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著者	Wang Jianqiao, Yamada Yuto, Notake Akira, Todoroki Yasushi, Tokumoto Toshinobu, Dong Jing, Thomas Peter, Hirai Hirofumi, Kawagishi Hirokazu
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Research papers

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

Jianqiao Wang[a], Yuto Yamada[b], Akira Notake[b] , Yasushi Todoroki[b, c], Toshinobu Tokumoto[a], Jing Dong[d], Peter Thomas[d], Hirofumi Hirai[b, c*], Hirokazu Kawagishi[a, b, c]

[a] Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

[b] Graduate School of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

[c] Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

[d] Marine Science Institute, University of Texas at Austin, 750 Channel View Drive, Port Aransas, TX 78373, USA

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

* Corresponding author: Hirofumi Hirai
Mailing address: Graduate School of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan.
Tel. & Fax: +81 54 238 4853
E-mail address: ahhirai@ipc.shizuoka.ac.jp

Abstract

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

1. Introduction

Endocrine disrupting compounds (EDCs) alter the function of the endocrine system of both wildlife and humans (Colborn et al., 1993). Bisphenol A (2,2-bis(4-hydroxyphenyl)propane; BPA), is an industrial chemical synthesized by condensation of two phenol groups and on acetone molecule. While BPA was first synthesized in 1891, it is used in many consumer products, including lacquers applied as food-can linings and dental composite fillings and sealants (Brotons et al., 1995; Olea et al., 1996). The global demand for BPA is predicted to grow from 3.9 million tons (in 2006) to about 5 million tons in 2010 (Ballesteros-Gómez et al., 2009). Due to the use of bisphenol A in the manufacture of many products, BPA often has been detected in many soil and water environments (Kitada et al., 2008; Matsumura et al., 2009).

A white-rot fungi have the unique ability to degrade lignin to the level of CO_2 (Kirk and Farrell, 1987), and the white-rot fungi have been shown to degrade recalcitrant persistent toxic organic chemicals, such as polychlorinated dibenzodioxin (Kamei et al., 2005), lindene (Bumpus et al., 1985), dieldrin (Xiao et al., 2011a), benzo[*a*]pyrene (Hadibarata and Kristanti, 2012), mycotoxin aflatoxin B_1 (Wang et al., 2011), and the neonicotinoid insecticide acetamiprid (Wang et al., 2012a).

Up until now, biotransformation of BPA also has been reported by many researchers. In rat hepatocytes, BPA glucuronide is the major metabolite of BPA (Nakagawa and Tayama, 2000). In plants, cells of *Eucalyptus perriniana* and *Portulaca oleracea* cv. metabolize BPA to its hydroxylated products (Hamada et al., 2002; Watanabe et al., 2012). Proposed pathways for bacterial degradation of BPA were summarized by Zhang et al. (2013). Another possible 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 BPA-*O*-β-D-glucopyranoside by *Aspergillus fumigatus* (Yim et al., 2003). Ligninolytic enzymes generate BPA phenoxy radicals, resulting in radical couplings and polymerization (Tsutsumi et al., 2001; Wang et al., 2012b; Wang et al., 2013a). Unfortunately, these BPA dimers can be decomposed by various reactions to yield toxic BPA. Therefore, effective BPA elimination requires processes distinct from BPA dimerization.

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 (hydroxy-BPA) as the primary metabolite (Wang et al., 2013b). In the present study, the further degradation of the hydroxy-BPA by *P. sordida* YK-624 was determined to clarify the metabolism of BPA by *P. sordida* YK-624 under non-ligninolytic condition.

2. Materials and methods

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 purification.

