The Journal of Antibiotics (2013) 66, 465–471

www.nature.com/ja

ORIGINAL ARTICLE

KB425796-A, a novel antifungal antibiotic produced by *Paenibacillus* sp. 530603

Hirohito Kai[1], Midori Yamashita[1], Shigehiro Takase[1], Michizane Hashimoto[2], Hideyuki Muramatsu[2], Ikuko Nakamura[1], Koji Yoshikawa[1], Masami Ezaki[1], Kumiko Nitta[1], Masato Watanabe[1], Noriaki Inamura[1] and Akihiko Fujie[1]

The novel antifungal macrocyclic lipopeptidolactone, KB425796-A (1), was isolated from the fermentation broth of bacterial strain 530603, which was identified as a new *Paenibacillus* species based on morphological and physiological characteristics, and 16S rRNA sequences. KB425796-A (1) was isolated as white powder by solvent extraction, HP-20 and ODS-B column chromatography, and lyophilization, and was determined to have the molecular formula $C_{79}H_{115}N_{19}O_{18}$. KB425796-A (1) showed antifungal activities against *Aspergillus fumigatus* and the micafungin-resistant infectious fungi *Trichosporon asahii*, *Rhizopus oryza*e, *Pseudallescheria boydii* and *Cryptococcus neoformans*.
The Journal of Antibiotics (2013) **66,** 465–471; doi:10.1038/ja.2013.63; published online 19 June 2013

Keywords: antifungal; fermentation; isolation; KB425796-A; *Paenibacillus* sp

INTRODUCTION

Life-threatening infections caused by *Aspergillus fumigatus* are increasing in prevalence, particularly in immunocompromised and neutropic patients.[1] However, antifungal therapies are currently limited to a small number of compounds. For example, toxicity is an issue for treatments based on amphotericin B, and a high potential for drug–drug interactions has been reported between azoles and anticancer agents or immunosuppressants, and causes difficulty in clinical use.[2] Thus, a need exists for effective novel antifungal compounds that are easy to use in clinical practice.

The fungal cell wall, which is composed of β-glucan, chitin and mannan, is essential to fungi and is fundamentally different from mammalian cell wall, making it an ideal target for antifungal drugs. In particular, 1,3-β-glucan is a promising antifungal target, which is an essential structural component of chytridiomycetes, ascomycetes, basidiomycetes and deuteromycetes.[3] In recent years, three new antifungal drugs, micafungin,[4,5] caspofungin[6] and anidulafungin,[7] which inhibit fungal 1,3-β-glucan synthase, have been launched; however, these drugs are fungistatic against *A. fumigatus* and have limited efficacy.[8]

In a course of searching for inhibitors of fungal cell wall synthesis from microorganisms, we isolated the novel lipopeptide KB425796-A (1) from the cultured broth of *Paenibacillus* sp. 530603. KB425796-A (1) is a 40-membered macrocyclic lipopeptidolactone consisting of 12 amino acids and a 3-hydroxy-13-methylmyristoyl moiety (Figure 1). In the present paper, we studied the taxonomy of the KB425796-A-producing strain, the fermentation, isolation, physicochemical properties, structure and preliminary biological properties of KB425796-A (1).

RESULTS

Taxonomic study

The cultural and physiological characteristics of strain 530603 are summarized in Table 1. Strain No. 530603 was a Gram-positive motile bacterium and had a temperature range for growth of 10–45 °C, with an optimum between 30–35 °C. Strain 530603 gave positive results for oxidase and catalase, and acid production from glucose and arabinose. Colonies on Luria-Bertani (LB) agar medium grew rapidly and were translucent, mucous and convex with a diameter of ~7 mm after 3 days of culture. Microscopic observation showed rod-shaped, single or short chains of cells (0.5–0.8 × 2.5–15 μm). Oval endospores (0.5–1.2 × 1.5–2.5 μm) were located terminally within swollen sporulating cells. The 16S ribosomal RNA gene sequence of strain 530603 determined in this study was almost complete, comprising 1502 nucleotides (nt) and having >99% similarity with those of *Paenibacillus* species. The phylogenetic tree constructed by the neighbor-joining method on the basis of almost-complete 16S rRNA gene sequences showed the relationship between strain 530603 and closely related valid species of the genus *Paenibacillus* (Figure 2). The morphological and physiological characteristics of strain 530603 were also highly similar to those of *Paenibacillus* species.

