Biological Evaluation of Phellinsin A Analogues as Chitin Synthase II Inhibitors

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Chitin is the β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc) that constitutes an important structural component of the cell walls of nearly all zoopathogenic and phytopathogenic fungi, and plays a crucial role in the determination of cell morphology.[1] Chitin have been known to be synthesized by chitin synthase I, II, and III in *Saccharomyces cerevisiae*.[2] Chitin synthase II is an essential enzyme for the formation of primary septum between mother and daughter cells,[3] and chitin synthase III is responsible for the formation of chitin ring at bud emergence,[4] whereas chitin synthase I is a repair enzyme of the damaged cells on cell division.[5] Accordingly, specific inhibitors of chitin synthase II and III are expected to be an attractive target for the development of antifungal agents.

In the course of exploring a chitin synthase II inhibitor from microbial sources, we isolated a new phenolic lignan bearing a γ-lactone ring from the cultured broth of *Phellinus* sp. PL3.[6] The lignan compound named phellinsin A (**3a**) exhibited inhibition of chitin synthase II with an IC_{50} value of 27 μg/mL and showed 2.5 times stronger inhibitory activity than polyoxin D, and its structure was established by NMR analysis and synthesis.[7] Our efforts toward the development of a potent antifungal agent have focused on examining structure-activity relationships for phellinsin A. We have modified the aryl group in phellinsin A by changing the number of phenolic OH groups in order to investigate the effect of the number of free phenolic OH groups in γ-lactone analogues of phellinsin A (**3a**) on chitin synthase II inhibitory activity. Herein, we describe the biological evaluation of phellinsin A analogues **3**.

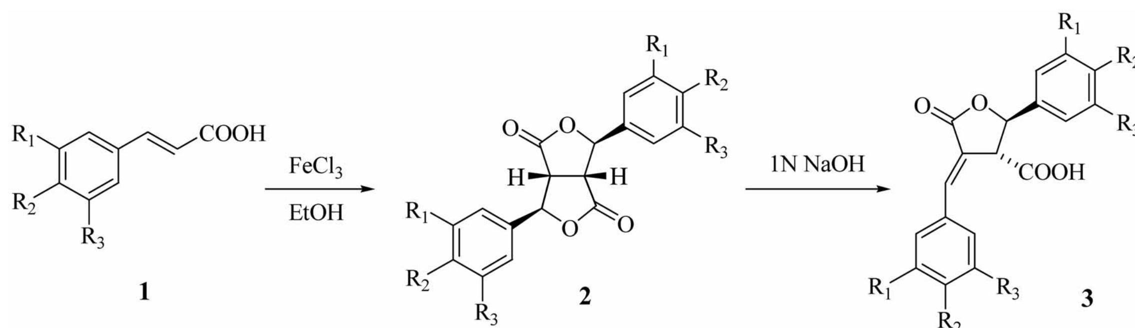
The γ-lactone analogues **3c-f** of phellinsin A were prepared by oxidative dimerization of cinnamic acid derivatives **1** into the corresponding dilactones **2**, followed by monohydrolysis of the dilactones as shown in Scheme 1.[7,8] The γ-lactones **3a** and **3b** were prepared by demethylation of compounds **3d** and **3e** using BBr_3, respectively.

The γ-lactone analogues **3** of phellinsin A were examined for structure-activity relationships of phellinsin A (**3a**). Inhibitory activities of the compounds against chitin synthase II were evaluated by measurement of the formation of chitin with UDP-[^{14}C]-*N*-acetyl-D-glucosamine. Table 1 showed the inhibitory activities of phellinsin A analogues at 140 and 280 μg/mL concentrations. The activities were largely dependent on the presence of free phenolic OH substituents in γ-lactone analogues **3**. Generally, the compound **3f** with non-phenolic OH groups did not show

Table 1. Inhibitory activities of phellinsin A analog against chitin synthase II

compound	% Inhibition (140 μg/mL)[a]	% Inhibition (280 μg/mL)[b]
3a	83.2	–[c]
3b	87.8	–[c]
3c	NA[d]	36.0
3d	3.6	40.1
3e	10.1	44.4
3f	NA[d]	10.1

[a]Percent inhibition at 140 μg/mL. [b]Percent inhibition at 280 μg/mL. [c]Not determined. [d]NA, no inhibitory activity.



a. R_1=H, R_2=OH, R_3=OH; **b.** R_1=OH, R_2=OH, R_3=OH; **c.** R_1=H, R_2=OH, R_3=H;
d. R_1=H, R_2=OH, R_3=OCH_3; **e.** R_1=OCH_3, R_2=OH, R_3=OCH_3; **f.** R_1=H, R_2=H, R_3=H

Scheme 1

Table 2. IC_{50} values of selected compounds

compound	IC_{50} (μg/mL)
3a	27.0
3b	9.6
PD[a]	70.0

[a]Polyoxin D as a reference.

