohydrate by elementary analysis and loss of weight on drying *in vacuo* at 60°C.

An aqueous solution at 1,000 mcg/ml was adjusted to pH 2.2, 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0 and kept at 60°C or 100°C. As shown in Table 4, the antibiotic is labile in alkaline solution.

Bicyclomycin was crystallized in different crystal forms according to the conditions used in crystallization process. It was shown by the X-ray diffraction that the crystal produced in type A is rhombic form and the crystal in type B is monoclinic form.

The rhombic crystal melts at 187~189°C (decomp.). The infra-red spectrum suspended in Nujol mull is shown in Fig. 4, giving peaks at the following frequencies: 3400, 3300, 3160, 2900, 2840, 1703, 1670, 1450, 1390, 1370, 1295, 1260, 1220, 1195, 1140, 1120, 1080, 1030, 970, 960, 935, 895, 870, 820, 785, 725, 680 cm^{-1}.

The monoclinic crystal melts at 188°~191°C (decomp.). The infra-red spectrum suspended in Nujol mull is shown in Fig. 5, giving peaks at the following frequecies: 3500, 3400, 3270, 2900, 2840, 1685, 1640, 1455, 1417, 1375, 1330, 1300, 1260, 1245, 1210, 1160, 1135, 1125, 1085, 1050, 1010, 995, 980, 940, 920, 900, 885, 870, 840, 805, 795, 760, 725, 690, 675 cm^{-1}.

The different crystals are easily interconverted by adding a small piece of one crystal type to the solution of the other form just before crystallization. Infra-red spectrum of bicyclomycin monohydrate crystal obtained from hot water in Nujol mull is shown in Fig. 6.

Fig. 3. Ultraviolet absorption spectrum of bicyclomycin in methanol.

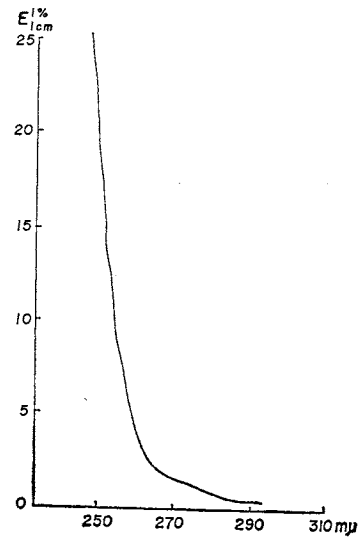


Table 4. Stability of bicyclomycin in solution.

Condition	Percent of residual activity						
	pH 2.2	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0
100°C for 10 min.	70	50	50	0	0	0	0
60°C for 60 min.	100	90	90	75	50	20	0

Fig. 4. Infrared absorption spectrum of bicyclomycin (rhombic form) as nujol mull.

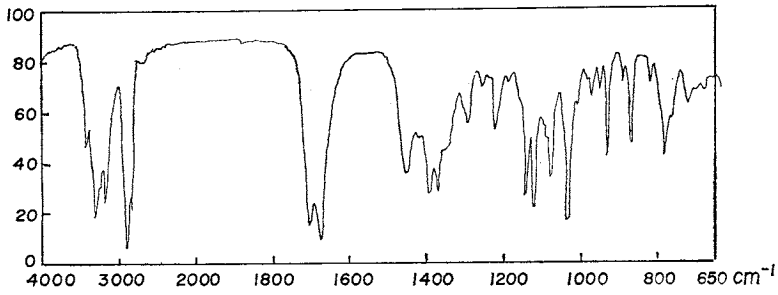


Fig. 5. Infrared absorption spectrum of bicyclomycin (monoclinic form) as nujol mull.

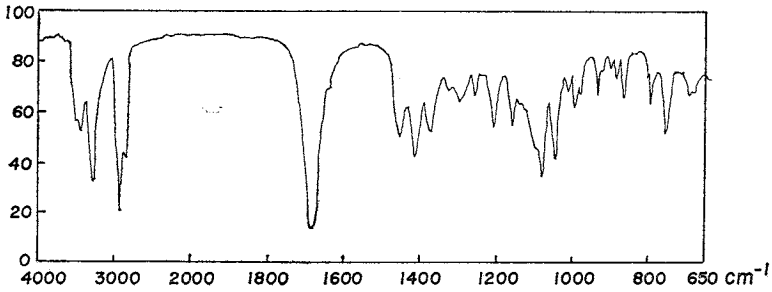
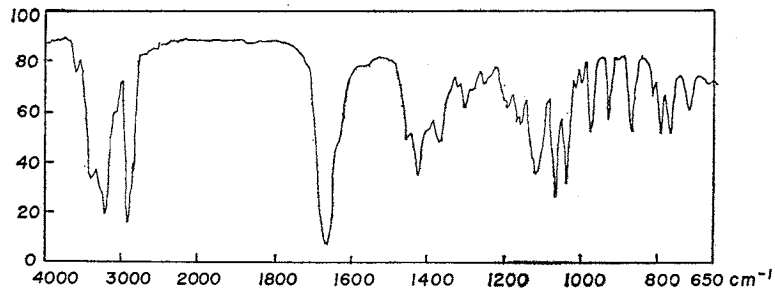


