

Dimerization of Bisphenol A by Hyper
Lignin-Degrading Fungus *Phanerochaete sordida*
YK-624 Under Ligninolytic Condition

メタデータ	言語: eng 出版者: 公開日: 2014-06-13 キーワード (Ja): キーワード (En): 作成者: Wang, Jianqiao, Yamamoto, Yotaro, Hirai, Hirofumi, Kawagishi, Hirokazu メールアドレス: 所属:
URL	http://hdl.handle.net/10297/7836

1 Original Paper

2

3 **Dimerization of Bisphenol A by hyper lignin-degrading fungus *Phanerochaete***
4 ***sordida* YK-624 under ligninolytic condition**

5 Jianqiao Wang¹, Yotaro Yamamoto², Hirofumi Hirai^{2*}, Hirokazu Kawagishi^{1,2}

6

7 ¹ Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku,
8 Shizuoka 422-8529, Japan

9 ² Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836
10 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

11

12 Running title: Dimerization of BPA

13

14 * Corresponding author: Hirofumi Hirai

15 Mailing address: Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka
16 University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan.

17 Tel. & Fax: +81 54 238 4853

18 *E-mail address:* ahhirai@ipc.shizuoka.ac.jp

Dimerization of Bisphenol A by hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 under ligninolytic condition

1 Bisphenol A (BPA) was treated with hyper lignin-degrading fungus *Phanerochaete sordida*
2 YK-624 under ligninolytic condition. After preculturing *P. sordida* YK-624 for 4 days, BPA (final
3 concentration, 0.1 mM and 1.0 mM) was added to cultures. Both 1 mM and 0.1 mM BPA were
4 effectively decreased within a 24 h treatment and two metabolites were detected. Two metabolites
5 (5,5'-bis-[1-(4-hydroxy-phenyl)1-methyl-ethyl]-biphenyl-2,2'-diol and 4-(2-(4-hydroxy- phenyl)
6 propan-2-yl)-2-(4-(2-(4-hydroxyphenyl) propan-2-yl) phenoxy)phenol) were identified by ESI-MS
7 and NMR analysis. These results indicated that BPA was oxidized to BPA phenoxy radicals by
8 ligninolytic enzymes and then dimerized at extracellular region.

1 **Introduction**

2 Endocrine-disrupting compounds (EDCs) are organic compounds widely used, which are
3 ubiquitous in the environment. Many synthetic chemicals released into the environment, such as
4 plasticizers, pesticides, antifouling paints, raw materials for polymer and hormonal
5 pharmaceuticals, have been identified to be potent EDCs and their environmental occurrence and
6 ecotoxicological effects are being actively studied in recent years [4, 11]. One of the
7 representative compounds of the EDCs group is bisphenol A (2,2-bis(4-hydroxyphenyl)propane;
8 BPA). BPA, a monomer component of polycarbonate plastics, is used in many consumer products,
9 including lacquers applied as food-can linings and dental composite fillings and sealants [3, 14].
10 Many countries throughout the world have large production capacities for BPA, especially
11 Germany, the Netherlands, the USA and Japan. BPA is one of the highest volume chemicals
12 produced worldwide, with an estimated production of 5 million tons in 2010 [1]. As a result, BPA
13 is often detected in many soil and water environments [10, 12].

14 Lignin-degrading white-rot fungi, which have the unique ability to degrade lignin to the
15 level of CO₂ [9], and their ligninolytic enzymes have also attracted interest for the
16 biotransformation of contaminants because of their industrial potential for degrading and
17 detoxifying recalcitrant environmental pollutants such as aflatoxin [24], neonicotinoid insecticides
18 [22], polycyclic aromatic hydrocarbons [2, 6], and dyes [15].

19 In recent years, the degradation of EDCs by ligninolytic enzymes has attracted the attention
20 of many researchers [8, 16, 18, 19]. More recently, we also reported that the removal of BPA by
21 lignin peroxidase from hyper lignin-degrading fungus *P. sordida* YK-624 [23]. However,
22 degradation of BPA using these fungi is also limited [5, 17] and the detail mechanisms are still
23 unknown. In the present study, we examined the removal of BPA by *P. sordida* YK-624 under
24 ligninolytic condition. We also detected the metabolites from BPA and proposed a metabolic
25 pathway for the metabolism of BPA by *P. sordida* YK-624.