The 16S rRNA gene sequence of strain 530603 showed the highest similarity (99.85%) to that of *Paenibacillus dendritiformis*. Thus, to determine the species of strain 530603, cells of this strain were compared with *P. dendritriformis* T168T (BGSC 30A1).[9] Several differences were detected between the two strains: tip-splitting colony morphology, cell size, spore position, salt tolerance and acid

[1]Drug Discovery Research, Astellas Pharma Inc., Tsukuba-shi, Ibaraki, Japan and [2]Fermentation Research Division, Astellas Research Technologies Co., Ltd., Tsukuba-shi, Ibaraki, Japan
Correspondence: H Kai, Drug Discovery Research, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan.
E-mail: hirohito.kai@astellas.com
Received 5 December 2012; revised 26 March 2013; accepted 2 April 2013; published online 19 June 2013

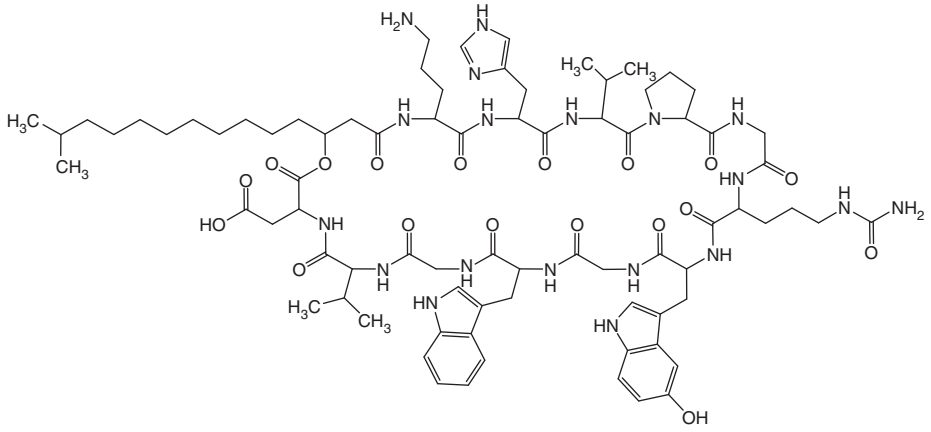

Figure 1 Chemical structure of KB425796-A (**1**).

Table 1 Comparison of strain 530603 and *Paenibacillus dendritriformis* T168T

Characteristic	*strain 530603*	*P. dendritriformis T168T*
Cell size (μm)	0.5–0.8 by 2.5–15	0.5–1.0 by 2.0–3.0
Branch pattern formation on peptone agar	−	+
Spore shape	oval	oval
Spore position	terminal	subterminal of terminal
Swollen sporangia	+	+
Anaerobic growth	+	+
Catalase	+	+
Oxidase	+	+
Production of:		
Indole	−	+
Voges-Proskauer test	−	+
Hydrolysis of:		
Casein	+	+
Starch	+	+
Acid production from:		
Glucose	+	+
Arabinose	+	+
Mannitol	−	−
Xylose	−	−
45 °C	+	+
65 °C	−	−
pH 5.7	+	+
5% NaCl	+	+
7% NaCl	−	+

+, positive.
−, negative.

production from several carbon sources (Table 1). On the basis of results of the 16S rRNA gene and phenotypic analyses, we considered that strain 530603 belongs to the genus *Paenibacillus*. Further study will be needed to determine the species of this strain, though there is a possibility that this strain belongs to a new species of the genus *Paenibacillus*. This strain has been deposited at the National Institute of Advanced Industrial Science and Technology, Japan, as FERM BP-10803, and the GenBank/EMBL/DDBJ with the accession number for the 16S rRNA gene sequence of strain 530603 being AB746175.

Isolation and purification of KB425796-A (1)

KB425796-A was isolated from the culture broth of *Paenibacillus* sp. 530603 using the following procedure. First, an equal volume of acetone was added to the culture broth and the resulting mixture was filtered through diatomaceous earth. The filtrate was diluted with an equal volume of water and passed through a DIAION HP-20 column (5.0 l; Mitsubishi Chemical Co., Ltd., Tokyo, Japan) packed with water. The column was washed with water (15 l) and 50% aqueous methanol (15 l), and eluted with methanol (17 l). The active fraction (0–15 l) was diluted with an equal volume of water and then loaded into a Daisogel SP-120-ODS-B column (15/30 μm, 2 l; Daiso Co., Ltd., Osaka, Japan) packed with water. The column was washed with water (5 l) and eluted with 45% aqueous acetonitrile containing 0.1% TFA (4.5 l). The active fraction (0.5–4.0 l) was diluted with an equal volume of water and then loaded into a Daisogel SP-120-ODS-B column (15/30 μm, 2 l; Daiso Co., Ltd.) packed with water. The column was washed with water (5 l) and eluted with 42% aqueous acetonitrile containing 0.1% TFA (4 l). The active fraction (2–3 l) was diluted with an equal volume of water and then loaded into a Daisogel SP-120-ODS-B column (15/30 μm, Daiso Co., Ltd.) packed with water. The column was washed with water (5 l) and eluted with 40% aqueous acetonitrile containing 0.1% TFA (5 l). The active fraction (1.5–3.5 l) was diluted with an equal volume of water and then loaded into a Daisogel SP-120-ODS-B column (15/30 μm, 0.2 l; Daiso Co., Ltd.) packed with water. The column was washed with water (1 l) and eluted with methanol (0.2 l).

