Inhibitory Activity for Chitin Synthase II from *Saccharomyces cerevisiae* by Tannins and Related Compounds

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Abstract: **In the course of search for potent inhibitors of chitin synthase II from natural resources, seven tannins and related compounds were isolated from the aerial part of *Euphorbia pekinensis* and identified as gallic acid (1), methyl gallate (2), 3-*O*-galloyl-(–)-shikimic acid (3), corilagin (4), geraniin (5), quercetin-3-*O*-(2″-*O*-galloyl)-β-D-glucoside (6), and kaempferol-3-*O*-(2″-*O*-galloyl)-β-D-glucoside (7). These and nine related compounds, (–)-quinic acid (8), (–)-shikimic acid (9), ellagic acid (10), kaempferol (11), quercetin (12), quercitrin (13), rutin (14), quercetin-3-*O*-(2″-*O*-galloyl)-β-D-rutinoside (15) and 1,3,4,6-tetra-*O*-galloyl-β-D-glucose (16), were evaluated for the inhibitory activity against chitin synthase II and III. They inhibited chitin synthase II with IC_{50} values of 18–206 μM, except for two organic acids, (–)-quinic acid (8) and (–)-shikimic acid (9). Among them, 3-*O*-galloyl-(–)-shikimic acid (3) was the most potent inhibitor against chitin synthase II of *Saccharomyces cerevisiae* with an IC_{50} value of 18 μM. The inhibition appears to be selective for chitin synthase II, as they did not appreciably inhibit chitin synthase III.**

Key words: ***Euphorbia pekinensis*, Euphorbiaceae, chitin synthase II, chitin synthase III, chitin synthase II inhibitor, tannins, antifungal agents.**

Introduction

Chitin is an important structural polysaccharide of fungal cell walls. Although the content of chitin in the cell wall varies from species to species, it has been shown to be indispensable for the maintenance of the fungal cell wall integrity. Its synthesis constitutes a model for morphogenesis and provides a potential target in antifungal chemotherapy (1). Chitin is synthesized by chitin synthases I, II, and III in *Saccharomyces cerevisiae* (2). Chitin synthase I is involved in the repair of damaged chitin, chitin synthase II plays important roles in primary septum formation, and chitin synthase III is required for all other chitin synthases, including the formation of glucan-chitin linkage. Thus, chitin synthases II and III are essential enzymes for cell division, whereas chitin synthase I is not. Therefore, specific inhibitors of chitin synthases II and III might be interesting lead compounds for the development of effective antifungal agents.

As a part of our continuing efforts to discover naturally occurring new antifungal agents, we have screened for chitin synthase II inhibitors in higher plants. We report herein on the isolation, structural elucidation, and chitin synthases II and III activities of the isolates from *Euphorbia pekinensis* Rupr.

Materials and Methods

Chemicals

UDP-[U-^{14}C]-*N*-acetylglucosamine (400,000 cpm/μmol) was purchased from New England Nuclear (NEN) (DuPont, U.S.A). Trypin, trypsin inhibitor, and *N*-acetylglucosamine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). Cobalt acetate was purchased from Showa Chemical Inc. (Japan). All other chemicals used in this study were of analytical grade.

Chitin synthases II and III assay

The assays of chitin synthases II and III prepared from recombinant *S. cerevisiae* ECY38 – 38A (pAS6) and ECY38 – 38A (pWJC6), respectively, were conducted according to the method of Choi and Cabib (3). The reaction mixtures contained 32 mM Tris-HCl (pH 8.0), 1.6 mM cobalt acetate, 1.0 mM UDP-[U-^{14}C]-*N*-acetylglucosamine, 2 μL of trypsin (2 mg/mL), and 20 μL of membrane suspension in a total volume of 46 μL. Mixtures were incubated for 15 min at 30 °C. Proteolysis was stopped by addition of a 2.0-fold excess of soybean trypsin inhibitor, and *N*-acetylglucosamine was added to a final concentration of 32 mM, followed by incubation at 30 °C for 90 min. The insoluble chitin formed was assayed by the measurement of radioactivity after addition of 1 mL of cold 10% trichloroacetic acid and filtration through a glass fiber filter (Whatman GF/C). For chitin synthase III activity (3), the assay was performed the same as for chitin synthase II, except that 32 mM Tris-HCl (pH 7.5) and 4.3 mM magnesium acetate were used. Blank values were obtained from the reaction containing solvents only. Percent inhibition of chitin synthases II and III was calculated by substracting the blank value from the control or test sample values.

