Title

Novel Pathway of Metabolic Activation of Bisphenol A-related Compounds for Estrogenic Activity

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Running title
Metabolic Activation of Bisphenol A-related Compounds

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Abbreviations
BPA, bisphenol A; MBP, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene; EDC, endocrine-disrupting chemical; BPB, bisphenol B, 2,2-bis(4-hydroxyphenyl)butane; DMBPA, 3,3’-dimethylbisphenol A, 2,2-bis(3-methyl-4-hydroxyphenyl)propane; TMBPA, 3,3’,5,5’-tetramethylbisphenol A, 2,2-bis(3,5-dimethyl-4-hydroxyphenyl)propane; BPF, bisphenol F, 4,4’-dihydroxydiphenylmethane; BPAD, bisphenol AD, ethylidenebisphenol; LC, liquid chromatography; MS, mass spectrometry; BPCH, 1,1-bis(4-hydroxyphenyl)cyclohexane; CP, 4-α-cumylphenol; DP, 2,2-Diphenylpropane; CPRG, chlorophenol red-β-galactopyranoside; YES, yeast estrogen screening; ERα, estrogen receptor α; ERE, estrogen-responsive element; CYP, cytochrome P450
Abstract

We previously demonstrated that estrogenic activity of bisphenol A (BPA) in the yeast estrogen screening (YES) assay was increased several-fold after incubation with rat liver S9 fraction in the presence of a NADPH-generating system. In this study, we investigated whether eight BPA-related compounds are similarly metabolically activated by rat liver S9 fraction. Three of the analogues exhibited an increase of estrogenic activity after incubation with rat liver S9 fraction, but not with microsomal or cytosolic fraction alone. The structures of the metabolites formed were examined by liquid chromatography/mass spectrometry (LC/MS). In addition to oxidized metabolites, such as catechols, we found novel dimer-type metabolites. Some of the putative metabolites were chemically synthesized to confirm their structures. The structural requirements for formation of the metabolites, some of which showed more potent estrogenic activity than the parent substrates, were examined. We have uncovered a new pathway of metabolic activation of certain phenolic compounds, such as BPA analogues, to estrogenic dimer-type compounds.
Introduction

Bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane) is a well-known endocrine-disrupting chemical (EDC). It has been used as a plasticizer of polycarbonate and epoxy resin, which are extensively used in the manufacture of consumer goods and products, such as food containers, dental sealants, and protective coatings in metal cans for food and beverages (Brotons et al., 1995; Olea et al., 1996; Yamamoto and Yasuhara, 1999). Consequently, there is a high risk of human exposure. Indeed, BPA was detected in the serum of pregnant women, as well as serum of fetuses, placental tissue, amniotic fluid, follicular fluid and breast milk (Ikezuki et al., 2002; Schönfelder et al., 2002; Ye et al., 2006; Kuruto-Niwa et al., 2007). Moreover, significant amounts of BPA were detected in saliva of dental patients treated with fissure sealants (Olea et al., 1996).

Many BPA analogues, including bisphenol B (BPB; 2,2-bis(4-hydroxyphenyl)butane), 3,3’-dimethylbisphenol A (DMBPA; 2,2-bis(3-methyl-4-hydroxyphenyl)propane), 3,3’,5,5’-tetramethylbisphenol A (TMBPA; 2,2-bis(3,5-dimethyl-4-hydroxyphenyl)propane), bisphenol F (BPF; 4,4’-dihydroxydiphenylmethane) and bisphenol AD (BPAD; ethylidenebisphenol), are also used in polycarbonate resin, and some of them have been detected in extracts from canned foods (Goodson et al., 2002; Grumetto et al., 2008; Gallart-Ayala et al., 2011). Kitamura et al. (2005) reported that BPB, BPF, BPAD, DMBPA and TMBPA, as well as BPA, exhibit estrogenic activity in a human breast cancer cell line. Furthermore, all of these compounds showed significant inhibitory effects on the androgenic activity of 5α-dihydrotestosterone in mouse fibroblast cell line NIH3T3 (Kitamura et al., 2005).

