ORIGINAL ARTICLE

AS2077715: a novel antifungal antibiotic produced by *Capnodium* sp. 339855

Keisuke Ohsumi¹, Teruhisa Masaki², Shigehiro Takase¹, Masato Watanabe¹ and Akihiko Fujie¹

A novel antifungal agent, AS2077715, was isolated from the fermentation broth of a fungal strain (339855) identified as a new *Capnodium* species based on morphological characteristics and large-subunit ribosomal DNA sequencing. AS2077715 was isolated as a white powder via solvent extraction, HP-20 and ODS-B column chromatography and crystallization, and was determined to have the molecular formula C₂₅H₄₁NO₇. AS2077715 has a structure related to that of funiculosin, an inhibitor of mitochondrial cytochrome *bc*₁ complex (complex III), and showed antifungal activity against *Trichophyton* species.

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INTRODUCTION

The prevalence of onychomycosis, also known as nail fungal infection and mainly caused by dermatophytes, has been estimated to range from 10 to 30% globally.¹ Dermatophytes—mainly *Trichophyton rubrum* and *Trichophyton mentagrophytes*—are the predominant causative agents of onychomycosis, accounting for ~50–90% of cases.^{2–5} Clinical management of onychomycosis includes prescription systemic and topical treatment with antifungals such as terbinafine (an allylamine), amorolfine (a morpholine), itraconazole (a triazole) and ciclopirox (a hydroxypyridone). However, despite the availability of several therapeutic options, onychomycosis remains difficult to treat, and new drugs with improved safety and efficacy are needed.⁶

In our search for new anti-*Trichophyton* agents from microbial products, we identified the novel antifungal compound AS2077715 in the culture broth of a strain belonging to the *Capnodium* species (Figure 1). AS2077715 is a structural relative of funiculosin, an inhibitor of mitochondrial cytochrome *bc*₁ complex (complex III). Here, we studied the taxonomy and fermentation of the AS2077715-producing strain, as well as the isolation, physico-chemical properties, structure and preliminary biological properties of AS2077715.

RESULTS

Taxonomy of strain 339855

Growth of strain 339855 on a potato dextrose agar was rather slow. Colonies grew to a diameter of 23–26 mm in 2 weeks at 25 °C. Surfaces of the colonies were felty and partially floccose. The entire margins of the colonies were in a circular pattern, and many radial sulcates were formed between the center and the margin. The felty parts of the colonies were grayish green (30F8), whereas the floccose parts were dull grayish green (29E4). The underside of each colony was overall dark grayish green (28F4). Colonies reached 6–7 mm in diameter in 2 weeks at 30 °C, whereas no growth was observed at 5 or

37 °C. Growth of the strain on a corn meal agar was also slow, and colonies grew to a diameter of 22–24 mm in 2 weeks at 25 °C. Surfaces of the colonies were velvety. The entire margins of the colonies were in a circular pattern, and sulcates were not formed on the colonies. The color of colonies was grayish green (29F8–29F4). The colonies were not undulated, but smooth. Synnematos conidiophore was observed on colonies, and drop-like conidial masses were formed on the apexes of conidiophores. The underside of each colony was overall dark grayish green (28F4). Colonies attained 6–9 mm in diameter in 2 weeks at 30 °C, whereas no growth was observed at 5 or 37 °C. The strain grew at temperatures between 12 and 25 °C, and the optimum growth temperature was in the range of 20–25 °C. Data on the growth temperatures were measured on potato dextrose agar. The pH at which the strain was capable of growing, measured in malt extract broth, was in the range of 2.0–11.5, and the optimum growth pH was in the range of 5.5–6.2.

Morphological observations of strain 339855 using an optical microscope are shown in Figure 2. Vegetative hyphae were brown and 2.3–4.0 μm thick. Chlamydospores were not observed. Conidiophores were not branched, but formed determinate synnemata. The overall length was 200–260 μm, and the thickness was 8–10 μm. The base of the synnemata formed stroma and widened to 30–40 μm. The synnemata was slightly swollen at the upper part and rather narrowed at the apex. Furthermore, several strands of setae were formed to surround the apex part. Colorless conidia were formed on the narrowed apex, and became a drop. The conidia were elliptical to cylindrical in shape (3.2–3.6 × 1.7–1.76 μm), with smooth surfaces.

