DB-2073, A NEW ALKYLRESORCINOL ANTIBIOTIC

I. TAXONOMY, ISOLATION AND CHARACTERIZATION

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(Received for publication August 7, 1975)

A new antibiotic, DB-2073, was isolated in crystalline form from the fermented broth of *Pseudomonas* sp. B-9004. The compound is a alkylresorcinol antibiotic. The antibiotic melts at 86~88°C. The molecular weight of 236 was determined by mass spectroscopy and the molecular formula was calculated as $C_{15}H_{24}O_2$. The antibiotic has antimicrobial activity against Gram-positive bacteria, mycobacteria and fungi.

During the course of our screening for new antibiotics, a bacterial strain No. B-9004 isolated from a soil sample was found to produce an antibacterial and antifungal antibiotic. The strain No. B-9004 belongs to the genus *Pseudomonas* and was tentatively named *Pseudomonas* sp. B-9004.

Isolation and purification of the antibiotic gave pure crystals. As the result of comparative studies of the antibiotic with other known antibiotics, the antibiotic was shown to be a new antibiotic and was named DB-2073. In this paper are described taxonomy of the strain, isolation, physico-chemical characteristics and biological properties of the antibiotic.

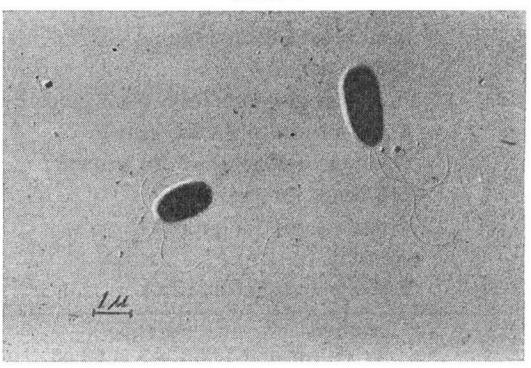
Description of the Producing Strain

1. Morphological Characteristics

Morphological observation of the strain No. B-9004 was carried out by both optical and electron microscopy with cells cultured mainly on nutrient agar for 18~24 hours at 26°C. The following results were obtained. The culture consists of short rods with rounded ends, 0.6~1.0×1.0~2.5 μ, occurring singly or sometimes in pairs. The strain is motile, possessing polar multitrichous flagella. It is Gram-negative, is not acid-fast and has no endospore.

The typical electronmicroscopic photograph of strain No. B-9004 is shown in Plate 1.

Plate 1



2. Cultural Characteristics

Cultural observation of the strain was carried out with the cultures on various media for 7~10 days. The following results were obtained.

(1) Colonies on nutrient agar plate (for 6 days at 26°C): Circular, convex, entire type

of 5~10 mm in diameter with smooth surface and pale purplish color.

(2) Nutrient agar slant (for 6 days at 26°C): Abundant growth, raised in center, smooth surface, glistening, at first pale yellow, later purple. No soluble pigment.

(3) Sucrose CZAPEK's agar slant (for 7 days at 26°C): Abundant growth, raised in center, smooth surface, glistening, at first pale yellow, later brownish purple, soluble purplish pigment.

(4) Gelatin stab (for 20 days at 20°C): Moderate growth, liquefaction in straight form. No soluble pigment.

(5) Litmus milk (for 10 days at 26°C): Moderate growth, pelicle. Positive peptonization without coagulation, an alkaline reaction to litmus.

(6) Nutrient broth (for 7 days at 26°C): Moderate growth, turbid, at first pelicle, later sedimentation occured. No soluble pigment.

(7) Potato plug (for 7 days at 26°C): Good growth with smooth and wet surface in brownish purple color. No soluble pigment.

3. Physiological Characteristics

Physiological characteristics of the strain are summarized in Tables 1, 2 and 3. Formation of 2-keto-gluconate from Na-gluconate was detected by ascending paper chromatography using the solvent systems of phenol-water (4:1), *n*-butanol-pyridine-water (3:2:1.5) and *iso*-butyric acid-acetic acid (9:2), and bromphenol blue (0.1 % in ethanol) and *p*-anisidine hydrochloride (1.0 % in *n*-butanol) were used as spray reagent.

