BE-40665D, a New Antibacterial Antibiotic Produced by an *Actinoplanes* sp.

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(Received for publication October 29, 1998)

In the course of our screening program for new antibacterial substances, an *Actinoplanes* strain A40665 isolated from a soil sample collected in Oita Prefecture, Japan, was found to produce an active compound. This compound, BE-40665D, was isolated from the mycelial cake of culture broth and the structure was elucidated as shown in Fig. 1. BE-40665D inhibited the growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). In this paper, the producing organism, fermentation, isolation, physicochemical properties, structure determination and biological properties of BE-40665D are described.

Characterization of the producing strain followed the method adopted by the International Streptomyces Project (ISP)[1] as well as several other tests. Strain A40665 had a deep orange vegetative mycelium and subglobose or irregular sporangia with a diameter of $6 \sim 10 \times 10 \sim 12\,\mu m$ on the surface of the agar media (Fig. 2). In contact with water, motile spores were released from the sporangia. The culture characteristics of strain A40665 are summarized in Table 1. Chemotaxonomic analysis of strain A40665 revealed meso-diaminopimelic acid, 3-OH-diaminopimelic acid and glycine as distinguishing components of the cell wall. Xylose and arabinose were the major sugars in the whole-cell hydrolysate. These results indicated that the strain A40665 has a type IID cell wall of LECHEVALIER and LECHEVALIER[2]. The physiological properties and carbon utilization of strain A40665 are shown in Table 2. Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[3] on agar medium cultured at 28°C for 14 days. The above-mentioned characteristics of strain A40665 revealed that it belonged to the genus *Actinoplanes*. Strain A40665 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the name of *Actinoplanes* sp. A40665 under the accession No. FERM P-14069.

The *Actinoplanes* sp. A40665 was inoculated into three 500-ml Erlenmeyer flasks each containing 110 ml of a medium consisting of 0.2% glucose, 2.0% dextrin, 0.5% meat meal, 0.5% defatted rice bran, 0.2% defatted meat bone meal, 0.1% dry yeast, 0.05% magnesium sulfate, 0.05% sodium bromide, 0.5% sodium chloride, 0.5% potassium bromide, 0.1% potassium hydrogen phosphate, 0.002% calcium chloride, 0.0002% ferrous sulfate, 0.00004% cupric chloride, 0.00004% manganese chloride, 0.00004% cobalt chloride, 0.00008% zinc sulfate, 0.00008% sodium borate and 0.00024% ammonium molybdate (pH 7.2). The seed culture was incubated for 72 hours at 28°C on a rotary shaker (180 rpm). Two ml of the culture broth was inoculated to each of 100 500-ml Erlenmeyer flasks containing 110 ml of the same medium and cultured on a rotary shaker (180 rpm) at

Fig. 1. Structures of BE-40665D.

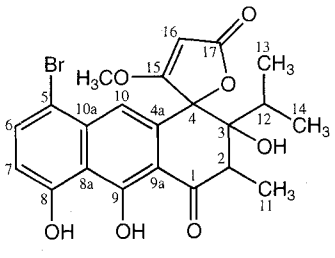


Fig. 2. Scanning electron micrograph of sporangia of *Actinoplanes* sp. A40665.

Bar represents 30 μm.

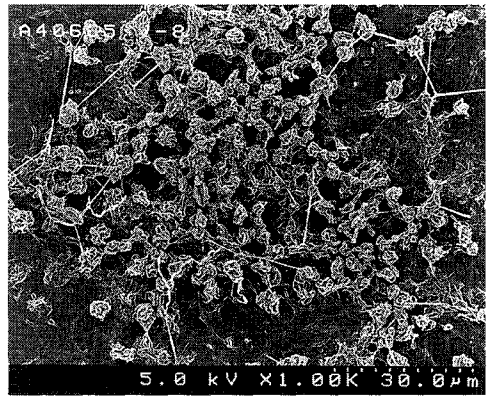


Table 1. Culture characteristics of strain A40665.

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Sporangium	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	None	Deep orange	None	Yellow
Oatmeal agar (ISP-3)	Good	None	Deep orange	Poor	Yellow
Inorganic salts-starch agar (ISP-4)	Good	None	Deep orange	Moderate	Yellow
Glycerol-asparagine agar (ISP-5)	Good	None	Deep orange	Moderate	Yellow
Peptone-yeast extract-iron agar (ISP-6)	Moderate	None	Light orange	None	None
Tyrosine agar (ISP-7)	Good	None	Deep brown	None	Brown
Nutrient agar	Moderate	None	Light yellowish pink	None	Yellow
Sucrose-nitrate agar	Good	None	Deep orange	None	Yellow
Glucose-asparagine agar	Moderate	None	Light yellowish pink	None	Yellow

Table 2. Physiological properties and carbon utilization of strain A40665.

