Chitin Synthase 2 Inhibitory Activity of *O*-Methyl Pisiferic Acid and 8,20-Dihydroxy-9(11),13-abietadien-12-one, Isolated from *Chamaecyparis pisifera*

Tae Hoon KANG,[a] Eui Il HWANG,[b] Bong Sik YUN,[a] Chul Soo SHIN,[c] and Sung Uk KIM*,[a]

[a] *Korea Research Institute of Bioscience and Biotechnology; P.O. Box 115, Yusung, Daejeon 305–806, Korea:* [b] *Ginseng Science Research Group, KT&G Central Research Institute; Daejeon 305–805, Korea: and* [c] *Department of Biotechnology, College of Engineering, Yonsei University; Seoul 120–749, Korea.*

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In the course of search for potent chitin synthase inhibitors from plant extracts, the chitin synthase 2 inhibitors, *O*-methyl pisiferic acid and 8,20-dihydroxy-9(11),13-abietadien-12-one which have diterpene skeleton, were isolated from the leaves of *Chamaecyparis pisifera*. These compounds inhibited chitin synthase 2 of *Saccharomyces cerevisiae* with the IC_{50} values of 5.8 and 226.4 μM, respectively. Especially, *O*-methyl pisiferic acid showed 15.3-fold stronger inhibitory activity than polyoxin D (IC_{50}=88.6 μM), a well-known chitin synthase inhibitor. These compounds exhibited weaker inhibitory activities against chitin synthase 1 than chitin synthase 2, whereas it showed no inhibitory activity for chitin synthase 3. The compound exhibited mixed competitive inhibition with respect to UDP-*N*-acetyl-D-glucosamine as substrate (K_i=5 μM). These results indicated that *O*-methyl pisiferic acid is a specific inhibitor of chitin synthase 2. The compound also inhibited chitin synthase 1 of *Candida albicans*, which represents analogues to chitin synthase 2 of *S. cerevisiae*, with an IC_{50} of 75.6 μM, which represents 1.8-fold weaker activity than that of polyoxin D. Although *O*-methyl pisiferic acid has been reported for antibacterial and insecticidal activities, the present study is the first report on its inhibitory activity against chitin synthase 2.

Key words *Chamaecyparis pisifera*; *O*-methyl pisiferic acid; chitin synthase inhibitor; antifungal activity

The cell wall, a structure essential to fungi and absent in mammalian cells, protects the cell from the hazards of the environment, acts as a filter permitting the passage of some molecules while excluding others,[1] and relays signals for invasion and infection of a likely plant, animal, or human host. [2] In addition, the cell walls maintain the shape of fungal cells and are essential for their integrity,[3] Furthermore, the biosynthesis of fungal cell walls constitutes a good model for fungal morphogenesis at the molecular level, and serves as a potential target in antifungal chemotherapy.

Chitin, the β-(1,4)-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), is an important structural component of the cell walls of nearly all pathogenic and phytopathogenic fungi and plays a major role in the determination of cell morphology.[4] Its synthesis constitutes a model for studying fungal morphogenesis and is a potential target in manipulating fungal growth. Chitin is synthesized by chitin synthase 1 (ScCHS1p), 2 (ScCHS2p), and 3 (ScCHS3p) in *Saccharomyces cerevisiae*.[5,6] Chitin synthase 1 is a nonessential repair enzyme of damaged chitin.[6] Chitin synthase 2 is an essential enzyme for primary septum formation and cell division,[7] whereas chitin synthase 3 is responsible for chitin in the ring at bud emergence and in the lateral cell wall.[8] Therefore, specific inhibitors of chitin synthase 2 and 3 might block the formation of fungal cell wall, and could be used as effective antifungal agents.

Chamaecyparis pisifera (SIEBOLD and ZUCCARINI) ENDLICHER, also known as Sawara Cypress or Sawara is a native tree of Japan. There is very little reported on the use of *Chamaecyparis* species in traditional medicine.[9] The Southern Kwakiutl Indians of British Columbia used the leaves, branch tips and bark of *C. nootkatensis* to treat sores, arthritis and rheumatism.[10] In Japan, the wood of *C. obtusa* is valued for use in the construction of important building such as temples and shrines and is also considered to have hygienic properties for use as counter tops in sushi bars.[11] Several species of *Chamaecyparis* have been shown to possess insecticidal,[12] antibacterial,[13,14] antifungal activities,[14] and antiviral activity against herpes simplex virus type 2.[15] Recently, acaricidal activity[16] and antiviral activity against herpes simples virus type 1[17] were reported from the genus *Chamaecyparis*.