The active fraction was concentrated *in vacuo* to an aqueous solution and lyophilized to yield 1.62 g of crude powder KB425796-A (1). The powder was dissolved in 2 ml methanol and applied to the top of a silica gel 60 column (40/50 μm, 50 ml; Kanto Chemical Co., Ltd., Tokyo, Japan) packed with chloroform. The column was eluted with a mixture of chloroform-methanol (10:1, 150 ml), and fractions containing KB425796-A (1) were collected and dried to give 1.50 g of white powder KB425796-A (1).

Physicochemical properties and structural elucidation of KB425796-A (1)

The physicochemical properties of KB425796-A (1) are summarized in Table 2. KB425796-A (1) was soluble in methanol and dimethyl sulfoxide, sparingly soluble in chloroform and acetone, and insoluble in water and ethyl acetate. KB425796-A (1) displayed positive color reactions to iodine vapor, ceric sulfate and ninhydrin, but had negative color reactions to Molish, Dragendorff and ferric chloride.

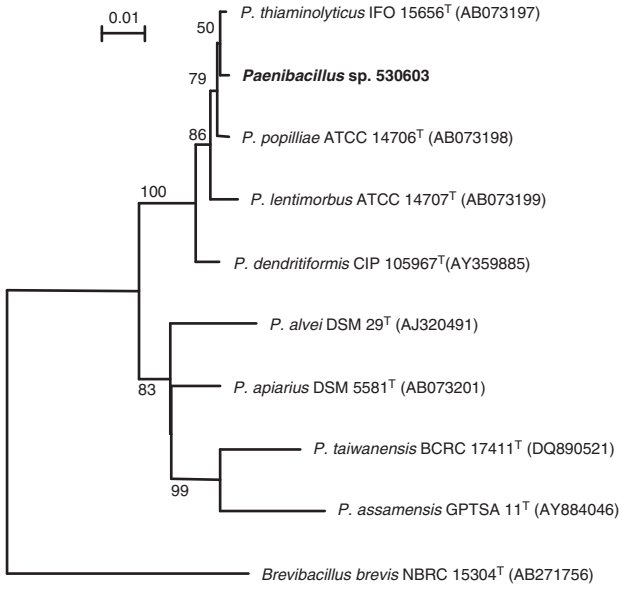

Figure 2 Neighbor-joining phylogenetic tree on the basis of almost-complete 16S rRNA gene sequences showing the relationship between strain 530603 and closely related valid species of the genus *Paenibacillus*. Bootstrap value (>50%) on the basis of 1000 replicates are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

Table 2 Physicochemical properties of KB425796-A(1)

Appearance	*White powder*
Specific rotation $[\alpha]_D^{23}$	−20.1 (c0.5, MeOH)
HR-LC-MS (*m/z*)	
Found (*m/z*)	1618.8745 $[M+H]^+$, 1616.8600 $[M-H]^-$
Calcd (*m/z*)	1618.8740 $[M+H]^+$, 1616.8595 $[M-H]^-$
Molecular formula	$C_{79}H_{115}N_{19}O_{18}$
UV λ_{max}^{MeOH} nm (ε)	290(10700), 280(12900)
Color test	
Positive	I_2, $Ce(SO_4)_2$-H_2SO_4, Ninhydrin
Negative	Molish, Dragendorff, $FeCl_3$
Solubility	
Soluble	methanol, DMSO
Insoluble	H_2O, ethyl acetate
IR λ_{max} (KBr) cm^{-1}	3300, 2930, 2860, 2360, 1650, 1540, 1460, 1400, 1200
TLC (Rf value)[a]	0.45

[a]Silica gel 60 NH2 F254s (MERCK):methanol-chloroform (1:1).

The UV absorption maxima of purified KB425796-A (1) occurred at 280 and 290 nm, and a molecular ion peak at *m/z* 1619 ($[M+H]^+$) was detected in the ESI-MS spectrum (data not shown).

The exact molecular mass of KB425796-A (1) (founded by ESI-TOF-MS) was 1618.8745 Da ($[M+H]^+$) and 1616.8600 Da ($[M-H]^-$)), which corresponded to the molecular formula $C_{79}H_{115}N_{19}O_{18}$ (theoretical: $[M+H]^+$ 1618.8740 Da and $[M-H]^-$ 1616.8595 Da) and was consistent with the NMR data (Table 3 and Supplementary Information).

The preparation of KB425796-A (**1**) contained several structurally related minor congeners. The use of DMSO-d_6 was avoided in the NMR measurement because of the difficulty of recovering material for bioassays. The ^1H NMR spectra measured in CD_3OD displayed sharp and well-resolved signals. As an amide NH proton was useful in assigning the sequence of a target peptide, CD_3OH was also employed for a series of NMR spectra.

