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F2928-1 and -2, New Antifungal Antibiotics from *Cladobotryum* sp.

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Abstract Two new antifungal antibiotics, F2928-1 (**1**) and -2 (**2**), were isolated from the culture broth of *Cladobotryum* sp. These compounds were purified by solvent extraction, silica gel column chromatography and preparative HPLC, consecutively. The structures of these compounds were assigned as a decalin compound on the basis of various spectral analyses. These compounds showed antimicrobial activity against fungi including clinically important fungus, *Aspergillus fumigatus*.

Keywords F2928, antifungal, decaline

Introduction

In the past 20 years, fungal infections have increased dramatically [1, 2]. Fungal infections occur more frequently in people, who undergo an organ transplant operation, who have human immunodeficiency virus infection, or who are treated with cancer chemotherapy. These infections are often life-threatening.

Amphotericin B [3, 4] is the most conventional drug to treat fungal infections. However, this drug has many side effects; nephrotoxicity is the major side effect. Therefore, new fungicidal drugs with low toxicity are expected to be discovered and developed.

In the course of our screening program for new antifungal agents, we have found that the extract of culture broth of *Cladobotryum* sp. showed activity against *A. fumigatus*. Two active compounds were isolated and characterized by spectroscopic methods.

In this paper, we describe producing strain, fermentation, isolation, physico-chemical properties, structure determination and antimicrobial activities of these compounds.

Materials and Methods

Producing Strain

The producing strain, TAMA 85 was isolated by single spore isolation with the aid of Skerman's manipulator from ascocarps growing on the decayed fruiting body of *Trametes versicolor* (L.: Fr.) Pilát. This material was collected in Iiyama, Atsugi, Kanagawa, Japan on 16 October, 2000. Taxonomic study was done according to the method of Rogerson and Samuels [5].

Fermentation

A slant culture of strain TAMA 85 grown on potato

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dextrose agar was used to inoculate into a 24 mm i.d. test tube containing 10 ml of a sterile seed medium consisting of potato extract 10%, V8 vegetable juice 10%, soluble starch 2.0%, soybean flour 1.5%, glucose 1.2%, malt extract 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%. pH was adjusted to 6.5 before autoclaving. The test tube was shaken on a reciprocal shaker at 25°C for 72 hours.

A half ml of the first seed culture was transferred to 100-ml Erlenmeyer Flasks containing 10 ml of a sterile producing medium consisting of glucose 3.5%, soluble starch 1.0%, soybean flour 1.0%, dried locust meal 1.0%, polypeptone 0.5%, meat extract 0.5%, corn steep liquor 0.5%, yeast extract 0.3%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05% (pH 6.5 before autoclaving). Fermentation was carried out for 120 hours at 25°C and 200 rpm on a rotary shaker.

Isolation

A culture broth (1.0 liters) was extracted with *n*-BuOH (1.0 liters). After evaporation of the solvent *in vacuo*, the oily residue was suspended in water, and then the suspension was extracted with hexane. The organic layer was concentrated under reduced pressure, and then the resulting extract was applied onto a silica gel column (Wakogel C-200, 25 mm i.d. ×300 mm), which was eluted with a mixture of hexane - EtOAc (1 : 3, v/v). Active fractions were monitored by TLC and/or HPLC analysis. Evaporation of the active fractions yielded crude mixture of **1** and **2**. The crude mixture were dissolved in DMSO and applied to preparative HPLC (Senshu Pak PEGASIL ODS II 20 mm i.d. ×250 mm). **1** and **2** were eluted with MeCN - 0.1% AcOH (57 : 43, v/v) at a flow rate of 9 ml/minute. The fractions containing **1** (t_R 23.5 minutes) were concentrated. **1** (25.6 mg) was isolated as pale yellow powder. The fractions containing **2** (t_R 31.2 minutes) were concentrated, giving **2** (9.8 mg) as pale yellow powder.

General Methods

UV spectra were determined on a HITACHI U-3200 spectrophotometer. Melting point data were obtained with a BÜCHI 535 Melting point apparatus. Specific rotations were measured with a HORIBA SEPA-300 polarimeter. ^1H and ^{13}C NMR spectra were recorded with a JEOL JNM-A500 spectrometer. ESI mass spectra were obtained with a FINNIGAN LCQ^{DUO} apparatus. HRFAB mass spectra were measured with a JOEL JMS-SX102 mass spectrometer. HPLC was performed on a Shimadzu LC-10AD system.