2.2. Synthesis of hydroxy-BPA

As shown in Scheme 1, the synthesis of hydroxy-BPA commenced from the acetylation reaction of *p*-hydroxyacetophenone. *p*-Hydroxyacetophenone (5 g) was stirred with 25 mL pyridine until fully dissolved. After adding 12.5 mL acetic anhydride, the reaction mixture was stirred for 16 h at room temperature. The solution then was extracted with ethyl acetate (EtOAc). The organic layer was washed with H_2O, and then purified by silica gel column chromatography with 30% EtOAc in hexane to obtain 4-acetoxyacetophenone (6 g, 33.6 mmol). The methylenation of 4-acetoxyacetophenone was achieved by a Witting reation with methyltriphenyl-phosphonium bromide. Methyltriphenyl-phosphonium bromide (21.05 g) in 227.6 mL anhydrous tetrahydrofuran was added dropwise, under argon, at 0 °C in the presence of 35 mL of *n*-butyllithium. The solution turned orange as the addition proceeded. After stirring at 0 °C for an additional half hour, a solution of 5.69 g 4-acetylphenyl acetate in 75.68 mL anhydrous tetrahydrofuran was added dropwise, the resulting mixture was warmed to room temperature and stirred for 36 h. The solution then was extracted with EtOAc. The organic layer was washed with H_2O, and then purified by silica gel column chromatography with a 10% EtOAc in hexane to obtain 4-isopropenylphenol (1.73 g, 12.89 mmol). Finally, the hydroxyl-BPA was formed by the reaction of 4-isopropenylphenol and catechol. Catechol (1.67 g), 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 (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 EtOAc. The organic layer was washed with brine, and then purified by silica gel

column chromatography with a 20% EtOAc in hexane to obtain hydroxy-BPA (1.72 g, 7.05 mmol, 20.21%). ^{1}H-nuclear magnetic resonance (NMR, 500 MHz, CD_3OD) yielded the following parameters: δ 1.54 (s, OMe), 6.57 (dd, H-5), 6.62 (d, H-3), 6.66 (dd, H-6, 3″, 5″), 7.02 (dd, H-2″, 6″), ^{13}C-NMR (500 MHz, CD_3OD): δ 31.6 (OMe), 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 (C-4), 143.7 (C-1), 144.4 (C-1″), 145.4 (C-2), 155.7 (C-4″). High-resolution 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 $C_{15}H_{15}O_3$ $[M-H]^-$.

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 μL 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).

2.4. Identification of metabolites of hydroxy-BPA

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 300 mM hydroxy-BPA (to a final concentration of 0.3 mM) was added to these cultures. The cultures were further incubated for 7 d and evaporated to about 250 mL. The remaining cultures were extracted 4 times with an equal amount of EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was separated by HPLC (column: C30-UG-15/30; 50 x 500 mm; 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; 4.6 x 250 mm; Nomura Chemical, Seto, Japan). Silica gel plates (Merck F254; Merck, 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, Japan) using 70% MeOH. The purified metabolites were analyzed by HR-ESI-MS and NMR, including distorsionless enhancement by polarization transfer (DEPT), 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. ^{1}H-NMR spectra were recorded using a Jeol Lambda-500 spectrometer at 500 MHz, while ^{13}C-NMR spectra were recorded on the same instrument at 125 MHz.

2.5. Quantitative analysis

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

2.6. Membrane estrogen receptor competition assay

Cultures of HEK231 cells were stably transfected with a construct encoding the membrane estrogen receptor (GPR30) and estrogen receptor competition studies were carried out as described before (Thomas and Dong, 2006). Briefly, human HEK293 cells stably transfected with the human GPR30-encoding construct were cultured in Dulbecco's 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 that reached 80% confluence after 3 d were used in the experiments.

Procedures for the preparation of plasma membrane fractions and assay of steroid membrane receptors were performed as previously described (Tokumoto et al., 2007) with the following modifications. We assayed [^{3}H] estradiol (E_2) binding to plasma membranes of HEK293 cells transfected with a construct encoding goldfish mPRα. Presoaking with Tween 80 was omitted. Radio-labeled steroid [2, 4, 6, 7-^{3}H]E_2 ([^{3}H]E_2, ~89 Ci mmol^{-1}) was 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 1 nM to 10 μM. After incubation (30 min, 4 °C) with the membrane fractions, the reaction was stopped by filtration (Whatman GF/B filters). Following washing of the filters, bound radioactivity was measured by scintillation counting. The displacement of [^{3}H]E_2 binding by 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*

YK-624 was cultured in PDB medium, hydroxy-BPA levels fell by 66% after 7 d of incubation (Fig. 1). These results suggested that *P. sordida* YK-624 can metabolize hydroxy-BPA under non-ligninolytic condition.

