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A Novel *Helicosporium* Isolate and Its Antimicrobial and Cytotoxic Pigment

Choi, Hye Jung[1], Sang Myeong Lee[2], Sun-Hee Kim[3], Dong Wan Kim[4], Young Whan Choi[5], and Woo Hong Joo[1]*

[1]*Interdisplinary Program for Biotechnology and Department of Biology, Changwon National University, Changwon 641-773, Korea*
[2]*SM Biovision Co., Ltd., Business Incubation Center, Gyeongsang National University, Jinju 660-701, Korea*
[3]*Department of Biochemistry, Pusan National University School of Medicine, Yangsan 626-870, Korea*
[4]*Department of Microbiology, Changwon National University, Changwon 641-773, Korea*
[5]*Department of Horticultural Bioscience, Pusan National University, Miryang 627-706, Korea*

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One *Helicosporium* strain, isolated from a wilted chestnut tree, evidenced *in vitro* antimicrobial activity against various types of bacteria and fungi, and generated a diffusible pigment. The antimicrobial compounds and the diffusible pigment of the *Helicosporium* sp. isolate were purified *via* solvent fractionation, column chromatography, and recycling preparative chromatography. Both the major antimicrobial compound and the diffusible pigment were identified as 2-methylresorcinol *via* nuclear magnetic resonance spectroscopy. Therefore, 2-methylresorcinol, a diffusible pigment generated by *Helicosporium* sp., appears to be an active antimicrobial principle. This pigment also exhibited considerable cytotoxicity against mammalian cells.

Keywords: *Helicosporium*, diffusible pigment, 2-methyl resorcinol, antimicrobial principle, mammalian cell toxicity

As certain ascomycetous imperfect filamentous fungi have been shown to cause the softening of wood surfaces, the term "soft-rot fungi" has been applied to the type of decaying fungi that grow within the walls of wood, destroy wood cell walls, and eventually destroy the hardwood itself. Members of the *Helicosporium* genus, like most helicosporous fungi, are recognized as soft decayers of wood and plant material, and also as plant parasites and phytopathogens [1].

The only functional component thus far detected in *Helicosporium* sp. is a 15 kDa protein that inhibits the neurite outgrowth of cortical neurons [3]. The antimicrobial potential of *Helicosporium* was reported as early as 1995 [4], but its active compounds had never been elucidated until now. On the other hand, only a few *Helicosporium* species have been known to produce diffusible pigment [2]. However, little is currently known regarding the pigment produced by the *Helicosporium* species.

In this paper, we report the isolation of an antagonistic *Helicosporium* strain. The purification and identification of the active compound and the pigment of the *Helicosporium* sp. isolate were carried out by different solvent fractionations, column chromatography, recycling preparative chromatography, and nuclear magnetic resonance spectrometry. The antimicrobial activity and cell toxicity of the bioactive compound were also evaluated.

Materials and Methods

Isolation and Identification of *Helicosporium* sp. Strain

Fungal strains were isolated from various plant samples collected in Gyeongsangnam-do Province, Korea. The inhibitory abilities of each isolate were measured against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans,* and *Aspergillus niger* on potato dextrose agar. The antagonistic *Helicosporium* sp. isolate was selected from the antagonistic fungal isolates. Identification was carried out through the investigation of colony colors, colony shapes, and morphological features of conidia and conidiophores [2].

Purification and Structure Determination of the Antimicrobial Substance and Pigment

The isolate was grown in potato dextrose broth for 5 days at 25°C. The supernatant was partitioned with ethylacetate. A wine-colored residue was obtained and purified *via* column chromatography on silica gel eluted first with ethylacetate/methanol [2:1 (v/v)], then with methanol, and finally with chloroform/methanol [19:1 (v/v)] as solvents. The final purification of the antimicrobial compound was assessed *via* recycling preparative high-performance liquid chromatography (JAI, Japan).

The diffusible pigment was also purified in accordance with the method described above on the basis of visible color. Gas

*Corresponding author
Phone: +82 55 2133453; Fax: +82 55 2133459;
E-mail: whjoo@changwon.ac.kr

chromatography-mass spectrometer (GC-MS) data of the isolated antimicrobial substance and pigment were obtained using a gas chromatography-single stage mass spectrometer (GC-MS Agilent 6890 GC coupled with Agilent 5973 MS, Santa Clara, USA). Nuclear magnetic resonance spectrometry (NMR) spectra were recorded on a Brucker spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C in methanol-d_4. (MeOD). DEPT NMR spectral data were also recorded on a Brucker 400 MHz spectrometer in MeOD, using trimethylsilane as an internal standard.

