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Biological Evaluation of Dilactone Lignan Analogues of Phellinsin A as Chitin Synthase II Inhibitors

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Chitin is a structural polymer that constitutes the cell walls of nearly all fungi, and plays a crucial role in the determination of cell morphology.[1] There are three different chitin synthases in *Saccharomyces cerevisiae*.[2] Chitin synthase I is nonessential repair enzyme of damaged chitin.[3] Chitin synthase II is an essential enzyme for the formation of primary septum formation and cell division.[4] and chitin synthase III makes more than 90% of cellular chitin including the chitin synthesized during mating and spoluration.[5] Accordingly, specific inhibitors of chitin synthase II are expected to be an attractive target for the development of antifungal agents.

In the course of our search for a potent antifungal agent from microbial sources, phellinsin A (**1**) was isolated from the cultured broth of *Phellinus* sp. PL3.[5] The compound exhibited inhibition of chitin synthase II with an IC_{50} value of 28 μg/mL and showed 2.5 times stronger inhibitory activity than polyoxin D The structure of phellinsin A was elucidated by NMR analysis and its total synthesis.[6] Structure-activity relationships for phellinsin A by changing the number of phenolic OH groups showed that compounds possessing three free phenolic OH groups in each aryl group of phellinsin A exhibited more potent activity than those of one OH group or two OH groups.[7] Our efforts toward the development of a potent antifungal agent have attention to dilactone lignan analogues of phellinsin A. Herein, we describe the biological evaluation of dilactone lignan analogues of phellinsin A.

Dilactone lignan analogues **2** of phellinsin A were prepared by oxidative dimerization of the corresponding cinnamic acid derivatives using either $FeCl_3$[8] or PbO_2[9] as an oxidant. Thermodynamically more stable *cis*-ring fused-dilactones positioning *exo*-orientation of the aryl groups were formed. The dilactones (**2a**, **2c**, **2d**, **2e**) involving *para*-hydroxy-substituted aryl moieties except **2b** were prepared by $FeCl_3$, whereas non-phenolic dilactones (**2f** and **2g**) and compound **2b** were prepared using PbO_2.[6] Dilactones **2h** and **2i** were prepared from **2d** and **2e** by treatment with diazomethane, respectively.

Mono-, di-, or tri-phenolic OH substituted dilactones **2a-e** and non-phenolic analogues **2f-i** were examined in order to investigate the effect of the number of free phenolic OH groups in dilactone analogues **2** of phellinsin A on chitin synthase II inhibitory activity. Inhibitory activities of chitin synthase II for the compounds were evaluated by measurement of the formation of chitin with UDP-[^{14}C]-*N*-acetyl-D-glucosamine. Table 1 showed the inhibitory activities of dilactone analogues at 140 and 280 μg/mL concentrations. The activities were largely dependent on the presence of free phenolic OH substituents in dilactone analogues **2**. Generally the compounds with non-phenolic OH groups (**2f**, **2g**, **2h**, **2i**) did not show inhibitory activities at 140 μg/mL concentration. Compounds with two and three phenolic OH groups (**2a** and **2b**) exhibited potent activities, whereas compounds with one phenolic OH group (**2c**) did not show inhibitory activities at 140 μg/mL concentration. Interestingly, dilactone **2d** possessing one phenolic OH group and an additional methoxy group at the each aryl moiety showed good inhibitory activity (68.9%) at 140 μg/mL concentration. However, dilactone **2e** with one phenolic OH group and two additional methoxy groups did not show activities at 140 μg/mL concentration. Inhibitory activities of compounds exhibiting potent activities at 140 μg/mL concentration were confirmed by the positive control with polyoxin D (Table 2). As

