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YM-193221, a Novel Antifungal Antibiotic Produced by *Pseudallescheria ellipsoidea*

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A novel antifungal antibiotic, YM-193221, was found in the culture broth of a fungus, *Pseudallescheria ellipsoidea*. The structure of the antibiotic was determined through several spectroscopic experiments as 2-dimethylamino-1-(4-hydroxyphenyl)-8,10-dimethyl-6-dodecene-3-one. YM-193221 exhibited potent antifungal activity against *Candida albicans* and also inhibited mannan synthesis in the yeast cell wall.

Opportunistic infections caused by pathogenic fungi such as *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* have increased recently and become a serious problem. Patients with compromised immune systems, those who receive organ transplants and cancer chemotherapy, and those infected by the human immunodeficiency virus (HIV), are particularly prone to such infections[1]. Although fluconazole is one of the most commonly used antifungal agents, the isolation of fluconazole-resistant strain of *Candida albicans* has been reported[2]. In light of this, new antifungal agents with a different mechanism of action have been sought extensively.

Mannan is a yeast glycoprotein with a high mannose content, and one of the major cell wall components[3]. Mannan is involved in the pathogenicity and adherance of the pathogenic fungi *Candida albicans*[4,5], *Cryptococcus neoformans*[6,7], and *Aspergillus fumigatus*[8,9]. Most yeast mannosylation mutants have been reported to be sensitive to aminoglycoside antibiotics[10]. The reason may be that their permeability to aminoglycoside antibiotics is enhanced due to defects in glycosylation in the yeast cell wall[10]. We hypothesized that an inhibitor of mannan synthesis could sensitize yeasts to aminoglycosides. Based on this hypothesis, activators of yeast sensitivity to hygromycin B, one of the aminoglycosides, were sought by screening microbial metabolites, leading to the discovery of YM-193221 in the culture broth of a fungus, *Pseudallescheria ellipsoidea* CBS 128.78.

In this paper, we describe the fermentation, isolation, physico-chemical properties, structure elucidation, and biological activities of YM-193221.

Materials and Methods

Producing Microorganism

The producing strain, the fungus *Pseudallescheria ellipsoidea* CBS 128.78, was obtained from the Centraalbureau voor Schimmelcultures (CBS).

Fermentation

Pseudallescheria ellipsoidea CBS 128.78 was cultured on a potato dextrose agar slant and inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a seed medium consisting of 1% glucose, 2% potato starch, 0.5% Polypepton (Nihon Pharmaceutical Co., Ltd.), 0.5% yeast extract, and 0.4% $CaCO_3$ (pH 7.0). The seed culture was incubated at 24°C for 4 days on a rotary shaker set at 200 rpm. Three milliliters of the first seed culture broth were inoculated into four 500-ml baffled Erlenmeyer flasks containing the same seed medium. The flasks were cultured at 24°C for 3 days on a rotary shaker set at 200 rpm. Three milliliters of the second seed culture broth were transferred

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into each of two hundred 500-ml Erlenmeyer flasks containing a production medium consisting of 60 g brown rice, 60 mg yeast extract, 60 mg K_2HPO_4, 60 mg sodium tartrate, and 100 ml distilled water. The production culture was incubated under static conditions for 20 days at 24°C.

Isolation

The fermentation broth (20 liters) was extracted with 80% aq acetone (100 liters), and filtered with a filter paper (Advantec, Pore size: 6 μm). The filtrate was evaporated *in vacuo* to remove acetone, and the aqueous residual was extracted with EtOAc (10 liters). The organic layer was dried and subjected to chromatography on a SSC-ODS-7515-12A column (Senshu Scientific co., ltd.), washed with 70%, and the 80% aq MeOH (100 ml) and eluted with 90% aq MeOH (100 ml). The elute fraction was subjected to Sephadex LH-20 (Amersham Biosciences Corp., 30 i.d.× 900 mm), and eluted with MeOH. The active fractions were subjected to L-column ODS (Chemical Evaluation and Research Institute, Japan, 20 i.d.×250 mm, flow rate: 7.0 ml/minute) and eluted with 90% aq acetonitrile to separate the single peak of YM-193221. The yield of YM-193221 was 7.2 mg.