3.2. Identification of the metabolites from hydroxy-BPA

To determine the structure of metabolites produced during the transformation of 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 metabolites were detected. Using HR-ESI-MS, the molecular formula of compound I was 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). This formula suggested that a hydroxy group of hydroxy-BPA had been converted to a methoxy moiety. The proton and carbon spectra of compound I were roughly in accordance with the hydroxy-BPA. Table 1 lists the chemical-shift assignment data for compound I. HMBC correlations (2-OCH_3/C-2) confirmed that compound I was methylated hydroxy-BPA, 4-(2-(4-hydroxyphenyl)propan-2-yl)-2-methoxyphenol (methoxy-BPA, Fig. 3a). Using HR-ESI-MS, the molecular formula of compound II was determined as $C_{17}H_{20}O_3$, with an *m/z* 295.1289$[M+Na]^+$ (calculated for $C_{17}H_{20}NaO_3$, 295.1310). This formula suggested that the metabolite might be a dimethoxy form of hydroxy-BPA. The complete assignment of all the protons and carbons was accomplished by DEPT, HMQC, COSY, and HMBC analyses (Table 2). HMBC correlations (3-OCH_3/C-3, 4-OCH_3/C-4) confirmed that compound II was 4-(2-(3, 4-dimethoxyphenyl)propan-2-yl)phenol (dimethoxy-BPA, Fig. 3b).

3.3. Quantity of the metabolites from BPA by P. sordida YK-624 under non-ligninolytic 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. Most of the decreased BPA was transformed to hydroxy-BPA, indicating hydroxy-BPA was the main product. Subsequently, the methoxy-BPA and the dimethoxy-BPA forms were detected at low concentrations starting at 4 d, with levels remaining at or below 4 μM by 7 d.

3.4. Steroid binding assay of BPA and the metabolites

We evaluated the estrogenic activities of BPA metabolites using a steroid binding assay. BPA exhibited significant competition with estrogen, with a IC_{50} of 1.8 μM. Affinities of

hydroxy-BPA, methoxy-BPA, and dimethoxy-BPA were reduced 67, 100, and 83% (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.

Suspected effects of the disruption of endocrine function may include reduced fertility and increased incidence of cancer in estrogen-responsive tissues (Colborn et al., 1993). The primary concern for the biodegradation of an EDC is the loss of estrogenic activity. Estrogenic activity of BPA was first reported in 1993 (Krishnan et al., 1993). In the present 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 methylation of BPA by *P. sordida* YK-624 is expected to diminish possible environmental estrogenic activity of BPA.

As noted above, the metabolism of BPA has been studied by many researchers. Based on the results of the present study, a novel metabolic pathway is proposed for BPA degradation by *P. sordida* YK-624 under non-ligninolytic condition (Fig. 6). Firstly, we have observed the generation of a BPA metabolite that was hydroxylated at the *ortho*-position of BPA (Wang et 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 white-rot fungi has been studied because cytochrome P450s play an important role on 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 inhibited by piperonyl butoxide, which is a common inhibitor of cytochrome P450s, under non-ligninolytic conditions (Wang et al., 2013b). In the present study, hydroxy-BPA was further metabolized by this fungus, generating two additional methylated metabolites. *O*-methyltransferases are involved in the biosynthesis of veratryl alcohol (VA), which is a secondary metabolite produced by many species of white-rot fungi (Harper et al., 1996). Identification of 3-*O*-methyltransferase and 4-*O*-methyltransferase activities has been reported in *P. chrysosporium* (Coulter et al., 1993; Jeffers et al., 1997). Moreover, *P. sordida*

YK-624 also produces VA under ligninolytic and non-ligninolytic conditions (data not shown). Hence, we hypothesize that these methylations of hydroxy-BPA are mediated in *P. sordida* YK-624 by intracellular *O*-methyltransferases.

In this report, we showed that *P. sordida* YK-624 can metabolize BPA to yield derivatives with reduced estrogenic activity. Based on the structures of these metabolites, the proposed mechanism is as follows: BPA is monooxygenated (by cytochrome P450) to form hydroxy-BPA; this primary metabolite then is methylated to generated two novel compounds, 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 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.

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.

Fig. 3 COSY and HMBC correlations for the identified hydroxy-BPA metabolites.

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.