The morphological features of strain 339855 resemble the genus *Leptoxiphium*⁷ or *Capnodium*.^{8,9} Both strains are known as plant diseases. We drew the phylogenetic tree using large-subunit rDNA sequences (Figure 3). Phylogenetic analysis showed that strain 339855 belongs to the clade of the genus *Capnodium*, so we identified strain 339855 as *Capnodium* sp.

¹Drug Discovery Research, Astellas Pharma Inc., Ibaraki, Japan and ²Fermentation Research Division, Astellas Research Technologies Co., Ltd., Ibaraki, Japan
Correspondence: K Ohsumi, Drug Discovery Research, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan.
E-mail: keisuke.ohsumi@astellas.com

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Figure 1 Chemical structure of AS2077715 (1) and funiculosin (2).

Figure 2 Morphological properties of strain 339855. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Isolation and purification of AS2077715

To isolate AS2077715 from the culture broth of *Capnodium* sp. 339855, an equal volume of acetone was added to the culture broth, and the resulting mixture was filtered with the aid of diatomaceous earth. The filtrate was diluted with twice the volume of water and passed through a DIAION HP-20 column (6.0 l; Mitsubishi Chemical, Tokyo, Japan) packed with water. The column was washed with 35%

acetone and eluted with 60% acetone. The eluate (25 l) was then diluted with twice the volume of water and passed through a Daisogel SP-120-ODS-B column (15/30 μm, 8.0 l; Daiso, Osaka, Japan) packed with 25% acetonitrile. The column was eluted with 50% acetonitrile containing 0.01 N HCl. The active fractions were then diluted with an equal volume of water and passed through a Sepabeads SP850 column (1.0 l; Mitsubishi Chemical) packed with 25% acetonitrile. The column was washed with water and eluted with ethyl acetate. The eluate was dried over Na₂SO₄ and concentrated *in vacuo*. The residue was dissolved in 5 ml of methanol and 5 ml of ethyl acetate, and combined with 50 ml of *n*-hexane to give crystals. The crystals were filtered and dried to give 17.6 g of AS2077715 as a white powder.

Physico-chemical properties and structural elucidation of AS2077715

The physico-chemical properties of AS2077715 are summarized in Table 1. AS2077715 was soluble in methanol, acetonitrile, chloroform, ethyl acetate and dimethyl sulfoxide and insoluble in water and *n*-hexane. The UV absorption maxima of purified AS2077715 occurred at 290.5 nm, and a molecular ion peak at *m/z* 468 ([M + H]⁺) was detected in the ESI-MS spectrum (data not shown).

The molecular formula of AS2077715 was determined to be C₂₅H₄₁NO₇ based on HRESI-MS and ¹³C NMR data (Table 2). ¹H NMR spectrum of AS2077715 showed the compound to be a natural product, structurally related to funiculosin, an antibiotic isolated from *Penicillium funiculosum* Thom.¹⁰ The ¹³C NMR data of 5-(2,3,4,5-tetrahydrocyclopentyl)-4-hydroxy-2-pyridinone moiety of AS2077715 were in good agreement with those of funiculosin. The difference between AS2077715 and funiculosin resided in the alkyl side chain. Therefore, elucidation of the structure of AS2077715 was focused on the side chain, although ¹H signal overlapping of 8-H₂, 9-H₂, 10-H₂ and 13-H₂ made structure elucidation difficult. Analysis of HSQC-TOCSY data coupled with COSY and HSQC revealed the sequence CH(7)-CH₂(8)-CH₂(9)-CH₂(10)-CH(11)-CH₂(12)-CH₂(13)-. Oxymethine proton 7-H at 4.94 p.p.m. showed HMBC correlation to C-11 (δ_C 80.0 (d)) and 11-H at 3.51 p.p.m.

