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Research papers

Metabolism of bisphenol A by hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under non-ligninolytic condition

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Keywords: bisphenol A; *Phanerochaete sordida* YK-624; estrogenic activity; metabolic pathway

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Abstract

- 1 Recently, we reported the conversion of bisphenol A (BPA) to
- 2 4-(2-(4-hydroxyphenyl)propan-2-yl)benzene-1,2-diol (hydroxy-BPA) by hyper
- 3 lignin-degrading fungus Phanerochaete sordida YK-624 under non-ligninolytic condition. In
- 4 the present study, the metabolism of hydroxy-BPA by *P. sordida* YK-624 was demonstrated
- 5 under non-ligninolytic condition. Under these conditions, approximately 66% of
- 6 hydroxy-BPA was degraded after 7 d of incubation. High-resolution electrospray ionization
- 7 mass spectra and nuclear magnetic resonance analyses of the metabolites isolated from the
- 8 culture broth indicated that hydroxy-BPA was metabolized to
- 9 4-(2-(4-hydroxyphenyl)propan-2-yl)-2-methoxyphenol (methoxy-BPA) and to
- 10 4-(2-(3,4-dimethoxyphenyl)propan-2-yl)phenol (dimethoxy-BPA) by sequential methylation
- 11 events. These metabolites showed reduced estrogenic activity compared to BPA. These
- 12 results suggested that the hydroxy BPA is methylated to 2 low toxic-methylation metabolites.

1. Introduction

13 Endocrine disrupting compounds (EDCs) alter the function of the endocrine system of 14 both wildlife and humans (Colborn et al., 1993). Bisphenol A 15 (2,2-bis(4-hydroxyphenyl)propane; BPA), is an industrial chemical synthesized by 16 condensation of two phenol groups and on acetone molecule. While BPA was first 17 synthesized in 1891, it is used in many consumer products, including lacquers applied as 18 food-can linings and dental composite fillings and sealants (Brotons et al., 1995; Olea et al., 19 1996). The global demand for BPA is predicted to grow from 3.9 million tons (in 2006) to 20 about 5 million tons in 2010 (Ballesteros-Gómez et al., 2009). Due to the use of bisphenol A 21 in the manufacture of many products, BPA often has been detected in many soil and water 22 environments (Kitada et al., 2008; Matsumura et al., 2009). 23 A white-rot fungi have the unique ability to degrade lignin to the level of CO₂ (Kirk and Farrell, 1987), and the white-rot fungi have been shown to degrade recalcitrant persistent 24 25 toxic organic chemicals, such as polychlorinated dibenzodioxin (Kamei et al., 2005), lindene 26 (Bumpus et al., 1985), dieldrin (Xiao et al., 2011a), benzo[a]pyrene (Hadibarata and Kristanti, 27 2012), mycotoxin aflatoxin B_1 (Wang et al., 2011), and the neonicotinoid insecticide 28 acetamiprid (Wang et al., 2012a). 29 Up until now, biotransformation of BPA also has been reported by many researchers. In 30 rat hepatocytes, BPA glucuronide is the major metabolite of BPA (Nakagawa and Tayama, 31 2000). In plants, cells of Eucalyptus perriniana and Portulaca oleracea cv. metabolize BPA 32 to its hydroxylated products (Hamada et al., 2002; Watanabe et al., 2012). Proposed pathways 33 for bacterial degradation of BPA were summarized by Zhang et al. (2013). Another possible 34 method of BPA removal involves the application of white-rot fungi or their enzymatic systems to the biodegradation of BPA. Among the fungi, BPA is metabolized to 35 36 BPA-*O*-β-D-glucopyranoside by *Aspergillus fumigatus* (Yim et al., 2003). Ligninolytic enzymes generate BPA phenoxy radicals, resulting in radical couplings and polymerization 37 38 (Tsutsumi et al., 2001; Wang et al., 2012b; Wang et al., 2013a). Unfortunately, these BPA 39 dimers can be decomposed by various reactions to yield toxic BPA. Therefore, effective BPA 40 elimination requires processes distinct from BPA dimerization. 41 Recently, we have examined the removal of BPA by P. sordida YK-624 under non-ligninolytic condition and detected 4-(2-(4-hydroxyphenyl)propan-2-yl)benzene-1,2-diol 42 43 (hydroxy-BPA) as the primary metabolite (Wang et al., 2013b). In the present study, the 44 further degradation of the hydroxy-BPA by P. sordida YK-624 was determined to clarify the 45 metabolism of BPA by *P. sordida* YK-624 under non-ligninolytic condition.

