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YM-215343, a Novel Antifungal Compound from *Phoma* sp. QN04621

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Through our screening for novel antifungal compounds, YM-215343 was found in the culture extract of *Phoma* sp. QN04621. The structure of YM-215343 was determined by several spectroscopic experiments as a novel compound closely related to apiosporamide and fischerin. YM-215343 exhibited antifungal activity against the pathogenic fungi, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* with MIC values of 2~16 μg/ml. It also showed cytotoxicity against HeLa S3 cells with an IC_{50} of 3.4 μg/ml.

In the course of our screening for novel antifungal compounds from microbial metabolites, we found a novel compound YM-215343 (**1**) from the culture extract of a fungal strain QN04621. This antibiotic inhibited the growth of yeasts and fungi including pathogenic strains. Herein, we report the identification of the producing organism, fermentation, isolation, physico-chemical properties, structure elucidation and biological activities of **1**.

Materials and Methods

Taxonomic Studies

The YM-215343 producing fungus, strain QN04621, was isolated from a dead leaf sample collected in Iriomote Island, Okinawa Prefecture, Japan. The following media were used for the identification of the fungus: potato dextrose agar (PDA), cornmeal agar (CMA), Miura's medium (LCA) and CZAPEK's agar. Morphological properties were observed under an optical microscope (Nikon OPTIPHOT-2).

Physico-chemical Properties and Structure Elucidation

IR spectra were recorded on a Perkin Elmer 2000 FT-IR spectrophotometer. Optical rotation was determined on a Horiba SEPA-200 polarimeter. Fast atom bombardment mass spectra (FAB-MS) were obtained with a JOEL JMS-700T using a glycerol matrix. UV spectra were recorded on a Shimadzu UV-2200 photometer. ^{1}H and ^{13}C NMR spectra were recorded on a JOEL JNM-ALPHA500 FT NMR spectrometer.

Biological Activities

Antifungal activity (MIC) was evaluated by the microbroth dilution method using RPMI-1640 medium buffered with MOPS described in the National Committee for Clinical Laboratory Standards (NCCLS) documents, M27-A[1] and M38-P[2].

Cytotoxicity (IC_{50}) against HeLa S3 cells was determined by a cell counting kit of Wako Pure Chemical Industries, Ltd. 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 antibiotics at 37°C for 3 days in a

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Fig. 1. Structure of YM-215343 (**1**).

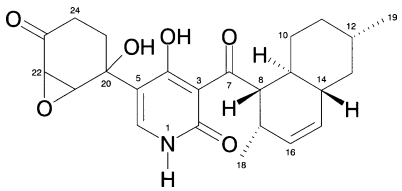
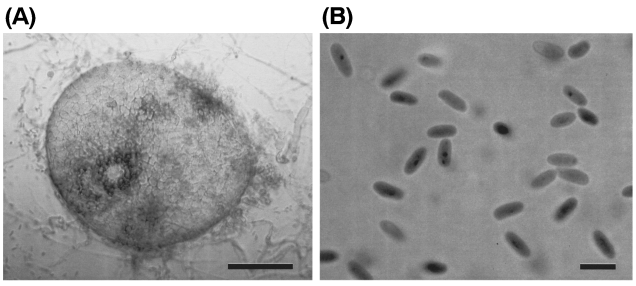


Fig. 2. Optical micrographs of strain QN04621.



(A) Pycnidium. Bar represents 50 μm. (B) Conidia. Bar represents 5 μm.

humidified atmosphere containing 5% CO_2.

Results and Discussion

Taxonomy of the Producing Strain

Cultural and morphological characteristics of strain QN04621 were as follows: colonies were quite fast-growing, reaching 80 mm in diameter in 14 days at 24°C on PDA. The colony surface was felty, velvety, and pale brown to olivaceous gray in color. The reverse color was dark brown. Brownish soluble pigment was produced around the colonies. The fungus abundantly formed pycnidia in the center of the colonies. The pycnidia were orange to dark brown in color, mostly globose to subglobose, 80~200 μm in diameter, with a single ostiole (Fig. 2A). Conidia were single-celled, hyaline, smooth, ellipsoidal, 3.5~6×1.5~2.5 μm (Fig. 2B). Chlamydospore was not observed.

Based on cultural and microscopic characteristics described above, strain QN04621 was considered to belong to coelomycete genus *Phoma*[3,4]. Thus, we identified this isolate as one strain of the genus *Phoma*, and named it as *Phoma* sp. QN04621.

Fermentation

A loopful of mycelia of strain QN04621 grown on an agar slant was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 1%, potato starch 1%, yeast extract 0.5%, Polypepton (Nihon Pharmaceuticals Co., Ltd.) 0.5% and $CaCO_3$ 0.4%. The pH of the medium was adjusted to 7.0 before sterilization. The seed culture was incubated at 24°C for 72 hours on a rotary shaker at 200 rpm. Two ml of the seed culture was transferred to each of twenty-five 500-ml Erlenmeyer flasks containing oatmeal 20 g, cellulose 10 g, yeast extract 0.05 g, sodium tartarate 0.03 g K_2HPO_4 0.03 g and 90 ml of distilled water. After the inoculation the flasks were incubated under static conditions for 8 days at 24°C.