Figure 3 Phylogenetic tree based on large-subunit ribosomal DNA sequences. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 1 Physico-chemical properties of AS2077715

Appearance	White powder
Molecular formula	C ₂₅ H ₄₁ NO ₇
Molecular mass (g/mol)	467
<i>HR-MS (m/z)</i>	
Found (M + H) ⁺	468.2965
Calculated (M + H) ⁺	468.2961
[α] _D (23 °C, MeOH)	−120° (c 0.5)
UV λ _{max} (MeOH) nm (ε)	233(sh), 290.5 (6100)
Solubility: soluble	CH ₃ OH, CH ₃ CN, EtOAc, CHCl ₃ and DMSO
insoluble	<i>n</i> -hexane, water
HPLC retention time (min) ^a	7.0
IR ν _{max} cm ^{−1}	3480, 2980, 2930, 1730, 1710, 1650, 1620, 1460, 1380, 1160 and 1140

^aRetention time obtained by Mightysil RP-18 GP 150-4.6 (5 μm, Kanto Chemical), 55% aq. CH₃CN, flow rate 1 ml min^{−1}.

showed HMBC cross-peak to C-7 (δ_C 78.1 (d)), indicating the presence of a tetrahydropyran ring. HMBC coupling from 14-CH₃ (δ_H 0.83) to the methylene at C-13 (δ_C 33.2 (t)) completed the side chain structure as shown in Figure 4. The linkage between C-3 and C-7 was indicated by HMBC couplings from 7-H to C-2 (δ_C 161.4 (s)), C-3 (δ_C 110.1 (s)) and C-4 (δ_C 162.5 (s)), completing elucidation of the gross planar structure of AS2077715 (Figure 1).

Antifungal activity of AS2077715

The antifungal activity of AS2077715 against several fungi is shown in Table 3. AS2077715 showed potent antifungal activity against *Trichophyton* species but except for *Cryptococcus neoformans*, not against any other fungi tested at levels up to 2.5 μg ml^{−1}. AS2077715 and funiculosin had the same spectrum of antifungal activity.

DISCUSSION

In this paper, we presented the isolation and characterization of a novel antifungal antibiotic, AS2077715, isolated from the fermentation broth of the strain 339855, which was identified as a species of *Capnodium* based on morphological characterization and DNA sequence results. We identified the structure of the novel antifungal

compound AS2077715 in the culture broth of strain 339855 and demonstrated that this compound has strong activity against *Trichophyton* species.

AS2077715 was shown to be an analog of funiculosin, an antibiotic isolated from *Penicillium funiculosum* Thom.¹⁰ AS2077715 and funiculosin have the same pyridone and cyclopentane rings, differing only in the alkyl side chain. Similar to funiculosin, AS2077715 showed potent antifungal activities against *Trichophyton* species and *C. neoformans* and no activities against other fungi. These compounds showed no activities against facultative Gram-positive cocci or facultative and aerobic Gram-negative bacteria, with MICs of > 12.5 μg ml^{−1} (data not shown). These results suggest that the difference in the side chains of these compounds induce no differences in antimicrobial activities.

From the structural similarity to funiculosin, AS2077715 is expected to be an inhibitor of complex III. We plan to evaluate the *in vitro* and *in vivo* activities of AS2077715 against *T. mentagrophytes*, and will describe the findings in a subsequent paper.

METHODS

Taxonomic study

Strain 339855 was isolated using a moist chamber method from a fallen leaf from *Zelkova serrata*, which was collected at Youroukeikoku in Otaki-Machi, Isumi-gun, Chiba, Japan. Media for the taxonomic study of the strain were purchased from Becton, Dickinson, and Company (Franklin Lakes, NJ, USA). The morphological observations were made from cultures grown on corn meal agar after 14 days' cultivation at 25 °C, using an optical microscope. The color descriptions were based on the *Methuen Handbook of Colour*.¹¹ The large-subunit rDNA sequence was determined via the method described by O'Donnell.¹² The large-subunit rDNA sequences of type strains were obtained from DDBJ database (<http://www.ddbj.nig.ac.jp/>), and the MEGA program (version 5.2.2, <http://www.megasoftware.net/>) was used to draw the phylogenetic tree.