inhibitory activities at 140 μg/mL concentration. Compounds **3a-b** with two and three phenolic OH groups exhibited potent activities, whereas compound **3c** with one phenolic OH group did not show inhibitory activities at 140 μg/mL concentration. However, compounds **3d-e** with one phenolic OH group and additional methoxy groups showed mild inhibitory activities at 140 and 280 μg/mL concentration, and **3e** with two additional methoxy groups showed stronger activity than **3d** with one additional methoxy group. Compound **3b** possessing three phenolic OH groups showed most potent activity. Compounds **3a** and **3b**, which showed potent inhibitory activities at 140 μg/mL concentration, were further evaluated with polyoxin D as positive control (Table 2). As shown in Table 2, the γ-lactone analogue **3b** (IC_{50} = 9.6 μg/mL) possessing three phenolic OH groups at each aryl moiety exhibited about 3 times stronger inhibitory activity than phellinsin A (**3a**) (IC_{50} = 27.0 μg/mL) possessing two phenolic OH groups. Compound **3b** showed about 7 times more potent activity than polyoxin D (IC_{50} = 70.0 μg/mL). Structure-activity analysis indicated that the number of free phenolic OH groups in each aryl group of phellinsin A analogues contributed to increase the activity.

In conclusion, the effects of the number of free phenolic OH groups in phellinsin A analogues on inhibitory activities of chitin synthase II were examined for structure-activity relationships. Phellinsin A analogues lacking free phenolic OH functionality exhibited poor inhibitory activities. Compounds possessing three free phenolic OH groups in each aryl group of γ-lactones **3** showed more potent activity than those of one OH group or two OH groups. Compound **3b** exhibited about 3 times stronger inhibitory activity than phellinsin A (**3a**) and about 7 times more potent activity than polyoxin D.

Experimental Section

Typical procedure for preparation of compound 3b. To a solution of iron(III) chloride (6.4 g, 28.5 mmol) in ethanol (70 mL) was added a solution of 3,5-dimethoxy-4-hydroxycinnamic acid (**1b**, 4.0 g, 17.8 mmol) in ethanol (20 mL) at room temperature. The reaction mixture was stirred for 1 h and concentrated *in vacuo*. The residue was diluted with water, extracted with ethyl acetate, washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting residue was chromatographed on silica gel (1:1 hexane-EtOAc) to afford 4.9 g (61%) of dilactone **2b** as a pale brown solid: ^{1}H NMR (300 MHz, acetone-d_6) δ 7.47 (s, 2H), 6.75 (s, 4H), 5.76 (brs, 2H), 4.12 (brs, 2H), 3.84 (s, 12H); ^{13}C NMR (75 MHz, acetone-d_6) δ 176.7, 149.7, 138.2, 130.5, 105.0, 84.1, 57.4, 49.8; HRMS (FAB) *m/z* 447.1288 $[(M+H)^+$, calcd for $C_{22}H_{23}O_{10}$ 447.1291]. To a solution of dilactone **2b** (1.0 g, 2.23 mmol) in THF (10 mL) was added 1 N NaOH (50 mL) at room temperature. The reaction mixture was stirred for 5 min, acidified with 2 N HCl to pH 4, extracted with ethyl acetate, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting residue was purified by flash chromatography on silica gel (9:1 CH_2Cl_2-CH_3OH) to afford 0.6 g (60%) of **3e** as a yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (d, J = 1.8 Hz, 1H), 6.77 (s, 2H), 6.51 (s, 2H), 5.69 (d, J = 2.4 Hz, 1H), 4.12 (m, 1H), 3.85 (s, 6H), 3.84 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.5, 171.4, 147.4, 147.2, 141.9, 137.7, 135.2, 130.2, 124.4, 118.1, 107.7, 101.9, 80.5, 56.4, 56.3, 53.5; HRMS (FAB) *m/z* 447.1286 $[(M+H)^+$, calcd for $C_{22}H_{23}O_{10}$ 447.1291]. To solution of compound **3e** (100 mg, 0.22 mmol) in methylene chloride (8 mL) at 0 °C was added 1 M BBr_3 in CH_2Cl_2 (5 mL, 5.0 mmol). The mixture was stirred for 2 h at 0 °C, quenched with 0.1 N HCl, extracted with ethyl acetate, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting residue was purified by a YMC ODS-H80 column chromatography (2:3 H_2O-CH_3OH) to afford 20 mg (22%) of **3b** as a brown solid: ^{1}H NMR (300 MHz, CD_3OD) δ 7.41 (d, J = 1.8 Hz, 1H), 6.69 (s, 2H), 6.30 (s, 2H), 5.50 (d, J = 2.4 Hz, 1H), 3.98 (m, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 177.7 174.9, 147.3, 147.0, 137.7, 132.9, 126.3, 122.1, 111.3, 105.5, 84.0, 57.5; HRMS (FAB) *m/z* 391.0678 $[(M+H)^+$, calcd for $C_{18}H_{15}O_{10}$ 391.0665].

