ISOLATION OF A NEW PHENAZINE ANTIBIOTIC, DOB-41, FROM *PSEUDOMONAS* SPECIES

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A new phenazine antibiotic, DOB-41, was isolated from the culture broth of a *Pseudomonas* strain. The antibiotic obtained as yellow crystals showed UV maxima at 255 nm and 370 nm. A molecular formula, $C_{19}H_{18}N_2O_6$, was indicated by elemental analysis and mass spectrometry. The structure was elucidated by X-ray diffraction analysis. The antibiotic exhibited inhibitory activity against Gram-positive bacteria, and antitumor effect against leukemia P388 in mice.

In the course of our screening work for new antibiotics from bacterial strains, a *Pseudomonas* strain, DOB-41, was found to produce an antibiotic active against Gram-positive bacteria and a murine leukemia P388. The antibiotic was isolated and determined to be a new member of phenazine antibiotics.

The taxonomy of the producing strain, the isolation, physico-chemical properties, and structure elucidation of the antibiotic as well as the biological activities are presented in this paper.

Taxonomy

The producing organism numbered DOB-41 was isolated from a soil sample collected in Yakushima, Kagoshima Prefecture, Japan.

The organism is Gram-negative, non-sporulating rods ($0.5\sim0.7\times1.0\sim1.2$ μm) with rounded ends. Motile by one to two polar flagella. On nutrient agar, it forms circular, entire, wet and glistening colonies with yellowish cream color. Fluorescent pigments are formed on King's B medium. Poly-β-hydroxybutyrate is accumulated as an intracellular carbon reserve.

The organism, aerobic, showed good growth at 28°C but not at 5, 37 and 42°C. Other physiological characteristics are shown in Table 1. On cleavage of carbohydrates, acid formation was observed from D-glucose, D-galactose, D-mannose, cellobiose, lactose, maltose and trehalose, but not from DL-arabinose, D-xylose, D-fructose, L-rhamnose, sucrose and D-mannitol. No gas formation was observed from the above carbohydrates.

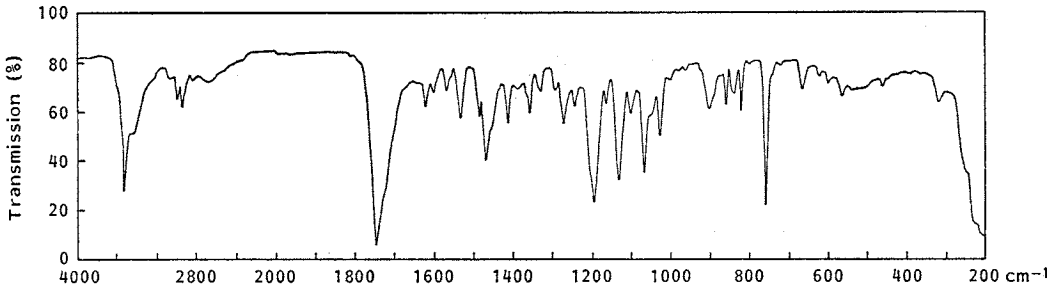
D-Glucose, trehalose, 2-ketogluconate, inositol, L-valine, D-alanine, and DL-arginine are used as sole carbon sources, but geramiol is not.

From comparison of these characteristics with those of bacteria registered in the Volume 1 of BERGEY's Manual of Systematic Bacteriology[1], the organism should be ascribed to the genus *Pseudomonas*. According to further comparison with eight species of the genus, the organism is similar to *Pseudomonas fluorescens* biovars, but different by poly-β-hydroxybutyrate accumulation and in-

Table 1. Physiological characteristics of strain DOB-41.

Properties observed	Result	Properties observed	Result
Catalase test	+	Arginine dihydrolase test	−
Oxidase test	+	Lysine decarboxylase test	+
OF-test	Oxidative	Ornithine decarboxylase test	+
Peptonization of milk	+	β-Galactosidase test	−
Coagulation of milk	−	Urease test	−
Haemolysis	+	Acylamidase test	+
Gelatin liquefaction	+	Phosphatase test	+
Esculin hydrolysis	+	Voges-Proskauer test	−
Starch degradation	−	Methyl red test	−
Levan formation	−	Nitrate reduction	+
Indole production	−	Denitrification	−
H_2S production	−	Citrate utilization	+

Fig. 1. IR spectrum of DOB-41 (KBr).



ability of growing at 5°C.