2. Materials and methods

46 2.1. Fungi and chemicals

P. sordida YK-624 (ATCC 90872), originally isolated from rotted wood, was
used for the present study (Hirai et al., 1994). The fungus was maintained on potato
dextrose agar (PDA) slants at 4 °C.

BPA was purchased from Alfa Aesar, A Johnson Matthey (Lancashire, United Kingdom).
All other chemicals were of analytical-grade purity and were used without further

52 purification.

53 2.2. Synthesis of hydroxy-BPA

54 As shown in Scheme 1, the synthesis of hydroxy-BPA commenced from the 55 acetylation reaction of *p*-hydroxyacetophenone. *p*-Hydroxyacetophenone (5 g) was stirred with 25 mL pyridine until fully dissolved. After adding 12.5 mL acetic 56 anhydride, the reaction mixture was stirred for 16 h at room temperature. The solution 57 then was extracted with ethyl acetate (EtOAc). The organic layer was washed with 58 H₂O, and then purified by silica gel column chromatography with 30% EtOAc in 59 60 hexane to obtain 4-acetoxyacetophenone (6 g, 33.6 mmol). The methylenation of 4-acetoxyacetophenone was achieved by a Witting reation with 61 62 methyltriphenyl-phosphonium bromide. Methyltriphenyl-phosphonium bromide (21.05 g) in 227.6 mL anhydrous tetrahydrofuran was added dropwise, under argon, at 63 0 °C in the presence of 35 mL of *n*-butyllithium. The solution turned orange as the 64 addition proceeded. After stirring at 0 °C for an additional half hour, a solution of 65 5.69 g 4-acetylphenyl acetate in 75.68 mL anhydrous tetrahydrofuran was added 66 dropwise, the resulting mixture was warmed to room temperature and stirred for 36 h. 67 The solution then was extracted with EtOAc. The organic layer was washed with H₂O, 68 and then purified by silica gel column chromatography with a 10% EtOAc in hexane 69 to obtain 4-isopropenylphenol (1.73 g, 12.89 mmol). Finally, the hydroxyl-BPA was 70 71 formed by the reaction of 4-isopropenylphenol and catechol. Catechol (1.67 g), 72 together with 6.75 mL of benzene-methanol (MeOH) (90:10) and 0.05 mL of 36% HCl, was stirred at 30 °C. 4-isopropenylphenol (1.73 g) in 7.61 mL benzene-MeOH 73 74 (90:10) was added dropwise over 1 h, and stirred for 5 h at 30 °C. The solution then was cooled to room temperature, 1.11 mL 1 M NaOH was added, extracted with 75 EtOAc. The organic layer was washed with brine, and then purified by silica gel 76

column chromatography with a 20% EtOAc in hexane to obtain hydroxy-BPA (1.72 g,

78 7.05 mmol, 20.21%). ¹H-nuclear magnetic resonance (NMR, 500 MHz, CD₃OD)

79 yielded the following parameters: δ 1.54 (s, OMe), 6.57 (dd, H-5), 6.62 (d, H-3), 6.66

80 (dd, H-6, 3", 5"), 7.02 (dd, H-2", 6"), ¹³C-NMR (500 MHz, CD₃OD): δ 31.6 (OMe),

81 42.5 (C-2'), 115.5 (C-6, 3", 5"), 115.6 (C-3), 118.9 (C-5), 128.7 (C-2", 6"), 143.5

82 (C-4), 143.7 (C-1), 144.4 (C-1"), 145.4 (C-2), 155.7 (C-4"). High-resolution

83 electrospray ionization mass spectra (HR-ESI-MS, negative mode) of the final

product was detected at 243.1021, consistent with the calculated value of 243.1015 for

85 $C_{15}H_{15}O_3 [M-H]^-$.