26

27 **Materials and methods**

28 **Fungi.**

29 *P. sordida* YK-624 (ATCC 90872), which has been isolated from rotted wood, was used in
30 the present study [7]. The fungus was maintained on potato dextrose agar (PDA) slants at 4°C.

31 **Chemicals.**

32 BPA was purchased from Alfa Aesar, A Johnson Matthey Company (Lancashire, United
33 Kingdom). All other chemicals were analytical pure grade and were used without further
34 purification.

1 **Elimination experiments.**

2 Nitrogen-limited (NL) medium described by Tien and Kirk [20] was used for BPA
3 elimination experiments. The fungus was incubated on a PDA plate at 30°C for 3 days, and then
4 10 mm-diameter disks were punched out from the growing edge of mycelium. Two disks were
5 each placed into a 100-mL Erlenmeyer flask containing 10 mL of NL medium. After statically
6 incubating the flasks at 30°C for 4 days, 100 µL of 10 or 100 mM BPA/DMSO were added to the
7 cultures (final concentration at 0.1 or 1 mM, respectively), and then further incubated for 24, 48,
8 72 hours (each in triplicate). The culture was filtrated with a 0.2-µm membrane filter, and the
9 filtrate was then subjected to high-performance liquid chromatography (HPLC) for the
10 quantification of BPA under the following conditions: column, Wakosil-II 5C18HG (4.6 x 150
11 mm; Wako Pure Chemical Industries); mobile phase, 60% MeOH in 1% acetic acid aq.; flow rate,
12 1 mL/min; and UV wavelength, 277 nm.

13 **Metabolism experiment.**

14 Inoculated cultures (1 L of NL medium) of *P. sordida* YK-624 were prepared under the
15 conditions described above. After static incubation at 30 °C for 4 days, BPA (final concentration,
16 1 mM) as added to these cultures. The cultures were further incubated for 24 hours and then
17 homogenized 2 times by Waring blender for 30 seconds with low speed. The cell suspensions
18 were extracted 3 times with equal amount of ethyl acetate (EtOAc). The EtOAc extract was dried
19 over anhydrous sodium sulfate and then evaporated to dryness. The residue was cleaned on a silica
20 gel chromatography (silica gel 60N, φ40 × 600 mm) and eluted with
21 dichloromethane/acetone/MeOH (9/1/0, 8/2/0, 5/5/0, 0/7/3, 0/5/5, and 0/0/10; vol/vol/vol) to
22 obtain 11 fractions. Each fraction was analyzed by thin-layer chromatography (TLC). Silica gel
23 plates (Merck F254; Merck, Darmstadt, Germany) were used for analytical TLC. The metabolite
24 was further separated by HPLC (column: C18; Wakosil-II 5C18HG Prep, 50 × 250 mm, Wako
25 Pure Chemicals, Japan) using 60% MeOH. The purified metabolite was analyzed by HR-ESI-MS
26 and NMR, including DEPT, COSY, HMQC, and HMBC experiments. The HR-ESI-MS data were
27 measured using a JMS-T100LC mass spectrometer. ¹H-NMR spectra were recorded using a Jeol
28 Lambda-500 spectrometer at 500 MHz, while ¹³C-NMR spectra were recorded on the same
29 instrument at 125 MHz.

30

31 **Results and Discussion**

32 We have previously investigated the removal of BPA by lignin peroxidase from hyper
33 lignin-degrading fungus *P. sordida* YK-624 [22]. In the present study, NL media were used for the
34 elimination experiment of BPA. After preculturing by *P. sordida* YK-624, BPA (final

1 concentration, 1 mM or 0.1 mM) was added to cultures. After a 24 h treatment, 60% or 100% of
2 BPA were eliminated, respectively (Fig. 1).