The DDBJ/GenBank/EMBL accession number for the large-subunit rDNA sequence of strain 339855 is AB894361.

Fermentation

A loopful of the slant culture of strain 339855 was inoculated into 30 ml of sterilized seed culture medium, consisting of 2% cornstarch, 1% glycerin, 1% sucrose, 1% gluten meal, 1% pharmamedia (Archer Daniels Midland, Decatur, IL, USA), and 0.25% Tween80, in a wide-mouth 100-ml Erlenmeyer flask.

Table 2 ^1H (500 MHz) and ^{13}C (125 MHz) NMR data of AS2077715 in CDCl_3

Position	AS2077715	
	δ_{C} (m)	δ_{H} (m, J)
2	161.4 (s)	
3	110.1 (s)	
4	162.5 (s)	
5	107.2 (s)	
6	137.2 (d)	7.87 (s)
7	78.1 (d)	4.94 (dd, 11 and 2)
8	30.2 (t)	2.04 (m), 1.45 (m)
9	23.1 (t)	1.88 (m), 1.70 (m)
10	31.4 (t)	1.70 (m), 1.30 (m)
11	80.0 (d)	3.51 (m)
12	33.9 (t)	1.54 (m), 1.52 (m)
13	33.2 (t)	1.37 (m), 1.12 (m)
14	30.1 (d)	1.45 (m)
15	44.1 (t)	1.06 (m), 1.05 (m)
16	31.7 (d)	1.38 (m)
17	30.4 (t)	1.26 (m), 1.13 (m)
18	11.4 (q)	0.86 (3H, t, 7.5)
1'	39.7 (d)	2.88 (m)
2', 5'	74.4 (d)	4.40 (2H, m)
	74.4 (d)	
3', 4'	70.0 (d)	4.10 (2H, m)
	70.0 (d)	
N-CH ₃	37.2 (q)	3.49 (3H, s)
14-CH ₃	19.4 (q)	0.83 (3H, d, 6.5)
16-CH ₃	19.0 (q)	0.80 (3H, d, 6.5)
4-OH		10.09 (s)
2'-OH		3.40 (d, 5)
5'-OH		3.39 (d, 5)
3'-OH		3.53 (d, 5)
4'-OH		3.50 (d, 5)

Figure 4 Structure elucidation of the side chain of AS2077715.

The flask was incubated at 25 °C for 4 days on a rotary shaker (220 r.p.m., 5.1-cm throw) and then inoculated (2%) into 150 ml of the same sterilized medium in each of three 500-ml Erlenmeyer flasks, which were then incubated at 25 °C for 3 days on a rotary shaker (220 r.p.m., 5.1-cm throw). The resultant seed culture was inoculated into a 30-l stainless steel jar fermenter containing 20 l of sterile production medium, which consisted of 6% soluble starch, 4% glucose, 2% yeast extract, 1% K_2HPO_4 , 0.02% MgSO_4 , 0.05% Adekanol LG-109 defoaming agent (ADEKA, Tokyo, Japan) and 0.05% Silicone KM-70 defoaming agent (Shin-Etsu Chemical, Tokyo, Japan). Fermentation was

Table 3 Antifungal activity of AS2077715 against *Trichophyton* spp. and other fungi