We previously demonstrated that the estrogenic activity of BPA in the yeast estrogen screening (YES) assay was increased several-fold after incubation with rat liver S9 fraction in the presence of a NADPH generating system (Yoshihara et al., 2001). The active metabolite of BPA was determined to be 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) by NMR and LC/MS/MS analysis (Yoshihara et al., 2004). MBP exhibited more potent estrogenic activity than BPA by a factor of tens to over one thousand times in several in vitro assays (Yoshihara et al., 2004). Subsequently, it was
shown that the estrogenicity of MBP is approximately 200- and 10,000 fold higher than that of BPA in larvae and adult fish (*Oryzias latipes*), respectively, as judged from the ability to induce vitellogenin production (Ishibashi et al., 2005; Yamaguchi et al., 2005). Furthermore, we recently reported that MBP also exhibits potent estrogenic activity in rat in vivo uterotrophic assay; indeed, its activity is several hundred times stronger than that of BPA (Okuda et al., 2010). We have reported that MBP is formed by recombination of the radical fragment of BPA, which is the one-electron oxidation product of carbon-phenyl bond cleavage, and its formation required both microsomal and cytosolic fractions. A similar metabolic pathway of BPA accompanied with carbon-carbon bond cleavage has also been reported in MV-1, a Gram-negative aerobic bacterium isolated from enriched sludge taken from a wastewater treatment plant (Spivack et al., 1994). However, the present paper is the first to identify this as a mammalian metabolic pathway.

In this study, we investigated whether or not BPA-like metabolic activation by rat liver S9 fraction also occurs with eight BPA-related compounds (Fig. 1). We employed liquid chromatography/mass spectrometry (LC/MS) to investigate the structure of the metabolites, and chemically synthesized some of the candidate metabolites to confirm their structures.
Materials and Methods

Chemicals

The sources of materials were as follows: BPA, BPB, DMBPA, TMBPA, BPAD, BPF, 1,1-bis(4-hydroxyphenyl)cyclohexane (BPCH) and 4-α-cumylphenol (CP) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). 2,2-Diphenylpropane (DP) was purchased from Aldrich Chemical Co. (Milwaukee, WI), and chlorophenol red-β-galactopyranoside (CPRG) from Boehringer Mannheim GmbH (Germany). Chemical synthesis of MBP was performed as reported previously (Yoshihara et al., 2004). Other chemicals used were of the highest quality commercially available.

Animals and preparation of S9 fraction

Male Wistar rats (8-9 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were housed under controlled temperature, lighting (12 h light: 12 h darkness), and humidity for at least 1 week for acclimation. Rat livers were excised and homogenized in 3 volumes of 1.15% KCl. The liver S9 fraction, consisting of both microsomal and cytosolic fractions, was obtained by centrifugation of whole-liver homogenate at 9,000 x g for 20 min. All animal studies were approved by the Institutional Review Board for Biomedical Research using Laboratory Animals at Hiroshima International University, and the animals were handled according to the institutional guidelines and regulations.

Incubation of BPA and related compounds with S9 fraction

The incubation was performed as described previously (Yoshihara et al., 2004) with a minor modification. The incubation mixture consisted of 0.1 μmol of substrate in 2 μl acetonitrile, a NADPH-generating system (0.5 μmol of NADP+, 5 μmol of glucose-6-phosphate, 5 μmol of MgCl2), and 200 μl, 50 mg liver equivalent, of S9 fraction in a final volume of 1 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA. The incubation was carried out at 37°C for 60
min with shaking. After addition of 1 ml of 20% trichloroacetic acid, the quenched reaction mixture was allowed to stand for 15 min on ice, then centrifuged at 3,000 rpm for 10 min. The incubation system without a NADPH-generating system, or in which S9 had been inactivated by boiling (95°C for 10 min) prior to incubation, was used as the control. A 1.8-ml aliquot of the resultant supernatant was passed through a Sep-Pak Plus C18 cartridge (Waters Associates Inc., Milford, MA) preconditioned with 13 ml of ethanol, 10 ml of methanol and 20 ml of water for solid-phase extraction. The cartridge was washed with 10 ml of water. The adsorbed substances were eluted with 4 ml of ethanol, and the eluate was evaporated to dryness under reduced pressure. The residue was dissolved in 200 µl of 50% aqueous acetonitrile, then 10 µl of the sample solution was transferred to a well of a microtiter plate for YES assay. A 20 µl aliquot of the sample was used for LC/MS analysis. All experiments were conducted in duplicate.