Fig. 5 Competition by BPA and three metabolites for binding to the membrane estrogen receptor. Samples were incubated with 4 nM [^{3}H]E_2 and 10 μM competitor. Competition rate is normalized as a percentage of the displacement of [^{3}H]E_2 binding by BPA. Values are the means±SD of triplicate samples.

Fig. 6 Proposed metabolic pathway for BPA metabolism by *P. sordida* YK-624 under non-ligninolytic condition.

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.

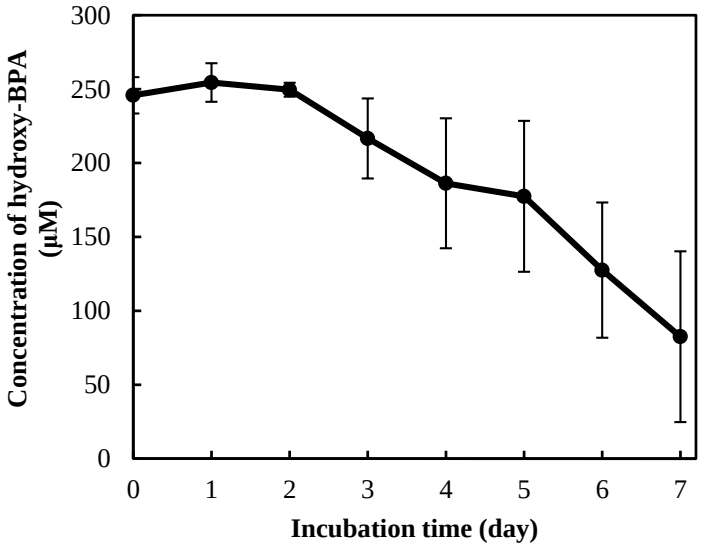

Concentration of hydroxy-BPA (μM)
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
Incubation time (day)

Fig. 1

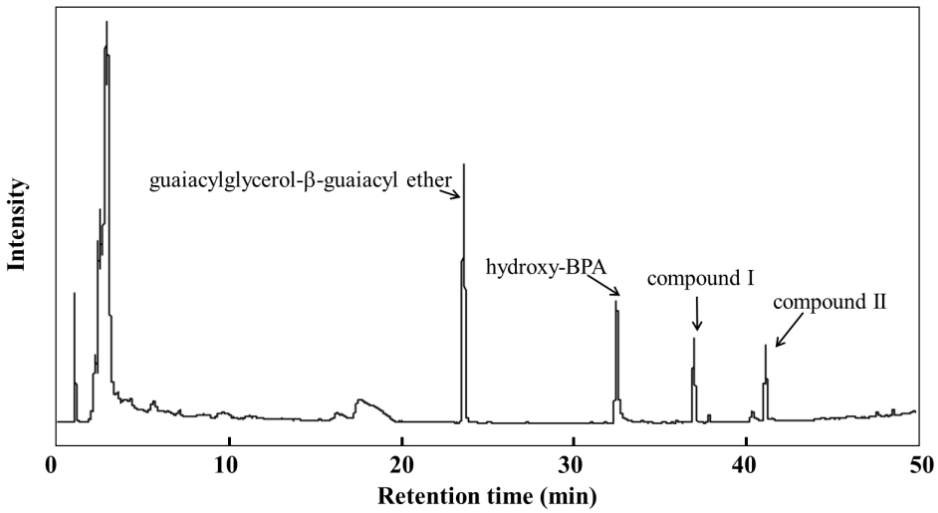
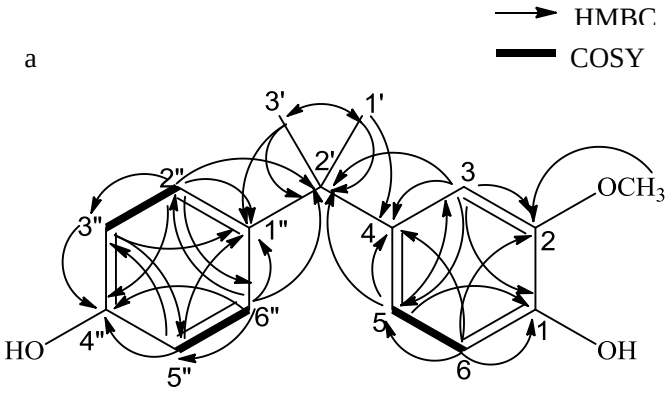

guaiacylglycerol-β-guaiacyl ether
hydroxy-BPA
compound I
compound II
Intensity
0
10
20
30
40
50
Retention time (min)