Melanoid formation	
Tryptone-yeast broth (ISP-1)	−
Peptone-yeast extract-iron agar (ISP-6)	−
Tyrosine agar (ISP-7)	+
Coagulation of milk	−
Peptonization of milk	−
Liquefaction of gelatin	+
Hydrolysis of starch	+
Reduction of nitrate	+
Decomposition of tyrosine	−
Decomposition of casein	+
NaCl tolerance	$\leqq$2%
Temperature range for growth	16~32°C
Carbon utilization	
D-Glucose	+
D-Xylose	+
L-Arabinose	+
L-Rhamnose	+
D-Fructose	+
Raffinose	+
D-Mannitol	+
i-Inositol	+
Sucrose	+
D-Galactose	+
Salicin	+

28 °C for 240 hours.

The mycelium was obtained by filtration of the whole broth (*ca.* 10 liters). Extraction was carried out twice with 5 liters of methanol and the combined extracts were concentrated. The concentrated solution was extracted twice with 3 liters of ethyl acetate and the extract was evaporated under reduced pressure. The residue was extracted with 100 ml of hexane and the hexane was evaporated under reduced pressure. The residue was applied to a Silica gel column (Merck, 23 × 3.0 cm i.d.) and eluted with $CHCl_3$/MeOH/CH_3CO_2H (100 : 1 : 0.1). The fractions containing BE-40665D were concentrated *in vacuo*. Further purification by a TOYOPEARL HW-40S (TOSOH) column chromatography (46 × 3.0 cm i.d.) using CH_3OH as eluent yielded 3.5 mg of BE-40665D as a yellow powder.

BE-40665D is soluble in common organic solvents such as $CHCl_3$ and DMSO, but insoluble in water. Other properties are as follows: BE-40665D ($C_{22}H_{21}O_7Br$); HRFAB-MS *m/z* 477.0542 $(M+H)^+$, calcd *m/z* 477.0549 for $C_{22}H_{21}O_7Br$; UV λ_{max}^{MeOH} nm 225, 265, 317, 330, 405; IR ν_{max} (KBr) cm^{-1} 3429, 2927, 1739, 1628, 1452, 1387, 1355, 1261, 958, 760.

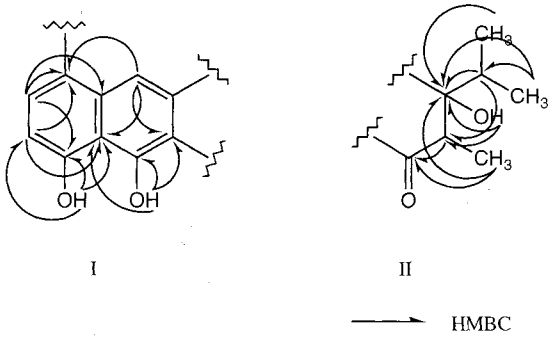
The ^1H and ^{13}C NMR data of BE-40665D are shown in Table 3. The molecular formula of BE-40665D was established as $C_{22}H_{21}O_7Br$ from the result of HRFAB-MS spectral data and ^{13}C NMR data. The ^{13}C NMR data and DEPT experiment revealed the presence of three methyl carbons, two methine carbons, two quaternary carbons, one methoxyl carbon, four sp_2 methine carbons, nine sp_2 quaternary carbons and one carbonyl carbon signals. The molecular formula agreed well with these data. In the ^1H NMR spectrum, a D_2O-exchangeable proton signal at δ_H 16.3 was observed. This result indicated the presence of a chelated phenolic proton. ^1H-^1H COSY and HMBC experiments of BE-40665D revealed two partial structures I and II as shown in Fig. 3. The D_2O-exchangeable proton (δ_H 2.36) in the partial structure II, which coupled to 2-H (δ_H 3.56) in the COSY spectrum, was shown to be a hydroxyl proton of a tertiary

Table 3. ^{13}C and ^{1}H NMR data for BE-40665D in $CDCl_3$.