In the course of our continuing search for potent inhibitors of chitin synthase 2 from higher plants, a strong inhibitory compound against chitin synthase 2 of *S. cerevisiae* was found in the methanol extract of *Chamaecyparis pisifera*, which was identified as *O*-methyl pisiferic acid and 8,20-dihydroxy-9(11),13-abietadien-12-one. Although *O*-methyl pisiferic acid has been reported to show antibacterial activities, this is the first report to describe the chitin synthase 2 inhibitory activity. Here, we describe the isolation of *O*-methyl pisiferic acid from the leaves of *Chamaecyparis pisifera* and their inhibitory activities against chitin synthases of *S. cerevisiae* and various pathogenic fungi.

MATERIALS AND METHODS

Strains and Culture Conditions The strains used in this study were *S. cerevisiae* YPH499 (*ura3-52 lys2-801*^{amber} *ade20101*^{ochre} *trp1-Δ63 his-Δ200 leu2-1*),[18] ECY38-38A (pAS6) (*MATa chs1-23 chs2*::*LEU2 call*/*csd2 ura3-52 trp1-1 leu2-2* pAS6),[19] and ECY38-38A (pWJC6) (*MATa chs1-23 chs2*::*LEU2 call*/*csd2 ura3-52 trp1-1 leu2-2* pWJC6),[19] respectively. *S. cerevisiae* YPH499, the wild type for all three synthases, was grown in YEPD [1% yeast extract, 2% Bacto peptone (Difco), 2% glucose]. *S. cerevisiae* ECY38-38A (pAS6) and ECY38-38A (pWJC6), which can only overexpress ScCHS2p and 3p, respectively, were grown in YPG

* To whom correspondence should be addressed. e-mail: kimsu@kribb.re.kr

[1% yeast extract, 2% Bacto peptone, 2% galactose] at 30 °C. In addition, the chitin synthase-deleted homologous mutant *chs2*Δ*chs3*Δ(*chs2*Δ::*hisG*/*chs2*Δ::*hisGchs3*Δ::*hisG*/*chs3*Δ::*hisG*) was used for *Candida albicans* chitin synthase 1 activity.[20] The strain was grown in Sabouraud dextrose medium at 30 °C.

Chemicals Uridine diphosphate (UDP)-[U-^{14}C]-*N*-acetyl-D-glucosamine (400000 cpm/μmol) was purchased from NEN Life Science Products (Boston, U.S.A.). Sabouraud agar and potato dextrose agar media were purchased from Difco (Sparks, U.S.A.). All other reagents were of the highest grade available and used without further purification.

Isolation of *O*-Methyl Pisiferic Acid The leaves of *C. pisifera* were collected at Yusung, Daejeon, Korea. A voucher specimen has been deposited under CFM-585 in the Korea Research Institute of Bioscience and Biotechnology. The leaves of *C. pisifera* (3 kg) were extracted twice with 20 l of methanol at room temperature for 5 d, filtered, and concentrated *in vacuo* to yield a dark brown sticky solid (400 g). After solvent extraction with chloroform, the chloroform layer (63 g) was subjected to silica gel column chromatography (E. Merck, Kieselgel 60, 230—400 mesh, 7.5×35 cm) and eluted stepwise with a gradient of *n*-hexane/ethyl acetate (10 : 0 to ethyl acetate only, v/v, each 8 l). The active fractions (6 : 4, v/v, 1.2 g) were subjected to Sephadex LH-20 column chromatography (Amersham Bioscience, Sweden) with methanol (3×150 cm, 0.7 ml/min, each 20 ml). The active fractions (fraction 51 to 75, 600 mg) were subjected to reverse phase silica gel column chromatography (E. Merck, LiChroprep RP-18 40—63 μm, 3.5×25 cm) and eluted stepwise with a gradient of water/methanol (6 : 4 to 0 : 10, v/v, each 2 l). Crystallization was induced at active fractions (90% methanol fraction). Crude crystal was resolved and finally purified by re-crystallization in methanol, after which pure compound (250 mg) was collected. Through the HPLC using an ODS column (Waters, Xterra C_{18}, 5 μm, 4.6×250 mm), a single peak with the retention time of 14 min was detected by a UV spectrometric detector at 254 and 210 nm. The column was eluted with CH_3CN/water (70 : 30 v/v) at a flow rate of 1.0 ml/min.