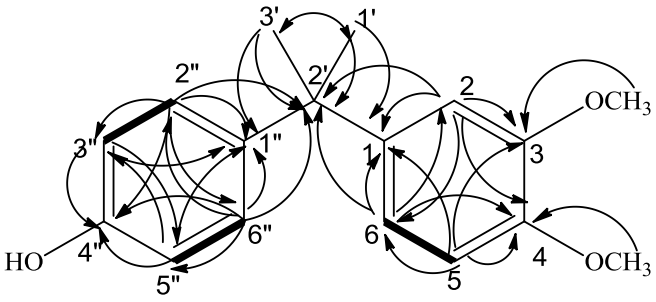
Fig. 2

a



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

b



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

Fig. 3

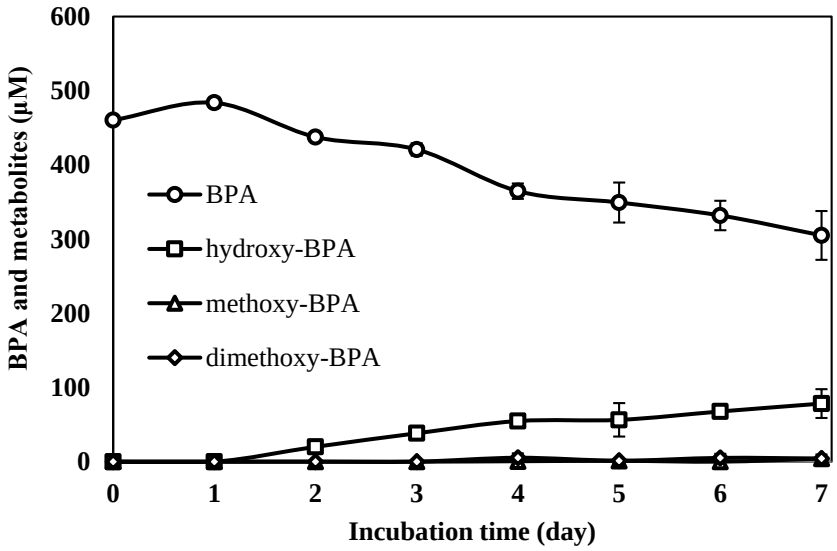

600
500
400
300
200
100
0
BPA and metabolites (μM)
BPA
hydroxy-BPA
methoxy-BPA
dimethoxy-BPA
0
1
2
3
4
5
6
7
Incubation time (day)

