BE-24566B, a New Antibiotic Produced by *Streptomyces violaceusniger*

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In the course of our screening program for new antibacterial compounds, a strain A24566 isolated from a lichen collected at Jyogasaki, Shizuoka prefecture, Japan, was found to produce a potent antibiotic. This compound, BE-24566B (**1**), was isolated from the mycelial cake of cultural broth, and was shown to have the unique structure in Fig. 1. BE-24566B possesses potent antibacterial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). This paper describes the strain, production, isolation, physico-chemical properties and structure elucidation of this new antibiotic.

Characterization of the strain followed the method adopted by the International Streptomyces Project (ISP)[1]. The strain A24566 formed well developed and branching substrate mycelia and aerial mycelia, but fragmentation of the substrate mycelia was not observed. The whole cell hydrolysate contained L,L-diaminopimelic acid. The spore chains of the strain were spirals and the spore surface was rugose. The spore mass was gray, becoming black and moist with maturity. Melanoid pigments and soluble pigments were absent. Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[2] on the agar medium culture at 28°C for 14 days. D-glucose, D-xylose, L-arabinose, L-rhamnose, D-fructose, raffinose, sucrose and D-galactose were utilized for growth. Utilization of inositol was doutful and salicin was not utilized by the strain.

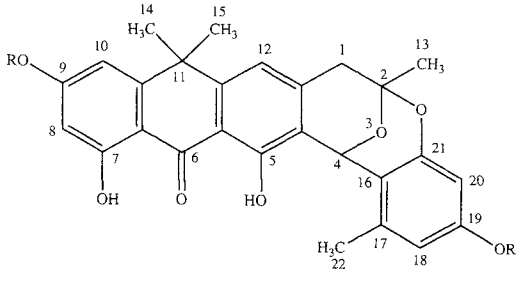
The above-mentioned combination of characteristics of strain A24566 indicated that it belonged to *Streptomyces violaceusniger*. A24566 has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan (No. FERM BP-3994).

The strain A24566, cultured on a slant agar medium, was inoculated into four 500-ml volume Erlenmeyer flasks which contained 110 ml of a culture medium comprising 0.2% glucose, 2.0% dextrin, 0.5% meat meal, 0.5% degreased rice bran, 0.2% degreased meat bone powder, 0.1% dry yeast, 0.05% magnesium sulfate, 0.05% sodium bromide, 0.5% sodium chloride, 0.1% potassium hydrogen phosphate, 0.002% calcium chloride, 0.00004% cuprous chloride, 0.00004% manganese chloride, 0.00004% cobalt chloride, 0.00008% zinc sulfate, 0.00008% sodium borate, 0.00024% ammonium molybdate and 0.0002% ferrous sulfate (pH 7.2) and shaken on a rotary shaker (180 rpm) at 28°C for 72 hours. Two ml each of the culture broth was inoculated into 100 of 500-ml volume Erlenmeyer flasks containing 110 ml of the foregoing culture medium and placed on a rotary shaker (180 rpm) at 28°C for 120 hours.

The broth (*ca.* 11 liters) so obtained was filtered to give mycelium. Methanol (8 liters) was added to the resulting mycelium and stirred at room temperature for 30 minutes, then the mycelium was filtered yielding the methanol extract. The methanol extract was concentrated to about 1 liter under reduced pressure and then 1.5 liters of ethyl acetate was added for extraction. Fresh ethyl acetate (0.7 liter) was added to the resulting aqueous layer to perform second extraction and the ethyl acetate extracts were combined. The extract was washed twice with water (1 liter × 2) and then concentrated under reduced pressure. To the resulting residue 240 ml of methanol and 400 ml of n-hexane were added and the methanol layer was separated. To the n-hexane layer 50 ml of methanol was added and methanol layer was separated. The two methanol layers were combined and evaporated under reduced pressure. The resulting residue was dissolved with 200 ml of chloroform and applied to a column of silica gel (3 × 38 cm) and the column was developed with solvent mixture (chloroform - methanol = 20 : 1 to 1 : 1). The active material containing fraction were combined and evaporated under reduced pressure to obtain crude BE-24566B. Final purification of BE-24566B was achieved Sephadex LH-20 column (2 × 88 cm) by elution with methanol. The active eluate upon evaporation afforded 209.6 mg of BE-24566B.