4. Comparison of strain No. B-9004 with closely related bacteria

According to BERGEY's Manual of Determinative Bacteriology[1] and IIZUKA,[2] strain No. B-9004 was considered to belong to genus *Pseudomonas* from those characteristics described above.

Among known species of *Pseudomonas* described in literatures, the strain is similar to

Table 1. Physiological characteristics of *Pseudomonas* strain No. B-9004.

Liquefaction of gelatin	+
Hydolysis of starch	−
Nitrite formation from nitrate	+
Acid formation in litmus milk medium	−
Hydrogen sulfate formation	−
Indol formation	−
VOGES-PROSKAUER test	−
Methyl red test	−
Catalase activity	+
Oxidase test	+
Acetic acid formation from gluconate	−
2-Keto-gluconic acid formation from ethanol	+
Ammonium formation	+
Urease activity	+
Acid and gas formation from glucose and lactose*	−
Temperature range for growth	5°~33°C
Optimum temperature for growth	26°~30°C
pH range for growth	5~10
Optimum pH for growth	6~ 8
Aerobic	+
Pathogenicity (mouse ip.)	−

+: positive −: negative
*: By HUGH and LEIFSON's method (HUGH R. & E. LEIFSON: J. Bact. 62: 377, 1951)

Table 2. Cleavage of carbohydrate by *Pseudomonas* strain No. B-9004.

Carbon source	Acid formation	Gas formation
Starch	−	−
Dextrin	−	−
Inulin	−	−
Lactose	−	−
Maltose	−	−
Sucrose	+	−
D-Glucose	+	−
D-Mannose	+	−
D-Galactose	+	−
D-Fructose	+	−
D-Mannitol	+	−
D-Sorbitol	−	−
L-Arabinose	+	−
D-Xylose	+	−
Glycerol	+	−
Inositol	+	−

+: positive
−: negative

Table 3. Utilization of carbon sources by *Pseudomonas* strain No. 9004.

Carbon source	Utilization
L-Arabinose	+
D-Xylose	+
D-Glucose	###
D-Fructose	##
Maltose	−
D-Mannose	###
Sucrose	###
D-Galactose	−
Lactose	−
D-Mannitol	###
Glycerol	###
Inositol	##
D-Sorbitol	−
Starch	−

###: good growth ##~#: moderate growth
+: poor growth −: no growth
Basal medium: carbon source 1 %, NH_4NO_3 0.1 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, agar 1.5 %, pH 7.2

Table 4. Comparison of *Pseudomonas* strain No. B-9004 with other related species.

	Ps. fluorescens	*Ps. iodinum*	*Ps. pyrrolnitrica*	Poly-β-hydroxy-butyric acid producing strain	No. B-9004
Flagellum	Polar multi-trichous	None	Polar	Polar two to eight	Polar multi-trichous
Motility	Positive	Negative	Positive	Positive	Positive
Color of colony on nutrient agar	At first reddish, later reddish gray	Gray-white	At first pale yellow to olive, later purple	At first yellow, later reddish purple*	At first pale yellow, later purple
Color of colony on potato medium	Grayish yellow	Creamy	Pale olive	—	Brownish purple
Milk medium	No coagula-tion, alkaline	Alkaline	Coagulation, weak acid	—	No coagula-tion, alkaline
Growth at 37°C	Positive, occasionally negative	Positive	Positive	Positive	Negative
Product	—	Iodinin	Pyrrolnitrin	Poly-β-hydroxy-butyric acid	Antibiotic DB-2073