86 2.3. Metabolism of hydroxy-BPA

The detailed static incubation was described in our previous study (Wang et al., 2013b). After static incubation of the flasks at 30 °C for 3 d, 100 μ L of 30 mM hydroxy-BPA in dimethyl sulfoxide (DMSO) were added to each flask (to a final hydroxy-BPA concentration of 0.3 mM), and the cultures were further incubated (in triplicate) for up to 7 d. The homogenization and analysis method were as described in our previous study (Wang et al., 2013b).

93 2.4. Identification of metabolites of hydroxy-BPA

94 Inoculated cultures (total 5 L PDB medium) of P. sordida YK-624 were prepared under the conditions described above. After static incubation at 30 °C for 3 d, 5 mL of 95 300 mM hydroxy-BPA (to a final concentration of 0.3 mM) was added to these 96 cultures. The cultures were further incubated for 7 d and evaporated to about 250 mL. 97 98 The remaining cultures were extracted 4 times with an equal amount of EtOAc. The 99 EtOAc extract was dried over anhydrous sodium sulfate and then evaporated to 100 dryness. The residue was separated by HPLC (column: C30-UG-15/30; 50 x 500 mm; 101 Nomura Chemical, Seto, Japan) using 70% MeOH to obtain 5 fractions. Each fraction was analyzed by thin-layer chromatography (TLC) and HPLC (column: C30-UG-5; 102 4.6 x 250 mm; Nomura Chemical, Seto, Japan). Silica gel plates (Merck F254; Merck, 103 104 Darmstadt, Germany) were used for analytical TLC. The metabolites were further separated by HPLC (column: C30-UG-5; 20 x 250 mm; Nomura Chemical, Seto, 105 106 Japan) using 70% MeOH. The purified metabolites were analyzed by HR-ESI-MS and NMR, including distorsionless enhancement by polarization transfer (DEPT), 107 108 correlated spectroscopy (COSY), heteronuclear multi quantum correlation (HMQC),

and heteronuclear multiple bond correlation (HMBC) experiments. The HR-ESI-MS
 data were measured using a JMS-T100LC mass spectrometer. ¹H-NMR spectra were
 recorded using a Jeol Lambda-500 spectrometer at 500 MHz, while ¹³C-NMR spectra
 were recorded on the same instrument at 125 MHz.

113 2.5. *Quantitative analysis*

114 After preculturing *P. sordida* YK-624 under the conditions described in our 115 previous study (Wang et al., 2013b) for 3 d, 100 μ L of 50 mM BPA in DMSO were 116 added to the cultures (to a final concentration of 0.5 mM BPA), which were then 117 further incubated for up to 7 d (each in triplicate). The homogenization and analysis 118 method were as described in our previous study (Wang et al., 2013b).

119 *2.6. Membrane estrogen receptor competition assay*

120 Cultures of HEK231 cells were stably transfected with a construct encoding the 121 membrane estrogen receptor (GPR30) and estrogen receptor competition studies were carried out as described before (Thomas and Dong, 2006). Briefly, human HEK293 cells stably 122 123 transfected with the human GPR30-encoding construct were cultured in Dulbecco's 124 modification of Eagle's medium/Ham's F-12 medium with 5% fetal bovine serum and 100 µg mL⁻¹ of G418 in 150-mm diameter plates. The medium was replaced every 1–2 d and the cells 125 that reached 80% confluence after 3 d were used in the experiments. 126 127 Procedures for the preparation of plasma membrane fractions and assay of steroid 128 membrane receptors were performed as previously described (Tokumoto et al., 2007) with the following modifications. We assayed [³H] estradiol (E₂) binding to plasma membranes of 129

