Sesquiterpene Furan Compound CJ-01, a Novel Chitin Synthase 2 Inhibitor from *Chloranthus japonicus* Sieb.

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Received October 1, 2007; accepted January 30, 2008; published online February 5, 2008

A novel sesquiterpene furan compound CJ-01 was isolated from the methanol extract of the whole plant of *Chloranthus japonicus* Sieb. by monitoring the inhibitory activity of chitin synthase 2 from *Saccharomyces cerevisiae*. Based on spectroscopic analysis, the structure of compound CJ-01 was determined as 3,4,8a-trimethyl-4a,7,8,8a-tetrahydro-4a-naphto[2,3-b]furan-9-one. The compound inhibited chitin synthase 2 of *Saccharomyces cerevisiae* in a dose-dependent manner with an IC_{50} of 39.6 μg/ml, whereas it exhibited no inhibitory activities against chitin synthase 1 and 3 of *S. cerevisiae* up to 280 μg/ml. CJ-01 has 1.7-fold stronger inhibitory activity than polyoxin D (IC_{50}=70 μg/ml), a well-known chitin synthase inhibitor. These results indicate that the compound is a specific inhibitor of chitin synthase 2 from *S. cerevisiae*. In addition, CJ-01 showed antifungal activities against various human and phytopathogenic fungi. Therefore, the compound might be an interesting lead to develop effective antifungal agents.

Key words fungal cell wall; chitin synthase 2 inhibitor; *Chloranthus japonicus*; sesquiterpene furan compound CJ-01; antifungal activity

Fungal cell walls have essential roles in the life of the fungal cell. They protect mechanically the cell from the environment, prevent osmotic bursting of the cell by the tugor pressure and act as a sieve for large molecules that might harm the cell membrane.[1)]

Chitin, along with (1,3)-β-D-glucan, represents the main structural components of the fungal cell wall. As a crystalline polymer composed of β-1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc), chitin contributes to the rigidity of the cell wall.[2)] It is synthesized on the cytoplasmic surface of the plasma membrane, extruded perpendicularly to the cell surface as microfibrils and crystallized outside the cell through extensive hydrogen bonding as α-chitin (the polyGlcNAc chains run antiparallel).[3)] Chitin is synthesized by chitin synthase 1 (ScCHS1p), 2 (ScCHS2p), and 3 (ScCHS3p) in *Saccharomyces cerevisiae*.[4,5)] Among them, chitin synthase 1 is a nonessential repair enzyme,[5)] whereas chitin synthase 2 and 3 are essential enzymes in primary septum formation[6)] and bud ring formation,[7)] respectively. Although the content of chitin in the fungal cell wall varies from species to species,[8)] its synthesis constitutes a model for fungal morphogenesis. Thus, specific inhibitors of chitin synthase 2 and 3 might serve as interesting lead compounds for the development of effective antifungal agents.

The *Chloranthus japonicus* (Chloranthaceae) is a perennial herb that grows in the southern part of Korea, Japan, and China. This plant has been long recognized to be useful for boils, dermatopathy, and enteric fever in Korea as a folk remedy.[9)] Recently, shizukaol B, cycloshizukaol A, and shizukaol F isolated from the roots of *Chloranthus japonicus* were reported as cell adhesion inhibitors.[10)] In addition, many plants of the genus *Chloranthus* have been used in Chinese folk medicine for the treatment of bone fractures.[11)] A number of sesquiterpenoids[12,13)] and sesquiterpenoid oligomers[14,15)] have been isolated from the genus *Chloranthus*, and a quite recent study showed that chloraholides A and B isolated from *Chloranthus holostegius* were potent and selective blockers of potassium channel.[16)]