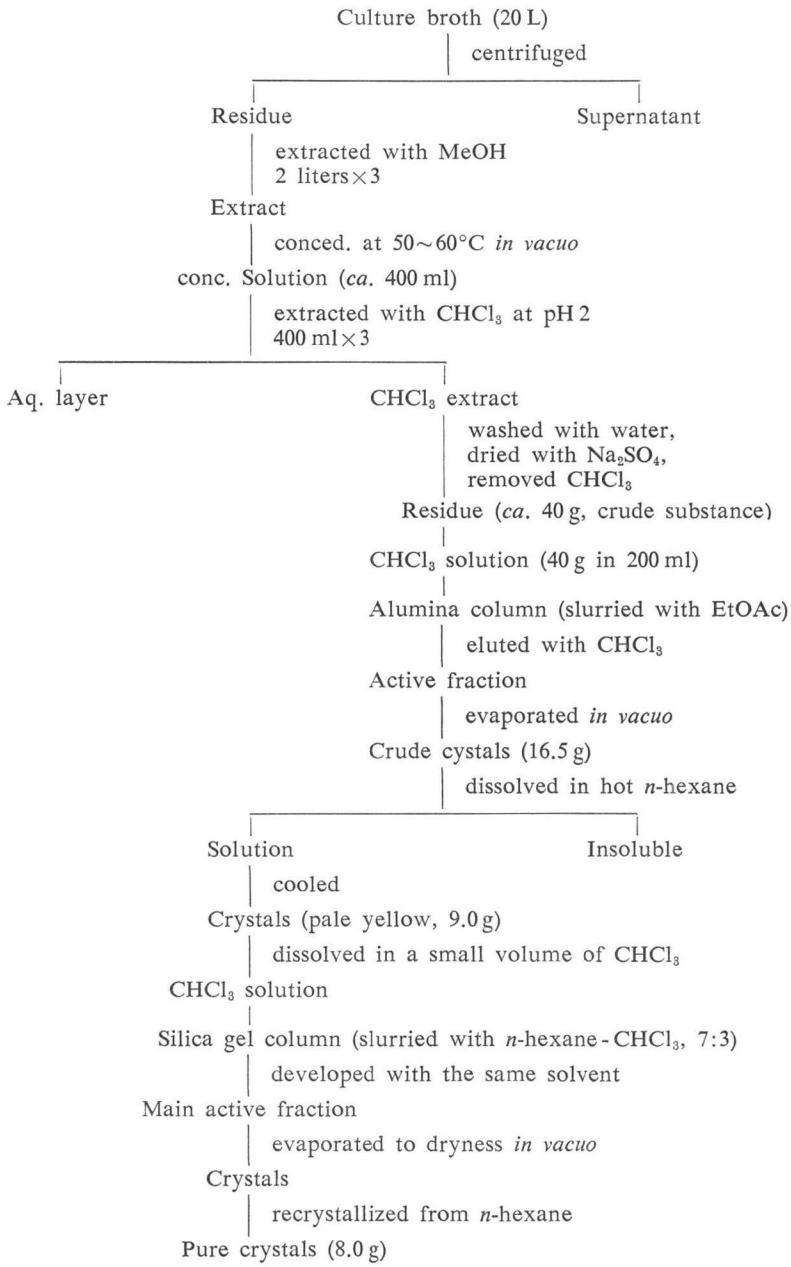
*: Color of colony on glucose-'Marmite'-peptone agar.

Pseudomonas fluorescens,[1] *Pseudomonas pyrrolnitrica*,[3] *Pseudomonas iodinum*[1] and poly-β-hydroxy-butyric acid producing strain (*Pseudomonas* species).[4] The comparison of strain No. B-9004 with these species is summarized in Table 4.

As shown in Table 4, strain B-9004 is different from these known species in some of morphological, cultural and physiological characteristics. Especially important were two characteristics of the strain, namely one was that the strain did not grow at temperatures above 37°C and the another one was that the strain produced a new antibiotic DB-2073.

These characteristics of strain B-9004, however, were not enough to designate the strain to be a new species. Therefore, strain B-9004 was tentatively named *Pseudomonas* sp. B-9004.

Chart 1. Isolation and purification procedure for DB-2073.



