Inhibition of Chitin Synthases and Antifungal Activities by 2′-Benzoyloxycinnamaldehyde from *Pleuropterus ciliinervis* and Its Derivatives

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In the course of search for potent chitin synthase inhibitors from natural resources, a novel chitin synthases inhibitor, 2′-benzoyloxycinnamaldehyde (2′-BCA) (I), was isolated from the aerial parts of *Pleuropterus ciliinervis* NAKAI. 2′-BCA inhibited chitin synthase 1 and 2 of *Saccharomyces cerevisiae* with the IC_{50}s of 54.9 and 70.8 μg/ml, respectively, whereas it exhibited no inhibitory activity for chitin synthase 3 up to 280 μg/ml. Its derivatives, 2′-chloro- (V) and 2(-bromo-cinnamaldehyde (VI), each showed 1.9 and 2.7-fold stronger inhibitory activities than 2′-BCA, with the IC_{50}s of 37.2 and 26.6 μg/ml, respectively. Especially, the IC_{50} of compound VI against chitin synthase 2 represented 1.7-fold more potent inhibitory activity than polyoxin D, a well-known chitin synthase inhibitor. Furthermore, compounds V and VI showed potent antifungal activities against various fungi including human pathogenic fungi, with a particularly strong inhibitory activity against *Cryptococcus neoformans* (MIC=16 μg/ml). Although the chemical synthesis of this compound has been reported, the present study is the first report to describe the isolation of 2′-BCA from natural resources and chitin synthases inhibitory activities of its derivatives. These results suggested that 2′-BCA and its derivatives can potentially serve as useful lead compounds for development of antifungal agents.

Key words *Pleuropterus ciliinervis*; 2′-benzoyloxycinnamaldehyde; chitin synthase inhibitor; antifungal activity

Fungal cells, like their bacterial and plant counterparts but unlike animal cells, are encased in the cell wall, which is essential for their survival.[1)] The fungal cell wall acts as an exoskeleton that provides the cell with resistance to tugor pressure, while maintaining their shape. As the most external structure of fungal cells, it is in direct contact with the external medium, including the physiological defence mechanisms of the host.[2)] To ensure the continuous integrity of the cell wall without interfering with its plasticity and constant growth, elaborate control mechanisms must be operating, which need to be strictly coordinated with those governing the cell cycle.[1)] Accordingly, the biosynthesis of fungal cell walls constitutes a good model for morphogenesis at the molecular level, and serve as a potential target in antifungal chemotherapy because it is unique to the pathogen.[3)]

Chitin, the β-(1,4)-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), is one of the most important structural component of the cell walls of nearly all fungi and plays a major role in the determination of cell morphology.[4)] Although the content of chitin in the cell wall varied among species, it is important for cell integrity.[5)] Chitin is absent from plant and mammalian species, while it is abundant in arthropods, most fungi, and other eukaryotes.[6)] Mutations that affect chitin synthesis cause osmotic sensitivity,[7)] abnormal morphology, aggregation, and growth arrest with elongated buds.[8,9)] Thus, its biosynthesis has become a more and more attractive target for the design of antifungal agents.

Chitin is synthesized by chitin synthase 1 (ScCHS1p), 2 (ScCHS2p) and 3 (ScCHS3p) in *Saccharomyces cerevisiae*,[9,10)] for which the most comprehensive work including biochemical and genetic studies on fungal cell walls has been carried out. Chitin synthase 2 is an essential enzyme for primary septum formation and cell division,[11)] whereas chitin synthase 1 is a nonessential repair enzyme of damaged chitin.[10)] Chitin synthase 3, on the other hand, is responsible for the synthesis of chitin during bud emergence and growth, mating, and spore formation.[12)] As in *S. cerevisiae, Candida albicans* harbours three chitin synthases 1 (CaCHS1p), 2 (CaCHS2p) and 3 (CaCHS3p), which are analogous to the *S. cerevisiae* chitin synthases 2, 1 and 3, respectively.[13)] Therefore, specific inhibitors of chitin synthase 2 and 3 from *S. cerevisiae* might be interesting leads to develop effective antifungal agents.