Table 1. Inhibitory activities of chitin synthase II for dilactone lignan **2**

R_1	R_2	R_3	**2**	% Inhibition[a]	% Inhibition[b]
H	OH	OH	**2a**	89.7	-[c]
OH	OH	OH	**2b**	79.9	-[c]
H	OH	H	**2c**	NA	18.6
H	OH	OCH_3	**2d**	68.9	-[c]
OCH_3	OH	OCH_3	**2e**	NA	6.8
H	H	H	**2f**	NA	9.6
H	OC_2H_5	H	**2g**	NA	14.1
H	OCH_3	OCH_3	**2h**	NA	10.1
OCH_3	OCH_3	OCH_3	**2i**	NA	7.9

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

Table 2. IC_{50} values of selected compounds

Compound	IC_{50} (μg/mL)
2a	8.2
2b	40.8
PA[a]	27.0
PD[b]	70.0

[a]Phellinsin A as a reference. [b]Polyoxin D as a reference.

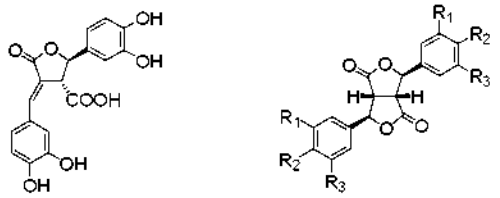

Figure 1. Phellinsin A (**1**) and dilactone analogues **2**.

showed in Table 2, dilactone **2a** possessing two phenolic OH groups exhibited stronger activity than dilactone **2b** possessing three phenolic OH groups. Among the tested compounds, dihydroxyphenolic dilactone **2a** showed the most potent activity with an IC_{50} value of 8.2 μg/mL, which is about 3 times stronger inhibitory activity than phellinsin A (**1**). Structure-activity analysis indicated that the presence of two free phenolic OH groups in each aryl group of phellinsin A dilactone analogues contributed to increase the activity. And, the dilactone form **2a** of phellinsin A possessing two phenolic OH groups increased the activity as compared to the corresponding mono-lactone form, phellinsin A (**1**).

In conclusion, the effects of the number of free phenolic OH groups in dilactone lignan analogues of phellinsin A on inhibitory activities of chitin synthase II were examined for structure-activity relationships. Dilactone analogues lacking free phenolic OH functionality exhibited poor inhibitory activities. Compounds possessing two free phenolic OH groups in each aryl group of dilactone **2** showed more potent activity than those of one OH group or three OH groups. Dihydroxyphenolic dilactone **2a** exhibited about 3 times stronger inhibitory activity than phellinsin A (**1**) and about 8 times more potent activity than polyoxin D.

Experimental Section

Compound 2a. To a solution of ferric chloride (9.2 g, 56.7 mmol) in ethanol (100 mL) was added a solution of 3,4-dihydroxycinnamic acid (4.6 g, 25.5 mmol) in ethanol (30 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 5.0 g (55%) of **2a** as a yellow solid: mp 176 - 178 °C; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.17 (s, 2H), 8.06 (s, 2H), 6.90 (dd, J = 8.1, 1.8 Hz, 2H), 6.85 (s, 2H), 6.81 (dd, J = 8.1, 1.8 Hz, 2H), 5.72 (brs, 2H), 3.99 (brs, 2H); ^{13}C NMR (75 MHz, acetone-d_6) δ 176.0, 146.8, 146.5, 131.3, 118.4, 116.5, 113.9, 83.1, 49.3; HRMS (FAB) *m/z* 359.0771 [(M+H)$^+$, calcd for $C_{18}H_{15}O_8$ 359.0767].

Compound 2b. To a solution of trifluoroacetic acid (3 mL) in methylene chloride (18 mL) was added 3,4,5-trihydroxycinnamic acid (1.0 g, 5.2 mmol) at 0 °C. Then, PbO_2 (1.24 g, 5.2 mmol) was added and the reaction mixture was sirred for 1 h. The mixture was poured into ethyl acetate (100 mL), and the organic layer was washed in succession with water, saturated $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated *in vacuo*. The resulting residue was purified by flash chromatography on silica gel (5:1 CH_2Cl_2-MeOH) to afford 0.84 g (42%) of **2b** as a dark brown solid: mp decomp.; ^{1}H NMR (300 MHz, CD_3OD) δ 6.33 (s, 4H), 5.61 (s, 2H), 3.75 (s, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 175.9, 151.6, 137.9, 132.1, 104.5, 83.2, 48.6; HRMS (FAB) *m/z* 391.0671 [(M+H)$^+$, calcd for $C_{18}H_{15}O_{10}$ 391.0665].