- 130 HEK293 cells transfected with a construct encoding goldfish mPR α . Presoaking with Tween
- 131 80 was omitted. Radio-labeled steroid $[2, 4, 6, 7-{}^{3}H]E_{2}$ ($[{}^{3}H]E_{2}$, ~89 Ci mmol⁻¹) was
- 132 purchased from PerkinElmer Life & Analytical Sciences (Waltham, MA). The competitive
- binding assay tubes contained $[^{3}H]E_{2}$ at 4 nM and competitors at concentrations ranging from
- 134 1 nM to 10 μ M. After incubation (30 min, 4 °C) with the membrane fractions, the reaction
- 135 was stopped by filtration (Whatman GF/B filters). Following washing of the filters, bound
- 136 radioactivity was measured by scintillation counting. The displacement of $[^{3}H]E_{2}$ binding by
- 137 the competitors was expressed as a percentage of the maximum specific binding of E_2

3. Results

*3.1. Metabolism of hydroxy-BPA by P. sordida YK-624 under non-ligninolytic condition*PDB medium was used for assaying the metabolism of hydroxy-BPA. When *P. sordida*

140 YK-624 was cultured in PDB medium, hydroxy-BPA levels fell by 66% after 7 d of

141 incubation (Fig. 1). These results suggested that *P. sordida* YK-624 can metabolize

142 hydroxy-BPA under non-ligninolytic condition.

143 3.2. Identification of the metabolites from hydroxy-BPA

144 To determine the structure of metabolites produced during the transformation of 145 hydroxy-BPA, 7-day cultures of *P. sordida* YK-624 in PDB medium supplemented with 0.3 mM hydroxy-BPA were subjected to TLC and HPLC (Fig. 2); two major hydroxy-BPA 146 147 metabolites were detected. Using HR-ESI-MS, the molecular formula of compound I was 148 determined as $C_{16}H_{18}O_3$, with an m/z 281.1133[M+Na]⁺ (calculated for $C_{16}H_{18}NaO_3$, 281.1154). 149 This formula suggested that a hydroxy group of hydroxy-BPA had been converted to a methoxy 150 moiety. The proton and carbon spectra of compound I were roughly in accordance with the 151 hydroxy-BPA. Table 1 lists the chemical-shift assignment data for compound I. HMBC 152 correlations (2-OCH₃/C-2) confirmed that compound I was methylated hydroxy-BPA, 153 4-(2-(4-hydroxyphenyl)propan-2-yl)-2-methoxyphenol (methoxy-BPA, Fig. 3a). Using 154 HR-ESI-MS, the molecular formula of compound II was determined as $C_{17}H_{20}O_3$, with an m/z295.1289[M+Na]⁺ (calculated for $C_{17}H_{20}NaO_3$, 295.1310). This formula suggested that the 155 metabolite might be a dimethoxy form of hydroxy-BPA. The complete assignment of all the 156 157 protons and carbons was accomplished by DEPT, HMQC, COSY, and HMBC analyses (Table 2). HMBC correlations (3-OCH₃/C-3, 4-OCH₃/C-4) confirmed that compound II was 4-(2-(3, 158

159 4-dimethoxyphenyl)propan-2-yl)phenol (dimethoxy-BPA, Fig. 3b).

160 *3.3. Quantity of the metabolites from BPA by P. sordida YK-624 under non-ligninolytic*

161 *condition*

To evaluate the transformation of BPA by *P. sordida* YK-624 under non-ligninolytic condition, the time course of BPA metabolism was investigated (Fig. 4). Decreases in the concentration of BPA were detected starting at 2 d, with transformation to hydroxy-BPA.

- 165 Most of the decreased BPA was transformed to hydroxy-BPA, indicating hydroxy-BPA was
- 166 the main product. Subsequently, the methoxy-BPA and the dimethoxy-BPA forms were detected
- 167 at low concentrations starting at 4 d, with levels remaining at or below 4 μ M by 7 d.