3 The removal of EDCs' estrogenic activities by ligninolytic enzymes from white-rot fungi
4 has been reported [18, 19, 22]. However, there have been no studies focusing on the metabolic
5 product of these ECDs by fungi. Therefore, we attempted to identify the metabolites of BPA, and
6 two metabolites were detected in the analysis of TLC and HPLC (data not shown). Molecular
7 formula of compound 1 was determined as C₃₀H₃₀O₄ by HR-ESI-MS, *m/z* 453.2062[M-H]⁻(calcd.
8 for C₃₀H₂₉O₄, 453.2022). This formula suggested that the metabolite might be a dimer of BPA.
9 The complete assignment of all the protons and carbons was accomplished by DEPT, HMQC,
10 COSY and HMBC experiments. The structure of compound 1 was identified as
11 5,5'-bis-[1-(4-hydroxy-phenyl)1-methyl-ethyl]-biphenyl-2,2'-diol (Fig. 2a). Compound 1 was the
12 same as the metabolite which has been detected by the treatment of BPA by lignin peroxidase
13 from hyper lignin-degrading fungus *P. sordida* YK-624, and we have demonstrated that the
14 estrogenic activity of the metabolite was lower than that of BPA [22]. Molecular formula of
15 compound 2 was the same to compound 1. The complete assignment of all the protons and
16 carbons was accomplished by DEPT, HMQC, COSY and HMBC experiments. The DEPT
17 experiment indicated the presence of 4 methyls, 9 methylenes, and 11 quaternary carbons (data not
18 shown). Compound 2 also had 1,2,4-trisubstituted phenyl group and 1,4-substituted group. The
19 complete structure was determined by interpreting HMBC correlations (Fig. 2b) (H-1''',
20 3'''/phenol C-4', C-2'', hydroxyphenyl C-1', H-1'', 3'''/phenol C-4, C-2'', hydroxyphenyl C-1),
21 and the connection to phenol C-1 was not determined. The structure of compound 2 was identified
22 as 4-(2-(4-hydroxy-phenyl)propan-2-yl)-2-(4-(2-(4-hydroxyphenyl)propan-2-yl)
23 phenoxy)phenol. This is the first report that compound 2 was chemically identified. We have
24 assayed the formation of compound I and II by HPLC. However, trace amounts of two metabolites
25 have detected. This result suggests that further radical coupling would occur during the fungal
26 treatments. Tsutsumi et al. have reported that BPA tetramers were formed by manganese
27 peroxidase treatment (21). Similarly, it is thought that BPA tetramers are formed as main products
28 by fungal treatment, following the dimerization of BPA.

29 The estrogenic activity of more than 500 chemicals were tested by the yeast two-hybrid
30 system (13), deducing that most of the compounds which have a phenol ring with substitution of a
31 bulky moiety at the *ortho*-position reduces the activity. We propose that the estrogenic activity of
32 two metabolites should be reduced because the radical coupling occurred at the *ortho*-position.

33 Compound 1 was completely disappeared after 48 h treatment under ligninolytic condition
34 (data not shown). It is possible that compound II is also removed under the condition. Furthermore,

1 a proteomic differential display technique was utilized to study cellular responses of this fungus
2 exposed to BPA, and both BPA and BPA polymers were not incorporated into intracellular region
3 under ligninolytic condition since proteome map of cytosolic fraction of this fungus grown in the
4 presence of BPA is same as that grown in the absence of BPA (data not shown). Therefore, we
5 propose that BPA is oxidized to these phenoxy radicals by ligninolytic enzymes, and BPA dimers
6 are formed by radical couplings at extracellular region without the incorporation into the cell of *P.*
7 *sordida* YK-624. Moreover, BPA dimer is converted to BPA tetramer, and then, the toxicity is
8 decreased by the polymerization of BPA.