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$$\% \text{ Inhibition} = \left[1 - \frac{\text{Sample (cpm)} - \text{Blank (cpm)}}{\text{Control (cpm)} - \text{Blank (cpm)}}\right] \times 100$$

Each test compound was solubilized in 25% MeOH or 25% DMSO to make stock solution (1 mg/mL) and 14 μL of the stock solution were used to give the final concentration of 280 μg/mL. Also, to determine IC_{50} values, a 2-fold serial dilution was made with each test compound (4). The inhibitory activities are shown as average values in duplicates obtained from two independent experiments

Plant material

The aerial part of *Euphorbia pekinensis* Rupr. [= *Galarhoeus pekinensis* (Rupr.) Hara] was collected at Mt. Sobaeck of Gangwon Province, Korea. A voucher specimen has been deposited under No. CNUP-151 in College of Pharmacy, Chungbuk National University, Korea.

Extraction and isolation

Melting points were determined on a Büchi 510-K melting point apparatus. FAB-MS were measured on a VG Trio 2 instrument. Optical rotations were measured on a Jasco DIP-181 digital polarimeter. The NMR spectra were obtained on a Bruker AMX 300 spectrometer (300 MHz) using acetone-d_6/D_2O and DMSO-d_6/D_2O. Chemical shifts are given in ppm using TMS as internal standard. Sephadex LH-20 (20 – 100 μ) (Pharmacia Fine Chemical Co.), MCI-gel CHP 20P (75 – 100 μ) (Mitsubishi Chemical Industries Co.), TSK-gel Toyopearl HW 40F (30 – 60 μ) (Tosoh Co.), and Avicel cellulose (Funakoshi) were used for column chromatography.

The dried and milled sample (9 kg) was soaked in 80% aqueous acetone (40 L) at room temperature for 5 days. The acetone was removed *in vacuo* and insoluble materials such as chlorophylls and waxes in aqueous phase were eliminated by filtration. The filtrate was concentrated *in vacuo* to yield a dark residue (298 g) having inhibitory activity for chitin synthase II by 50–60% at 280 μg/mL. The residue was suspended in 50% MeOH and subjected to Sephadex LH-20 column chromatography (300 g) with a gradient of H_2O/MeOH (1 : 1 to 100% MeOH, v/v, 1 L each eluent) to give two active fractions, which inhibited chitin synthase II by more than 60% at 280 μg/ml; fraction 1 (50% MeOH eluent, 35 g) and fraction 2 (70% MeOH eluent, 43 g). Fraction 1 was recharged on MCI-gel CHP 20P (350 g) and eluted stepwise with a gradient of H_2O/MeOH (2 : 8 to 100% MeOH, v/v, 1 L each eluent) to give two active fractions, which inhibited chitin synthase II by 70% at 280 μg/ml; subfractions 1 – 1 (50% MeOH eluent, 1.8 g) and 1 – 2 (50–60% MeOH eluent, 750 mg). Subfraction 1 – 1 was further purified by Sephadex LH-20 chromatography (180 g) with a gradient of H_2O/MeOH (2 : 8 to 100% MeOH, v/v, 1 L each eluent) to give compound **1** (50% MeOH eluent, 761 mg) and **2** (60% MeOH eluent, 288 mg). Subfraction 1 – 2 was further purified by Sephadex LH-20 (75 g) eluting with 50% MeOH (1 L) to give compounds **3** (150 mg) and **4** (63 mg). Fraction 2 was recharged on MCI-gel CHP 20P (430 g) and eluted stepwise with a gradient of H_2O/MeOH (2 : 8 to 100% MeOH, v/v, 1 L each eluent) to give two active fractions, which inhibited by more than 70% at 280 μg/mL; subfractions 2 – 1 (80% MeOH eluent, 7.9 g), and 2 – 2 (90%–100% MeOH eluent, 2.0 g). Subfraction 2 – 1 was further purified by Sephadex LH-20 (150 g) column chromatography with 50% MeOH (1.5 L) to give compound **5** (7.0 g) and one active fraction (800 mg). The active fraction was chromatographed on TSK-gel Toyopearl HW 40F (40 g) using H_2O/EtOH (2 : 8 to 100% EtOH, v/v, 500 mL each eluent) to give compound **6** (80% EtOH eluent, 321 mg). Subfraction 2 – 2 was further purified by Sephadex LH-20 (150 g) with EtOH (1 L) to give one active fraction (1.5 g). The active fraction was chromatographed on TSK-gel Toyopearl HW 40F (45 g) using H_2O/EtOH (1 : 1 to 100% EtOH, v/v, 500 ml each eluent), and the 80% EtOH eluate (1.2 g) was rechromatographed on Avicel cellulose (36 g) using H_2O/AcOH (98 : 2, v/v, 400 ml eluent) column chromatography to give compound **7** (0.9 g).