Preparation of Crude Membranes Membranes of *S. cerevisiae* YPH499, ECY38-38A (pAS6), ECY38-38A (pWJC6), and *C. albicans chs2Δchs3Δ* were prepared as described previously.[21] The *S. cerevisiae* YPH499, recombinant ECY38-38A strains, and *C. albicans* strain were cultivated at 30 °C overnight to reach the absorbance of 0.7 at 600 nm. Cells suspended in 50 mM Tris–HCl (pH 7.5) containing 5 mM magnesium acetate were broken by vortex mixing with glass beads. Cell walls were sedimented at 4000×***g*** for 5 min and the supernatant fluid was centrifuged at 130000×***g*** for 45 min. The membrane pellet was suspended in the 50 mM Tris–HCl (pH 7.5) containing 33% glycerol used in the breakage, to a final volume of 1.6 ml/g (wet weight) of cells.

Chitin Synthases Assays The assays of chitin synthase 2 and 3 prepared from recombinant *S. cerevisiae* ECY38-38A (pAS6) and ECY38-38A (pWJC6), respectively, were conducted according to the method of Choi and Cabib.[22] Chitin synthase 2 activity was measured by the procedure described previously.[22] For the proteolytic activation step, reaction mixtures contained 32 mM Tris–HCl (pH 8.0), 1.6 mM cobalt acetate, 1.0 mM UDP-[^{14}C]-*N*-acetyl-D-glucosamine (400000 cpm/μmol, NEN), 2 μl of trypsin at the optimal concentration for activation (2.0 mg/ml), 20 μl of membrane suspensions, and 14 μl of sample in a total volume of 46 μl. The mixtures were incubated for 15 min at 30 °C. Proteolysis was stopped by adding 2 μl of a soybean trypsin inhibitor solution (4.0 mg/ml) at a concentration 2 times of the trypsin solution used, and tubes were placed on ice for 10 min. *N*-acetyl-D-glucosamine was added to a final concentration of 32 mM, and incubation at 30 °C was carried out for 90 min. For chitin synthase 3 activity,[19,22] the assay was performed as for chitin synthase 2, except that 32 mM Tris–HCl (pH 7.5) and 4.3 mM magnesium acetate were used. For the assay of chitin synthase 1 activity,[22] reaction mixtures contained 37 mM Tris–HCl (pH 7.5), 0.12% digitonin, 4.8 mM magnesium acetate, 2 μl of trypsin (1.0 mg/ml), 6 μl of membrane suspension, and 14 μl of the test sample in a total volume of 41 μl. After 15 min of incubation at 30 °C, 2 μl of trypsin inhibitor (2.0 mg/ml) was added, and the tubes were placed on ice. GlcNAc (32 mM) and 1.0 mM UDP-[^{14}C]-*N*-acetyl-D-glucosamine were added as for the chitin synthase 2 and 3 assays, and the mixtures were incubated for 30 min at 30 °C. In addition, the assay of chitin synthase 1 prepared from the chitin synthase-deleted homologous mutant *C. albicans chs2*Δ*chs3*Δ was conducted according to the method of Choi.[23] For chitin synthase 1 activity of *C. albicans*,[19,21] the assay was performed as for chitin synthase 2, except that 32 mM Tris–HCl (pH 7.5) and 2 mM cobalt acetate were used. In all cases, the reaction was stopped by the addition of 10% trichloroacetic acid, and radioactivity of the insoluble chitin formed was counted after filtration through glass fiber filter (GF/C, Whatman). The concentration of protein was measured by the method of Lowry.[24] Blank values were measured with addition of 25% aqueous MeOH instead of both enzyme and sample. Percent inhibition of chitin synthase activity was calculated by subtracting the blank values from both control and test sample values.