The structural assignments of the standard amino acids, (Orn (***a***), His (***b***), Val (***c***), Pro (***d***), Gly (***e***), Gly (***h***), Trp (***i***), Gly (***j***), Val (***k***) and Asp (***l***)), as shown in Figure 3, were made without difficulty. In addition, analysis of the NMR data, using 1D (^1H, ^{13}C and DEPT) and 2D (COSY, TOCSY, HSQC, HMBC and ROESY) techniques, revealed the presence of two uncommon non-proteinogenic amino acids, citrulline (Cit (***f***)) and 5-hydroxytryptophan (HOTrp (***g***)), and an acyl component (***m***) (Figure 3). Cit (***f***): the COSY and TOCSY experiments indicated that **1** possessed a spin system similar to Orn. The terminal nitrogen-bearing methylene protons (δ_H 2.90 and 2.84) showed HMBC correlations with a carbonyl group (δ_C 162.3), reminiscent of an ureido group. The presence of an ureido group was further supported from ROE correlation between $-NH_2$ (δ_H 5.45 (2H, br s)) and –NH (δ_H 5.90 (1H, br t)). The presence of the ureido group and Orn-like residue identified the subunit (***f***) as a citrulline residue. HOTrp (***g***): the low-field shifted NH signal at 10.13 p.p.m. was due to Trp indole-NH (***i***). Another low-field NH signal at 10.08 p.p.m. was indicative of a Trp-like residue. The three benzene proton signals (δ_H 7.18 (d, $J=8.5$ Hz), 7.03 (d, $J=1.5$ Hz), and 6.78 (dd, $J=8.5$ and 1.5 Hz)) suggested that the residue was 5-hydroxytryptophan or 6-hydroxytryptophan. ROE correlation between β-CH_2 (δ_H 3.47) and the benzene signal (δ_H 7.03) identified the subunit (***g***) as 5-hydroxytryptophan. Acyl component (***m***): 2-methylpropyl and 3-hydroxybutyryl units were evident from the COSY, HSQC and HMBC analyses. Seven aliphatic methylene resonances around δ_H 1.30–1.20 (δ_C 30.9 (t), 30.7 (t), 30.6 (t), 30.5 (t), 30.4 (t), 28.5 (t), and 27.0 (t)), together with the identified 2-methylpropyl and 3-hydroxybutyryl units indicated the presence of a 3-hydroxy-13-methylmyristoyl moiety (***m***). The 12 amino acids and acyl component accounted for all of the atoms present in **1** and for 31 out of the 32 degrees of unsaturation required by the molecular formula. The remaining unsaturation was due to the cyclic nature of **1**.

Subunits ***a*∼*m*** were assembled into two fragments, acyl(***m***)-Orn(***a***)-His(***b***)-Val(***c***)- and –Gly(***e***)-Cit(***f***)-OHTrp(***g***)-Gly(***h***)-Trp(***i***)-Gly(***j***)-Val(***k***)-Asp(***l***)- by extensive consideration of the HMBC correlations (Figure 4). These proposed sequences were further supported by the ROE results (Figure 4). Additionally, ROE connectivities between Val(***c***) αH (δ_H 4.30) and Pro(***d***) δ-CH_2 (δ_H 3.60), and between Pro(***d***) αH (δ_H 4.32) and Gly(***e***) NH (δ_H 7.33) indicated the presence of a –Val(***c***)-Pro(***d***)-Gly(***e***) fragment. The ROE results allowed us to connect the identified fragments to assemble the gross sequence of **1**. The ^1H and ^{13}C chemical shifts (δ_H 5.08, δ_C 72.8) of C-3 in subunit (***m***) were characteristic of an acyloxymethine. Macrocyclic lactone was indicated by the HMBC correlation from H-3 (***m***) to Asp(***l***) carbonyl. On the basis of the above described analyses, the structure of KB425796-A was elucidated to be **1**, as depicted in Figure 1.

Biological activity

The antifungal activity of KB425796-A (1) against several fungi is shown in Table 4. KB425796-A (1) exhibited potent antifungal activity against *A. fumigatus* and a variety of micafungin-resistant fungal species *Trichosporon asahii*, *Rhizopus oryza*e, *Pseudallescheria boydii* and *Cryptococcus neoformans*, with particularly high efficiency against *T. asahii*, but was inactive against *Candida albicans*. Microscopic observation of *A. fumigatus* cells after treatment with KB425796-A (1) revealed several morphological changes, including swelling and bulging of hyphae (Figure 5a), in comparison with the