Isolation

The culture was extracted with 80% aqueous acetone. After removal of the organic solvent, the aqueous solution was adjusted to pH 3.0 with 1 N HCl and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to dryness *in vacuo* (7.6 g). The extract was subjected to ODS flash chromatography (YMC-GEL ODS-A 120-130/70, YMC) and eluted with a step gradient from 60% to 100% MeOH. The active fraction eluted with 90% MeOH was evaporated to dryness (2.6 g). The residue was subjected to Silica gel flash chromatography (Kieselgel 60 0.040~0.063 mm, MERCK), eluted with a step gradient of $CHCl_3$ - MeOH. The active fraction eluted with $CHCl_3$/MeOH (90 : 10) was evaporated to dryness (247 mg). The residue was further separated on a Silica gel column (Kieselgel 60 0.063~0.200 mm, MERCK), developed with $CHCl_3$/MeOH (10 : 1). The active fractions were combined and evaporated to dryness (34 mg). The residue was finally purified by ODS HPLC on YMC-PACK Pro C18 with MeCN/H_2O/TFA (80 : 20 : 0.05) to yield 15 mg of **1** as a white powder.

Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of **1** are listed in Table 1. The molecular formula of **1** was determined to be $C_{24}H_{29}NO_6$ by high-resolution FAB-MS and NMR data,

which indicated eleven degrees of unsaturation. The IR spectrum suggested the presence of hydroxyl groups (3400 cm^{-1}) and carbonyl groups (1700, 1650 cm^{-1}). The ^1H NMR and DEPT spectra revealed the presence of two methyls, five methylenes, seven methines, one oxygenated quaternary carbon, three olefinic methines, three carbonyls and three other sp^2 quaternary carbons, which accounted for six of the eleven degrees of unsaturation. The remaining five degrees of unsaturation were attributed to five rings. The ^1H and ^{13}C NMR chemical shifts are shown in Table 2.

Analysis of one- and two-dimensional NMR spectra including COSY, HMQC and HMBC led to the assignment of three partial structures, **a**, **b** and **c**, as shown in Fig. 3. In partial structure **a**, a cyclohexanone ring was established by a COSY correlation (H-24/H-25) and HMBC correlations (H-21/C-20, C-25; H-22/C-23, C-24; H-24/C-20, C-23, C-25; H-25/C-20, C-21, C-23, C-24). The presence of an epoxide was disclosed by the chemical shifts of C-21 (δ_C 58.6, δ_H 3.78) and C-22 (δ_C 55.7, δ_H 3.41). Oxygenation of C-20 was inferred from its chemical shift at δ_C 71.5. Partial

Table 1. Physico-chemical properties of YM-215343 (**1**).

Appearance	White powder
Molecular weight	427
Molecular formula	$C_{24}H_{29}NO_6$
HRFAB-MS (m/z)	
Found:	428.2099 $(M+H)^+$
Calcd:	428.2073
$[\alpha]^{25}_D$	- 44.0° (c 0.10, MeOH)
UV (MeOH) λ_{max} nm(ε)	231 (15300), 279 (5200), 330 (8900)
IR νmax (KBr) cm^{-1}	3400, 2950, 2910, 1700, 1650, 1600, 1540, 1460, 1210

Fig. 3. Partial structures of YM-215343 (**1**).

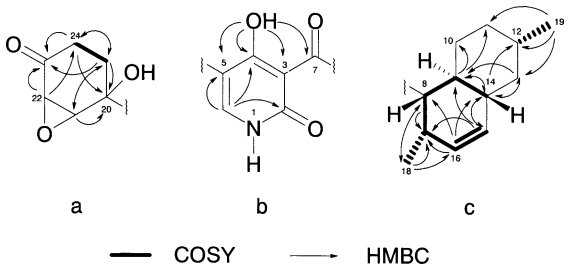


Table 2. ^1H and ^{13}C NMR data of YM-215343 (**1**) in $CDCl_3$.