Compounds 2c-i. Compounds **2c-i** were prepared by the reported method.[6]

2c: A yellow solid; mp 185 - 188 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 7.23 (dd, J = 6.6, 1.8 Hz, 4H), 6.82 (dd, J = 6.6, 1.8 Hz, 4H), 5.75 (s, 2H), 3.93 (s, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 177.3, 159.5, 130.6, 128.3, 116.8, 84.1, 49.7; HRMS (FAB) *m/z* 327.0851 [(M+H)$^+$, calcd for $C_{18}H_{15}O_6$ 327.0869].

2d: A white solid; mp 206 - 208 °C; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.86 (s, 2H), 7.06 (d, J = 1.8 Hz, 2H), 6.92 (dd, J = 7.8, 1.8 Hz, 2H), 6.86 (d, J = 7.8 Hz, 2H), 5.78 (brs, 2H), 4.09 (brs, 2H), 3.87 (s, 6H); ^{13}C NMR (75 MHz, acetone-d_6) δ 176.1, 148.9, 148.4, 131.0, 119.7, 116.2, 110.5, 83.3, 56.5, 49.2; HRMS (FAB) *m/z* 387.1092 [(M+H)$^-$, calcd for $C_{20}H_{19}O_8$ 387.1080].

2e: A pale brown solid; mp 230 - 234 °C; ^{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].

2f: A pale brown solid; mp 161 - 163 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44-7.30 (m, 10H), 5.93 (s, 2H), 3.56 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.8, 138.0, 129.2, 129.1, 124.5, 81.7, 48.2; HRMS (FAB) *m/z* 295.0982 [(M+H)$^-$, calcd for $C_{18}H_{15}O_4$ 295.0970].

2g: A white solid; mp 163 - 164 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21 (d, J = 8.7 Hz, 4H), 6.90 (dd, J = 6.6, 1.8 Hz, 4H), 5.88 (s, 2H), 4.02 (q, J = 6.9 Hz, 4H), 3.56 (s, 2H), 1.41 (t, J = 6.9 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.9, 159.6, 129.8, 126.2, 115.1, 81.9, 63.7, 48.3, 14.7; HRMS (FAB) *m/z* 383.1485 [(M+H)$^-$, calcd for $C_{22}H_{23}O_6$ 383.1495].

2h: A white solid; mp 200 - 203 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.03-7.00 (m, 6H), 5.80 (s, 2H), 4.22 (s, 2H), 3.79 (s, 6H), 3.77 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.3, 149.4, 149.0, 130.5, 118.8, 111.7, 109.9, 81.7, 55.7, 55.6, 48.1; HRMS (FAB) *m/z* 415.1394 [(M+H)$^-$, calcd for $C_{22}H_{23}O_8$ 415.1393].

2i: A white solid; mp 200 - 202 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.73 (s, 4H), 5.79 (s, 2H), 4.26 (s, 2H), 3.81 (s, 12H), 3.66 (s, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 175.3, 153.2, 137.8, 133.9, 103.5, 81.6, 60.0, 56.1, 47.8; HRMS (FAB) *m/z* 475.1601 [(M+H)$^+$, calcd for $C_{24}H_{27}O_{10}$ 475.1604].

Chitin synthase II assay. The strain used in this study is a *Saccharomyces cerevisiae* ECY38-38A (*MATa chs1-23 chs2::LEU2 cal1/csd2 ura3-52 trp1-1 leu2-2* 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.[10] 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.[10] 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.[11] 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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