Physico-chemical Properties and Structure Elucidation

UV spectra were recorded on a Shimadzu UV-160A UV-visible spectrophotometer. FAB-MS spectra were obtained with a JEOL JMS-700T using glycerol as the matrix. ^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-ALPHA500 FT NMR spectrometer.

Bioassays

Antifungal activity (IC_{80}) against *Candida albicans* ATCC10231, *Cryptococcus neoformans* TIMM0362, *Aspergillus fumigatus* TIMM1776 and *Saccharomyces cerevisiae* YFC805 was determined by a micro dilution method developed by KUME and YAMAZAKI[11].

HeLa S3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 20 mM HEPES buffer. The cells were incubated in the presence or absence of the antibiotic for 3 days at 37°C in a humidified atmosphere containing 5% CO_2. Cytotoxicity on the cells was determined using a cell counting kit from Wako Pure Chemical Industries, Ltd.

During the screening program, *S. cerevisiae* YFC805 was incubated with screening samples in the presence or absence of hygromycin B (1 μg/ml) for 3 days at 24°C. The IC_{80} of hygromycin B against *S. cerevisiae* YFC805 was 100 μg/ml. YPD broth was used as the culture medium. Samples which exhibited a more than 10-fold increase in IC_{80} in the absence of the antibiotic as in its presence were selected as hit samples. The inhibition of the mannosylation was confirmed by the active staining of invertase[12].

Results and Discussion

Fermentation and Isolation

Pseudallescheria ellipsoidea CBS 128.78 was cultured under static conditions for 20 days at 24°C. The fermentation broth (20 liters) was extracted with 80% aq acetone and EtOAc. The crude extract was subjected to chromatography on an ODS column, Sephadex LH-20 column, and ODS-HPLC, sequentially. The yield of YM-193221 was 7.2 mg.

Physico-chemical Properties

The physico-chemical properties of YM-193221 are summarized in Table 1. The molecular formula of YM-193221 was determined to be $C_{22}H_{35}NO_2$, based on positive-ion HRFAB-MS data ($(M+H)^+$ *m/z* Calcd: 346.2746, found: 346.2745)

Structure Elucidation

The structure of YM-193221 was elucidated using ^1H NMR, ^{13}C NMR, DEPT, COSY, and HMBC. The ^1H and ^{13}C NMR spectral data of YM-193221 are summarized in Table 2. The ^{13}C NMR spectral data of YM-193221 exhibited nineteen carbon signals which, using a DEPT experiment, were assigned to four methyls, five methylenes, seven methines, and three quaternary carbons. The presence of two equivalent methines (δ_H 6.70, 2H, d; δ_C 115.4), (δ_H 7.01, 2H, d; δ_C 130.4) and two equivalent methyls (δ_H 2.34, 6H, s; δ_C 42.1) were determined by

Table 1. Physico-chemical properties of YM-193221.

Appearance	Yellow oil
Molecular formula	$C_{22}H_{35}NO_2$
HRFAB-MS (*m/z*)	
Calcd:	346.2746 $(M+H)^+$
Found:	346.2745 $(M+H)^+$
FAB-MS (*m/z*)	346 $(M+H)^+$, 344 $(M-H)^-$
UV λ_{max} nm (MeOH)	280, 226
Solublity	
Soluble:	$CHCl_3$, MeOH, DMSO
Insoluble:	Hexane, H_2O

Table 2. ^{1}H NMR and ^{13}C NMR data of YM-193221.