Table 3 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments of WB425796A

Position	δ*C (m)*	δ*H*
Orn (*a*)		
NH		8.97
α	52.5 (d)	4.80
β	31.3 (t)	1.98, 1.72
γ	23.5 (t)	1.78, 1.55
δ	40.7 (t)	2.90, 2.80
CO	173.3 (s)	
His (*b*)		
NH		8.66
α	54.7 (d)	4.91
β	30.9 (t)	3.07, 2.88
2	135.7 (d)	7.63
4	118.0 (d)	6.99
5	134.2 (s)	
CO	172.9 (s)	
Val (*c*)		
NH		8.68
α	57.6 (d)	4.30
β	31.8 (d)	1.75
γ	19.8 (g)	0.97
γ	19.2 (g)	0.81
CO	171.1 (s)	
Pro (*d*)		
N		
α	61.0 (d)	4.32
β	31.2 (t)	2.09, 1.80
γ	25.5 (t)	1.90, 1.73
δ	49.0 (t)	3.60, 3.60
CO	174.3 (s)	
Gly (*e*)		
NH		7.33
α	43.6 (t)	4.10, 3.73
CO	170.7 (s)	
Cit (*f*)		
NH		8.57
α	56.1 (d)	4.05
β	28.7 (t)	1.51, 1.51
γ	27.0 (t)	1.20, 0.98
δ	40.1 (t)	2.90, 2.84
CO	176.1 (s)	
NHCONH2	162.3 (s)	
NHCONH2		5.90
NHCONH2		5.45
OH Trp (*g*)		
NH		8.58
α	56.6 (d)	4.68
β	27.9 (t)	3.47, 3.02
NH		10.08
2	125.7 (d)	7.13
3	110.5 (s)	
4	103.6 (d)	7.03
5	151.5 (s)	
6	112.8 (d)	6.78
7	113.0 (d)	7.18
8	133.2 (s)	
9	129.1 (s)	
CO	174.1 (s)	

Table 3 (Continued)

Position	δ*C (m)*	δ*H*
Gly (*h*)		
NH		8.37
α	43.9 (t)	4.43, 3.80
CO	171.8 (s)	
Trp (*i*)		
NH		7.49
α	55.6 (d)	5.03
β	29.0 (t)	3.28, 2.91
NH		10.13
2	125.5 (d)	7.13
3	110.0 (s)	
4	119.4 (d)	7.45
5	119.6 (d)	6.93
6	122.1 (d)	7.00
7	112.2 (d)	7.28
8	137.8 (s)	
9	129.1 (s)	
CO	174.4 (s)	
Gly (*j*)		
NH		8.06
α	43.4 (t)	4.10, 3.00
CO	170.2 (s)	
Val (*k*)		
NH		8.17
α	58.6 (d)	5.05
β	33.4 (d)	1.85
γ	19.9 (g)	0.80
γ	19.0 (g)	0.77
CO	173.1 (s)	
Asp (*l*)		
NH		8.83
α	50.4 (d)	4.84
β	41.3 (t)	3.11, 2.43
CO	173.0 (s)	
γ-CO	177.5 (s)	
Acyl (*m*)		
1	171.5 (s)	
2	41.2 (t)	2.90, 2.25
3	72.9 (d)	5.08
4	33.2 (t)	1.58, 1.50
5	30.9 (t)	1.20
6	30.7 (t)	1.20
7	30.6 (t)	1.20
8	30.5 (t)	1.20
9	30.4 (t)	1.20
10	28.5 (t)	1.20
11	27.0 (t)	1.20
12	40.2 (t)	1.13
13	29.1 (d)	1.50
14	23.0 (q)	0.83
15	23.0 (q)	0.83

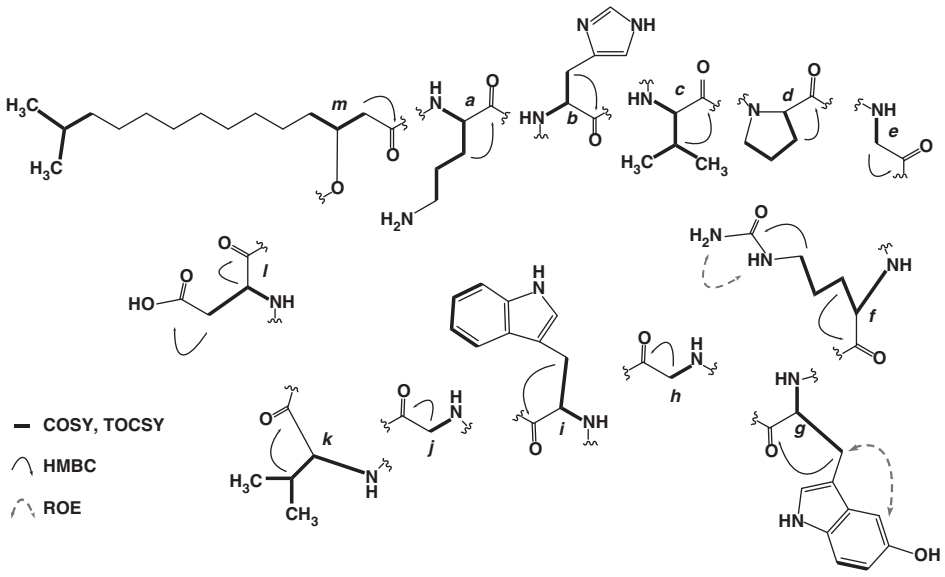

Figure 3 Subunits ***a*–*m*** of **1**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