In the course of search for chitin synthase 2 inhibitors from plant extracts, a novel type of chitin synthase 2 inhibitor was found in the methanol extract of the whole bodies of *C. japonicus* Sieb. Although the extract of *C. japonicus* has been long recognized to be useful as a folk remedy in Korea,[9)] this is the first report that the extract of *C. japonicus* could be used as antifungal agents by the inhibition of chitin synthase 2. Here, we describe the isolation and structure elucidation of a novel sesquiterpene furan compound CJ-01, and its inhibitory activities against chitin synthase 2 of *S. cerevisiae* and various human and phytopathogenic 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-*Δ*63 his-*Δ*200 leu2-1*),[17)] ECY38-38A (pAS6) (*MATa chs1-23 chs2*::*LEU2 cal1*/*csd2 ura3-52 trp1-1 leu2-2* pAS6),[18)] and ECY38-38A (pWJC6) (*MATa chs1-23 chs2*::*LEU2 cal1*/*csd2 ura3-52 trp1-1 leu2-2* pWJC6),[18)] respectively. *S. cerevisiae* YPH499, the wild type strain expressing 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 [1% yeast extract, 2% Bacto peptone, 2% galactose] at 30 °C.

Chemicals Uridine diphosphate (UDP)-[U-^{14}C]-*N*-acetyl-D-glucosamine (400,000 cpm/μmol) was purchased from NEN Life Science Products (Boston, U.S.A.). Trypsin, trypsin inhibitor, and *N*-acetyl-D-glucosamine (GlcNAc) were purchased from Sigma Chemical CO. (St. Louis, U.S.A.). Polyoxin D and nikkomycin Z were purchased from Calbiochem (San Diego, U.S.A.). Sabouraud agar and potato dextrose

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agar media were purchased from Difco (Sparks, U.S.A.). All other chemicals were of the highest grade available and used without further purification.

The UV and EI mass spectra were recorded on a Shimadzu UV265 UV–Visible spectrophotometer and Hewlett Packard 5989A, respectively. NMR spectra were obtained using a Bruker model AMX-500 spectrometer from samples dissolved in CD_3OD. Chemical shifts are given in ppm using tetramethylsilane (TMS) as internal standard.

Plant Materials The aerial parts of *Chloranthus japonicus* Sieb. were collected at Mt. Odae of Gangwon Province, Korea. A voucher specimen has been deposited under No. KRIBB-101 in Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea.

Isolation of Sesquiterpene Furan Compound CJ-01 The aerial parts of *C. japonicus* (750 g) were extracted twice with methanol (20 l) at room temperature for 7 d, filtrated, and concentrated *in vacuo* to yield a dark brown sticky residue (85 g). The residue was suspended in water and partitioned with *n*-hexane. The *n*-hexane layer (17.9 g) was subjected to silica gel column chromatography (Merck, Kieselgel 60, 230—400 mesh, 7.5×35 cm) and eluted stepwise with a gradient of *n*-hexane/ethyl acetate (*n*-hexane only, 9 : 1, 8 : 2, 7 : 3, 6 : 4, 5 : 5 and ethyl acetate only, v/v, each 2 l). The active fractions (8 : 2, v/v, 2.5 g) were subjected to ODS column chromatography (Merck, Lichroprep RP-18, 40—63 μm, 3.5×25 cm) and eluted stepwise with a gradient of water/methanol (5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9 and methanol only, v/v, each 500 ml). Fractions with activity (3 : 7 to 2 : 8, v/v, 380 mg) were then subjected to Sephadex LH-20 column chromatography (Amersham Bioscience, Sweden) with methanol (1.5×120 cm, 0.4 ml/min, each 8 ml). The active fractions (fraction 4 to 7, 42 mg) obtained were subjected to HPLC using an C_8 column (Agilent, Eclipse XDB-C_8, 4.6×150 mm) and the column was eluted with a mixture of water–methanol, 55 : 45 (v/v) at a flow rate of 1 ml/min. Through the HPLC, a single peak with the retention time of 22.3 min was detected by a UV spectrometric detector at 254 nm, which was concentrated *in vacuo* to give a yellow oil of pure compound CJ-01 (5 mg).

Preparation of Crude Membranes Membranes of *S. cerevisiae* YPH499, ECY38-38A (pAS6), and ECY38-38A (pWJC6) were prepared as described previously.[19] The recombinant strains 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.[18] The protein concentration in crude membrane was measured by the method of Lowry *et al.*[20] and used in chitin synthases assays after adjusting the concentration of protein.