**Yeast estrogen screening (YES) assay**

Recombinant yeast strain transfected with the human estrogen receptor α (ERα) gene, together with expression plasmid carrying the reporter gene lac-Z preceded by an estrogen-responsive element (ERE), was provided with permission by Prof. Sumpter (Brunel University, U.K.). The YES assay was conducted as described (Routledge and Sumpter, 1996; Routledge and Sumpter, 1997), with some minor modifications (Yoshihara et al., 2001). In brief, to check the suitability of the yeast, 10 µl aliquots of ethanol standard solutions of 1.0 x 10^{-10} and 1.0 x 10^{-9} M E2 were tested at the same time as other samples. For assay of estrogenic activity after incubation of BPA and related compounds with rat liver S9 fraction, 10 µl aliquots of 50% acetonitrile solution of solid-phase extracts from incubation mixtures were transferred to wells. Then 200 µl aliquots of the yeast assay medium containing recombinant yeast and CPRG, the chromogenic substrate of β-galactosidase reporter enzyme, were dispensed into each well. The plates were sealed with tape and incubated at 32°C in a dry incubator without shaking to avoid cross-contamination between wells. After 48 h, the absorbance of the red hydrolysis product of CPRG generated by β-galactosidase was read using a
microplate reader at 540 nm. All assays were carried out at least in duplicate and included a blank well containing the yeast assay medium alone. The data were corrected for turbidity using the absorbance reading at 620 nm, and values were calculated as follows: Net OD540 = (OD540 for test – OD620 for test) – (OD540 for blank – OD620 for blank).

**LC/MS**

An Agilent 1100 series HPLC system (Agilent, Santa Clara, CA) coupled to an AccuTOF JMS-T100LC (JEOL Ltd., Tokyo, Japan) equipped with negative-mode ESI was used. Separation was performed on a reversed-phase Inertsil ODS-SP column (2.1 x 50 mm, 3 µm; GL Sciences Inc., Torrance, CA) using a gradient of 30-50% acetonitrile in 0.06% acetic acid over 20 min, followed by a hold for 8 min; from 28 min, acetonitrile was reduced from 50% to 30% over 1 min, followed by a hold for 6 min at a flow rate of 0.2 ml/min. The conditions of ionization were as follows: needle voltage -1000 V, ring lens voltage -16 V, orifice 1 voltage -85 V, orifice 2 voltage -5 V, de-solvent chamber temperature 250°C, orifice 1 temperature 80°C, detector voltage 2600 V.

**Chemical synthesis**

Chemical synthesis of the putative metabolites of some of the BPA-related compounds was accomplished according to the literature (Chen et al., 2004) with a minor modification. Each compound was dissolved in more than 6 volumes of concentrated H₂SO₄ and the solution was allowed to stand for 15-30 min at room temperature, then slowly dropped into an excess of ice-chilled water. The aqueous mixture was extracted with dichloromethane 3 times and the combined extract was dried over Na₂SO₄. The solvent was removed under reduced pressure to afford a viscous oil, from which the desired product was separated by preparative HPLC as described below.

**(Z)-5-methyl-3,5-bis(4-hydroxyphenyl)hept-2-ene (Ia)**
Amorphous powder; m.p. 133-135, NMR (600 MHz / CDCl₃), \(^1\)H: \(\delta = 6.92 (d, J = 9.0 \text{ Hz}, 2\text{H}), 6.76 (d, J = 8.4 \text{ Hz}, 2\text{H}), 6.64 (d, J = 7.8 \text{ Hz}, 2\text{H}), 6.60 (d, J = 9.0 \text{ Hz}, 2\text{H}), 5.34 (q, J = 6.6 \text{ Hz}, 1\text{H}), 2.70 (d, J = 13.2 \text{ Hz}, 1\text{H}), 2.56 (d, J = 12.6 \text{ Hz}, 1\text{H}), 1.71-1.65 (m, 1\text{H}), 1.49 (d, J = 7.2 \text{ Hz}, 3\text{H}), 1.47-1.41 (m, 1\text{H}), 0.57 (t, J = 7.2 \text{ Hz}, 3\text{H}). \(^{13}\)C: \(\delta = 153.2, 152.6, 139.5, 138.2, 134.3, 130.0, 127.8, 124.7, 114.4, 114.3, 53.3, 41.4, 35.1, 23.3, 14.7, 8.6.\) MS (ESI -), m/z 295.2, 147.1, 133.1.