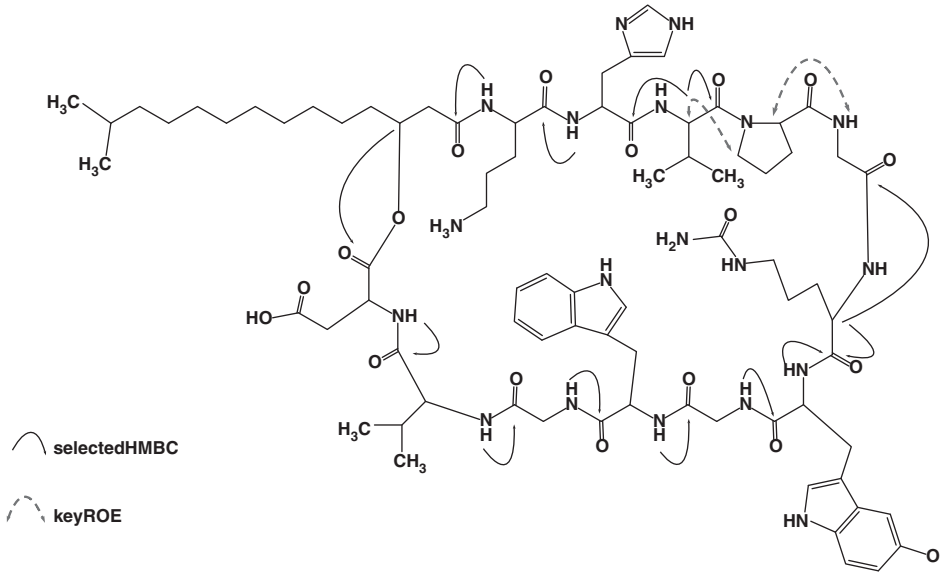

Figure 4 Key HMBC and ROE for the sequence analysis of **1**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

micafungin-treated hyphae (Figure 5b) and untreated control hyphae (Figure 5c). The cytotoxicity (IC_{50}) of KB425796-A (1) for EL-4 cells was $>50\,\mu g\,ml^{-1}$.

DISCUSSION

In this paper, we have presented the isolation and characterization of the novel antifungal lipopeptide, KB425796-A, isolated from the fermentation broth of a soil isolate (530603), which was determined to be a species of *Paenibacillus* based on morphological and physiological characterization. The 16S rRNA gene sequence indicated that strain 530603 was closely affiliated with *P. dendritiformus*, although a few phenotypic differences were detected between the type strain T168T and strain 530603. Based on these analyses, we concluded that strain 530603 is a novel species within the genus *Paenibacillus*. We identified the novel antifungal compound KB425796-A (1) in the culture broth of strain No. 530603 and demonstrated that this compound had strong activity against *A. fumigatus*.

Table 4 Antifungal activity of KB425796-A (1) and micafungin

	MEC ($\mu g\,ml^{-1}$)	
Test organism	*KB425796-A*	*micafungin*
Aspergillus fumigatus FP1305	6.25	0.05
Candida albicans FP633	>50	0.01
Cryptococcus neoformans YC203	3.13	>50
Trichosporon asahii FP2044	1.56	50
Rhizopus oryzae FP1988	3.13	50
Pseudallescheria boydii FP1987	6.25	>50

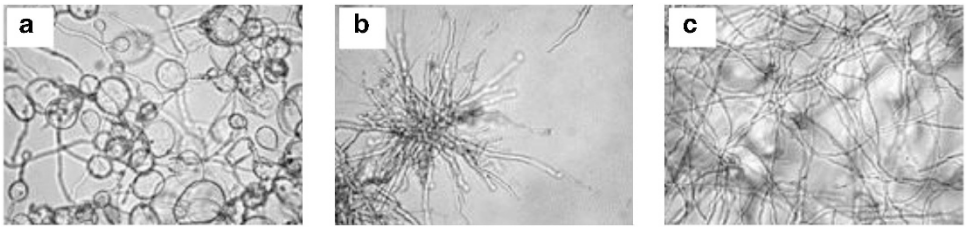

Figure 5 Hyphal morphology of *A. fumigatus* after treatment with KB425796-A (1) and micafungin. (**a**) 6.25 μg ml^{-1} KB425796-A, (**b**) 0.05 μg ml^{-1} micafungin and (**c**) control. Scale bar, 100 μm. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Among microbial secondary metabolites characterized to date, two 40-membered macrocyclic lipopeptidolactones consisting of 12 amino acids have been identified: WAP-8294 (Tcherpakov *et al.*[9]) and FR901469.[10,11] WAP-8294 was discovered as an anti-methicillin-resistant *Staphylococcus aureus* (MRSA) agent but did not exhibit antifungal activity, whereas FR901469 was a 1,3-β-glucan synthase inhibitor that was produced by fungal strain 11243. Similar to micafungin, which has 1,3-β-glucan synthase inhibitory activity, FR901469 induces the formation of short, stubby and highly branched hyphae in the infectious fungus *A. fumigatus*.[10,12] In contrast to the actions of these agents, KB425796-A (1) induced swelling and bulging of *A. fumigatus* hyphae (Figure 5a), suggesting that KB425796-A (1) has a different mode of action from that of FR901469. In addition, KB425796-A (1) had antifungal activities against the micafungin-resistant infectious fungi *T. asahii*, *R. oryza*e, *P. boydii* and *C. neoformans*. Although the effectiveness of micafungin against infection by species of *Candida* and *Aspergillus* has been established,[13] micafungin-resistant fungi remain a problematic issue in clinical practice.[14,15] Although being preliminary, our present findings suggest that KB425796-A (1) has the potential utility to treat infections caused by these fungi. To investigate the efficacy of KB425796-A (1) against micafungin-resistant fungi, further antifungal studies with an *in vivo* infection model will be needed.