Antimicrobial Assays

The minimal inhibitory concentrations (MICs) of the purified compound were determined against four Gram-positive bacterial strains, seven Gram-negative bacterial strains, and three fungal strains by the agar dilution method according to Clinical and Laboratory. Standards Institute (CLSI) recommendations. All assays conducted herein were carried out in triplicate.

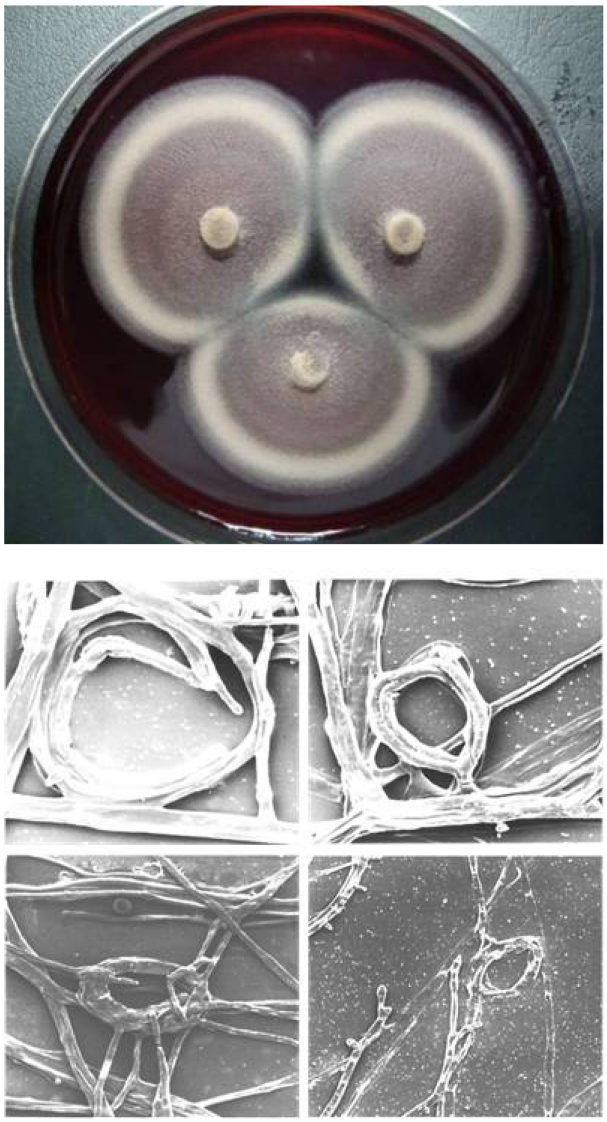

Fig. 1. Culture plate and scanning electron microphotograph of *Helicosporium* sp. KCTC 0635BP.

Cell Culture and Mammalian Cell Toxicity

Human hepatoblastoma cell line HepG2, human breast adenocarcinoma cell line MCF-7, and human colon cancer cell line KM12 were cultured in DMEM, and the human erythromyeloblastoid leukemia cell line K562 and human T cell lymphoblast-like cell line CCRF-CEM were cultured in RPMI1640 medium. All media were supplemented with 10% fetal bovine serum. The cells were maintained at 37°C, 5% CO_2. Cell toxicity was measured using the MTT colorimetric dye-reduction method. Exponentially growing cells (2×10^4 cells/well) were plated in 96-well plates and incubated in growth medium treated with various concentrations of purified compound at 37°C. After 96 h, the medium was aspirated using centrifugation, and MTT-formazan crystals that had formed in the MTT reaction were solubilized in 100 µl of DMSO. The absorbance was determined at 570 nm using an ELISA reader. All experiments were repeated with at least two experiments in triplicate.

RESULTS

Identification of *Helicosporium* sp. Isolate

Among the antagonistic fungal strains collected in Gyeongsangnam-do Province, Korea, one putative *Helicosporium* sp. strain was selected for further study. The isolate had branched mycelia and helical conidia (Fig. 1). After 5 days of incubation, the isolate released characteristic reddish wine-colored pigments on PDA medium (Fig. 1). Based on the above morphological characteristics, the isolate was identified as *Helicosporium* sp. and deposited in the Korean Collection for Type Cultures (KCTC) as *Helicosporium* sp. KCTC 0635BP.