$$\%\ \text{inhibition} = \left[1 - \frac{\text{sample (cpm)} - \text{blank (cpm)}}{\text{control (cpm)} - \text{blank (cpm)}}\right] \times 100$$

The chitin synthase 1, 2, and 3 activities of the enzyme were confirmed by positive control with polyoxin D and nikkomycin Z (Calbiochem Co.) to compare the potency of our compound against chitin synthases. Each of the isolated and control compounds was solubilized in 25% MeOH and distilled water to make a stock solution (1 mg/ml), respectively, and an aliquot (14 μl) of the stock was used for each reaction to give the final concentration of 280 μg/ml. The inhibitory activities were represented as average values in duplicates obtained from two independent experiments.

Determination of Minimum Inhibitory Concentrations (MICs) MICs were determined by the method of CLSI (formerly NCCLS, U.S.A.)[25] using RPMI1640 medium for human pathogenic fungi including *S. cerevisiae* and the agar dilution method using potato dextrose agar medium for phytopathogenic fungi.[26] *O*-Methyl pisiferic acid and 8,20-dihydroxy-9(11),13-abieta-dien-12-one were dissolved in 25% MeOH, while polyoxin D and nikkomycin Z were dissolved in distilled water. Human pathogenic fungi were grown on Sabouraud agar medium, and plant pathogenic fungi were

grown on potato dextrose agar medium. The inoculum sizes of yeasts and spore-forming fungi were 5.0×10^2 to 2.5×10^3 colony forming unit (CFU)/ml and 10^5 spore/spot, respectively. Antifungal activity was observed after 24 h incubation at 35 °C for yeasts and 48 h incubation at 25 °C for fungi. The MIC was defined as the lowest concentration of compounds which completely inhibited the growth of the organism when compared to a control containing no compounds.

RESULTS AND DISCUSSION

The bioactive compounds from the leaves of *Chamaecyparis pisifera* were purified by solvent partition, silica gel and Sephadex LH-20 column chromatographies, and HPLC. Structure analyses of isolated compounds with EI-MS and various NMR techniques including ^{1}H–^{1}H COSY, HMQC, and HMBC identified the compound as *O*-methyl pisiferic acid and 8,20-dihydroxy-9(11),13-abietadien-12-one. The mass spectroscopic and nuclear magnetic resonance data for the purified compounds were in good agreement with the spectral data for both compounds published previously.[27,28]

Although several chitin synthase inhibitors have been isolated from microbes and higher plants, *O*-methyl pisiferic acid and 8,20-dihydroxy-9(11),13-abietadien-12-one have not been reported as chitin synthase inhibitors (Fig. 1). The inhibitory activities of these compounds on chitin synthase isozymes were examined by the filter binding assay using UDP-[^{14}C]-*N*-acetyl-D-glucosamine as substrate. As shown in Table 1, *O*-methyl pisiferic acid strongly inhibited the chitin synthase 2 of *S. cerevisiae* in a dose-dependent manner with an IC_{50} of 5.8 μM, whereas 8,20-dihydroxy-9(11),13-abietadien-12-one showed a very weak inhibitory activity (IC_{50}=226.4 μM) for ScCHS2p. The IC_{50} value of *O*-methyl pisiferic acid for ScCHS2p represented 15.3-fold stronger inhibitory activity than polyoxin D, a well-known chitin synthase inhibitor. To examine whether the observed inhibition was specific for chitin synthase 2, the effects of both compounds on chitin synthase 1 and 3 from *S. cerevisiae* were also determined. These compounds showed no effects on chitin synthase 3 activity, whereas they exhibited very weak inhibitory activities on ScCHS1p (IC_{50}>424 μM). In addition, *O*-methyl pisiferic acid also inhibited the chitin synthase 1 of *C. albicans* with an IC_{50} of 75.6 μM, which represents 1.8-fold weaker activity than that of polyoxin D. From these results, it was suggested that the *O*-methyl pisiferic acid is a specific inhibitor of chitin synthase 2 from *S. cerevisiae*. The mechanism of inhibition of *O*-methyl pisiferic acid on ScCHS2p was investigated in a kinetic analysis of the inhibition with a Lineweaver–Burk plot. Double reciprocal plots of the data demonstrated that *O*-methyl pisiferic acid acted as a mixed type competitive inhibitor with respect to the substrate, UDP-*N*-acetyl-D-glucosamine. The K_i value of *O*-methyl pisiferic acid was calculated to be 5 μM (Fig. 2). Based on these results, this compound inhibited chitin synthase 2 activity by mixed competition with UDP-*N*-acetyl-D-glucosamine, indicating that chitin synthase 2 is one of the cellular targets for the inhibitory activity of *O*-methyl pisiferic acid. Unfortunately, in spite of a potent inhibitory activity of *O*-methyl pisiferic acid on chitin synthase 2, the compound showed only a weak inhibitory activity against *Pythium ultimum* at the concentration of 128 μg/ml, whereas 8,20-dihydroxy-9(11),13-abietadien-12-one with mild activ-