Antimicrobial Activities

Antifungal activities were determined by broth dilution method using RPMI1640 medium. After the addition of test compounds, *Aspergillus fumigatus* and *Candida albicans* were grown at 35°C for 48 hours and at 35°C for 24 hours, respectively. MIC was defined as the lowest concentration in which 80% inhibition of growth was observed.

Antibacterial activity was determined by broth dilution method using Modified Mueller Hinton Medium. All bacteria were grown at 37°C for 20 hours. MIC was defined as the lowest concentration in which visible inhibition of growth was observed.

Results

Characterization of the Producing Strain

Colonies of TAMA 85 on Miura medium (LCA), Malt extract agar (MA) and Oatmeal agar (OA) grow rapidly at 25°C, floccose, hyaline forming a loose or dense hyphal network in the center or margin, but never matured to ascocarps. Conidiogenesis was observed, and conidiophores were born from aerial hyphae and irregularly branched (Fig. 2). Conidiogenous cells were born laterally or as a main branch of conidiophores, solitary or sometimes in a whorl, hyaline, 88～234×3.0～4.5 μm, lanceolate, gradually tapered to the apex of 1.5～3.5 μm in width. Conidia are retrogressively born in short imbricate chains from the apex of conidiogenous cells, hyaline, 2～3 septate, clavate to long ellipsoidal, slightly asymmetrical, smooth, 23.5～34.5×5.5～9.0 μm, average 25.3×6.7 μm, (L/W ratio, 2.73～4.83, average 3.79). Chlamydospores were produced in chains.

Based on cultural and microscopic characteristics described above, the strain TAMA 85 was considered to belong to the genus *Cladobotryum*.

Isolation of 1 and 2

Two new antifungal antibiotics, F2928-1 (**1**) and -2 (**2**), were isolated by bioassay-guided separation as described in Materials and Methods.

Structure Determination of 1

Physico-chemical properties of **1** were summarized in Table 1. This compound was readily soluble in MeOH, $CHCl_3$, EtOAc and DMSO and practically insoluble in water. In the ESI mass positive ion mode measurement, **1** showed the peak of *m*/*z* 473. In the ESI mass negative ion mode measurement, **1** showed the peak of *m*/*z* 471. These results suggested the molecular weight of **1** is 472. The molecular formula of **1** was determined to be $C_{27}H_{36}O_7$ on the basis of HRFAB mass measurement and NMR spectral analyses.

The ^{13}C-NMR and DEPT spectra of **1** displayed 27

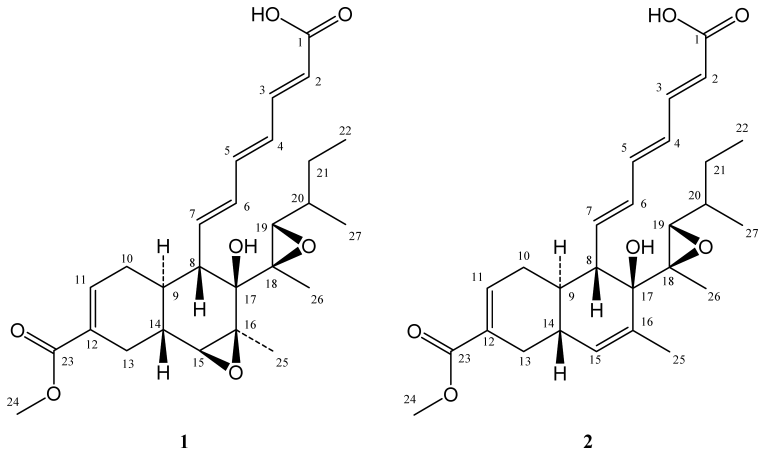

Fig. 1 Relative structures of F2928-1(**1**) and -2 (**2**).