Chitin Synthases Assays Chitin is biosynthesized by the serial reaction involving protease activation of the chitin synthase and uridine diphosphate (UDP) *N*-acetyl-D-glucosamine as a substrate. The bioassays for chitin synthases were performed by the measurement of amount of radiolabeled insoluble chitin formed to examine whether the biosynthesis of chitin by activated chitin synthase and UDP-[U-^{14}C]-*N*-acetyl-D-glucosamine is occurred or not.

The assays of chitin synthase 2 and 3 from recombinant *S. cerevisiae* ECY38-38A (pAS6) and ECY38-38A (pWJC6), which can only overexpress chitin synthase 2 and 3, respectively, were conducted by the methods as described previously.[18,21] For the proteolytic activation of chitin synthase 2,[18,21] 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 (400,000 cpm/mmol 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 twice that 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 the mixture was incubated at 30 °C for 90 min. For chitin synthase 3 activity,[18,21] the assay was carried out as for chitin synthase 2, except that 32 mM Tris–HCl (pH 7.5) and 4.3 mM magnesium acetate were used. The assay of chitin synthase 1 in the presence of other chitin synthase 2 and 3 from wild type *S. cerevisiae* YPH499 was performed by adding Mg^{2+} ion and 0.1% digitonin in assay mixture.[18,21] For chitin synthase 1 activity,[18,21] 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 sample in a total volume 41 μl. After 15 min of incubation at 30 °C, 2 μl of trypsin inhibitor (2.0 mg/ml) were 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 all cases, the reaction was stopped by the addition of 10% trichloroacetic acid (TCA), and radioactivity of the insoluble chitin formed was counted after filtration through a glass fiber filter (GF/C, Whatman). Blank values were measured with addition of 25% aqueous MeOH instead of both enzyme and sample. Percentage inhibition of chitin synthase activity was calculated by subtracting the blank values from both control and sample values.

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

The chitin synthases activities were confirmed by positive control with polyoxin D and nikkomycin Z 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 Minimal Inhibitory Concentrations (MICs) Human pathogenic fungi including *Aspergillus fumigatus*, *Candida albicans*, *C. krusei*, *C. lusitaniae*, *C. tropicalis*, and *Cryptococcus neoformans* were purchased from American Type Culture Collection (ATCC, Rockville, U.S.A.) except *C. albicans* A207 (clinical isolate) and used for antifungal assays. In addition, antifungal activities against

phytopathogenic fungi were performed by using various fungi such as *Alternaria kikuchiana* (pear black spot), *Magnaporthe grisea* (rice blast), *Botrytis cinerea* (cucumber gray mold), *Colletotrichum lagenarium* (watermelon anthracnose), *Fusarium oxysporum* (cucumber fusarium wilt), *Pythium ultimum* (rice damping-off), and *Rhizoctonia solani* (rice sheath blight). All of the phytopathogenic fungi used are field isolates and used by providing from Korea Research Institute of Chemical Technology (Daejeon, Korea) and Rural Development Administration (Suwon, Korea).

MICs were determined by a two-fold serial broth microdilution method for yeasts using Sabouraud dextrose broth and agar dilution method using potato dextrose agar medium for spore-forming fungi.[22] Isolated compound CJ-01 was dissolved in 25% dimethyl sulfoxide (DMSO), 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 30 °C for yeasts and 48 h incubation for fungi at 25 °C. The MIC was defined as the lowest concentration of compounds which completely inhibited the growth of the organism when compared to a control well containing no compounds.

RESULTS AND DISCUSSION

A novel sesquiterpene furan compound CJ-01 from the whole bodies of *C. japonicus* was purified by solvent fractionation, silica gel and Sephadex LH-20 column chromatographies, and HPLC as a yellow oil. Purification of *C. japonicus* was carried out by monitoring chitin synthase 2 inhibitory activity. The structure of isolated compound was determined by EI-MS and various NMR spectroscopic analyses including ^{1}H, ^{13}C, distortionless enhancement by polarization transfer (DEPT), ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC).