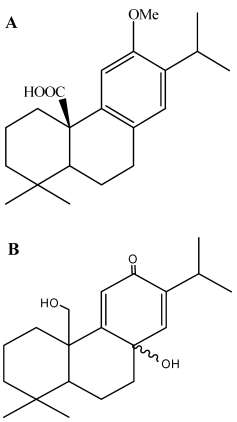

Fig. 1. Structures of *O*-Methyl Pisiferic Acid (A) and 8,20-Dihydroxy-9(11),13-abietadien-12-one (B)

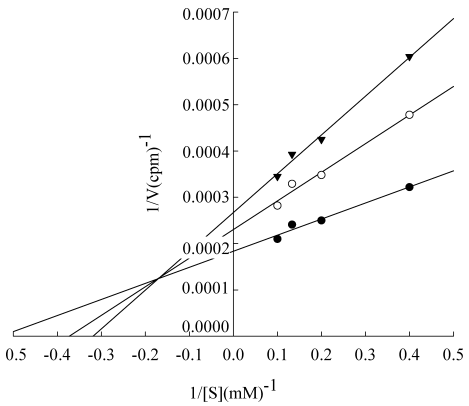

Fig. 2. Lineweaver–Burk Plot of the Inhibition of ScCHS2p by *O*-Methyl Pisiferic Acid

Reaction mixtures contained 0.1 mM UDP-[^{14}C]-*N*-acetyl-D-glucosamine and varying amounts of UDP-*N*-acetyl-D-glucosamine (0.25, 0.5, 0.75, 1.0 mM), and *O*-methyl pisiferic acid (●, 0; ○, 26; ▼, 53 μM), and were incubated as in Materials and Methods.

Table 1. Effects of *O*-Methyl Pisiferic Acid and 8,20-Dihydroxy-9(11),13-abietadien-12-one on Chitin Synthase Isozymes in Our Assay System

Isozyme[a)]	Strain	IC_{50} (μM)			
		O-Methyl pisiferic acid	DHA[c)]	PD[c)]	NZ[c)]
ScCHS1p	YPH449	424.0	440.0	7.1	1.1
ScCHS2p	ECY38-38A (pAS6)	5.8	226.4	88.6	354.7
ScCHS3p	ECY38-38A (pWJC6)	—[b)]	—	5.9	2.0
CaCHS1p	*chs2*Δ*chs3*Δ	75.6	170.9	42.7	15

a) ScCHS1p was prepared from wild type *S. cerevisiae* YPH499. pAS6 is a high-copy-number plasmid carrying *CHS2* on a vector containing a *TRP* marker. pWJC6 is a high-copy-number plasmid carrying *CAL/CSD2* (complete gene) under the control of the *GAL1* promoter. *b*) No inhibitory activity. *c*) DHA: 8,20-dihydroxy-9(11),13-abietadien-12-one, PD: polyoxin D, NZ: nikkomycin Z.