168 3.4. Steroid binding assay of BPA and the metabolites

169 We evaluated the estrogenic activities of BPA metabolites using a steroid binding assay.

170 BPA exhibited significant competition with estrogen, with a IC_{50} of 1.8 μ M. Affinities of

- hydroxy-BPA, methoxy-BPA, and dimethoxy-BPA were reduced 67, 100, and 83%
- 172 (respectively) compared to that of the parent compound (Fig. 5).

4. Discussion

White-rot fungi are capable of degrading a wide variety of recalcitrant aromatic compounds, including polymeric lignin and environmentally persistent pollutants. In the present study, we synthesized the hydroxy-BPA and determined whether hydroxy-BPA was futher-metabolized by *P. sordida* YK-624 under non-ligninolytic condition. We detected two metabolites of hydroxy-BPA, methoxy-BPA and dimethoxy-BPA. To the best of our knowledge, this work represents the first identification of these two compounds as BPA metabolites.

180 Suspected effects of the disruption of endocrine function may include reduced fertility 181 and increased incidence of cancer in estrogen-responsive tissues (Colborn et al., 1993). The 182 primary concern for the biodegradation of an EDC is the loss of estrogenic activity. 183 Estrogenic activity of BPA was first reported in 1993 (Krishnan et al., 1993). In the present 184 study, just a few affinities for binding to the membrane estrogen receptor were detected in three BPA metabolites compared to the BPA (Fig. 5). Thus, the sequential hydroxylation and 185 186 methylation of BPA by P. sordida YK-624 is expected to diminish possible environmental 187 estrogenic activity of BPA.

188 As noted above, the metabolism of BPA has been studied by many researchers. Based on 189 the results of the present study, a novel metabolic pathway is proposed for BPA degradation 190 by P. sordida YK-624 under non-ligninolytic condition (Fig. 6). Firstly, we have observed the 191 generation of a BPA metabolite that was hydroxylated at the ortho-position of BPA (Wang et 192 al., 2013b). Under non-ligninolytic condition, no lignolytic enzymes activities in the culture of P. sordida YK-624 have been detected. The functional diversity of cytochrome P450s in 193 194 white-rot fungi has been studied because cytochrome P450s play an important role on 195 degrading a wide variety of recalcitrant aromatic compounds (Kamei et al., 2005; Xiao et al., 2011b; Wang et al., 2012a). Our previous study showed that the transformation of BPA was 196 inhibited by piperonyl butoxide, which is a common inhibitor of cytochrome P450s, under 197 198 non-ligninolytic conditions (Wang et al., 2013b). In the present study, hydroxy-BPA was further metabolized by this fungus, generating two additional methylated metabolites. 199 200 O-methyltransferases are involved in the biosynthesis of veratryl alcohol (VA), which is a 201 secondary metabolite produced by many species of white-rot fungi (Harper et al., 1996). 202 Identification of 3-O-methyltransferase and 4-O-methyltransferase activities has been 203 reported in P. chrysosporium (Coulter et al., 1993; Jeffers et al., 1997). Moreover, P. sordida 204 YK-624 also produces VA under ligninolytic and non-ligninolytic conditions (data not shown).

- 205 Hence, we hypothesize that these methylations of hydroxy-BPA are mediated in *P. sordida*
- 206 YK-624 by intracellular *O*-methyltransferases.
- 207 In this report, we showed that *P. sordida* YK-624 can metabolize BPA to yield
- 208 derivatives with reduced estrogenic activity. Based on the structures of these metabolites, the
- 209 proposed mechanism is as follows: BPA is monooxygenated (by cytochrome P450) to form
- 210 hydroxy-BPA; this primary metabolite then is methylated to generated two novel compounds,
- 211 methoxy-BPA and dimethoxy-BPA (Fig. 6). To the best of our knowledge, this work
- represents the first elucidation of the hydroxylation and then methylation of BPA by a
- 213 white-rot fungus.