Other test compounds

To obtain further information on the inhibitory activity for chitin synthases II and III, two tannins, quercetin-3-*O*-(2″-galloyl)-*β*-D-rutinoside (**15**), and 1,3,4,6-tetra-*O*-galloyl-*β*-D-glucose (**16**) were obtained from College of Pharmacy, Chungbuk National University. (–)-Quinic acid (**8**), (–)-shikimic acid (**9**), ellagic acid (**10**), kaempferol (**11**), quercetin (**12**), quercitrin (**13**), and rutin (**14**) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). Polyoxin D and nikkomycin Z, purchased from Calbiochem Co. (U.S.A), were used as standard compounds in this study. Polyoxin D and nikkomycin Z were dissolved in distilled water (4).

*3-O-Galloyl-(–)-shikimic acid (**3**)*: m. p. 225 °C. $[\alpha]_D^{29}$: –110.9° (*c* 0.4, acetone).

*Corilagin (**4**)*: m. p. 211–212 °C. $[\alpha]_D^{26}$: –230.2° (*c* 0.9, MeOH).

*Geraniin (**5**)*: m. p. 218–221 °C. $[\alpha]_D^{28}$: –147.8° (*c* 0.9, MeOH).

Quercetin-3-O-(2″-O-galloyl)-β-D-glucoside (**6**): m. p. 205 °C. $[\alpha]_D^{20}$: –129° (c 0.1, MeOH)

Kaempferol-3-O-(2″-O-galloyl)-β-D-glucoside (**7**): m. p. 227–229 °C. $[\alpha]_D^{20}$: –85° (*c* 0.9, MeOH).

Results and Discussion

In the course of our screening program for chitin synthase II inhibitors from natural resources, we found that the 80% aqueous acetone extract of aerial parts of *Euphorbia pekinensis* strongly inhibited chitin synthase II from *Saccharomyces cerevisiae*. Chitin synthase II assay-directed separation yielded seven tannins and related compounds; gallic acid (**1**), methyl gallate (**2**), 3-*O*-galloyl-(–)-shikimic acid (**3**), corilagin (**4**), geraniin (**5**), quercetin-3-*O*-(2″-*O*-galloyl)-*β*-D-glucoside (**6**), and kaempferol-3-*O*-(2″-*O*-galloyl)-*β*-D-glucoside (**7**). The structures of compounds **1**–**7** were previously reported (3–8). Among them, compounds **6** and **7** gave a positive response in $FeCl_3$ and Mg-HCl tests and showed absorption bands for a glycosidic linkage at 1050 cm^{-1} (glycosidic C – O) in their IR spectra, indicating a flavonoid glycoside. Acid hydrolysis of both compounds with 5% H_2SO_4 yielded glucose and gallic acid along with quercetin (**12**) from compound **6**, and kaempferol (**11**) for compound **7**. The ^{1}H-NMR spectrum of compound **6** showed an anomeric proton signal at 6.02 (1H, H-1″) and a galloyl proton signal at δ = 7.32 (2H, galloyl H-2 and H-