The root of *Pleuropterus ciliinervis* NAKAI (Polygonaceae) has been used in a traditional Chinese folk medicine, "Hasuo", which is used to treat inflammation, bacterial infections, suppurative dermatititis and gonorrhea in China as well as in Korea.[14,15)] Stilbenes, anthraquinones, and flavoids have been isolated from the genus *Pleuropterus*.[16)] Recently, a new naphthopyrone, pleuropyrone A with antioxidant activity was reported from *Pleuropterus ciliinervis*.[17)]

In the course of our continuing search for potent inhibitors of chitin synthase 2 from the higher plants, a strong inhibitory compound against chitin synthase 2 of *S. cerevisiae* was found in the methanol extract of *Pleuropterus ciliinervis*, which was identified as 2′-benzoyloxycinnamaldehyde (2′-BCA). Although this compound, which is synthesized by chemical synthesis, has been reported to demonstrate antitumor activity,[18,19)] here, we describe the isolation of 2′-BCA from the aerial parts of *Pleuropterus ciliinervis* and the synthesis of its derivatives, and their inhibitory activities against chitin synthases of *S. cerevisiae* and various fungi including several human pathogenic fungi.

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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-Δ1*),[20] ECY38-38A (pAS6) (*MAT**a** chs1-23 chs2::LEU2 cal1/cds2 ura3-52 trp1-1 leu2-2* pAS6) and ECY38-38A (pWJC6) (*MAT**a** chs1-23 chs2::LEU2 cal1/cds2 ura3-52 trp1-1 leu2-2* pWJC6),[21] which were used as sources of ScCHS1p, 2p and 3p activity, 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 [1% yeast extract, 2% Bacto peptone, 2% galactose] at 30 °C. The strain was grown in Sabouraud dextrose medium at 30 °C.

Chemicals Cinnamaldehyde (compound **II**) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, U.S.A.). Uridine diphosphate (UDP)-[U-^{14}C]-GlcNAc (400000 cpm/μmol) was purchased from NEN Life Science Products (Boston, U.S.A.). All other reagents were of the highest grade available and used without further purification.

Isolation of 2′-Benzoyloxycinnamaldehyde (I) The aerial part of *P. ciliinervis* Nakai was collected at Mt. Deokyu, Muju, Korea. A voucher specimen has been deposited under No. CFM-580 in the Korea Research Institute of Bioscience and Biotechnology. The aerial parts of *P. ciliinervis* (2 kg) was 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 (83 g). After solvent extraction with ethyl acetate, the ethyl acetate layer (53 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 (5 : 5 to ethyl acetate only, v/v, each 6 l). The active fractions (5 : 5, v/v, 400 mg) were subjected to Sephadex LH-20 column chromatography (Amersham Bioscience, Sweden) with methanol (1.5×120 cm, 0.4 ml/min, each 8 ml). Crystallization was induced at active fractions (fraction 14—23). Crude crystal was resolved and finally purified by re-crystallization in methanol, after which pure compound (100 mg) was collected. Through the HPLC using an ODS column (Waters, Xterra C_{18}, 5 μm, 250×4.6 mm), a single peak with the retention time of 10.2 min was detected by a UV spectrometric detector (254 nm). The column was then eluted with CH_3CN/water (50 : 50 v/v) at a flow rate of 1.0 ml/min.

Syntheses of 2′-BCA Derivatives 2′-BCA derivatives (compound **III**—**VI**) were synthesized using the standard literature methods. For the synthesis of 2′-substituted cinnamaldehydes (**III**—**VI**), the commercially available 2′-substituted cinnamic acids were converted to 2′-substituted methyl cinnamates by methylation with diazomethane, which was prepared from Diazal. The methyl esters were reduced by DIBAL-H (diisobutyl aluminum hydride) in tetrahydrofuran (THF) at −40 °C to give 2′-substituted cinnamyl alcohols. Finally, the treatment of cinnamyl alcohols with MnO_2 gave 2′-cinnamaldehydes (**III**—**VI**).[22]

Preparation of Crude Membranes Membranes of *S. cerevisiae* YPH499, ECY38-38A (pAS6) and ECY38-38A (pWJC6) were prepared as described previously.[23] The *S. cerevisiae* YPH499 and recombinant ECY38-38A 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.