No.	δ_C	δ_H
1	30.5	2.76 (dd, 13.4, 4.3), 2.92 (dd, 13.4, 9.8)
2	74.7	3.34 (dd, 9.8, 4.3)
3	210.4	
4	42.6	2.22 (m), 2.53 (m)
5	26.4	2.10 (2H, m)
6	126.6	5.23 (dt, 15.3, 6.1)
7	137.3	5.16 (m)
8	34.3	2.10 (m)
9	44.4	0.96 (m), 1.18 (m)
10	31.8	1.28 (m)
11	29.9	1.10 (m), 1.28 (m)
12	11.3	0.83 (3H, t, 7.3)
1'	130.9	
2'	130.4	7.01 (2H, d, 8.5)
3'	115.4	6.70 (2H, d, 8.5)
4'	154.1	
2-N-CH_3	42.1	2.34 (6H, s)
8-CH_3	21.6	0.89 (3H, d, 6.1)
10-CH_3	19.0	0.79 (3H, dd, 6.1, 1.2)

^{1}H NMR spectra and ^{13}C NMR spectra of YM-193221 were recorded at 500 MHz and 125 MHz in $CDCl_3$, respectively. The proton resonance multiplicity and coupling constant (J = Hz) are in parenthesis.

Fig. 1. ^{1}H-^{1}H DOF COSY and HMBC experiments for YM-193221.

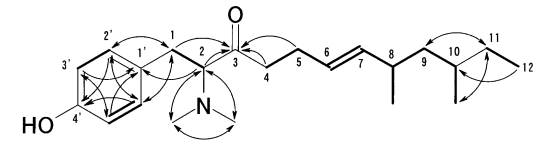
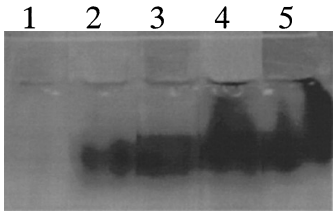


Fig. 2. Mobility of invertase treated with YM-193221.



S. cerevisiae YFC805 cultured with 5 μg/ml of tunicamycin (lane 1), as well as 5 μg/ml (lane 2), 0.5 μg/ml (lane 3), and 0.05 μg/ml (lane 4) of YM-193221, and also without treatment (lane 5).

performing ^{1}H NMR integration and using HMQC data. A YM-193221 ^{1}H-^{1}H DQF COSY experiment revealed four spin networks, from 1-H to 2-H, from 4-H to 10-H, from 11-H to 12-H, and from 2′-H to 3′-H. The HMBC spectrum displayed ^{1}H-^{13}C long-range couplings indicating the connections of the four partial structures as shown in Fig. 1. The long-range couplings from methyl protons (δ_H 2.34) to C-2 methine (δ_C 74.7) and to the equivalent methyl carbon (δ_C 42.1), and the chemical shift of the methyl carbons (δ_C 42.1) indicated that the two equivalent methyls and C-2 methine were connected by a nitrogen atom. The molecular formula of YM-193221 and the chemical shift of C-4′ (δ_C 154.4) indicated that a hydroxyl was connected to C-4′. The geometry of the disubstituted double bond C-6 was determined to be 6*E* by the coupling constant $J_{6,7}$=15.3 Hz. From the structural elucidation above, the formula of YM-193221 was determined to be 2-dimethylamino-1-(4-hydroxyphenyl)-8,10-dimethyl-6-dodecene-3-one.

Biological Activities

Antifungal activity (IC_{80}) was determined by the micro dilution methed[9]. The IC_{80}s of YM-193221 against *C. albicans* ATCC10231, *C. neoformans* TIMM0362 and *A. fumigatus* TIMM1776 were 0.16 μg/ml, 10 μg/ml and 20 μg/ml, respectively. Cytotoxicity (IC_{50}) of YM-193221 against HeLa S3 cells *in vitro* was 21 μg/ml. Although YM-193221 was inactive against *S. cerevisiae* YFC805 at the concentration of 20 μg/ml and in the absence of hygromycin B, the IC_{80} of YM-193221 against *S. cerevisiae* YFC805 was 0.02 μg/ml in the presence of hygromycin B (1 μg/ml). This may be because YM-193221 enhances yeast cell permeability for hygromycin B.

S. cerevisiae YFC805 was incubated with YM-193221 for 17 hours at 24°C, and its invertase, which is a mannnoprotein in the cell wall, was extracted using glass beads. YM-193221 slightly inhibited the mannosylation of invertase at the concentration of 0.5 and 5 μg/ml, and the molecular weight of invertase was smaller than the control, as shown in Fig. 2. From these results, we can see that the inhibition of glycosylation by YM-193221 could change the permeability of hygromycin B[10].

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