Fermentation

When *Pseudomonas* sp. B-9004 was grown on the natural or synthetic agar medium, crystals of antibiotic DB-2073 were formed in the medium. To obtain large amounts of the antibiotic, the bacteria were grown as a submerged culture. The cell suspension from the agar slant culture of the bacteria were inoculated into 500 ml SAKAGUCHI-flasks each containing 100 ml of the medium consisted of the following ingredients: glucose 1 %, peptone 1 %, meat extract 1 % and NaCl 0.2 % (initial pH 7.0).

After 48 hours of preculture at 30°C on a reciprocal shaker, 1 liter of the preculture was inoculated into 30-liter stainless steel fermentor containing 20 liters of the following medium: glucose 4 %, peptone 1 %, meat extract 1 % and NaCl 0.2 %. Fermentation was conducted at 30°C, the agitation was at 250 rpm, and aeration was at 6 liters/min.

The maximum antibiotic activity was obtained at 90 hours of fermentation. The antimicrobial activity of the culture broth was determined by paper disk method. Ten ml of the culture broth was centrifuged at 3,000 rpm for 10 minutes to separate the cells, and the cells were extracted with 3 ml methanol. The methanol extract was assayed against *Trichophyton gypseum*.

Isolation and Purification

The isolation and purification of the antibiotic were carried out by the procedure shown in the Chart 1. Eight grams of the pure crystals were obtained from 20 liters of culture broth. According to the later studies, the amount of antibiotic produced in a semisynthetic medium was more abundant and more constant than in the organic medium.

Physico-chemical Properties

Antibiotic DB-2073 was obtained as colorless or pale yellow crystals. It gradually turns yellow on exposure to light and/or air. It melts at 86~88°C, and has no optical rotation (1 % in MeOH). The elemental analysis gave C 76.20, H 10.43, O 13.37 % (diff.), but no nitrogene, halogen or sulfur. The molecular weight of 236 was determined by mass spectroscopy. The ultraviolet absorption spectrum in methanol had a large maximum at 212 nm ($E^{1\%}_{1cm}$ 1,120), and small peaks at 272 nm ($E^{1\%}_{1cm}$ 49) and 281 nm ($E^{1\%}_{1cm}$ 45) as shown in Fig. 1. As shown in Fig. 2, the infrared spectrum shows main peaks at 3340, 3410, 3280, 2960, 2925, 2860, 1635, 1590, 1525, 1470, 1445, 1340, 1230, 1170, 1118, 1020, and 845 cm^{-1}. The nmr spectrum shows that the molecule contains aromatic hydrogens (δ 6.20) and methyl groups on saturated carbons (δ 0.85~0.90) (Fig. 3).

Fig. 1. UV spectrum of DB-2073.

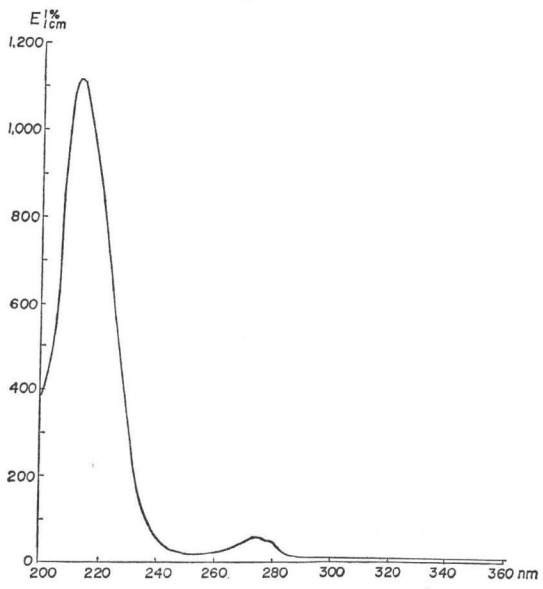


Fig. 2. IR spectrum of DB-2073 (KBr).