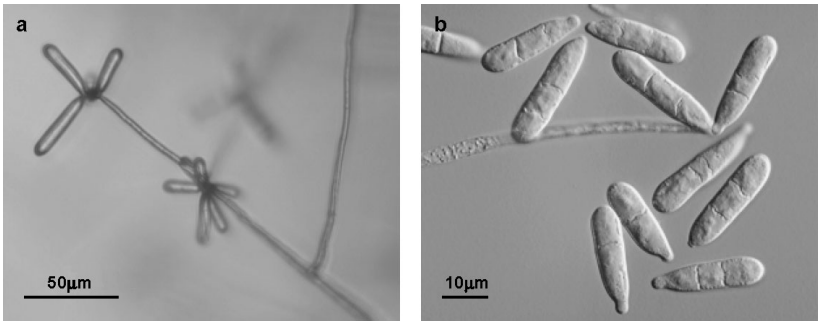

Fig. 2 Optical micrographs of strain TAMA 85. a. Conidiogeneous cells with imbricate chains of conidia; b. Conidia

Table 1 Physico-chemical properties of F2928s

	F2928-1 (**1**)	F2928-2 (**2**)
Appearance	Pale yellow powder	Pale yellow powder
Molecular formula	$C_{27}H_{36}O_7$	$C_{27}H_{36}O_6$
ESI-MS	473 $[M+H]^+$, 471 $[M-H]^-$	457 $[M+H]^+$, 455 $[M-H]^-$
HRFAB-MS (*m*/*z*)		
Calcd	471.2383 for $C_{27}H_{35}O_7$	455.2434 for $C_{27}H_{35}O_6$
Found	471.2372 $[M-H]^-$	455.2423 $[M-H]^-$
Melting point	131°C	120°C
$[\alpha]_D^{25}$	−58° (*c* 1.0, MeCN)	−58° (*c* 1.0, MeCN)
UV λ_{max} nm in MeOH (ε)	216 (12400), 290 (38600)	292 (57900)

signals composed of four methyl carbons, three methylene carbons, four methine carbons, one methoxy carbon, two oxymethine carbons, three oxygenated quaternary carbons, one sp^2 quaternary carbon, seven sp^2 methine carbons, and two ester carbons.

Analyses of ^{1}H-NMR, ^{13}C-NMR, DQFCOSY and HMQC experiments on **1** showed three structural fragments. The geometries of the three double bonds were determined to be 2*E*, 4*E* and 6*E* on the basis of the coupling constants ($J_{2,3}$=15.3 Hz, $J_{4,5}$=15.0 and $J_{6,7}$=14.7, respectively). The presence of two oxirane rings was indicated by the chemical shift (C-15: δ_C 63.3, C-19: δ_C 63.6) and characteristic coupling constants (C-15: $^1J_{CH}$=172.8 Hz, C-19: $^1J_{CH}$=170.6 Hz).