Fig. 4

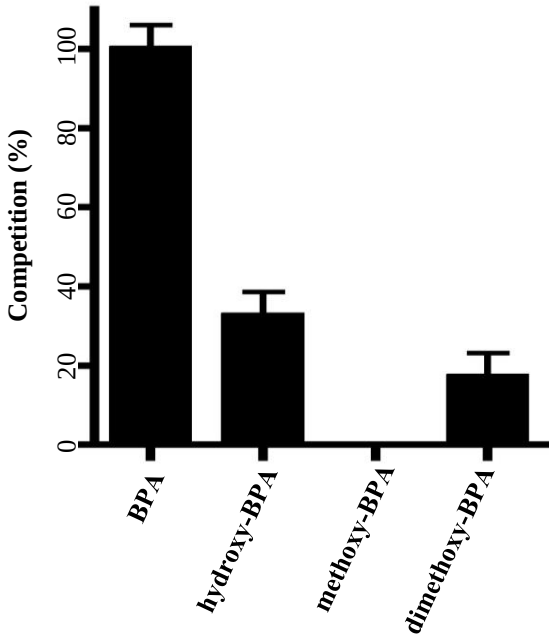

Competition (%)
0
20
40
60
80
100
BPA
hydroxy-BPA
methoxy-BPA
dimethoxy-BPA

Fig. 5

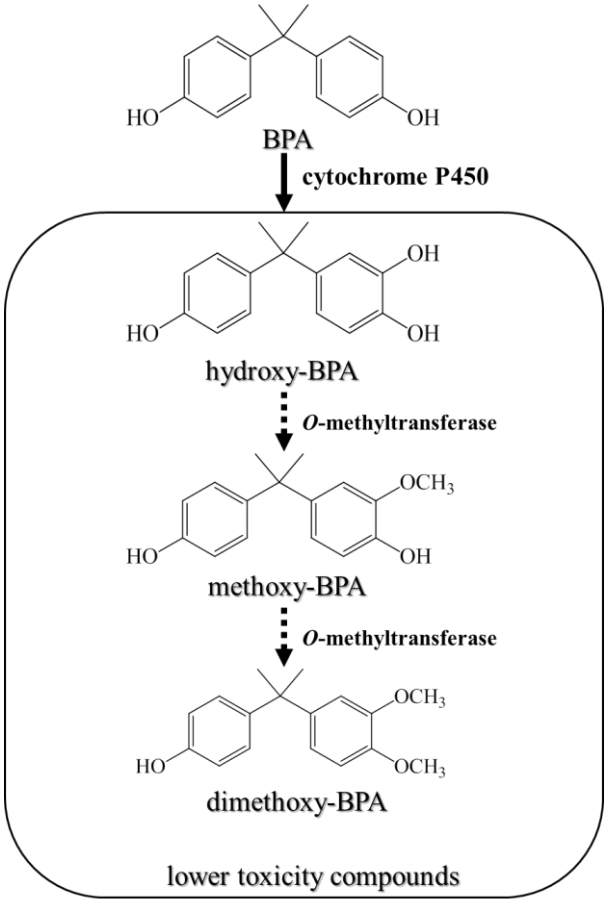
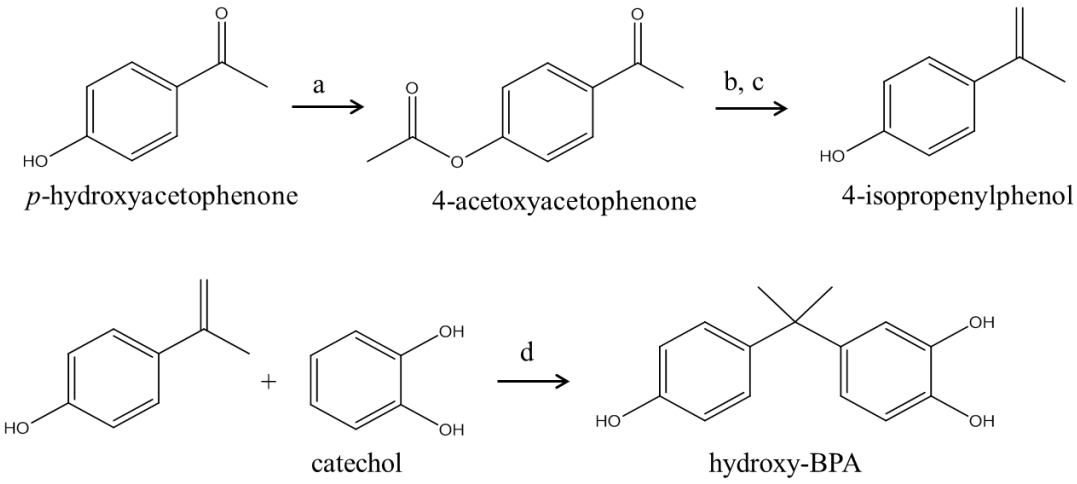

HO
OH
BPA
cytochrome P450
OH
HO
OH
hydroxy-BPA
O-methyltransferase
OCH3
HO
OH
methoxy-BPA
O-methyltransferase
OCH3
HO
OCH3
dimethoxy-BPA
lower toxicity compounds

Fig. 6



p-hydroxyacetophenone 4-acetoxyacetophenone 4-isopropenylphenol

catechol hydroxy-BPA

Scheme 1

Table 1 ^{1}H- and ^{13}C-NMR data for compound I (methoxy-BPA) (in $CDCl_3$)

Position	^{1}H	^{13}C
	δ_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 2 ^{1}H- and^{13}C-NMR data for compound II (dimethoxy-BPA) (in CD_3OD)

Position	^{1}H	^{13}C
	δ_H (mult, *J* 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