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.[24] Chitin synthase 2 activity was measured by the procedure described previously.[24] 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 ml 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,[21,24] 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,[24] 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. For the chitin synthase 2 and 3 assays, 32 mM *N*-acetyl-D-glucosamine and 1.0 mM UDP-[^{14}C]-*N*-acetyl-D-glucosamine were added, and the mixtures were incubated for 30 min at 30 °C. In all cases, the reaction was stopped by 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.[25] 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 substracting 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 activity 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 isolated and control compounds were 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. The preparation of microsome and chitin synthase 1 activity of *C. albicans* were conducted by the method described previously.[26] For CaCHS1p activity, the assay was performed the same as that for ScCHS2p except that 32 mM Tris–HCl (pH 7.5) and 2 mM cobalt chloride were used. Nikkomycin Z was used as a positive control for CaCHS1p inhibition. Each isolated and control compounds were solubilized in 25% dimethyl sulfoxide (DMSO) and distilled water to make a stock solution (1 mg/ml), respectively.

Determination of Minimum Inhibitory Concentrations (MICs) MICs were determined by a two-fold serial broth microdilution method using Sabouraud dextrose broth.[27] 2′-BCA and its derivatives were dissolved in 25% MeOH, while polyoxin D and nikkomycin Z were dissolved in distilled water. The inoculum size of yeasts was 10^3 colony forming unit (CFU)/ml. Antifungal activity was observed after 24 to 48 h incubation at 30 °C. The MIC was defined as the lowest concentration of antibiotics which completely inhibited the growth of the organism when compared to a control well containing no antibiotics.

RESULTS AND DISCUSSION

The bioactive compound from the aerial part of *P. ciliinervis* was purified by solvent partition, silica gel and Sephadex LH-20 column chromatographies, crystallization, and HPLC. The structure of isolated compound was determined by EI-MS and various NMR spectroscopic analyses including ^{1}H, ^{13}C, DEPT, ^{1}H–^{1}H COSY, HMQC and HMBC.

The ^{1}H-NMR (500 MHz, CD_3OD) of compound **I** (Table 1) revealed an aldehyde proton appearing as doublets at δ 9.55 (J=8 Hz, H-1) and several aromatic protons (9H, δ 6.78—8.23). The multiple signals at δ 8.23 (2H), 7.72 (1H) and 7.58 (2H) were attributed to benzoyl hydrogens. Signals at δ 151.17, 131.46, 129.38, 128.56, 127.82 and 124.66 in the ^{13}C-NMR (125 MHz, CD_3OD) data and those at δ 133.38 and 147.40, together with correlation indicated by the HMBC spectrum between H-2 and H-3 with the carbonyl group at 195.82, were typical of a 2′-hydroxylcinnamaldehyde (Fig. 1). The signals at δ 166.25, 135.32, 131.23 and 130.05 were assigned to the benzoyl carbons from C-1″ to C-7″, which are connected to 2′-hydroxyl. The presence of a benzoyl moiety in compound **I** was confirmed from peaks at *m*/*z* 105 and 77 in the EIMS. Structure analyses with EI-MS spectrum (*m*/*z* 252, $[M]^+$) and various NMR techniques including ^{1}H–^{1}H COSY, HMQC, and HMBC revealed that the isolated compound had molecular formular $C_{16}H_{12}O_3$ and was identified as 3-(2′-benzoyloxy phenyl)-propenal (2′-benzoyloxycinnamaldehyde). The melting point of the compound was 78—80 °C.