Table 2. *In Vitro* Antifungal Activities of *O*-Methyl Pisiferic Acid and 8,20-Dihydroxy-9(11),13-abietadien-12-one against Various Fungi (Unit: μg/ml)

Strains	Compounds (MICs)[b]				
	O-MPA	DHA	PD	NZ	Amp B
S. cerevisiae YPH499	>128	>128	>100	>100	0.125
S. cerevisiae ECY38-38A (pAS6)	>128	128	>100	>100	0.25
S. cerevisiae ECY38-38A (pWJC6)	>128	>128	>100	>100	0.5
Candida albicans ATCC10231	>128	>128	>100	>100	0.25
Candida albicans A207[a]	>128	>128	>100	>100	0.25
Candida krusei ATCC6258	>128	>128	>100	>100	1
Candida tropicalis ATCC13803	>128	>128	>100	>100	0.5
Candida parapsilosis ATCC34136	>128	>128	>100	>100	0.5
Candida lusitaniae ATCC42720	>128	>128	100	>100	0.125
Candida glabrata ATCC48435	>128	>128	>100	>100	0.5
Cryptococcus neoformans ATCC36556	>128	128	>100	>100	0.06
Aspergillus fumigatus ATCC16424	>128	>128	>100	>100	8
Trichophyton mentagrophytes ATCC9533	>128	>128	>100	100	4
Magnaporthe grisea	>128	>128	100	100	8
Botrytis cinerea	>128	>128	>100	>100	16
Colletotrichum lagenarium	>128	>128	>100	>100	2
Fusarium oxysporum	>128	>128	>100	>100	32
Pythium ultimum	128	>128	>100	>100	8
Phytophthora capsici	>128	>128	>100	>100	>128
Rhizoctonia solani	>128	>128	50	100	4

a) *C. albicans* A207: clinical isolate. *b*) *O*-MPA: *O*-methyl pisiferic acid, DHA: 8,20-dihydroxy-9(11),13-abietadien-12-one, PD: polyoxin D, NZ: nikkomycin Z, Amp B: amphotericin B.

ity on chitin synthase 2 exhibited weak inhibitory activities against *S. cerevisiae* ECY38-38A (pAS6) and *Cryptococcus neoformans* ATCC36556 (Table 2). Interestingly, the antifungal activity of 8,20-dihydroxy-9(11),13-abietadien-12-one against *S. cerevisiae* ECY38-38A (pAS6), which is used as source of chitin synthase 2, was stronger than that of *O*-methyl pisiferic acid regardless of 39-fold lower inhibitory activity against chitin synthase 2. Considering that *O*-methyl pisiferic acid acted as a mixed competitive inhibitor with respect to UDP-*N*-acetyl-D-glucosamine, the weak antifungal activities might be due to the difference in permeability for the fungal cell walls of various strains because chitin synthase 2 is a fungal membrane protein and the compound should penetrate into the cell wall to inhibit the chitin synthase of fungi. In addition, it is reported that *C. albicans* and other medically important fungi are resistant to nikkomycins owing to their transport across the cell membrane.[29,30] However, the detailed mode of action of the compound remains to be investigated.

On the other hand, *O*-methyl pisiferic acid and its derivatives including methyl pisiferate, pisiferic acid, and methyl *O*-methyl pisiferic acid were reported to have, antibacterial,[13,14] antifungal activities,[14] and cytoxicity against HeLa cell,[14] while 8,20-dihydroxy-9(11),13-abietadien-12-one showed cytotoxicity against human oral carcinoma KB cells.[28] In particular, pisiferic acid and methyl pisiferate inhibited strongly spore germinations of *Magnaporthe grisea* (formerly *Pyricularia oryzae*, a causative agent of rice blast fungus) with inhibitory activities of 85 and 90% at the concentration of 100 μg/ml, respectively, whereas *O*-methyl pisiferic acid showed no spore germination.[14] These results suggest very interesting facts that the antifungal activities between methyl pisiferic acid and *O*-methyl pisiferic acid are significantly different from each other in spite of the same molecular weight and molecular formula except the mutual replacement of hydroxyl and methyl groups in 12th and 20th carbon position.

Considering that *O*-methyl pisiferic acid has diverse biological activities such as antibacterial activity,[13,14] mite growth and development regulatory activities,[31] and cytotoxicity against human carcinoma cell,[14] other than differential inhibitory activity on chitin synthase 2 of *S. cerevisiae*, this compound may have multiple target sites. Although the mode of action of *O*-methyl pisiferic acid still remains to be investigated, this is the first report to describe an inhibitory activity on chitin synthase 2 from *S. cerevisiae*. The present study also suggest that it may serve as a useful lead compound for development of antifungal agents if further studies on structure–activity relationship of *O*-methyl pisiferic acid will be performed.

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