\((E)-5\)-methyl-3,5-bis(4-hydroxyphenyl)hept-2-ene \((1b)\)

Colorless oil, NMR (600 MHz / CDCl₃), \(^1\)H: \(\delta = 7.03 (d, J = 8.4 \text{ Hz}, 2\text{H}), 7.00 (d, J = 8.4 \text{ Hz}, 2\text{H}), 6.65 (d, J = 9.0 \text{ Hz}, 2\text{H}), 6.65 (d, J = 8.4 \text{ Hz}, 2\text{H}), 5.54 (q, J = 6.6 \text{ Hz}, 1\text{H}), 2.75 (d, J = 13.8 \text{ Hz}, 1\text{H}), 2.68 (d, J = 13.8 \text{ Hz}, 1\text{H}), 1.80-1.75 (m, 1\text{H}), 1.49 (d, J = 6.6 \text{ Hz}, 3\text{H}), 1.45-1.39 (m, 1\text{H}), 0.56 (t, J = 7.2 \text{ Hz}, 3\text{H}). \(^{13}\)C: \(\delta = 153.6, 152.9, 139.5, 138.9, 138.5, 127.9, 127.8, 126.1, 114.6, 114.4, 44.0, 42.6, 34.5, 23.5, 14.7, 8.7.\) MS (ESI -), m/z 295.2, 147.1, 133.1.

4-methyl-2,4-bis(4-hydroxy-3-methylphenyl)pent-1-ene \((2a)\)

Pale yellow oil, NMR (600 MHz / CDCl₃), \(^1\)H: \(\delta = 6.98-6.94 (m, 3\text{H}), 6.91 (d, J = 2.4 \text{ Hz}, 1\text{H}), 6.62 (s, 1\text{H}), 6.61 (s, 1\text{H}), 5.06 (d, J = 1.8 \text{ Hz}, 1\text{H}), 4.73 (s, 1\text{H}), 4.59 (s, 1\text{H}), 4.49 (s, 1\text{H}), 2.72 (s, 2\text{H}), 2.18 (s, 3\text{H}), 2.17 (s, 3\text{H}), 1.19 (s, 6\text{H}).\) MS (ESI -), m/z 295.2, 147.1.

(Z)-2-methyl-2,4-bis(4-hydroxy-3-methylphenyl)pent-3-ene \((2b)\)

Pale yellow oil, NMR (600 MHz / CDCl₃), \(^1\)H: \(\delta = 7.16 (d, J = 1.8 \text{ Hz}, 1\text{H}), 7.13 (d, J = 1.8 \text{ Hz}, 1\text{H}), 7.12-7.08 (m, 2\text{H}), 6.72 (d, J = 8.4 \text{ Hz}, 1\text{H}), 6.70 (d, J = 9.0 \text{ Hz}, 1\text{H}), 6.00 (s, 1\text{H}), 4.64 (s, 1\text{H}), 4.54 (s, 1\text{H}), 2.26 (s, 3\text{H}), 2.24 (s, 3\text{H}), 1.46 (s, 6\text{H}).\) MS (ESI -), m/z 295.2, 280.2.

3-(4-hydroxy-3-methylphenyl)-1,1,3,4-tetramethylindan-5-ol \((2c)\)

Colorless oil, NMR (600 MHz / CDCl₃), \(^1\)H: \(\delta = 6.94 (d, J = 3.0 \text{ Hz}, 1\text{H}), 6.92 (d, J = 8.4 \text{ Hz}, 1\text{H}), 6.85 (dd, J = 2.4, 8.4 \text{ Hz}, 1\text{H}), 6.75 (d, J = 7.8 \text{ Hz}, 1\text{H}), 6.64 (d, J = 7.8 \text{ Hz}, 1\text{H}), 4.62 (s, 1\text{H}), 4.61 (s, 1\text{H}), 2.22 (d, J = 13.2 \text{ Hz}, 1\text{H}), 2.20 (s, 3\text{H}), 2.13 (d, J = 13.8 \text{ Hz}, 1\text{H}), 1.77 (s, 3\text{H}), 1.73 (s, 3\text{H}), 1.30 (s, 3\text{H}), 1.19 (s, 3\text{H}). \(^{13}\)C: \(\delta = 153.2, 151.4, 148.9, 145.1, 143.2, 128.8, 124.9, 123.1, 120.4, 120.0, 114.5, 114.4, 62.0, 50.7, 41.8, 31.8, 31.5, 27.9, 16.0, 11.7.\) MS (ESI -), m/z 295.2, 280.2, 187.1.
3-(4-hydroxy-3-methylphenyl)-1,1,3,6-tetramethylindan-5-ol (2d)