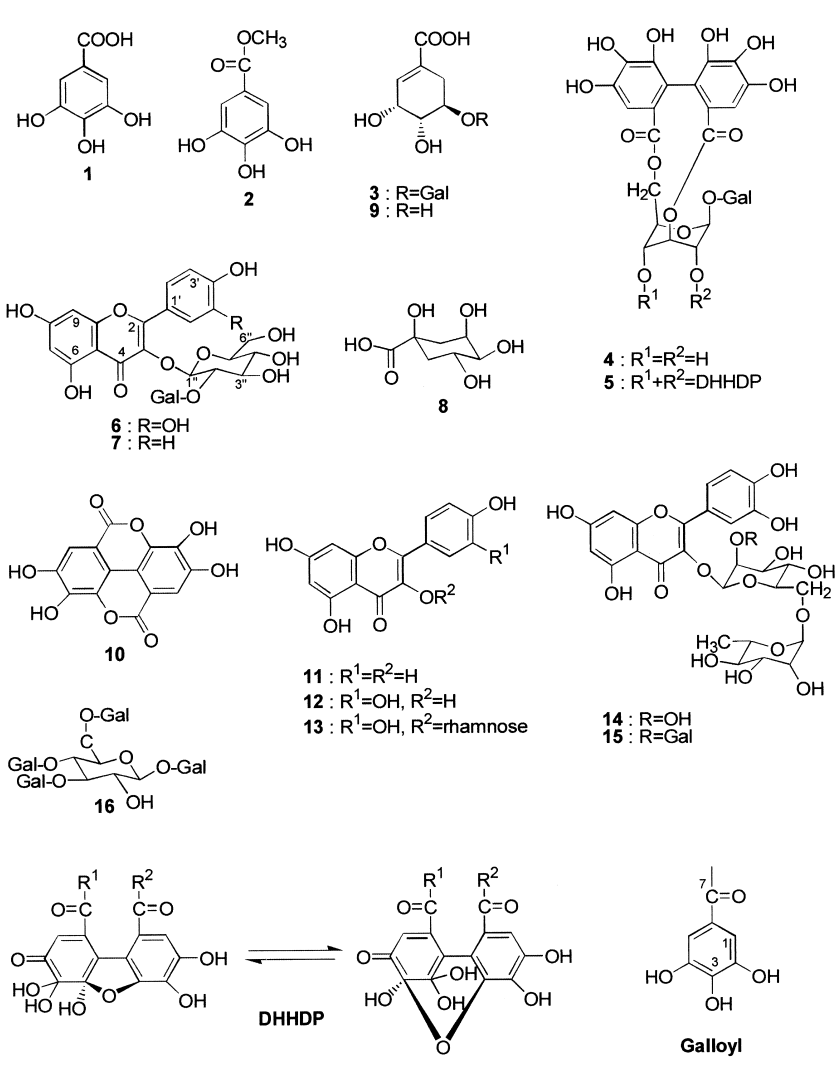

Fig. 1 Structures of compounds used in this study.

6), suggesting the presence of one mole of glucoside and galloyl. In addition, compound **6** showed five proton signals characteristic of quercetin, three aromatic signals at δ = 7.03 (1H, H-3′), 7.71 (1H, H-2′) and 7.91 (1H, H-6′) for 1,4,5-trisubstituted benzene (B ring), and two doublet signals at δ = 6.31 (1H, H-7) and 6.60 (1H, H-9) for the A-ring of flavonoid. The ^{1}H-NMR spectrum of compound **7** was similar to that of compound **6** except for H-5′ (δ = 6.92, 2H). On the basis of the UV spectrum, ^{1}H- and ^{13}C-NMR spectra, and ^{1}H-^{1}H COSY spectrum, for compounds **6** and **7,** it was suggested that the C-2″ of glucoside was ester-linked to gallic acid. Therefore, compounds **6** and **7** were determined to be quercetin-3-*O*-(2″-*O*-galloyl)-β-D-glucoside and kaempferol-3-*O*-(2″-*O*-galloyl)-β-D-glucoside, respectively, whose spectral data were consistent with those reported in the literature (5 – 10).