	^{13}C	^{1}H
1	204.7	
2	46.0	3.56 (1H, dq, J=1.8, 7.0 Hz)[a]
3	79.2	
3-OH		2.36 (1H, d, J=1.8 Hz)
4	86.8	
4a	134.1	
5	111.5	
6	136.1	7.77 (1H, d, J=8.2 Hz)
7	113.4	6.84 (1H, d, J=8.2 Hz)
8	157.9	
8-OH		9.91 (1H, s)
8a	115.1	
9	164.5	
9-OH		16.3 (1H, s)
9a	108.6	
10	116.3	7.33 (1H, s)
10a	135.8	
11	8.5	1.46 (3H, d, J=7.0 Hz)
12	35.4	2.29 (1H, m)
13	17.4	1.11 (3H, d, J=7.3 Hz)
14	18.3	1.14 (3H, d, J=7.3 Hz)
15	181.4	
16	93.1	5.54 (1H, s)
17	169.6	
15-OCH_3	59.9	4.09 (3H, s)

^{1}H and ^{13}C NMR spectra were measured at 500 MHz and 125 MHz, respectively.
[a] Multiplicity, J in Hz.

Fig. 3. Partial structures of BE-40665D.

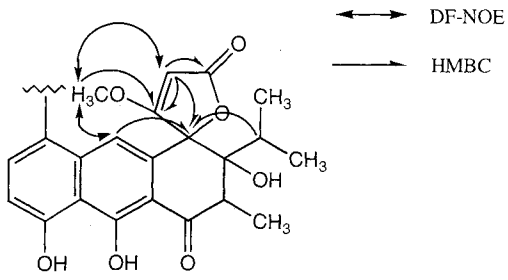


alcohol by analysis of the HMBC spectrum. The observed $^{4}J_{H-H}$ coupling (1.8 Hz) through an oxygen atom was previously observed in bafilomycins[4] and L-681,110[5]. It was deduced that carbonyl carbon (δ_C 204.7) was linked to C-9a of partial structure I by the presence of a chelated phenolic proton (δ_H 16.3). Furthermore the HMBC spectrum showed that sp_3 quaternary carbon (δ_C 86.8)

Table 4. Antibacterial activities of BE-40665D.

Strain	MIC (μg/ml) BE-40665D
Bacillus cereus IFO3001	6.25
Staphylococcus aureus FDA209P	12.5
Staphylococcus aureus Smith	12.5
Staphylococcus aureus (MRSA) BB6117	12.5
Staphylococcus aureus (MRSA) BB6118	12.5
Enterococcus faecalis IFO12580	6.25
Streptococcus thermophillus IFO3535	12.5
Micrococcus luteus ATCC9341	12.5
Escherichia coli NIHJ JC-2	>50
Enterobacter cloacae IFO 13535	>50
Serratia marcescens IFO3736	>50
Morganella morganii IFO3848	>50
Flavobacterium meningosepticum IFO12535	>50
Acinetobacter calcoaceticus IFO12552	>50
Pseudomonas aeruginosa IFO 3445	>50

Fig. 4. Connectivities between partial structures I and II of BE-40665D.



was correlated to an aromatic proton (δ_H 7.33) and a methine proton (δ_H 2.29). From these results, the connectivity between partial structures I and II was determined as shown in Fig. 4. The ^{13}C NMR spectrum indicated the presence of the methoxyl group. The presence of α, β- unsaturated lactone was suggested by IR spectroscopy (1739 cm^{-1}). In the HMBC spectrum, an olefinic proton (δ_H 5.54) was correlated to a sp_3 quaternary carbon (δ_C 86.8) and sp_2 carbons (δ_C 181.4, 169.6). From these results, it was deduced that the remaining $C_4H_4O_3$ unit has spiro structure as shown in Fig. 4. In the differential NOE experiments, NOEs between the methoxyl protons and an olefinic proton (16-H) and between the methoxyl protons and 10-H were observed (Fig. 4). These results also supported the structure of Fig. 4. By default, it determined that the bromine must be attached to C-5. Thus the structure of BE-40665D was determined as shown in Fig. 1.

The antibacterial activity of BE-40665D was de-

termined using agar dilution method in Sensitivity Disk Agar-N (Nissui). About 5 μl of bacterial suspension (10^6 cfu/ml) was inoculated by inoculating apparatus (Sakuma, Tokyo). After 20 hours at 37 °C of incubation, the MIC values were recorded. Antibacterial activity of BE-40665D was shown in Table 4. BE-40665D showed moderate activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), and no activity against Gram-negative bacteria.

Acknowledgments

The authors thank Dr. AKIO SEINO in Research Center for Biological Function, The Kitasato Institute, for many useful discussions about taxonomical studies.

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