Colorless oil, NMR (600 MHz / CDCl\textsubscript{3}), \( ^1\text{H}: \delta= 6.96 \) (d, \( J= 2.4 \) Hz, 1H), 6.92 (s, 1H), 6.86 (dd, \( J= 2.4, 8.4 \) Hz, 1H), 6.62 (d, \( J= 8.4 \) Hz, 1H), 6.48 (s, 1H), 4.60 (s, 1H), 4.57 (s, 1H), 2.33 (d, \( J= 13.2 \) Hz, 1H), 2.27 (s, 3H), 2.19 (s, 3H), 2.12 (d, \( J= 13.2 \) Hz, 1H), 1.60 (s, 3H), 1.30 (s, 3H), 1.03 (s, 3H).
\(^{13}\text{C}: \delta=152.8, 151.5, 148.3, 144.4, 143.2, 129.2, 125.3, 124.6, 122.9, 122.6, 114.2, 110.9, 59.7, 49.8, 42.3, 31.0, 30.7, 16.1, 16.0. MS (ESI\textsuperscript{-}), m/z 295.2, 280.2.

4-methyl-2,4-bis(4-hydroxy-3,5-dimethylphenyl)pent-1-ene (3a)

Colorless oil, NMR (600 MHz / CDCl\textsubscript{3}), \( ^1\text{H}: \delta= 6.80 \) (s, 2H), 6.75 (s, 2H), 5.05 (d, \( J= 1.2 \) Hz, 1H), 4.76 (s, 1H), 4.53 (s, 1H), 4.42 (s, 1H), 2.70 (s, 2H), 2.19 (s, 6H), 2.16 (s, 6H), 1.20 (s, 6H). \(^{13}\text{C}: \delta= 151.0, 149.7, 146.8, 141.1, 135.3, 126.8, 126.4, 126.3, 122.0, 121.8, 114.8, 50.2, 37.5, 28.8, 16.1, 15.9. MS (ESI\textsuperscript{-}), m/z 323.2, 161.1.

(Z)-2-methyl-2,4-bis(4-hydroxy-3,5-dimethylphenyl)pent-3-ene (3b)

Colorless oil, NMR (600 MHz / CDCl\textsubscript{3}), \( ^1\text{H}: \delta= 7.02 \) (s, 2H), 6.99 (s, 2H), 5.98 (s, 1H), 4.56 (s, 1H), 4.46 (s, 1H), 2.26 (s, 6H), 2.23 (s, 6H), 1.56 (s, 3H), 1.45 (s, 6H). \(^{13}\text{C}: \delta= 154.5, 151.1, 149.7, 142.3, 137.2, 137.0, 135.3, 126.3, 125.9, 122.5, 122.3, 39.2, 31.8, 17.2, 16.2, 16.0. MS (ESI\textsuperscript{-}), m/z 323.2, 308.2.

3-(4-hydroxy-3,5-dimethylphenyl)-1,1,3,4,6-pentamethyl-5-indan-ol (3c)

Colorless oil, NMR (600 MHz / CDCl\textsubscript{3}), \( ^1\text{H}: \delta= 6.82 \) (s, 1H), 6.78 (s, 1H), 4.50 (s, 1H), 4.46 (s, 1H), 2.28 (s, 3H), 2.20 (d, \( J= 14.4 \) Hz, 1H), 2.19 (s, 6H), 2.10 (d, \( J= 13.2 \) Hz, 1H), 1.78 (s, 3H), 1.70 (s, 1H), 1.29 (s, 3H), 1.20 (s, 3H). \(^{13}\text{C}: \delta= 151.5, 149.7, 146.4, 144.4, 142.7, 126.4, 122.38, 122.37, 122.1, 121.8, 119.3, 62.0, 50.6, 41.9, 31.8, 31.5, 28.1, 16.5, 16.1, 11.9. MS (ESI\textsuperscript{-}), m/z 323.2.