Position	^{13}C	^1H (mult, J in Hz)	HMBC
1		10.68 (brs)	
2	162.7		
3	107.8		
4	178.5		
4-OH		18.42 (s)	C-3, C-4, C-5, C-7
5	115.7		
6	137.2	7.48 (s)	C-2, C-4, C-5, C-20
7	211.6		
8	52.8	4.35 (dd, 11.1, 6.0)	C-7, C-17, C-18
9	36.1	1.61 (brq, 11.1)	C-10, C-11, C-14
10ax	29.9	0.93 (m)	
10eq		1.90 (m)	
11ax	35.3	1.06 (m)	
11eq		1.75 (m)	
12	33.1	1.49 (m)	
13ax	41.7	0.83 (m)	
13eq		1.73 (m)	C-9, C-14
14	41.6	1.88 (m)	C-9, C-12, C-13, C-15
15	130.9	5.43 (d, 9.5)	C-9, C-14, C-17
16	131.1	5.60 (brd)	C-8, C-14, C-17
17	31.2	2.84 (m)	
18	18.1	0.86 (d, 6.4)	C-8, C-16, C-17
19	22.5	0.92 (d, 6.4)	C-11, C-12, C-13
20	71.5		
20-OH		4.42 (brs)	
21	58.6	3.78 (d, 3.2)	C-20. C-25
22	55.7	3.41 (d, 3.2)	C-23, C-24
23	203		
24	32.3	2.66 (dt, 19.1, 9.5), 2.41 (dt, 19.1, 4.0)	C-20, C-23, C-25
25	28.0	2.19 (dd, 9.5, 4.0)	C-20, C-21, C-23, C-24

structure **b** was deduced through analysis of chemical shifts and HMBC correlations for H-6 and 4-OH. The singlet methine proton H-6 gave cross peaks to C-2, C-4 and C-5. The hydrogen-bonded hydroxyl proton 4-OH was correlated to C-3, C-4, C-5 and C-7. The broad singlet NH proton (δ_H 10.68) did not give any cross peaks in COSY and HMBC spectra. The chemical shifts of the pyridone ring were consistent with those of a known compound, apiosporamide[5], so that the NH proton could be attached to C-2 and C-6. In partial structure **c**, the C^{14}–C^{9}–C^{8}–C^{17}(C^{18})–C^{16}=C^{15} and C^{12}–C^{19} portions were assigned by tracing cross peaks in the COSY spectrum. The *cis*-geometry of olefinic protons was deduced by the coupling constant for H-15/H-16 (9.5 Hz) and the olefinic protons H-15 and H-16 were correlated to C-14, indicating the presence of a cyclohexene ring moiety. An unsaturated decalin ring was disclosed by interpretation of HMBC correlations. The doublet methyl protons H-19 gave cross peaks to C-11, C-12 and C-13. The methine proton H-9 and the methylene protons H-13 were correlated to C-10, 11 and C-9, 14, respectively.

Connection of partial structures, **a**, **b** and **c**, was accomplished by interpretation of the HMBC spectrum. An HMBC correlation from H-6 to C-20 showed a connection between partial structures **a** and **b**. The methine proton H-8 was correlated to C-7, indicating a connection between partial structures **b** and **c**. The relative stereochemistry of the decalin ring was deduced by interpretation of $^3J_{HH}$ values and difference NOE spectra. The coupling constants for H-8/H-9 and H-9/H-14 (11.1 and 11.1 Hz) indicated *trans*-diaxial couplings. The coupling constant for H-8/H-17 (6.0 Hz) suggested that the configuration of H-17 was equatorial. A difference NOE from H-12 to H-14 indicated that the configuration of H-12 was axial. Thus, the structure of **1** was determined as shown in Fig. 1. Structurally, **1** is closely related to apiosporamide[5] and fischerin[6]. They possess a cyclohexanol ring moiety instead of a cyclohexanone ring moiety. Furthermore, fischerin contains a *cis*-decalin and an *N*-hydroxyl group but lacks the C-19 methyl group. The UV chromophore and IR data (Table 1) are consistent with the proposed structure. The relative stereochemistry of C-20, 21, 22 and the absolute stereochemistry will be determined at a later date.

Biological Activities

Antifungal activity of **1** was evaluated by the microbroth dilution method in comparison with itraconazole. As shown in Table 3, **1** exhibited antifungal activity against the pathogenic fungi, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* with MIC values of 2~16 μg/ml. It also showed cytotoxicity against HeLa S3 cells with an IC_{50} of 3.4 μg/ml. Apiosporamide[5] was produced by the coprophilous fungus *Apiospora montagnei*. It was reported to show antifungal and antibacterial activity against the coprophilous fungus *Ascobolus furfuraceus*, *Bacillus subtilis* and *Staphylococcus aureus*, but show no activity against *C. albicans* at 200 μg/disk. The difference in the activity of **1** and apiosporamide may depend on the methods used for the evaluation rather than the small difference in their structures.

Table 3. Antifungal activity of YM-215343 (**1**).

Organisms	MIC (μg/ml)	
	YM-215343	ITCZ
Candida albicans YFC 497	4	0.06
C.glabrata YFC 501	2	1
C.krusei YFC 827	16	0.5
Saccharomyces cerevisiae YFC 250	4	2
Cryptococcus neoformans YFC 513	16	0.5
Aspergillus fumigatus YFC 526	16	0.05

Acknowledgment

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