Table 2 ^{1}H and ^{13}C NMR data of F2928s in $CDCl_3$

Position	F2928-1 (**1**)				F2928-2 (**2**)			
	^{1}H		^{13}C (DEPT)		^{1}H		^{13}C (DEPT)	
1	—		169.5	s	—		169.4	s
2	5.67	(1H, d, J=15.3 Hz)	121.0	d	5.67	(1H, d, J=15.3 Hz)	120.7	d
3	7.15	(1H, dd, J=11.3, 15.3 Hz)	145.9	d	7.17	(1H, dd, J=11.6, 15.3 Hz)	146.1	d
4	6.15	(1H, dd, J=11.3, 15.0 Hz)	130.0	d	6.17	(1H, dd, J=11.6, 15.0 Hz)	129.6	d
5	6.51	(1H, dd, J=11.0, 15.0 Hz)	139.8	d	6.56	(1H, dd, J=10.7, 15.0 Hz)	140.3	d
6	6.12	(1H, dd, J=11.0, 14.7 Hz)	134.9	d	6.16	(1H, dd, J=10.7, 15.0 Hz)	134.0	d
7	5.88	(1H, dd, J=11.0, 14.7 Hz)	134.7	d	5.93	(1H, dd, J=11.0, 15.0 Hz)	136.0	d
8	2.08	(1H, dd, J=11.0, 11.3 Hz)	59.1	d	2.40	(1H, dd, J=11.0, 12.2 Hz)	58.7	d
9	1.83	(1H, m)	31.8	d	1.95	(1H, m)	37.4	d
10	1.59	(1H, m)	31.7	t	1.70	(1H, m)	32.2	t
	2.17	(1H, ddd, J=2.5, 6.7, 18.6 Hz)			2.20	(2H, m)		
11	6.90	(1H, dd, J=2.5, 2.5 Hz)	138.4	d	6.94	(1H, dd, J=2.7, 2.7 Hz)	139.3	d
12	—		129.9	s	—		130.2	s
13	2.27	(1H, dd, J=11.0, 17.1 Hz)	28.4	t	1.97	(1H, dd, J=4.4, 16.8 Hz)	31.4	t
	2.62	(1H, dd, J=4.6, 17.1 Hz)			2.62	(1H, dd, J=2.3, 16.8 Hz)		
14	1.83	(1H, ddd, J=4.6, 10.1, 11.0 Hz)	38.2	d	2.07	(1H, ddd, J=2.3, 4.4, 9.8 Hz)	37.8	d
15	2.94	(1H, s)	63.3	d	5.62	(1H, s)	131.4	d
16	—		62.0	s	—		134.7	s
17	—		72.7	s	—		75.6	s
17-OH	4.29	(1H, s) *			N.D.**			
18	—		64.1	s	—		65.0	s
19	3.19	(1H, d, J=9.5 Hz)	63.6	d	3.23	(1H, d, J=8.9 Hz)	63.8	d
20	1.44	(1H, m)	33.7	d	1.32	(1H, m)	34.2	d
21	1.34	(2H, m)	27.9	t	1.32	(2H, m)	27.8	t
	1.59				1.61			
22	0.96	(3H, t, J=7.3 Hz)	11.3	q	0.95	(3H, t, J=7.0 Hz)	11.2	q
23	-		167.4	s	—		167.6	s
24	3.74	(3H, s)	51.7	q	3.74	(3H, s)	51.7	q
25	1.38	(3H, s)	19.6	q	1.75	(3H, q, J=1.1 Hz)	18.2	q
26	1.65	(3H, s)	16.5	q	1.41	(3H, s)	15.7	q
27	1.02	(3H, d, J=6.7 Hz)	15.7	q	0.96	(3H, d, J=7.0 Hz)	15.4	q

Footnote: *Measured in DMSO-d_6, **Not detected.

The connection of these structural fragments was deduced from the observation of the HMBC correlations as shown in Fig. 3. The correlations from H-3 (δ_H 7.15) to C-1 (δ_C 169.5), and from H-2 (δ_H 5.67) to C-1 indicated the linkage of C-1 and C-2 to form a trienoic acid. The correlations from H-19 (δ_H 3.19) to C-17 (δ_C 72.7), C-18 (δ_C 64.1), C-20 (δ_C 33.7), C-21 (δ_C 27.9) and C-27 (δ_C 15.7), from H-22 (δ_H 0.96) to C-20 and C-21, from H-26 (δ_H 1.65) to C-17, C-18 and C-19, and from H-27 (δ_H 1.02) to C-19, C-20 and C-21 were observed. These correlations revealed that the 3-*sec*-butyl-2-methyl-oxiranyl moiety is bonded to C-17. The correlations from H-11 (δ_H 6.90) to C-9 (δ_C 31.8) and C-13 (δ_C 28.4) were observed. Moreover, correlations from H-10 (δ_H 1.59, 2.17) to C-12 (δ_C 129.9) and C-14 (δ_C 38.2), and from H-13 (δ_H 2.27, 2.62) to C-9 and C-11 (δ_C 138.4) were observed. These correlations revealed the existence of cyclohexene. Both H-11 and H-13 showed correlations to C-23 (δ_C 167.4). Moreover, correlations from H-24 (δ_H 3.74) to C-12 and C-23 were observed. These observations suggested methoxy carbonyl group is bonded to C-12. H-7 (δ_H 5.88) showed correlations to C-8 (δ_C 59.1), C-9 and C-17. Moreover, correlations from H-8 (δ_H 2.08) to C-6 (δ_C 134.9), C-7 (δ_C 134.7), C-9, C-14, C-17 and C-18 were observed. These results suggested that C-8 is bonded to C-9 and C-17. H-15 (δ_H 2.94) showed correlations to C-9, C-13 and C-14.