1 **References**

2

- 3 1 Ballesteros-Gómez A, Rubio S & Pérez-Bendito D (2009) Analytical methods for the
4 determination of bisphenol A in food. *J Chromatogr A* **1216**: 449–469.
- 5 2 Bezalel L, Hadar Y, Fu PP, Freeman JP & Cerniglia CE (1996) Initial Oxidation products in
6 the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by the white rot
7 fungus *Pleurotus ostreatus*. *Appl Environ Microbiol* **62**: 2554–2559.
- 8 3 Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V & Olea N (1995) Xenoestrogens
9 released from lacquer coatings in food cans. *Environ Health Perspect* **103**: 608–612.
- 10 4 Campbell CG, Borglin SE, Green FB, Grayson A, Wozel E & Stringfellow WT (2006)
11 Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine
12 disrupting compounds in water. *Chemosphere* **65**: 1265–1280.
- 13 5 Chai W, Handa Y, Suzuki M, Saito M, Kato N & Horiuchi CA (2005) Biodegradation of
14 bisphenol A by fungi. *Appl Biochem Biotechnol* **120**: 175–182.
- 15 6 Collins PJ, Kotterman M, Field JA & Dobson A (1996) Oxidation of anthracene and
16 benzo[a]pyrene by laccases from *Trametes versicolor*. *Appl Environ Microbiol* **62**: 4563–
17 4567.
- 18 7 Hirai H, Kondo R & Sakai K (1994) Screening of lignin-degrading fungi and their
19 ligninolytic enzyme activities during biological bleaching of kraft pulp. *Mokuzai Gakkaishi*
20 **40**: 980–986.
- 21 8 Hirano T, Honda Y, Watanabe T & Kuwahara M (2000) Degradation of bisphenol A by the
22 lignin-degrading enzyme, manganese peroxidase, produced by the white-rot basidiomycete,
23 *Pleurotus ostreatus*. *Biosci Biotechnol Biochem* **64**:1958–1962.
- 24 9 Kirk TK & Farrell RL (1987) Enzymatic “combustion”: the microbial degradation of lignin.
25 *Annu Rev Microbiol* **41**: 465–505.
- 26 10 Kitada Y, Kawahata H, Suzuki A & Oomori T (2008) Distribution of pesticides and bisphenol
27 A in sediments collected from rivers adjacent to coral reefs. *Chemosphere* **71**: 2082–2090.
- 28 11 Matozzo V, Gagne F, Marin MG, Ricciardi F & Blaise C (2008) Vitellogenin as a biomarker
29 of exposure to estrogenic compounds in aquatic invertebrates: a review. *Environ Int* **34**: 531–
30 545.
- 31 12 Matsumura Y, Hosokawa C, Sasaki-Mori M, Akahira A, Fukunaga K, Ikeuchi T, Oshiman K
32 & Tsuchido T (2009) Isolation and characterization of novel bisphenol-A--degrading bacteria
33 from soils. *Biocontrol Sci* **14**: 161–169.
- 34 13 Nishihara T, Nishikawa J, Kanayama T, Dakeyama F, Saito K, Imagawa M, Takatori S,

- 1 Kitagawa Y, Hori S & Utsumic H (2000) Estrogenic activities of 517 chemicals by yeast
2 two-hybrid assay. *J Health Sci* **46**: 282–298.
- 3 14 Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM
4 & Sonnenschein C (1996) Estrogenicity of resin-based composites and sealants used in
5 dentistry. *Environ Health Perspect* **104**: 298–305.
- 6 15 Ollikka P, Alhonmäki K, Leppänen VM, Glumoff T, Rajola T & Suominen I (1993)
7 Decolorization of azo, triphenyl methane, heterocyclic, and polymeric dyes by lignin
8 peroxidase isoenzymes from *Phanerochaete chrysosporium*. *Appl Environ Microbiol* **59**:
9 4010–4016.
- 10 16 Saito T, Kato K, Yokogawa Y, Nishida M & Yamashita N (2004) Detoxification of bisphenol
11 A and nonylphenol by purified extracellular laccase from a fungus isolated from soil. *J Biosci*
12 *Bioeng* **98**: 64–66.
- 13 17 Shin EH, Choi HT & Song HG (2007) Biodegradation of endocrine-disrupting bisphenol A
14 by white rot fungus *Irpex lacteus*. *J Microbiol Biotechnol* **17**:1147–1151.
- 15 18 Suzuki K, Hirai H, Murata H & Nishida T (2003) Removal of estrogenic activities of
16 17 β -estradiol and ethinylestradiol by ligninolytic enzymes from white rot fungi. *Water Res* **37**:
17 1972–1975.
- 18 19 Tamagawa Y, Yamaki R, Hirai H, Kawai S & Nishida T (2006) Removal of estrogenic
19 activity of natural steroidal hormone estrone by ligninolytic enzymes from white rot fungi.
20 *Chemosphere* **65**: 97–101.
- 21 20 Tien M & Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods*
22 *Enzymol* **161B**: 238–249.
- 23 21 Tsutsumi Y, Haneda T & Nishida T (2001) Removal of estrogenic activities of bisphenol A
24 and nonylphenol by oxidative enzymes from lignin-degrading basidiomycetes. *Chemosphere*
25 **42**:271–276.
- 26 22 Wang J, Hirai H & Kawagishi H (2012) Biotransformation of acetamiprid by the white-rot
27 fungus *Phanerochaete sordida* YK-624. *Appl Environ Microbiol* **59**: 1779–1785.
- 28 23 Wang J, Majima N, Hirai H & Kawagishi H (2012) Effective removal of
29 endocrine-disrupting compounds by lignin peroxidase from the white-rot fungus
30 *Phanerochaete sordida* YK-624. *Curr Microbiol* **64**: 300–303.
- 31 24 Wang J, Ogata M, Hirai H & Kawagishi H (2011) Detoxification of aflatoxin B₁ by
32 manganese peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624. *FEMS*
33 *Microbiol Lett* **314**: 164–169.