Structure Elucidation of Isolated Compound

Both the isolated compound and pigment had a molecular formula of $C_7H_8O_2$, as revealed by analysis of the positive-ion HRESI-MS data (data not shown). In the ^{1}H NMR spectra of the isolated compound and pigment (Fig. 2A, Table 1), two hydroxy moieties were identified by observation of signals at δ 5.18 (2H, *bs*, 1-OH and 3-OH, D_2O exchangeable). The proton of the benzene ring was also assigned on the basis of the signals at δ 6.09–6.11

Table 1. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), and DEPT NMR spectroscopic data of the compound isolated from *Helicosporium* sp. strain KCTC 0635BP in MeOD (δ in ppm, *J* in Hz).

No	δ_C	δ_H	DEPT
1	157.83		C
2	99.32	6.10, dd, *J* = 4.5/2.0	CH
3	157.83		C
4	107.25	6.15, dd, *J* = 3.0/1.0	CH
5	139.90		C
6	107.25	6.15, dd, *J* = 3.0/1.0	CH
7	20.27	2.18, s	CH_3

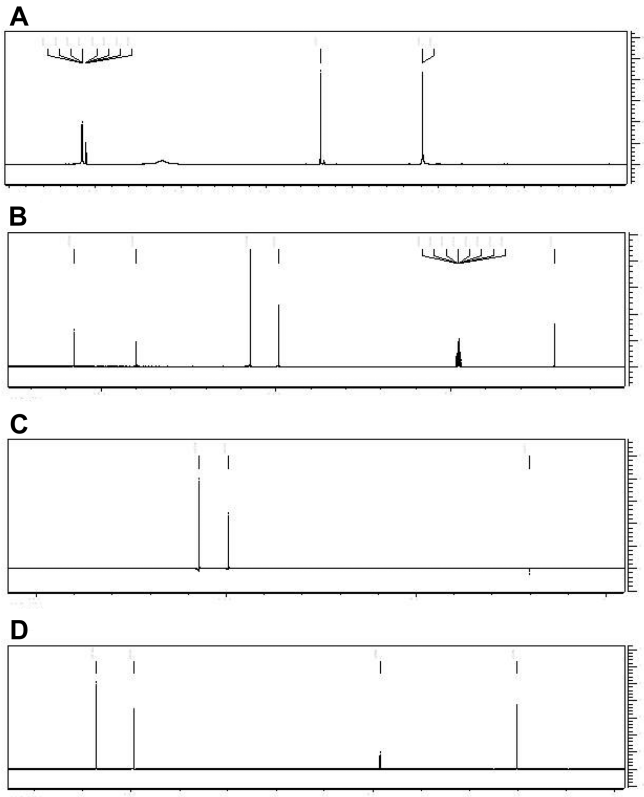

Fig. 2. NMR spectrum of the isolated compound and pigment. (**A**) ^{1}H-NMR spectrum; (**B**) ^{13}C-NMR spectrum; (**C**) DEPT 90 spectrum; (**D**) DEPT 135 spectrum.

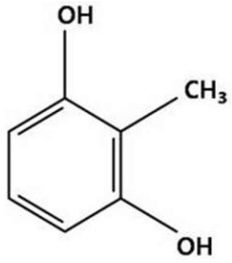

Fig. 3. Structure of the 2-methylresorcinol isolated from the fungus *Helicosporium* sp. KCTC 0635BP.

(1H, m, H-5) and δ 6.14–6.15 (2H, *m*, H-4 and H-6). Additionally, the ^{1}H NMR spectrum of the methyl group evidenced a singlet at δ 2.18 (3H, *s*, H-2). This finding was consistent with the corresponding ^{13}C NMR spectroscopic data in the literature, which suggested that 2-methylresorcinol might possess a resorcinol skeleton. This was inferred from the observed signals at δ 157.83 (C-1 and C-3), δ 139.90 (C-5), δ 107.25 (C-4), and δ 99.32 (C-2) for the benzene ring, and δ 20.27 (C-7) for methyl at the C-2 position observed in the ^{13}C NMR spectrum (Fig. 2B, Table 1). Data from the DEPT NMR experiment (Fig. 2C, 2D, Table 1) supported these conclusions and enabled completion of the structure of the isolated compound and pigment (Fig. 3).

Table 2. Antimicrobial activity and mammalian cell toxicity of the pure isolated compound.