Isolation

The producing organism was cultured in a medium consisting of glycerol 1.0% and Soytone 2.0%, pH 7.0, in the usual shaking manner using Sakaguchi flask at 27°C for 2 days.

To the culture broth (10 liters), methanol (5 liters) and Hyflo Super-Cel (200 g) were added, and the mixture was filtered. The filtrate was evaporated *in vacuo*, and extracted with ethyl acetate at pH 3.0. After washing with water, the antibiotic contained in the solvent layer was transferred to 1% sodium bicarbonate, and then it was re-extracted with ethyl acetate at pH 3.0. The ethyl acetate extract was washed with water, dehydrated with sodium sulfate and evaporated to give an oily residue. It was applied to a silica gel column (Merck, Silica gel 60, size; 2.2×40 cm) and eluted with chloroform - methanol (8 : 2). The active eluate was evaporated to give a residue

Table 2. ^{13}C and ^{1}H NMR data[a] of DOB-41.

δ_C (ppm)	m	$^1J_{C,H}$ (Hz)	Related ^1H
21.6	q	128	1.73 (3H, d, J=6.5 Hz)
57.4	q	141.5	3.36 (3H, s)
62.0	t	141	3.76 (1H, m), 3.71 (1H, m)
67.7	d	152	7.11 (1H, q, J=6.5 Hz)
81.8	d	146	4.01 (1H, m)
127.2	d	164	
128.0	d	168	
130.6	d	170	8~8.6 (6H)
132.4	d	166	
133.6	d	170	
134.3	d	166	
128.1	s		
139.5	s		
140.1	s		
140.2	s		
140.4	s		
141.5	s		
166.2	s		
169.9	s		

[a] Data were obtained at 24°C using DMSO-d_6 solution and internal TMS reference.
m: Multiplicity of signal.

(360 mg). It was then re-chromatographed on a silica gel column (Merck, Silica gel 60, size; 3.0×26 cm) with chloroform - methanol (19 : 1). The active eluate was concentrated and applied to a silica gel plates (Merck, Silica gel 60 F-254), which was developed with chloroform - methanol (19 : 1). The zone of the antibiotic detected by a UV lamp was extracted with chloroform - methanol (1 : 1). The extract was evaporated to dryness and dissolved in ethyl acetate. The solution was washed with water of pH 3.0 (HCl), and then concentrated and cooled to form yellow needles (170 mg) of the antibiotic.

Physico-chemical Properties and Structure Elucidation

The antibiotic, DOB-41, was obtained as yellow needles, showing the following properties: MP 201°C; soluble in dimethyl sulfoxide, methanol, acetone, ethyl acetate, chloroform, but insoluble in ethyl ether, *n*-hexane and water. Electron impact mass spectra (EI-MS) *m/z* 370 (M); UV $\lambda_{max}^{CHCl_3}$ nm (ε) 255 (76,700), 370 (15,700); IR (KBr) cm^{-1} 3530, 1742, 1470, 1193, 1130, 1065 (Fig. 1). The ^{13}C and ^1H NMR spectroscopic data are shown in Table 2.

Anal Calcd for $C_{19}H_{18}N_2O_6$: C 61.62, H 4.86, N 7.56.
Found: C 61.11, H 4.88, N 7.33.

The UV spectrum is similar to those of some of phenazine antibiotics[2~4]. The analysis of the NMR data including decoupling experiments suggest the presence of OCH_3 (δ_H 3.36; δ_C 57.4 (q)), $OCHCH_3$ (δ_H 7.11, 1.73; δ_C 67.7 (d), 21.6 (q)) and $OCHCH_2O$ (δ_H 4.01, 3.76 and 3.71; δ_C 81.8 (d), 62.0 (t)). Six aromatic protons, twelve aromatic carbons and two carbonyl carbons are also shown. These findings suggest that the antibiotic is a disubstituted phenazine.