We also identified numerous possible congeners of KB425796-A by HPLC analysis of the fermentation broth of strain 530603. As these congeners may also possess potent antifungal activity, isolation and antifungal studies of these compounds will be reported in a succeeding paper.

METHODS

Taxonomic Studies

Strain 530603 was originally isolated from a soil sample collected in Aomori Prefecture, Japan. Taxonomic studies were performed based on the methods described in Bergey's manual.[16] 16S rRNA gene sequences were analyzed according to the method of Muramatsu *et al.*[17] Phylogenic tree was generated by the neighbor-joining method based on the 16S rRNA gene sequences. Morphological observation was carried out using a light microscope with cells cultured on agar (Invitrogen Japan, Tokyo, Japan) for 3 days at 30 °C. Enzyme and carbohydrate acid production were detected using the API 50 CHB kit (BioMéruex, Lyon, France). Growth at various temperatures (5–50 °C) was evaluated on LB agar.

Fermentation

Culture medium (100 ml) consisting of 2% nutrient broth (Kyokuto, Tokyo, Japan) and 0.1% sodium pyruvate (Nacalai Tesque, Kyoto, Japan) was sterilized in 500 ml Erlenmeyer flasks at 120 °C for 30 min. After cooling, the culture medium was inoculated with a loopful of cells from a slant culture of strain 530603 and incubated at 30 °C for 48 h on a rotary shaker (250 r.p.m., 5.1-cm throw). The resultant seed culture was inoculated into a 30 l stainless steel jar-fermentor containing 20 l of a production medium consisting of 3% MS3600 (Nihon Shokuhin Kako, Tokyo, Japan), 2% peptone (Kyokuto), 1% CSL (Nihon Shokuhin Kako), 0.05% Adekanol defoaming agent (Asahi Denka Co., Ltd., Tokyo, Japan), and 0.05% Silicone KM-70 defoaming agent (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan). The fermentation was carried out at 30 °C for 4 days under aeration at 20 l min^{-1}, an inner pressure of 1.0 kg cm^{-2}, and agitation at 200 r.p.m.

HPLC analysis

Detection of KB425796-A (1) in the fermentation broth and column fractions during purification was performed by HPLC using a reverse phase column (L-column ODS, 250 mmϕ × 4.6 mm I.D.; Chemical Evaluation and Research Institute, Japan). An aqueous acetonitrile solution (42%) containing 0.1% TFA was used as the mobile phase at a flow rate of 1.0 ml/min. The detection wavelength was set at 210 nm.

General experimental procedures

High-resolution mass spectra were measured using a LCMS-IT-TOF spectrometer (Shimadzu, Kyoto, Japan), and UV/Vis spectra were recorded on a UV-2500 PC (Shimadzu). Optical rotations were measured on a SEPA-500 polarimeter (Horiba, Kyoto, Japan). Infrared spectra were recorded with a Spectrum 65 FT-IR spectrometer (PerkinElmer). NMR spectra, ^{1}H (500 MHz) and ^{13}C (125 MHz), were recorded on an AVANCE 500 spectrometer (Bruker Japan, Yokohama, Japan) equipped with a cryoprobe. Chemical shifts were given in δ (p.p.m.) with the residual CD_3OH solvent signal referenced to δ_H 3.31 and δ_C 49.15 as the internal reference.

Antimicrobial activity

C. albicans FP633, *T. asahii* FP2044, *R. oryza*e FP1988 and *P. boydii* FP1987, which are clinical isolates deposited in our laboratory,[18] were grown in yeast-maltose (YM) agar broth for 24 h at 30 °C under the standing condition. *C. neoformans* YC203 (deposited in our laboratory) was cultured in YM broth for 20 h at 30 °C with shaking at 250 r.p.m. A cell suspension was prepared by washing the cultured cells once with sterile saline. *A. fumigatus* FP1305 (deposited in our laboratory) was cultured on a potato dextrose agar (PDA) slant for 4 days at 37 °C, and spores were then harvested in sterile saline and filtered through gauze. The antifungal activity of KB425796-A (1) was measured by the micro-broth dilution method using 96-well culture plates and RPMI 1640 medium (Invitrogen) lacking sodium bicarbonate and supplemented with L-glutamine, buffered to pH 7.0 with 0.165 M MOPS. Yeast nitrogen base-glucose (YNBD) medium was used for measuring the antifungal activity of KB425796-A (1) against *C. neoformans*. For the measurements, the test fungus was inoculated into each well at a final concentration of 1×10^5 c.f.u. per well. The plates were incubated for 20 h (*C. albicans* FP633, *T. asahii* FP2044, *R. oryza*e FP1988, *P. boydii* FP1987 and *A. fumigatus* FP1305) at 37 °C or 48 h (*C. neoformans* YC203) at 37 °C. Minimum effective concentration (MEC), which was the lowest concentration causing a substantial reduction in fungal growth, was determined by microscopic observation.[19]

Cell cytotoxicity assay

Cytotoxicity was examined using EL-4 cells. After 3 days of incubation with a range of KB425796-A (1) concentrations, cell viability was determined colorimetrically at 550 nm, using 660 nm as a reference, using the methylthiazoletetrazolium (MTT) method[20]. Cytotoxicity is expressed as the concentration of a compound needed to reduce EL-4 cell viability by 50%.