The physico-chemical properties of BE-24566B are shown in Table 1. The BE-24566B (**1**) was obtained as a pale yellow powder, soluble in methanol and hardly soluble in water. The low resolution FAB mass spectrum

Fig. 1. Structures of BE-24566B (**1**) and (**2**).



R=H BE-24566B (**1**)

R=CH_3 (**2**)

Table 1. Physico-chemical properties of BE-24566B.

Appearance	Pale yellow powder
Rf	0.5 (Kiesel gel 60; $CHCl_3$/MeOH=10:1)
Molecular formula	$C_{27}H_{24}O_7$
UV λ_{max}^{MeOH} nm	204, 220, 273, 360
IR(KBr) cm^{-1}	3418, 1470, 1365, 1290, 1230, 1143, 1029, 1611, 1431, 1326, 1254, 1170, 1056, 834
HR FAB-MS m/z	Calcd for $C_{27}H_{25}O_7$: 461.1600 $(M+H)^+$
	Found: 461.1611

Table 2. ^{13}C and ^1H NMR data for BE-24566B (**1**) and (**2**).

	BE-24566B (**1**)[a]		(**2**)[b]	
	^{13}C(ppm)	^1H (ppm)	^{13}C (ppm)	^1H (ppm)
1	40.1	3.13 (1H, d, J=18.4Hz)	40.6	3.16 (1H, d, J=18.0Hz)
		3.32 (1H, d, J=18.4Hz)		3.24 (1H, d, J=18.0Hz)
2	97.7		97.9	
4	65.0	6.24 (1H, s)	65.3	6.33 (1H, s)
4a	123.6		123.4	
5	157.6		158.1	
5-OH		13.5 (1H, s)		13.4 (1H, s)
5a	111.3		111.6	
6	190.6		190.7	
6a	107.1		108.0	
7	165.7		165.8	
7-OH		12.8 (1H, s)		13.0 (1H, s)
8	101.2	6.31 (1H, d, J=2.1Hz)	98.7	6.39 (1H, d, J=2.4Hz)
9	165.6		166.1	
10	106.5	6.74 (1H, d, J=2.1Hz)	106.1	6.62 (1H, d, J=2.4Hz)
10a	154.8*		153.7*	
11	38.6		38.6	
11a	150.3*		150.1*	
12	117.4	7.13 (1H, s)	116.9	6.85 (1H, s)
12a	141.9		141.4	
13	27.1	1.64 (3H, s)	27.7	1.73 (3H, s)
14	33.2	1.67 (3H, s)	33.9	1.64 (3H, s)
15	33.3	1.60 (3H, s)	34.0	1.56 (3H, s)
16	113.8		114.9	
17	136.0		136.5	
18	111.0	6.26 (1H, d, J=2.5Hz)	109.1	6.34 (1H, d, J=2.6Hz)
19	157.0		159.2	
20	100.8	6.15 (1H, d, J=2.5Hz)	99.3	6.27 (1H, d, J=2.6Hz)
21	152.4		152.2	
22	18.7	2.44 (3H, s)	19.4	2.51 (3H, s)
9-OCH_3	-	-	55.5	3.88 (3H, s)
19-OCH_3	-	-	55.1	3.73 (3H, s)

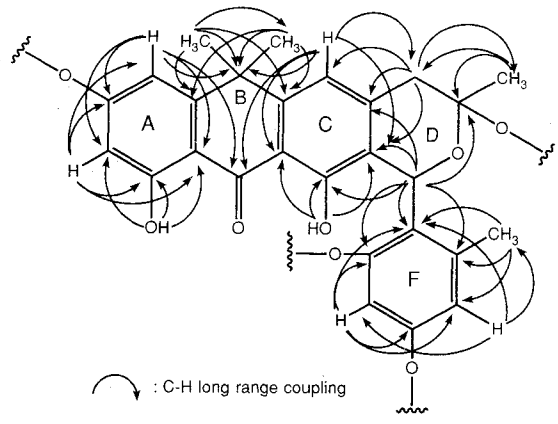
[a] in acetone-d_6.
[b] in $CDCl_3$.
* may be exchanged.

displayed an intense protonated $(M+H)^+$ peak at 461. Peak matching, using high resolution mass measurements, showed this elemental composition to be $C_{27}H_{24}O_7$ (calcd for $C_{27}H_{25}O_7$: m/z 461.1600 $(M+H)^+$; found: m/z 461.1611). The UV spectrum of this compound in methanol showed absorption maxima at 204, 220, 273 and 360 nm.