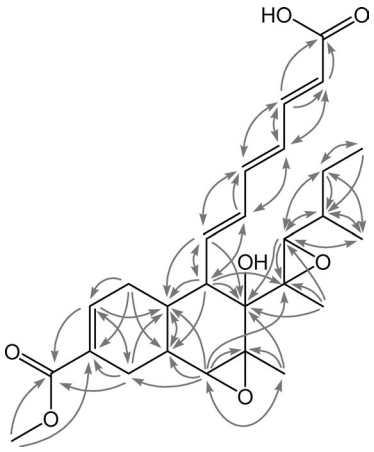

Fig. 3 HMBC correlations of **1**.

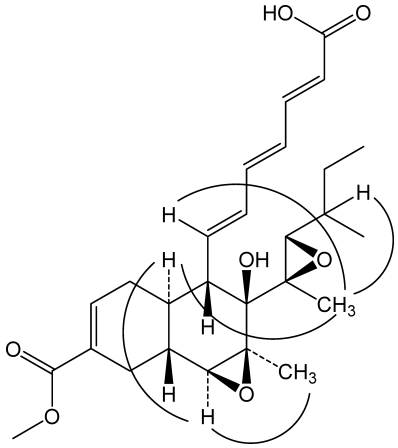

Fig. 4 NOE correlations of **1**.

Moreover, H-25 (δ_H 1.38) showed correlations to C-15, C-16 (δ_C 62.0) and C-17. These results revealed that **1** has epoxy decaline moiety in its structure.

Although hydroxyl proton was not observed in the ^{1}H-NMR experiment in $CDCl_3$, one hydroxyl proton (δ_H 4.29) was observed in DMSO-d_6. This proton signal showed HMBC correlation to C-16. This result and chemical shift at C-17 suggested that hydroxyl group is bonded to C-17. Thus, the planar structure of **1** was determined as shown in Fig. 3.

The relative stereochemistry of **1** was elucidated by the ^{1}H-^{1}H coupling pattern and NOE difference spectra (Fig. 4). A coupling constant between H-9 and H-14 (J = 10.1 Hz) indicated a trans junction for the decalin ring. The vicinal coupling constant between H-8 and H-9 (J = 11.3 Hz) also indicated diaxial relationship. On the other hand, no coupling was observed between H-14 and H-15. Probably the dihedral angle was nearly 90°C. NOEs were observed between H-15 and H-25, and between H-9 and H-15, respectively. These results indicated that H-15 and H-25 exist on the α side of the decalin ring and that epoxy ring exists on the β side of the decalin ring. NOEs were observed between H-7, H-9, H-20 and H-26. Therefore, hydroxyl group (17-OH) was assigned to equatorial orientation. Moreover, these results indicated that methyl group and *sec*-butyl group are located on the identical surface of epoxy ring.

Based on all of the observations described above, the structure of **1** was elucidated as shown in Fig. 1.

Structure Determination of 2

In the ESI mass positive ion mode measurement, **2** showed the peak of *m*/*z* 457. In the ESI mass negative ion mode measurement, **2** showed the peak of *m*/*z* 455. These results suggested the molecular weight of **2** is 456 and it was 16 mass units smaller than **1**. The molecular formula of **2** was determined to be $C_{27}H_{36}O_6$ the basis of HRFAB mass measurement and NMR spectral analyses. The UV-VIS spectrum indicated that **2** has the same chromophore as **1**.

Although the ^{1}H-NMR spectrum of **2** was similar to that of **1**, oxymethine proton, which was located at 15th position of **1**, was not observed. But sp^2 methine proton was observed instead. The ^{13}C-NMR and DEPT spectra of **2** were also similar to those of **1**. However, sp^2 methine carbon and sp^2 quaternary carbon were observed instead of epoxy carbons of **1**.

In the HMBC spectrum of **2**, the correlation pattern was very similar to that of **1** (Fig. 5). H-15 (δ_H 5.62) showed correlations to C-9 (δ_C 37.4), C-13 (δ_C 31.4), C-14 (δ_C 37.8) and C-17 (δ_C 75.6). Moreover, correlations from H-25 (δ_H 1.75) to C-15 (δ_C 131.4), C-16 (δ_C 134.7) and C-17 were observed. These results indicated that one of the epoxy groups present in **1** is replaced with an olefin group in **2**.

^{1}H-^{1}H coupling pattern and NOE data of **2** showed a close similarity to those of **1**. Therefore, the structure of **2** was proposed as shown in Fig. 1.