1 Wade, J. C. Treatment of fungal and other opportunistic infections in immunocompromised patients. *Leukemia* **11** (Suppl 4), S38–S39 (1997).
2 Thompson, G. R. 3rd, Cadena, J. & Patterson, T. F. Overview of antifungal agents. *Clin. Chest Med.* **30,** 203–215, (2009).
3 Liu, J. & Balasubramanian, M. K. 1,3-beta-Glucan synthase: a useful target for antifungal drugs. *Curr. Drug Targets* **1,** 159–169 (2001).
4 Tomishima, M. *et al.* FK463, a novel water-soluble echinocandin lipopeptide: synthesis and antifungal activity. *J. Antibiot.* **52,** 674–676 (1999).
5 Higashiyama, Y. & Kohno, S. Micafungin: a therapeutic review. *Expert Review of Anti-infective Therapy* **2,** 345–355 (2004).
6 Cornely, O. A., Schmitz, K. & Aisenbrey, S. The first echinocandin: caspofungin. *Mycoses* **45** (Suppl 3), 56–60 (2002).
7 Debono, M. *et al.* Semisynthetic chemical modification of the antifungal lipopeptide echinocandin B (ECB): structure-activity studies of the lipophilic and geometric parameters of polyarylated acyl analogs of ECB. *J. Med. Chem.* **38,** 3271–3281 (1995).
8 Mikulska, M. & Viscoli, C. Current role of echinocandins in the management of invasive aspergillosis. *Curr. Infect. Dis. Rep.* **13,** 517–527 (2011).
9 Tcherpakov, M., Ben-Jacob, E. & Gutnick, D. L. Paenibacillus dendritiformis sp. nov., proposal for a new pattern-forming species and its localization within a phylogenetic cluster. *Int. J.Syst. Bacteriol.* **49** (Pt 1), 239–246 (1999).
10 Fujie, A. *et al.* FR901469, a novel antifungal antibiotic from an unidentified fungus No.11243. I. Taxonomy, fermentation, isolation, physicochemical properties and biological properties. *J. Antibiot.* **53,** 912–919 (2000).
11 Fujie, A. *et al.* FR901469, a novel antifungal antibiotic from an unidentified fungus No.11243. II. *In vitro* and *in vivo* activities. *J. Antibiot.* **53,** 920–927 (2000).
12 Hatano, K., Morishita, Y., Nakai, T. & Ikeda, F. Antifungal mechanism of FK463 against *Candida albicans* and *Aspergillus fumigatus*. *J. Antibiot.* **55,** 219–222 (2002).
13 Hanadate, T. *et al.* Evaluation of the safety and efficacy of micafungin in Japanese patients with deep mycosis: a post-marketing survey report. *J. Infect. Chemother.* **17,** 622–632 (2011).
14 Uchida, K., Nishiyama, Y., Yokota, N. & Yamaguchi, H. In vitro antifungal activity of a novel lipopeptide antifungal agent, FK463, against various fungal pathogens. *J. Antibiot.* **53,** 1175–1181 (2000).
15 Matsue, K., Uryu, H., Koseki, M., Asada, N. & Takeuchi, M. Breakthrough trichosporonosis in patients with hematologic malignancies receiving micafungin. *Clin. Infect. Dis.* **42,** 753–757 (2006).
16 Sneath, PHA. *et al. Bergey's Manual of Systematic Bacteriology.* 2nd. edn. (William & Wilkins Co., Baltimore, MA, USA, 1986).
17 Muramatsu, H., Mokhtar, S., Katsuoka, M. & Ezaki, M. Phylogenic analysis of immunosuppressant FK506-producing streptomycete strains. *Actinomycetologica* **19,** 33–39 (2005).
18 Tawara, S. *et al.* In vitro activities of a new lipopeptide antifungal agent, FK463, against a variety of clinically important fungi. *Antimicrob. Agents Chemother.* **44,** 57–62 (2000).
19 Kanasaki, R. *et al.* FR220897 and FR220899, novel antifungal lipopeptides from Coleophoma empetri no. 14573. *J. Antibiot.* **59,** 149–157 (2006).
20 Berg, K., Hansen, M. B. & Nielsen, S. E. A new sensitive bioassay for precise quantification of interferon activity as measured via the mitochondrial dehydrogenase function in cells (MTT-method). *APMIS* **98,** 156–162 (1990).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)