With regard to the inhibitory activities of compounds **1**–**16** (Fig. **1**), chitin synthases II and III from *S. cerevisiae* ECY38 – 38A (pAS6) and *S. cerevisiae* ECY38 – 38A (pWJC6), respectively, were used, and the activities were measured by the formation of chitin from UDP-[U-^{14}C]-*N*-acetylglucosamine. The inhibitory activities of the compounds can be compared with the positive controls, polyoxin D and nikkomycin Z, known chitin synthase II and III inhibitors with IC_{50} values of 134 and 1 μM, respectively. The inhibitory activities of these compounds against chitin synthases II and III are shown in Table **1**. Compounds **1**–**7** and **10–16** dose-dependently inhibited chitin synthase II activity. Phenolic compounds **1**, **2**, and **10** showed weak or similar inhibitory activity against chitin synthase II with IC_{50} values of 87–206 μM. In addition, tannins (**3**–**7**, **15**, and **16**) and related flavonoids (**11**–**14**) exhibited strong inhibitory activities against chitin synthase II with IC_{50} values of 18–54 μM, which is 2.5–7.5 times stronger inhibitory activity than that of polyoxin D. On the other hand, two organic acids, (–)-quinic acid (**8**) and (–)-shikimic acid (**9**), did not exhibit inhibitory activity against chitin synthase II, whereas 3-*O*-galloyl-(–)-shikimic acid (**3**), having a (–)-shikimic acid moiety, was the most potent inhibitor with an IC_{50} value of 18 μM. In contrast, compounds **1**–**16** demonstrated little or no inhibitory activity against chitin synthase III.

From the results of these experiments, it was found that the degree of inhibition of chitin synthase II was in the order of tannins > flavonoids > phenolic acids. The results suggested these compounds (except that organic acids) could specifically inhibit the chitin synthase II. Therefore, compounds **1**–**7** and **10**–**16** may be useful lead compounds for development of antifungal agents through the control of chitin biosynthesis.

Table 1 Inhibitory activities of tannins and related compounds against chitin synthases II (Chs II) and III (Chs III)

Compounds	M. W.	IC_{50} against Chs II (μM)	Inhibitory activity against Chs III at 280 μg/ml (%)
Phenol and phenolic acids			
Gallic acid (**1**)	170	206	0
Methyl gallate (**2**)	184	87	0
Ellagic acid (**10**)	302	149	0
Organic acids			
(–)-Quinic acid (**8**)	192	0	< 10
(–)-Shikimic acid (**9**)	174	0	< 15
Flavonoids			
Kaempferol (**11**)	286	52	0
Quercetin (**12**)	302	36	0
Quercitrin (**13**)	448	45	0
Rutin (**14**)	610	54	0
Tannins			
3-*O*-galloyl-(–)-shikimic acid (**3**)	326	18	0
Corilagin (**4**)	634	25	0
Geraniin (**5**)	952	30	0
Quercetrin-3-*O*-(2″-*O*-galloyl)-β-D-glucoside (**6**)	616	21	0
Kaempferol-3-*O*-(2″-*O*-galloyl)-β-D-glucoside (**7**)	600	50	0
Quercetrin-3-*O*-(2″-*O*-galloyl)-β-D-rutinoside (**15**)	762	37	0
1,3,4,6-tetra-*O*-galloyl-β-D-glucoside (**16**)	788	23	0
Polyoxin D	521	134	> 95[a]
Nikkomycin Z	495	273	> 95[a]

[a] IC_{50} values for Chs III of both compounds were 2 and 1 μM, respectively.

Euphorbia pekinensis is known to be rich in tannins and has shown diverse biological and pharmacological actions such as antiviral, antibacterial and antioxidative activities. Compounds **1**–**16** have also shown antibacterial (11), anti-tyrosinase (12), antitumor (13), and astringent activity (14). However, this is the first report demonstrating these compounds specifically inhibit the chitin synthase II. In previous papers (15), (16), we have reported that several phenolic compounds such as flavonoids and triterpenoids from higher plants showed a specific inhibitory activity against chitin synthase II. Taken together, these phenolic compounds, as potential inhibitors of chitin synthase II, should be investigated in more detail.

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