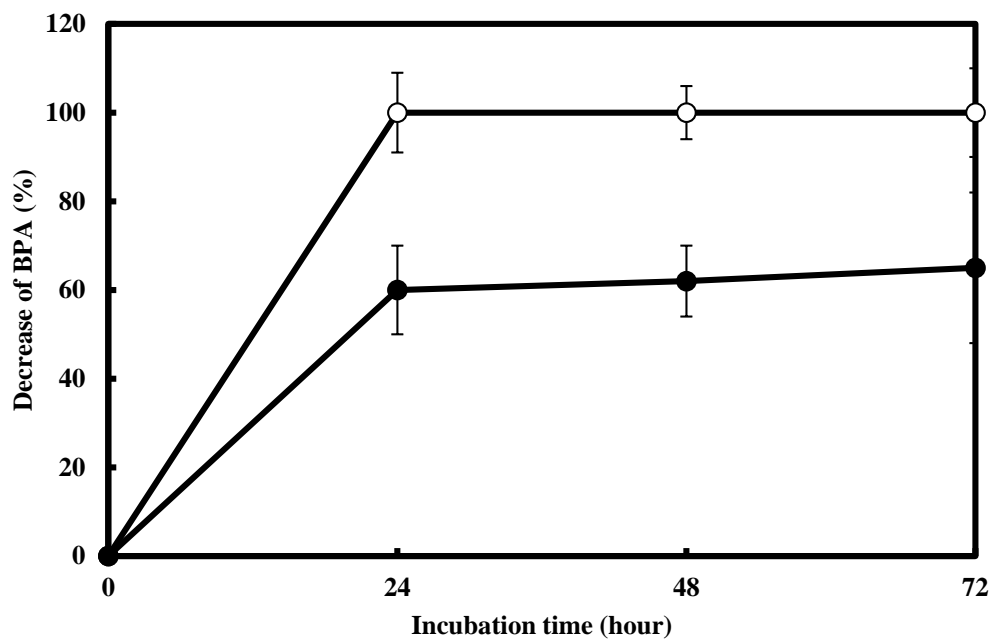


Fig. 1 Time course for BPA elimination by *P. sordida* YK-624. Closed circles 1 mM BPA, open circles 0.1 mM. Values are the means \pm SD of triplicate samples