Although several chitin synthase inhibitors have been isolated from microbes and higher plants,[28—32] 2′-BCA has not yet been reported as chitin synthase inhibitor. The inhibitory activities of this compound on chitin synthase isozymes were investigated by the filter binding assay using UDP-^{14}C-GlcNAC as substrate. As shown in Table 2, 2′-BCA inhibited the chitin synthase 1 and 2 of *S. cerevisiae* in a dose-dependent manner with IC_{50} values of 54.9 and 70.8 μg/ml, respectively, but it showed no inhibitory activity on chitin synthase 3 up to 280 μg/ml. The IC_{50} values of 2′-BCA for chitin synthase 1 and 2 of *S. cerevisiae* represented less inhibitory activities than polyoxin D, whereas the inhibitory activity of the compound against chitin synthase 2 showed 2.5-fold stronger inhibitory activity than nikkomycin Z. In addition, this compound showed more potent antifungal activities against several fungi such as *S. cerevisiae*, *C. neoformans*, *C. lusitaniae*, and *C. krusei* (MICs=16—64 μg/ml) than polyoxin D and nikkomycin Z, well-known chitin synthase inhibitors. Although polyoxin D and nikkomycin Z exhibited potent inhibitory activities against chitin synthases in assays performed *in vitro*, their antifungal activity would depend on the permeability, because the compounds should penetrate into the fungal cell wall to exhibit antifungal activities through the inhibition of chitin synthases, which are located on the cell membrane.[33,34]

To determine whether structural changes of the benzoyl group could enhance inhibitory activities, we synthesized cinnamaldehyde derivatives such as 2′-hydroxy- (**III**) and 2′-methoxy- (**IV**) cinnamaldehyde, based on the previously reported method.[35] In addition, according to the report that the incorporation of halogen atoms into a lead compound results in analogues that are more lipophilic, we have synthesized

Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of 2′-BCA

Carbon No.	^{1}H-NMR chemical shifts (ppm)	^{13}C-NMR chemical shifts (ppm)
1	9.55 (1H, d, J=8 Hz)	195.82
2	6.78 (1H, q)	133.38
3	7.71 (1H, m)	147.40
1′		128.56
2′		151.17
3′	7.30 (1H, dd, J=1 Hz)	124.66
4′	7.55 (1H, m)	129.38
5′	7.38 (1H, m)	127.82
6′	7.85 (1H, dd, J=2 Hz)	131.46
1″		166.25
2″		130.10
3″	8.23 (1H, m)	131.23
4″	7.58 (1H, m)	130.05
5″	7.72 (1H, m)	135.32
6″	7.58 (1H, m)	130.05
7″	8.23 (1H, m)	131.23

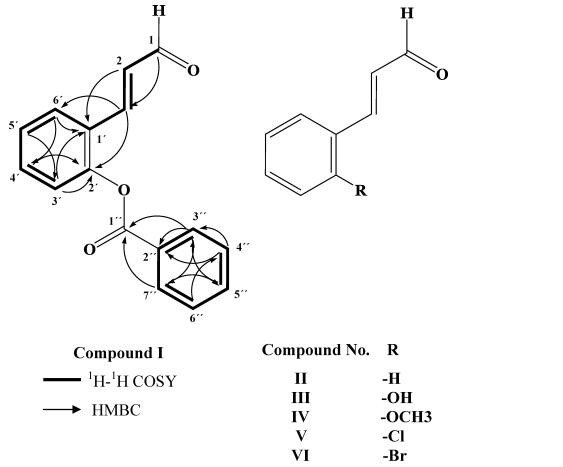

Fig. 1. ^{1}H–^{1}H COSY and HMBC Connectivity for 2′-Benzoyloxycinnamaldehyde and Its Derivatives