The molecular structure of DOB-41 was determined by X-ray analysis. Yellow needle crystals grown from methanol - 1-propanol (1 : 1) solution were suitable for the measurement.

Crystal data: Orthorhombic; space group $P2_12_12_1$; a=13.239(3), b=28.790(5), c=4.544(1) Å; Z=4. Intensity data were collected by ω-2θ scan on a Rigaku diffractometer with graphite-monochromatized CuKα radiation and a crystal of dimensions 0.05×0.05×0.30 mm. Intensities were measured in the range $\theta \leqq 70°$ with variable scan range $(2.0+0.2 \tan \theta)°$ and a constant scan speed of 5° min^{-1}. The 1890 independent intensities were corrected for Lorentz and polarization factors, but not for absorption effects.

The structure was solved by MULTAN 78[5]. Hydrogen atoms were located on a difference density map calculated after block-diagonal least-squares refinement. Successive refinement of the positional parameters of all the atoms and anisotropic thermal parameters of the non-hydrogen atoms gave an R value ($\sum|\Delta F|/\sum|F_o|$) of 0.054 for 1388 reflections. In the refinements the temperature factor of each hydrogen atom was set equal to B_{eq} of the bonded atom. The weighting scheme used was $\omega=[\sigma^2(F_o)+0.00051|F_o|^2]^{-1}$ for $\omega^{1/2}|F_c| \geqq 2$ and $\omega^{1/2}|\Delta F|<3$, $\omega=0$ otherwise.

Final positional parameters and equivalent isotropic temperature factors of the non-hydrogen atoms are listed in Table 3. The stereoscopic view of the relative configuration of the molecule, along with the atom-numbering system, is shown in Fig. 2[6]. The carboxyl group lies on the plane of the phenazine ring, of which OH group forms the intramolecular hydrogen bond of O(16) – N(13), 2.603(6) Å. The molecules related by 2_1 axis are linked by hydrogen bonds between O(25) and O(17), 2.790(6), to form an infinite chain extended along the B_{ax}.

Thus, the perspective view of the molecular structure was drew as shown in Fig. 2. The relative configurations at C(18) and C(23) were shown to be *S** and *R**, but the absolute configurations were not determined.

Table 3. Atomic coordinates ($\times 10^4$) and equivalent isotropic temperature factors ($\times 10^2$ Å^2) with their estimated standard deviations in parentheses.

	X	Y	Z	B_{eq}
C(1)	−980 (3)	−2164 (1)	−2135 (11)	318 (12)
C(2)	−281 (4)	−2335 (1)	−4098 (13)	404 (14)
C(3)	545 (4)	−2067 (2)	−5045 (13)	427 (14)
C(4)	690 (3)	−1619 (2)	−4113 (12)	345 (12)
C(5)	−11 (3)	−1429 (1)	−2031 (11)	271 (10)
N(6)	167 (3)	−1001 (1)	−1002 (9)	281 (9)
C(7)	−491 (3)	−830 (1)	987 (10)	253 (10)
C(8)	−290 (30)	−380 (1)	2263 (10)	267 (10)
C(9)	−975 (3)	−214 (1)	4234 (11)	322 (12)
C(10)	−1873 (4)	−455 (2)	4939 (12)	381 (13)
C(11)	−2081 (4)	−883 (2)	3787 (12)	361 (13)
C(12)	−1369 (3)	−1084 (1)	1809 (11)	284 (11)
N(13)	−1528 (3)	−1515 (1)	811 (9)	289 (9)
C(14)	− 848 (3)	−1697 (1)	−1103 (10)	275 (11)
C(15)	−1803 (4)	−2473 (1)	−1195 (13)	412 (14)
O(16)	−2456 (3)	−2312 (1)	811 (10)	509 (11)
O(17)	−1923 (3)	−2859 (1)	−2118 (10)	593 (12)
C(18)	677 (3)	−135 (1)	1486 (10)	280 (11)
C(19)	1597 (3)	−384 (1)	2656 (13)	371 (13)
O(20)	722 (2)	324 (1)	2804 (7)	332 (8)
C(21)	226 (3)	675 (1)	1487 (11)	314 (12)
O(22)	−343 (3)	631 (1)	−552 (8)	415 (9)
C(23)	583 (4)	1131 (1)	2825 (11)	340 (12)
C(24)	1674 (4)	1223 (1)	2262 (11)	341 (12)
O(25)	1890 (2)	1229 (1)	−809 (8)	357 (8)
O(26)	8 (3)	1506 (1)	1738 (9)	488 (11)
C(27)	−864 (5)	1607 (2)	3350 (18)	677 (23)

$B_{eq}=4/3 \sum_i \sum_j \beta_{ij} a_i \cdot a_j$.