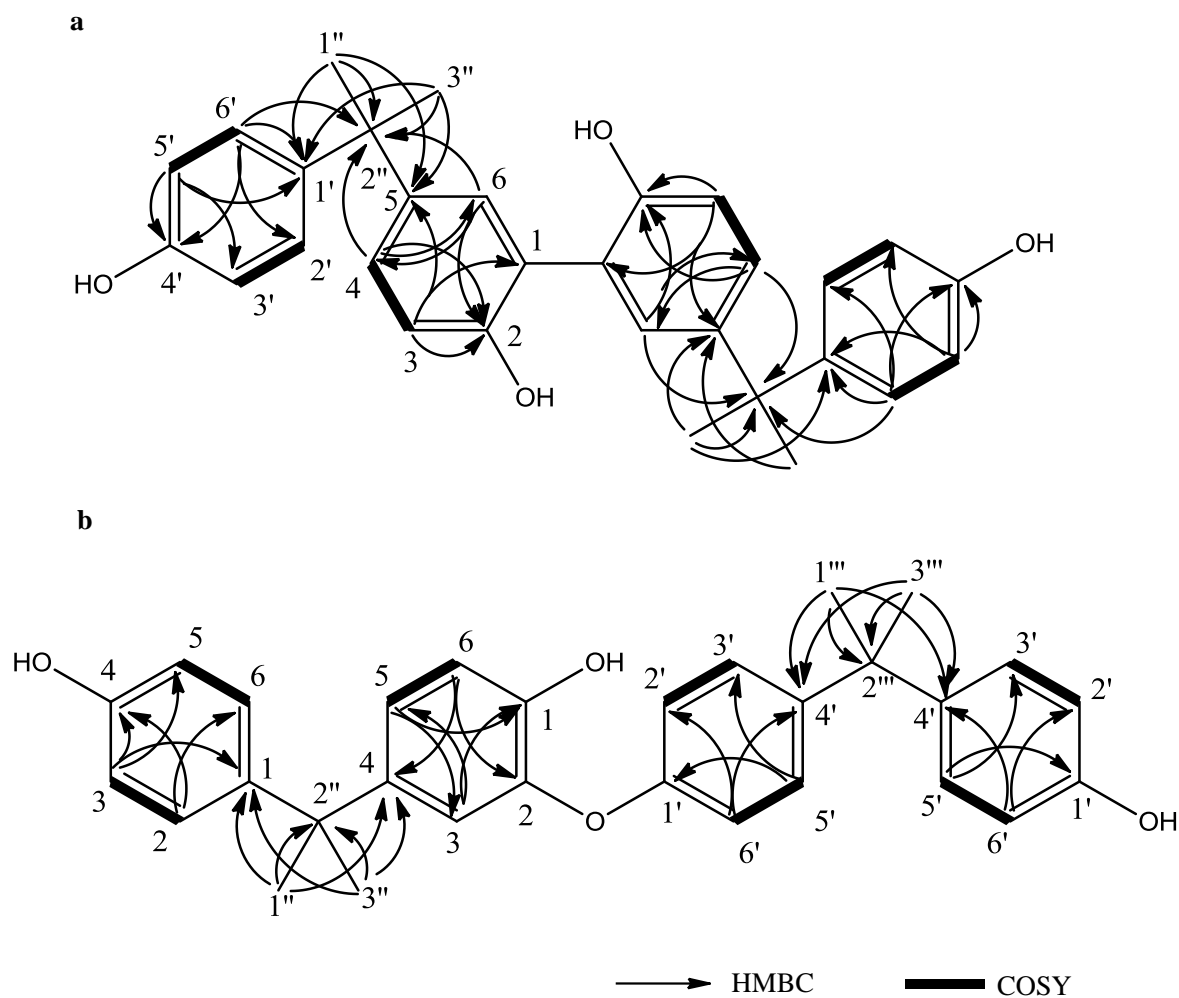


Fig. 2 COSY and HMBC correlations for the identified BPA metabolites

Table 1 ¹H- and ¹³C-NMR data for BPA metabolites (in CD₃OD)

	Position	¹ H δ _H (mult, <i>J</i> in Hz)	¹³ C δ _C
Compound 1	1	-	127.3
	2	-	152.7
	3	6.78(d, 8.2)	116.9
	4	7.05(dd, 8.2, 2.4)	127.8
	5	-	144.7
	6	6.89(d, 2.4)	131.5
	1'	-	143.2
	4'	-	156.2
	2', 6'	7.05(d, 8.6)	125.8
	3', 5'	6.68(d, 8.6)	115.6
	2''	-	42.6
	1'', 3''	1.57(s)	31.5
Compound 2	[phenol]		
	1	-	147.7
	2	-	144.8
	3	6.69(d, 2.0)	120.8
	4	-	144.3
	5	6.86(dd, 8.5, 2.0)	124.0
	6	6.80(d, 8.5)	141.4
	1'	-	156.1
	2', 6'	6.64(d, 8.5)	115.6
	3', 5'	7.01(d, 8.5)	128.8
	4'	-	143.1
	[hydroxyphenyl]		
	1	-	143.0
	2,6	6.99(d, 8.5)	128.7
	3,5	6.66(d, 8.5)	115.6
	4	-	156.1
	1'	-	146.5
	2', 6'	7.10(d, 8.5)	128.9
	3', 5'	6.72(d, 8.5)	117.3
	4'	-	157.2
	1'', 3''	1.53(s)	31.5
	2''	-	42.9
	1''', 3'''	1.59(s)	31.6
2'''	-	42.8	