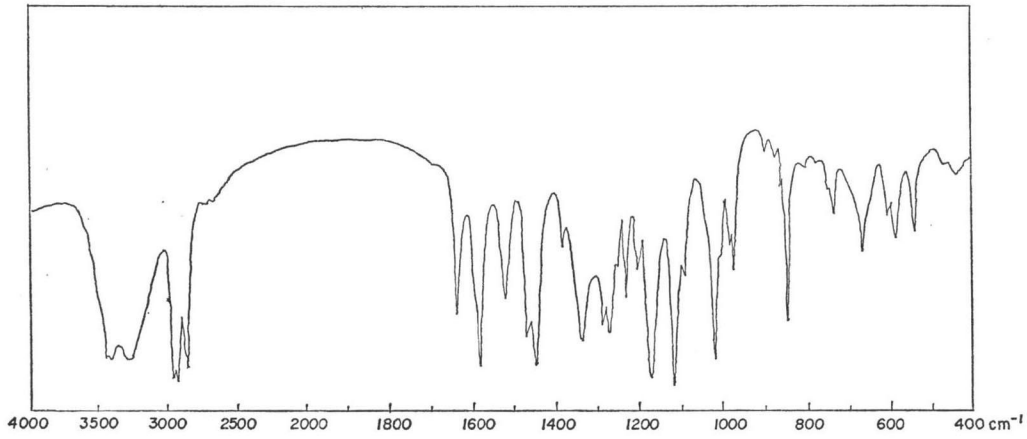
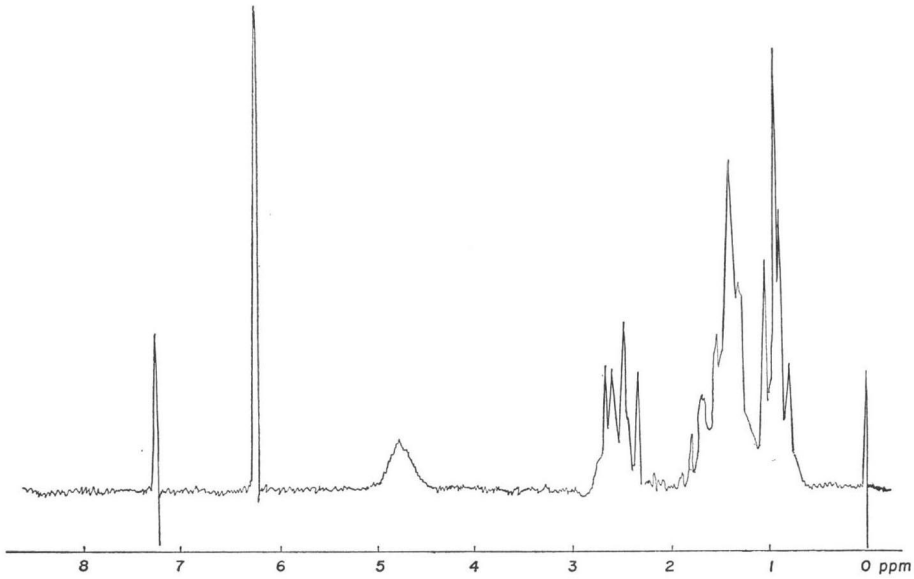


Fig. 3. NMR spectrum of DB-2073 (60 MHz, $CDCl_3$).



Antibiotic DB-2073 is easily soluble in many organic solvents such as methanol, ethanol, ethylether, chloroform, dioxan and dimethylsulfoxide, soluble in dichloromethane, dichloroethane, tetrachloromethane, benzene and toluene, slightly soluble in petroleum-ether, *n*-hexane and cyclohexane, and insoluble in water and acidic water.

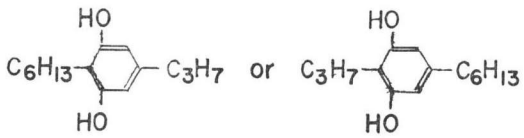
It changes gradually to purple or dark purple in alkaline water, but returns to pale yellow color in acidic condition. It decolorize potassium permanganate solution, reduces FEHLING's solution. It gives bluish purple color with ferric chloride in chloroform containing pyridine, which indicate that at least one phenolic hydroxyl group may exist. It shows negative anthrone, ninhydrin and biuret reactions.

The behavior on thin-layer chromatography of the antibiotic is described in Table 5. In every case a single spot was detected with iodine vapor and by bioautography on *Tricohophyton gypseum*.