The ^1H NMR spectrum (400 MHz, acetone-d_6) of **1** showed four methyl groups, one methylene proton, six methine protons and two chelated phenolic protons (Table 2). The ^{13}C NMR spectrum (100 MHz, acetone-d_6) of **1** revealed the presence of 27 carbon atoms, supporting the above elemental composition.

The ^1H-^1H COSY and ^1H-^{13}C spectra of **1** indicated

Fig. 2. HMBC experiment of **1**.



the presence of two isolated aromatic spin systems with meta-coupled protons. Remaining methine protons were an aromatic proton and an aliphatic methine proton. Based on the HMBC spectrum of **1**, the partial structure for A ring to D ring and F ring was deduced (Fig. 2).

Treatment of **1** with excess trimethylsilyl diazomethane in MeOH-benzene at room temperature gave the corresponding dimethyl ether derivative (**2**) (FAB-MS; m/z 489 $(M+H)^+$). The NMR data of **2** was listed in Table 2. In the NMR data of **2**, two methoxyl signals (δ_H 3.73, δ_C 55.1; δ_H 3.88, δ_C 55.5) were newly observed and other signals were similar to those of **1**. Furthermore, these two methoxyl protons were coupled to the carbons at δ_C 159.2 and δ_C 166.1 ppm, respectively, which carbons were assigned to C-19 carbon in F ring and C-9 carbon in A ring by analysis of the HMBC spectrum of **2**. These observation implied that non-chelated phenolic protons should exist at C-9 and C-19 in **1**. Taking into consideration of the molecular formula of **1**, a ketal structure for BE-24566B was supposed. Difference NOE spectra of **2** also supported the structure of **1** (Fig. 3). Recently, Benastatin A was isolated as an inhibitor of glutathione S-transferase from a *Streptomyces* sp.[3]. The ^{13}C NMR data of **1** in A, B and C ring was quite similar to that of corresponding structure in Benastatin A[4]. From the data described above, the structure of **1** was determined as shown in Fig. 1.

The antibacterial activity of BE-24566B was determined by a standard twofold serial dilution method recommended by the Japan Society of Chemotherapy. About 5 μl of bacterial suspension (10^6 cfu/ml) was inoculated by inoculating apparatus (Sakuma, Tokyo). After 18 hours of incubation, the MIC values were recorded. As shown in Table 3, BE-24566B was exhibited antimicrobial activity against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).

Single dose intraperitoneal administration of BE-24566B into CDF1 female mice caused no death at 100 mg/kg. BE-24566B may be a new lead as an antibacterial agents.

Fig. 3. Difference NOE experiments of **2**.

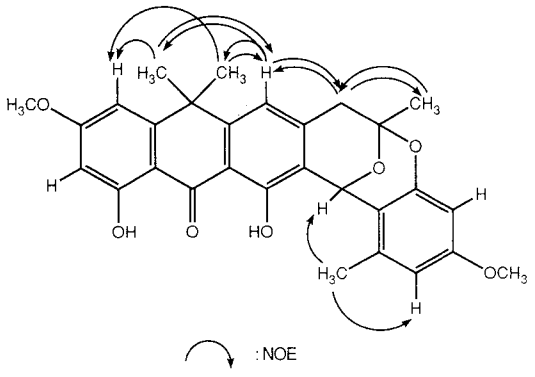


Table 3. Antimicrobial activity of BE-24566B.

Microorganism	MIC (μg/ml)
Bacillus subtilis ATCC 6633	1.56
Bacillus cereus IFO 3001	1.56
Staphylococcus aureus FDA 209P	1.56
Staphylococcus aureus Smith	3.13
Staphylococcus aureus BB 6152*	3.13
Micrococcus luteus ATCC 9341	1.56
Enterococcus faecalis IFO 12580	3.13
Streptococcus thermophilus IFO 3535	3.13

* Methicillin-resistant (MRSA).

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