Fig. 6. Infrared absorption spectrum of bicyclomycin monohydrate as nujol mull.



Biological Properties

The antimicrobial spectrum of bicyclomycin is shown in Table 5. This test was conducted by the agar dilution streak method, using heart infusion agar for bacteria, glycerin bouillon medium for *Mycobacterium* and malt extract medium for fungi and yeast which were incubated at 30°C for 24~72 hours.

The antibiotic had selective antibacterial activity, showing moderate activity against

Table 5. The antimicrobial spectrum of bicyclomycin.

Test organism	MIC (mcg/ml)
Staphylococcus aureus 209P JC-1	>1,000
Bacillus subtilis ATCC 6633	>1,000
Sarcina lutea PCI-100	250
Escherichia coli NIHJ JC-2	25
Klebsiella pneumoniae ST-101	25
Salmonella typhosa T-287	25
Shigella flexneri 1a-2W-A	25
Proteus vulgaris IAM 1025	>1,000
Pseudomonas aeruginosa IAM 1095	>1,000
Mycobacterium phlei	>1,000
Candida albicans	>1,000
Penicillium chrysogenum Q-176	>1,000

Gram-negative bacteria only, including *Escherichia coli*, *Klebsiella*, *Salmonella* and *Shigella* sp.. On the other hand, it appeared to have no activity against *Proteus*, *Pseudomonas* sp., and Gram-positive bacteria. The compound had no cross resistance with streptomycin, kanamycin, chloramphenicol, tetracycline, aminobenzyl penicillin and nalidixic acid.

The acute toxicity (LD_0) by intravenous injection into mice was greater than 2 g/kg and by subcutaneus, oral and intraperitoneal administration was greater than 4 g/kg.

Discussion

Bicyclomycin shows no UV absorption maximum and is mainly active against Gram-negative bacteria. Among known antibiotics produced by *Streptomyces* sp., materials with these properties are negamycin,[9] armentomycin,[10] and SOB-7.[11] Bicyclomycin may be differentiated from these compounds by the molecular formula and other physicochemical properties. Thus, bicyclomycin is considered to be a new antibiotic.

Achnowldgement

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References

1) WAKSMAN, S. A.: The Actinomycetes. Vol. 2. Classification, identification and descriptions of genera and species. The Williams & Wilkins Co., Baltimore, 1961
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Intern. J. Syst. Bact. 16: 313~340, 1966
3) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bact. 56: 107~114, 1948
4) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Intern. J. Syst. Bact. 18: 69~189, 1968
5) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Intern. J. Syst. Bact. 18: 279~392, 1968
6) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second third, and fourth studies. Intern. J. Syst. Bact. 19: 391~512, 1969
7) OKAMOTO, S.; M. MAYAMA, Y. TANAKA, K. TAWARA, H. KATO, N. SHIMAOKA, H. NISHIMURA, M. EBATA & H. OHTSUKA: Kobenomycin, a new polypeptide antibiotic. J. Antibiotics 21: 320~326, 1968
8) SUMIKI, Y.: Production of antibiotic, triculamin. Jap. Patent 45-9234, 1970
9) KANAGAWA, H.; K. MAEDA, T. OKAMI & H. UMEZAWA: A new antibiotic negamycin. J. Antibiotics 23: 170~171, 1970
10) ARGOUDELIS, A. D.; R. R. HERR & D. J. MASON: Production of new antibiotic, armentomycin. Jap. Patent 46-15676, 1971
11) ANDO, K.; H. SAKAI, H. OHISHI, T. YATO. K. KOYAMA & T. AKIBA: Abstracts of papers, Annual Meeting of the Agricultural Chemical Society of Japan, April, 1971, p. 362.