Table 5. Thin-layer chromatographic behaviour of DB-2073.

Solid phase	Solvent system	Rf
Cellulose (Avicel SF, Asahi Kasei)	Ethanol	0.96
〃	Methanol	0.86
Silica gel G (Merck)	$CHCl_3$-benzene (2:8)	0.27
〃	EtOH-benzene (1:9)	0.77

The analytical data indicate the molecular formula $C_{15}H_{24}O_2$ for DB-2073 and the molecular weight of 236 calculated for the formula coincided with the molecular ion in the mass spectrum. Its chemical structure, which will be described in the separate paper, was presumed to be as follows.



Biological Properties

Antibiotic DB-2073 is active against Gram-positive bacteria, mycobacteria, yeasts and fungi. No activity was observed against Gram-negative bacteria. The minimum inhibitory concentration (MIC) against a variety of microorganisms are shown in Table 6. The intraperitoneal injection of the antibiotic into mice were lethal at 1,200 mg/kg, but at 800 mg/kg all were tolerated.

Table 6. Antimicrobial spectrum of DB-2073*

Test microorganism	MIC (mcg/ml)
1. *Escherichia coli* 0111	>100
2. *Shigella dysenteriae* Hanabusa	>100
3. *Shigella flexneri* 253	>100
4. *Salmonella typhosa* H901	>100
5. *Pseudomonas aeruginosa* Tsuchijima	>100
6. *Proteus vulgaris* 3167	>100
7. *Staphylococcus aureus* Terajima	25
8. *Staphylococcus aureus* R_4	25
9. *Corynebacterium diphtheriae* PW-8	3.2
10. *Diplococcus pneumoniae* DP-1	3.2
11. *Diplococcus pneumoniae* DP-2	3.2
12. *Erysipelothrix indiosa* Chiran	12.5
13. *Streptococcus agalactiae* 9925	12.5
14. *Streptococcus dysgalactiae* 9926	12.5
15. *Streptococcus pyogenes* G-36	12.5
16. *Mycobaterium tuberculosis* $H_{37}RV$	12.5
17. *Mycobaterium* FDA 607	6.25
18. *Mycobaterium phlei*	6.25
19. *Mycobaterium avium*	6.25
20. *Candida albicans* YU-1200	50
21. *Cryptococcus neoformans*	25
22. *Aspergillus fumigatus*	50
23. *Trichophyton gypseum*	3.2
24. *Trichophyton mentagrophytes*	3.2
25. *Trichophyton rubrum*	3.2
26. *Microsporium gypseum*	25
27. *Sporotrichum gougeroti*	25
28. *Blastomyces dermatitidis*	3.2

* Agar dilution method

Discussion

The physico-chemical and biological properties of antibiotic DB-2073 were compared with those of known antibiotics, but no antibiotic could be found to be identical with DB-2073.

Of the antibiotics produced by *Pseudomonas*, iodinin,[5] pyocyanine,[6] chlororaphine,[7] phenazine-1-carboxylic acid,[8] and pyrrolnitrin[9] are well known. These antibiotics, however, contain nitrogen or nitrogen and chlorine in the molecule. In contrast, DB-2073 contains neither nitrogen nor chlorine in its molecule differing from these antibiotics.

Recently, AOYAGI *et al.*[10] reported an enzyme inhibitor, panosialin, produced by a streptomycete, but this is disulfonate of 5-alkylresorcinol. STODOLA *et al.*[11] reported that a compound, stemphol, was produced by *Stemphylium majusculum*. Stemphol is a dialkylresorcinol substance having the molecular formula $C_{15}H_{24}O_2$. Its biological activity has not been reported. The molecular formula of DB-2073 is same as that of stemphol, however, the chemical structure of

DB-2073, which will be described in a separate paper, is different from that of stemphol. From these comparison, antibiotic DB-2073 can be considered as a new antibiotic.

Acknowledgement

The authors are grateful to Mr. S. ŌGA and Mr. M. KŌNO, Research Institute, Daiichi Seiyaku Co., Ltd., for their kind help.

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