The ^{1}H-NMR spectrum of the compound CJ-01 showed one methine (7.55 ppm) from furan ring, one methine (5.47 ppm) from olefin, and three methyl proton peaks at 1.05, 1.78 and 2.04 ppm (Table 1). The ^{13}C-NMR spectrum displayed fifteen carbon signals (Table 1), which were assigned as three methyl, three methylene, three methine, and six quarternary carbons including a ketone (δ=194.3) with three carbon–carbon double bonds (δ=122.6, 123.1, 134.0, 140.4, 146.7, 147.5) by DEPT spectra. The ^{1}H–^{1}H COSY spectrum showed connectivities of C-1 to C-2, C-2 to C-3, C-5 to C-6 and an allylic coupling peak between C-12 and C-13 (Fig. 1). Furthermore, additional coupling with H-3, H-4, H-5 and H-15 gave clear indications of signals for vicinal or allylic group. The HMBC spectrum clearly demonstrated two sets of correlations from the methylene proton (H-6) to six carbons (C-4, C-5, C-7, C-8, C-10, C-11), and from methine proton (H-12) to three carbons (C-7, C-8, C-11). It indicated a –CH_2–CH_2–CH=C(–CH_3)–CH–CH_2– attached to the furan ring. In addition, correlations between the methyl protons (H-13, H-14, H-15) and carbons completed the structure of the compound CJ-01 as shown in Fig. 1. The regiostereochemistry of the compound CJ-01 was determined to be *trans* by NOESY experiment (Fig. 1). The methyl proton (H-14) exhibited a significant NOE correlation with H-6a but no correlation with H-5. Structural analyses with EI-MS spectrum (*m/z* 230, $[M]^+$) and various NMR techniques including ^{1}H–^{1}H COSY, HMQC, and HMBC revealed that the isolated compound had molecular formula $C_{15}H_{18}O_2$, and it was identified as a novel sesquiterpene furan, 3,4,8a-trimethyl-4a,7,8,8a-tetrahydro-4a-naphto[2,3-b]furan-9-one (Fig. 1). This compound showed a *Rf* value of 0.5 in methanol–water (8 : 2, v/v) on a ODS plate (Merck, Lichroprep RP-18, 40—63 μm). The UV spectrum of the compound in methanol exhibited the absorption maxima at 279 nm.

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of a Novel Sesquiterpene Furan Compound CJ-01

No.	^{1}H-NMR chemical shifts (ppm)	^{13}C-NMR chemical shifts (ppm)
1	1.48 (Ha, 1H, d, J=4.5 Hz) 2.13 (Hb, 1H, m)	29.1
2	2.13 (2H, m)	23.2
3	5.47 (1H, br s)	123.1
4		134.0
5	2.78 (1H, m)	46.7
6	2.49 (Ha, 1H, dd, J=17.1, 12.0 Hz) 2.95 (Hb, 1H, dd, J=17.1, 4.5 Hz)	22.9
7		140.4
8		147.5
9		194.3
10		45.8
11		122.6
12	7.55 (1H, s)	146.7
13	2.04 (3H, s)	7.6
14	1.05 (3H, s)	15.5
15	1.78 (3H, br s)	21.1

Measured in CD_3OD. m; multiplets, s: singlet, dd; doublet of doublets.

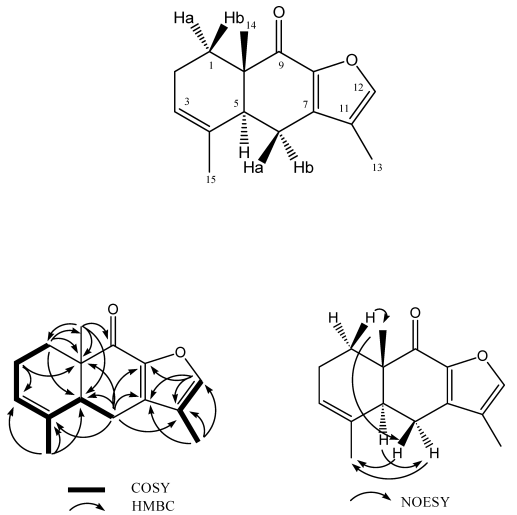

Fig. 1. ^{1}H–^{1}H COSY, HMBC (Left), and NOESY (Right) Connectivity for a Novel Sesquiterpene Furan Compound CJ-01

The compound CJ-01 inhibited the chitin synthase 2 of *S. cerevisiae* in a dose-dependent manner with an IC_{50} of 39.6 μg/ml (Table 2). The compound has 1.7-fold stronger inhibitory activity than polyoxin D (IC_{50}=70 μg/ml), a well-known chitin synthase inhibitor. However, this compound did not exhibit inhibitory activities against chitin synthase 1 and