Cells	2-Methylresorcinol
Bacterial strains	MIC (μg/ml)
Bacillus subtilis KACC 10111	25
Clavibacter michiganensis KACC 20122	20
Micrococcus luteus KACC 10488	30
Staphylococcus aureus IMSNU 11088	25
Escherichia coli IMSNU 10080	>500[a]
Klebsiella pneumonia KCTC 2208	250
Pseudomonas aeruginosa IMSNU 10191	>500
Proteus mirabilis KCTC 2566	62
Proteus vulgaris IMSNU 13025	20
Shigella sonnei KCTC 2518	50
Salmonella Typhimurium KCTC 2515	>500
Fugal strains MIC (μg/ml)	
Candida albicans KACC 30062	250
Filobasidium neoformans KCTC 7902	125
Aspergillus niger KACC 40280	>1,000[b]
Mammalian cells IC_{50} (ug/ml)	
HepG2	20.02 ± 0.97
MCF-7	5.39 ± 0.5
KM12	38.43 ± 1.88
K562	16.75 ± 0.71
CEM	18.78 ± 0.81

MIC indicates the minimal inhibitory concentration that inhibits microbial cell growth.

IC_{50} is defined as the concentration that inhibits 50% cell growth.

KACC, Korean Agricultural Culture Collection; KCTC, Korean Collection for Type Culture; IMSNU, Institute of Microbiology, Seoul National University.

[a]Not susceptible at up to 500 μg/ml of 2-methylresorcinol.

[b]Not susceptible at up to 1,000 μg/ml of 2-methylresorcinol.

All experiments were repeated with at least two experiments in triplicate.

Minimal Inhibitory Concentrations (MICs)

Helicosporium sp. evidenced antagonistic ability against various types of bacteria and fungi. The MICs of 2-methylresorcinol against test strains, determined *via* agar and broth dilution methods, are shown in Table 2. Gram-positive bacteria evidenced greater susceptibility to 2-methylresorcinol than Gram-negative bacteria. The majority of the Gram-positive bacterial strains were susceptible at 30 μg/ml of 2-methylresorcinol, but Gram-negative *K. pneumoniae* was susceptible at only 250 μg/ml of 2-methylresorcinol.

In particular, *P. aeruginosa*, *Salmonella* Typhimurium, and *E. coli* were not susceptible at up to 500 μg/ml of 2-

methylresorcinol. The MICs of 2-methylresorcinol against *F. neoformans* and *C. albicans* were more or less higher than the MICs of 2-methylresorcinol against Gram-positive bacteria.

Cellular Toxicity

Cell toxicity of 2-methylresorcinol was evaluated using MTT assay (Table 2). 2-Methylresorcinol showed cell toxicity toward a broad range of tumor cells. This material showed particularly strong cytotoxicity to human breast cancer cell MCF-7.

Discussion

We isolated one *Helicosporium* sp. strain from a wilted chestnut tree. The pinkish colony and microscopic morphological features of conidia and conidiophores of the *Helicosporium* isolate assessed herein strongly suggest that the isolate belongs to *Helicosporium pallidum* [2], but its taxonomic assignment will have to be conducted in a future phylogenetic study. The antimicrobial potential of *Helicosporium* was first described in 1995 [4]. However, the compounds with antimicrobial potential contained in or generated by *Helicosporium* remain to be clearly elucidated.

In this study, we purified the antimicrobial compound of *Helicosporium* sp. strain KCTC 0635BP. The main antimicrobial constituent of *Helicosporium* was identified as 2-methylresorcinol. 2-Methylresorcinol was identified for the first time as occurring in *Helicosporium* sp. fungi in this study, and has not been previously reported as an antimicrobial compound of any other known fungus. Variations of the resorcinol skeleton, however, have been identified by other researchers, including the antibacterial and antifungal 2-butyl-5-pentylresorcinol (stemphol) isolated from the anamorphic fungus *Stemphylium majusculum* [9] and *Pleospora herbarum* [6]. Four dialkylresorcinols have also been reported as antibacterial and antifungal compounds in *Pseudomonas* sp. [8]. Anti-methicillin-resistant *S. aureus* (MRSA) 5-heptadeca-8'Z,11'Z,16-trienylresorcinol and 5-heptadeca-9'E,11'Z,16-trienylresorcinol have been previously isolated from mushrooms [5].

2-Methylresorcinol was also identified as the principal diffusible pigment in *Helicosporium* and it is secreted into the extracellular milieu from cells. As a consequence, 2-methylresorcinol was diffused around the PDA plate. In addition, it evidenced antimicrobial activity. To the best of our knowledge, this is the first report of an antimicrobial pigment with a simpler resorcinol skeleton. Alkyl phenols have been known to show cell toxicity [5, 7]. 2-Methylresorcinol also shows cell toxicity and is more active against MCF-7 than against K562, CCRF-CEM, and HepG2. This first report on the cytotoxicity of 2-methylresorcinol originating from *Helicosporium* sp. may offer opportunities for the development of more potent analogs against human cancer cells by means of the large-scale production of 2-methylresorcinol.

Acknowledgments

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