Compound 3a. Compound **3a** was prepared from 4-hydroxy-3-methoxycinnamic acid according to the typical procedure: ^{1}H NMR (300 MHz, CD_3OD) δ 7.48 (s, 1H), 7.17 (d, J = 0.6 Hz, 1H), 7.05 (d, J = 5.1 Hz, 1H), 6.79 (d, J = 4.8 Hz, 1H), 6.73 (d, J = 5.1 Hz, 1H), 6.71 (d, J = 0.9 Hz, 1H), 6.64 (dd, J = 4.8, 0.9 Hz, 1H), 5.59 (d, J = 0.9 Hz, 1H), 3.97 (s, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 178.2, 175.2, 149.4, 146.8, 146.7, 146.5, 140.3, 133.6, 127.4, 125.5, 122.6, 118.3, 118.0, 116.6, 116.4, 113.4, 84.3, 57.7; HRMS (FAB) *m/z* 359.0771 $[(M+H)^+$, calcd for $C_{18}H_{15}O_8$ 359.0767].

Compound 3c: ^{1}H NMR (300 MHz, CD_3OD) δ 7.57 (s, 1H), 7.56 (d, J = 8.7 Hz, 2H), 7.12 (d, J = 9.0 Hz, 2H), 6.81 (d, J = 9.0 Hz, 2H), 6.76 (d, J = 8.7 Hz, 2H), 5.68 (d, J = 2.4 Hz, 1H), 4.00 (s, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 178.1, 174.9, 161.2, 159.0, 140.2, 134.0, 132.6, 128.0, 126.8, 122.2. 116.8, 116.6, 84.0, 57.1; HRMS (FAB) *m/z* 327.0857 $[(M+H)^+$, calcd for $C_{18}H_{15}O_6$ 327.0869].

Compound 3d: ^{1}H NMR (400 MHz, CD_3OD) δ 7.55 (s, 1H), 7.36 (s, 1H), 7.15 (d, J = 8.0 Hz, 1H), 6.86 (s, 1H), 6.81 (d, J = 8.0 Hz, 1H), 6.75 (bs, 2H), 5.69 (d, J = 2.0 Hz, 1H), 3.98 (bs, 1H), 3.87 (s, 3H), 3.78 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 178.8, 175.2, 150.4, 149.2, 149.1, 148.0, 140.2, 133.5, 127.5, 126.8, 123.1, 119.3, 116.5, 116.4, 114.6, 110.3, 84.4, 58.1, 56.7, 56.4; HRMS (FAB) *m/z* 387.1082 $[(M+H)^+$, calcd for $C_{20}H_{19}O_8$ 387.1080].

Compound 3f: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.50-7.28 (m, 10H), 5.81 (s, 1H), 4.25 (s, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 177.1, 174.6, 142.3, 139.4, 135.6, 131.7, 131.1, 129.9, 129.8, 129.4, 127.0, 126.3, 84.5,

58.7; HRMS (FAB) *m/z* 295.0974 $[(M+H)^+$, calcd for $C_{18}H_{15}O_4$ 295.0970].

Chitin synthase II assay: The strain used in this study is a *Saccharomyces cerevisiae* ECY38-38A (*MAT*a *chs*1-23 *chs*2::*LEU2 cal*1/*csd*2 *ura*3-52 *trp*1-1 *leu*2-2 pAS6), which can only overexpress the chitin synthase II, that was grown in YPG (yeast extract 1.0%, peptone 2.0%, galactose 2.0%). The cells suspended in 50 mM Tris-HCl (pH 7.5) containing 5 mM magnesium acetate were broken by vortex mixing with glass beads.[9] And the cell walls were sedimented at 3,000 × g for 5 min and supernatant fluid was centrifuged at 130,000 × g for 45 min. The membrane pellet was resuspended 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 used. The activity of chitin synthase II was measured by the described procedure.[9] 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]-GlcNAc (400,000 cpm/mmol, NEN), 2 mL of trypsin at the optimal concentration for activation (2.0 mg/mL), 20 μL of membrane suspension, and 14 μL of samples in a total volume of 46 μL. The mixtures were preincubated for 15 min at 30 °C. Proteolysis was stopped by adding 2 μL of a soybean trypsin inhibitor (4.0 mg/mL) at a concentration 2 times that of trypsin used, and mixtures were placed on ice for 10 min. GlcNAc was added to a final concentration of 32 mM and incubation at 30 °C was carried out for 90 min. The insoluble chitin formed was assayed by measurement of radioactivity after addition of 10% trichloroacetic acid and filtration through glass fiber filter (GF/C, Whatman). The concentration of protein was measured by the method of Lowry.[10] Blank values were measured with addition of 25% aqueous MeOH instead of both enzyme and sample. Percent inhibition of chitin synthase II activity was calculated by substracting the blank values from both control and test sample values using the following equation: Inhibition (%) = [1 − Sample (cpm)-Blank (cpm)/Control (cpm)-Blank (cpm)] × 100.

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