Table 2. Effects of a Novel Compound CJ-01 on Chitin Synthase Isozymes in Our Assay System

Isozymes[a]	Strains	Compounds (IC_{50}, μg/ml)		
		CJ-01	Polyoxin D	Nikkomycin Z
ScCHS1p	YPH449	—[b]	3.7	0.6
ScCHS2p	ECY38-38A (pAS6)	39.6	70.0	175.6
ScCHS3p	ECY38-38A (pWJC6)	—[b]	3.1	1.0

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 at concentration up to 280 μg/ml.

Table 3. *In Vitro* Antifungal Activities of CJ-01 against Various Phytopathogenic Fungi

Fungi	Compounds (MICs, μg/ml)		
	CJ-01	Polyoxin D	Nikkomycin Z
C. albicans ATCC10231	50	>100	>100
C. albicans A207[a]	100	>100	>100
C. krusei ATCC6258	>100	>100	>100
C. lusitaniae ATCC42720	>100	100	>100
C. tropicalis ATCC13803	>100	>100	>100
C. neoformans ATCC36556	100	>100	>100
A. fumigatus ATCC16424	>100	>100	>100
A. kikuchiana[b]	100	>100	>100
M. grisea[b]	100	100	>100
B. cinerea[b]	50	>100	>100
C. lagenarium[b]	100	>100	>100
F. oxysporum[b]	100	>100	>100
P. ultimum[b]	50	>100	>100
R. solani[b]	50	50	100

a) *C. albicans* A207: clinical isolate, *b*) *A. kikuchiana*, *M. grisea*, *B. cinerea*, *C. lagenarium*, *F. oxysporum*, *P. ultimum*, and *R. solani*: field isolates. The experiment was repeated three times and MICs are shown as average values of three independent determinations.

3 of *S. cerevisiae* up to 280 μg/ml. These results indicate that a novel compound CJ-01 is a specific inhibitor for chitin synthase 2 of *S. cerevisiae*. It also exhibited antifungal activities against various human and phytopathogenic fungi such as *C. albicans*, *Cryptococcus neoformans*, *Alternaria kikuchiana*, *Colletotrichum lagenarium*, *Fusarium oxysporum*, *Magnaporthe grisea*, *Botrytis cineria*, *Pythium ultimum*, and *Rhizoctonia solani* with MIC values of 50—100 μg/ml (Table 3). Among them, *P. ultimum* is the Oomycetes fungus, which had long been classified as one of the few fungi with cellulose walls, absent in chitin.[8] Later, both chitin and cellulose were found to be present in the cell walls of *P. ultimum*.[23] Our results suggest that the antifungal activities of the compound CJ-01 against various fungi might be caused in part by inhibitory activities for chitin synthase 2 because the growth of most of fungi were inhibited by the compound CJ-01 at the concentration of 50—100 μg/ml. However, we could not rule out the possibility that the antifungal activities of the compound are either due to the difference of permeability for the fungal cell walls of various strains because the compound should penetrated the cell wall to inhibit the chitin synthase of fungi,[24] or due to the inhibition on other cellular targets. The detailed mode of action of the compound remains to be investigated in future studies.

Although various sesquiterpenoids[12,13] and sesquiterpenoid oligomers[14,15] isolated from *C. japonicus* have biological activities such as cell adhesion inhibition[10] and growth inhibition against *Mucor* and *Rhizopus*,[25] this is the first report that a novel sesquiterpene furan compound CJ-01 specifically inhibited the chitin synthase 2 of *S. cerevisiae*. Therefore, the compound CJ-01 may be a novel lead compound for the development of antifungal agent through the control of chitin synthesis.

Acknowledgments We thank Dr. Enrico Cabib (NIH, Bethesda, MD, U.S.A.) and Dr. Hee Moon Park (Chungnam National University, Daejeon, Korea) for providing the recombinant *S. cerevisiae* ECY38-38A and *S. cerevisiae* YPH499, respectively. This work was supported by a grant from the Plant Diversity Research Center of 21st Century Frontier Research program funded by the Ministry of Science and Technology of the Korean government.

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