Antimicrobial Activities

As shown in Table 3, **1** and **2** were active against *A. fumigatus* and *C. albicans*. However, the activities of these compounds were weaker than those of amphotericin B or itraconazole. These compounds had no inhibitory activity on the growth of bacteria.

Discussion

The strain TAMA 85 was isolated by single spore isolation from ascocarps growing on the decayed fruiting body of

Trametes versicolor. At that time, two additional strains, TAMA 84 and TAMA 86 were also isolated from the same material by a mass spore isolation method. Colonies of TAMA 85 on LCA, MA and OA never matured to ascocarps, whereas colonies of TAMA 84 and TAMA 86 on OA produced abundant light yellow to yellow perithecia half immersed in the subiculum-like pale or light yellow hyphal mat (Munsell 5Y8-9/6-12). Perithecia were light yellow, obpyriform, 330～376×249～268 μm with a distinct papilla, 103～123×101～109 μm, with a flat apex bearing an ostiole, 16～27 μm in diameter. Asci were cylindrical, 89～122×5.0～7.5 μm, 8-spored. Ascospores were hyaline, fusiform, always equally two-celled, 12.5～18.0×4.5～6.0 μm, average 14.7×4.8 μm (L/W ratio, 2.13～4.00, average 3.08), verrucose with prominent warts, apiculate at both ends. Conidiogenesis was observed in all three isolates belonging to *Cladobotryum*.

The present strains showed some affinity to *Hypomyces corticiicola* K. Põldmaa, *H. polyporinus* Peck and *H. pseudocorticiicola* Tokiwa & Okuda in terms of morphology of ascospores and anamorph [5～8]. Among the three candidates, the first two species always produce aseptate ascospores as well. Conidia of *H. corticiilola* are less septate and smaller, and *H. polyporinus* forms narrower ascospores. The description of the last species however agreed well with that of our strains. We therefore identified the strains, TAMA 84, 85 and 86 as *Hypomyces pseudocorticiicola* with *Cladobotryum* anamorph. TAMA 85 unabled to form teleomorph, probably because it was an isolate derived from a single ascospore. This suggested that *H. pseudocorticiicola* is a hetelothalic species.

The structures of F2928s were determined as shown in Fig. 1. These compounds were structurally related to compactin (ML-236B) [9, 10], fusarielins [11], ICM0301s [12, 13] and some other polyketide compounds which possess a decalin moiety in their structures. In these compounds, F2928s, fusarielins and compactin had antifungal activity, while ICM0301s exhibited no antifungal activity at 100 μg/ml. From these results, it was revealed that only a decalin moiety does not cause antifungal activity.

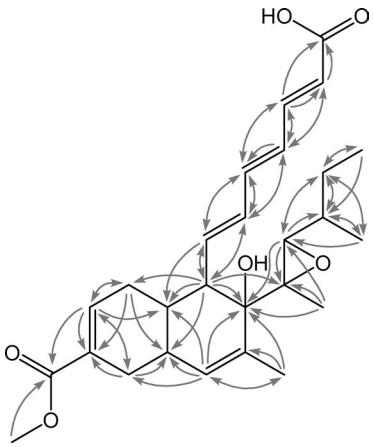

Fig. 5 HMBC correlations of **2**.

Table 3 Antimicrobial activities of F2928s.

Microorganism	MIC (μg/ml)				
	F2928-1	F2928-2	AMPH-B	FLCZ	ITCZ
Aspergillus fumigatus KA-26	25.0	25.0	0.1	>25	0.2
Candida albicans KC-03	6.3	3.1	0.003	0.05	0.002
Staphylococcus aureus Smith	>50	>50	N.T.	N.T.	N.T.
Escherichia coli NIHJ JC-2	>50	>50	N.T.	N.T.	N.T.
Klebsiella pneumoniae No.42	>50	>50	N.T.	N.T.	N.T.
Pseudomonas aeruginosa PAO-1	>50	>50	N.T.	N.T.	N.T.
Pseudomonas cepacia 23	>50	>50	N.T.	N.T.	N.T.

Abbreviation; AMPH-B: amphotericin B, FLCZ: fluconazole, ITCZ: itraconazle, N.T.: Not Tested

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