Test organism	MIC ($\mu\text{g ml}^{-1}$)		
	AS2077715	funiculosin	terbinafine
<i>Trichophyton mentagrophytes</i> FP2103	0.08	0.08	0.01
<i>T. rubrum</i> FP596	0.08	0.08	ND
<i>Aspergillus fumigatus</i> FP1305	>2.5	>2.5	ND
<i>Rhizopus oryzae</i> FP1988	>2.5	>2.5	ND
<i>Pseudallescheria boydii</i> FP1987	>2.5	>2.5	ND
<i>Fusarium solani</i> FP1930	>2.5	>2.5	ND
<i>Candida albicans</i> FP633	>2.5	>2.5	ND
<i>C. krusei</i> FP1979	>2.5	>2.5	ND
<i>C. glabrata</i> FP1944	>2.5	>2.5	ND
<i>C. tropicalis</i> FP1589	>2.5	>2.5	ND
<i>C. guilliermondii</i> FP2086	>2.5	>2.5	ND
<i>C. parapsilosis</i> FP1980	>2.5	>2.5	ND
<i>Cryptococcus neoformans</i> FP1739	1.3	2.5	ND

Abbreviation: ND, not determined.

carried out at 25 °C for 6 days under aeration of 20 l min⁻¹, an inner pressure of 1.0 kg cm⁻² and agitation at 200 r.p.m.

HPLC Analysis

Detection of AS2077715 from the fermentation broth and the fractions under purification was monitored via HPLC using a reverse-phase column (Mightysil RP-18 GP 150-4.6 (5 μm); Kanto Chemical, Tokyo, Japan). An aqueous acetonitrile solution (60%) was used as the mobile phase at a flow rate of 1.0 ml min⁻¹, and detection wavelength was set at 210 nm.

General experimental procedures

High-resolution mass spectra were measured using an LCMS-IT-TOF spectrometer (Shimadzu, Kyoto, Japan). UV/Visible spectra were recorded with a UV-2500 PC (Shimadzu). Optical rotations were measured with a SEPA-500 polarimeter (Horiba, Kyoto, Japan). IR spectra were recorded with a Spectrum 65 FT-IR spectrometer (PerkinElmer, Waltham, MA, USA). NMR spectra, ^1H (500 MHz) and ^{13}C (125 MHz), were recorded on an AVANCE 500 spectrometer (Bruker, Yokohama, Japan).

Antifungal activity

Funiculosin was prepared in our laboratories. *Rhizopus oryzae* FP1988, *Pseudallescheria boydii* FP1987, *Fusarium solani* FP1930, *Candida albicans* FP633, *C. krusei* FP1979, *C. glabrata* FP1944, *C. tropicalis* FP1589, *C. guilliermondii* FP2086 and *C. parapsilosis* FP1980, which are clinical isolates deposited in our laboratory,¹³ were incubated in yeast-maltose broth for 24 h at 30 °C without shaking. *Cryptococcus neoformans* FP1739 (deposited in our laboratory) was cultured in yeast-maltose broth for 20 h at 30 °C with shaking (250 r.p.m.). A cell suspension was prepared by washing the cultured cells once with sterile saline. *Aspergillus fumigatus* FP1305, *Trichophyton mentagrophytes* FP2103 and *T. rubrum* FP596 (deposited in our laboratory) were cultured on a potato dextrose agar slant for 4 days at 37 °C, and spores were then harvested in sterile saline and filtered through gauze. The antimicrobial activity of AS2077715 was measured via the micro-broth dilution method using 96-well culture plates and RPMI 1640 medium (Invitrogen, Tokyo, Japan) lacking sodium bicarbonate and supplemented with L-glutamine, buffered to pH 7.0 with 0.165 M MOPS. Yeast nitrogen base-glucose medium and modified Sabouraud dextrose medium, which was composed of 0.1% glucose, 0.2% bacto tryptone, 0.2% K_2HPO_4 , 0.005% MgSO_4 and 0.005% CaCl_2 , were used to measure the antifungal activity of AS2077715 against *C. neoformans* and *Trichophyton* species, respectively. For measurements, the test fungus was inoculated into each well at a final concentration of 1×10^5 colony-forming units per well. The plates were incubated for 48 h at 30 °C for *Trichophyton* species, 48 h at 37 °C for *C. neoformans* or 20 h at 37 °C for all other strains.

MIC, which was the lowest concentration needed to completely inhibit fungal growth, was determined by microscopic observation.

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