Fig. 2. Perspective view of the molecule of DOB-41.

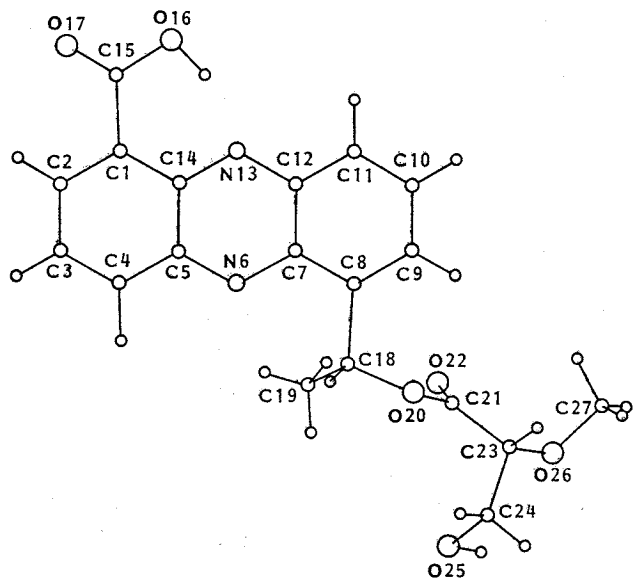
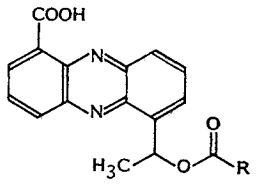


Fig. 3. Structure of DOB-41 and DC-86-M.



DOB-41 $R=CHCH_2OH$ (with OCH_3)
DC-86-M $R=CH_2OH$

This structure is closely similar to that of DC-86-M[4], an antitumor antibiotic recently isolated from *Streptomyces* sp. as illustrated in Fig. 3.

Biological Properties

DOB-41 showed strong inhibitory activity against Gram-positive bacteria as shown in Table 4. The antibiotic exhibited a moderate effect in prolonging the survival period of mice in which murine lymphatic leukemia P388 cells were implanted (Table 5). However, it exhibited only a weak effect in mice bearing leukemia L1210 (T/C: 129%) or B-16 melanoma (T/C: 113%).

Table 4. Antimicrobial activity of DOB-41.

Test organism	MIC (μg/ml)
Staphylococcus aureus FDA JC-1	0.39
S. aureus Smith	0.1
Streptococcus pyogenes C203	0.2
S. pneumoniae I	0.78
Micrococcus luteus ATCC 9341	0.78
Escherichia coli NIHJ JC-2	>50
Proteus mirabilis PR4	>50
Enterobacter cloacae ATCC 13047	>50
Serratia marcescens ATCC 13880	>50
Pseudomonas aeruginosa ATCC 15691	>50
Mycoplasma gallisepticum S-6	0.39

Table 5. Antitumor activity of DOB-41 against murine leukemia P388 (ip-ip).

Dose (mg/kg)	T/C (%)
0.2	113
0.5	123
1.0	127
2.0	133
5.0	145
10	153
20	124 (Toxic)

The drug is administered 1 day after tumor inoculation.

T/C: The ratio of mean survival days of the treated group divided by that of the control group.

Experimental

General Methodology

The UV absorption spectrum was measured with a Hitachi 323 spectrometer and the IR absorption spectrum with a Jasco DS-403G spectrometer. EI-MS was obtained with a Hitachi RMV-8GN mass spectrometer. The ^1H and ^{13}C NMR spectra were recorded with a Varian XL-200 spectrometer.

Acknowledgment

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