the derivatives with various halogen atoms such as 2′-chloro- (**V**) and 2′-bromo- (**VI**) cinnamaldehyde. The inhibitory activities of compounds **II** (cinnamaldehyde), **III** and **IV** against chitin synthases showed weaker activities than 2′-BCA (**I**), whereas compounds **V** and **VI** exhibited 1.9 and 2.7-fold stronger inhibitory activities than 2′-BCA, respectively (Table 2). Especially, the IC_{50} of compound **VI** against chitin synthase 2 represented 1.7-fold more potent inhibitory activity than polyoxin D. In addition, compound **V** and **VI** exhibited 2 to 4 fold stronger antifungal activities, depending on the species of fungi, than 2′-BCA (Table 3). These results suggest that the significant structural changes leading to increase inhibitory activities may be due to the introduction of halogen atoms at 2′-hydroxyl position in place of a benzoyl group. Interestingly, compounds **V** and **VI** showed the same antifungal activities as 2′-BCA against *S. cerevisiae* in spite of differential inhibitory activities on chitin synthase 2. Besides, 2′-BCA, compound **V** and **VI** exhibited potent antifungal activities against human pathogenic fungi including *Cryptococcus* and C*andida* species. The reason why these halogen compounds showed the increased antifungal activities might be their improved ability to penetrate the lipid membrane in fungal cell. Based on these results, the antifungal activities of 2′-BCA and its derivatives might be caused at least in part by potent inhibitory activities for chitin synthase 2 in *S. cerevisiae*. Besides we could not rule out the possibility that the antifungal activities of these compounds are due to the difference in permeability for the fungal cell wall of various strains or due to the inhibition on other cellular targets. However, the detailed mode of action of the compounds remains to be investigated.

Table 2. Effect of 2′-BCA and Its Derivatives on Chitin Synthase Isozymes

Isozymes[a]	Compounds (IC_{50})[b]							
	I	**II**	**III**	**IV**	**V**	**VI**	PD[c]	NZ[c]
ScCHS1p	54.9	223.1	124.3	160.5	78.6	181.9	3.7	0.6
ScCHS2p	70.8	>280	84.1	89.9	37.2	26.6	46.2	175.6
ScCHS3p	>280	>280	>280	>280	>280	>280	3.1	1.0

a) ScCHS1p was prepared from wild type *S. cerevisiae* YPH499. ScCHS2p: *S. cerevisiae* ECY38-38A (pAS6) is a high-copy-number plasmid carrying *CHS2* on a vector containing a *TRP* marker. ScCHS3p: *S. cerevisiae* ECY38-38A (pWJC6) is a high-copy-number plasmid carrying *CAL*/*CSD2* (complete gene) under the control of the *GAL1* promoter. *b*) Unit: μg/ml. *c*) PD: polyoxin D, NZ: nikkomycin Z.

Considering that chemically synthesized 2′-BCA and its derivatives have biological activity such as cytotoxicity against human tumor cells,[18,19,33] other than differential inhibitory activities on chitin synthase 1 and 2 of *S. cerevisiae*, this compound and its derivatives may have multiple target sites. Although the mode of action of 2′-BCA still remains to be investigated in the future, this is the first report to describe the isolation of 2′-BCA from natural resources and its chitin synthases inhibitory activities. This study also suggests that 2′-BCA and its derivatives may serve as useful lead compounds for development of antifungal agents.

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Table 3. *In Vitro* Antifungal Activities of 2′-BCA and Its Derivatives against Various Fungi

Fungi	Compounds (MICs)[b]							
	I	**II**	**III**	**IV**	**V**	**VI**	PD[c]	NZ[c]
S. cerevisiae YPH499	16	32	64	32	16	16	>100	>100
S. cerevisiae ECY38-38A (pAS6)	32	64	128	64	32	32	>100	>100
S. cerevisiae ECY38-38A (pWJC6)	32	64	128	64	32	32	>100	>100
C. albicans ATCC10231	>128	128	>128	128	32	32	>100	>100
C. albicans A207[a]	>128	>128	>128	128	32	32	>100	>100
C. krusei ATCC6258	64	128	>128	128	32	32	>100	>100
C. lusitaniae ATCC42720	64	128	128	128	32	64	100	>100
C. tropicalis ATCC13803	>128	>128	>128	128	64	64	>100	>100
C. neoformans ATCC36556	32	128	>128	64	16	16	>100	>100

The experiment was repeated three times and MICs were shown as average values of three independent determinations. *a*) *C. albicans* A207: clinical isolate. *b*) Unit: μg/ml. *c*) PD: polyoxin D, NZ: nikkomycin Z.

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