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- Fig. 1 Time course of hydroxy-BPA metabolism by *P. sordida* YK-624 under non-ligninolytic condition. Values are the means±SD of triplicate samples.
- 294

Fig. 2 Detection of the hydroxy-BPA metabolites by HPLC. These compounds were assayed by HPLC under the following conditions: column, Wakosil-II 5C18HG; mobile phase, 10 to 100% MeOH gradient for 60 min; flow rate, 1 mL min⁻¹; detection wavelength, 277 nm. Guaiacylglycerol- β -guaiacyl ether as an internal standard was used for the determination.

- 300
- Fig. 3 COSY and HMBC correlations for the identified hydroxy-BPA metabolites.
- 302
- Fig. 4 Time course for BPA elimination and metabolic transformation by *P. sordida* YK-624 under non-ligninolytic condition. Values are the means±SD of triplicate samples.
- 305
- 306 Fig. 5 Competition by BPA and three metabolites for binding to the membrane estrogen receptor.
- 307 Samples were incubated with 4 nM [³H]E₂ and 10 µM competitor. Competition rate is normalized
- 308 as a percentage of the displacement of $[^{3}H]E_{2}$ binding by BPA. Values are the means±SD of
- 309 triplicate samples.
- 310
- 311 Fig. 6 Proposed metabolic pathway for BPA metabolism by *P. sordida* YK-624 under 312 non-ligninolytic condition.
- 313
- 314 Scheme 1 Synthesis of hydroxy-BPA.
- a) acetic anhydride, pyridine; b) methyltriphenyl-phosphonium bromide, *n*-butyllithium, 0 °C;
- c) tetrahydrofuran, 0 °C to RT; d) benzene/MeOH, 36% HCl.
- 317
- 318







4-(2-(4-hydroxyphenyl)propan-2-yl)-2-methoxyphenol (methoxy-BPA)



4-(2-(3,4-dimethoxyphenyl)propan-2-yl)phenol (dimethoxy-BPA)





 Fig. 4







Scheme 1

Position	$^{1}\mathrm{H}$	¹³ C
	$\delta_{\rm H}$ (mult, J in Hz)	d
1	-	143.4
2	-	146.0
3	6.65 (d, 2.0)	110.0
4	-	143.1
5	6.76 (dd, 10.0, 2.0)	119.3
6	6.80 (d, 8.0)	113.6
2-OCH ₃	3.76 (s)	55.9
1″	-	143.1
2''	7.07 (d, 9.0)	127.9
3"	6.72 (d, 9.0)	114.7
4''	-	153.4
5''	6.72 (d, 9.0)	114.7
6″	7.07 (d, 9.0)	127.9
1'-CH ₃	1.61 (s)	31.1
2'	-	42.0
3'-CH ₃	1.61 (s)	31.1

Table 1 ¹H- and ¹³C-NMR data for compound I (methoxy-BPA) (in CDCl₃)

Position	$^{1}\mathrm{H}$	¹³ C
	$\delta_{\rm H}$ (mult, <i>J</i> in Hz)	d
1	-	145.6
2	6.70	112.7
3	-	149.8
4	-	148.3
5	6.79	112.5
6	6.79	119.9
3-OCH ₃	3.67 (s)	56.5
4-OCH ₃	3.75 (s)	56.4
1″	-	143.0
2''	7.02 (dd, 6.5, 2.0)	128.7
3"	6.66 (dd, 6.0, 2.0)	115.6
4″	-	156.0
5″	6.66 (dd, 6.0, 2.0)	115.6
6''	7.02 (dd, 6.5, 2.0)	128.7
1′-CH ₃	1.58 (s)	31.6
2'	-	42.9
3'-CH ₃	1.58 (s)	31.6

Table 2 ¹H- and ¹³C-NMR data for compound II (dimethoxy-BPA) (in CD₃OD)