Preparative HPLC for crude mixtures

Preparative HPLC was performed in a Shimadzu HPLC system equipped with a preparative reversed-phase column. For the product mixture from BPB, separation was performed with Inertsil ODS-3 (7.6 x 250 mm; GL Sciences Inc., Torrance, CA). Elution was performed with 50%
acetonitrile at 3 ml/min, and the chromatogram was monitored at 254 nm. The sample was dissolved in 50% acetonitrile at a concentration of 20 mg/ml and 100 μl aliquots were subjected to HPLC. The eluate from 26 to 34 min was fractionated at 0.1 min intervals, and the procedure was repeated 37 times. The fractions corresponding to each peak were combined and lyophilized after evaporation to remove acetonitrile. For the product mixture from DMBPA, separation was performed with Inertsil CLC-ODS(M) (4.6 x 250 mm; GL Sciences Inc., Torrance, CA). Elution was performed with 55% acetonitrile at 1 ml/min, and the chromatogram was monitored at 254 nm. The sample was dissolved in 50% acetonitrile at a concentration of 10 mg/ml and 10 μl aliquots were subjected to HPLC. The eluate from 24.4 to 34 min was collected at 0.2 min intervals, and the procedure was repeated 46 times. The fractions corresponding to each peak were combined and lyophilized after evaporation to remove acetonitrile. For the product mixture from TMBPA, separation was performed with Inertsil ODS-3 (7.6 x 250 mm; GL Sciences Inc., Torrance, CA). Elution was performed with 55% acetonitrile at 3 ml/min, and the chromatogram was monitored at 254 nm. The sample was dissolved in 50% acetonitrile at the concentration of 20 mg/ml and 100 μl aliquots were subjected to HPLC. The eluate from 38 to 47 min was collected at 0.2 min intervals, and the procedure was repeated 18 times. The fractions corresponding to each peak were combined and lyophilized after evaporation to remove acetonitrile.
Results

In agreement with previous reports, the estrogenic activity of BPA increased after incubation with rat liver S9 fraction in the presence of a NADPH-generating system, compared with that after incubation with a boil-denatured S9 fraction (Yoshihara et al., 2001; Yoshihara et al., 2004). Similar activation was observed for BPB, DMBPA and TMBPA (Fig. 2). The increases of estrogenic activity were about 1.6-, 1.9- and 1.4-fold, respectively. On the other hand, the estrogenic activities of BPCH, BPAD, BPF and CP were reduced by incubation with S9 fraction; in the case of CP, almost all of the estrogenic activity was lost. DP did not show estrogenic activity after incubation with either S9 fraction or boil-denatured S9.

In the LC/MS analysis, the metabolic extracts of BPA or its analogues were separated on an octadecylsilyl column, and passed to a time-of-flight mass spectrometer. We identified putative metabolite peaks by subtracting the peaks in the chromatogram of the extract from the incubation mixture incubated with boil-denatured S9 from those of the extract obtained with native S9. Table 1 showed the retention times of the parent compounds and detected metabolites, and the corresponding mass-to-charge ratio (m/z) values.

BPA was metabolized by rat liver S9 fraction to MBP and 3-hydroxy-BPA, in accordance with our previous report (Yoshihara et al., 2004). Catechols, such as 3-hydroxy-BPA, are general oxidative metabolites of phenolic compounds, and are eluted earlier than the parent compound in reversed-phase chromatography. MBP was eluted at a later retention time than that of BPA, suggesting increased hydrophobicity.

The metabolic pattern of BPB was similar to that of BPA. An unknown metabolite and catechol were eluted at early retention times, and the metabolite showed ions at m/z 295.1 and 147.0, suggesting a dimer-type structure. DMBPA metabolites were observed at 12.5, 24.8 and 25.4 min. Two of these metabolites, eluted 24.8 and 25.4 min, appeared to be dimer-type products from their mass spectra. TMBPA gave one dimer-type metabolite at 32.7 min.

The metabolites of BPF, BPCH, CP and DP were catechols. In addition to catechol, BPAD gave
an unknown metabolite that was eluted at a later retention time, but a dimer structure for this was ruled out by the mass spectrum.

All compounds showed metabolic activation in YES assay, suggesting that they were converted into dimer-type products, as in the case of MBP.

We synthesized the putative dimer-type metabolites and confirmed their structures by means of $^1$H, $^{13}$C, HMQC, HMBC, and DEPT 135 NMR methods (Table 2). The structure of cis-trans double bond isomers was confirmed by the NOESY method. With regard to compound 1a, NOE correlations between a proton at olefin and protons at both methyl groups and closer methylene could be observed while compound 1b showed only correlation against methyl group next to olefin.

The HPLC retention times and MS spectra of the synthesized compounds were compared with those of the metabolites. The BPB metabolites eluted at 22.1 and 23.1 min were identical with compounds 1a and 1b, respectively (Fig. 3). In the case of the DMBPA metabolites, the peak at 24.2 min was identical with compound 2d, and the peak at 23.5 min was identical with compound 2a (Fig. 4). The MS spectrum of compound 2a showed ions at m/z 295.2 ([M-H] -) and m/z 147.1, while that of compound 2b did not show m/z 147.1. Similarly, the TMBPA metabolites were identical with compound 3a and 3c. These compounds were not well separated under various analytical conditions, but the MS spectral patterns were similar to those of DMBPA metabolites (Fig. 5).

To evaluate estrogenic activity, the synthesized compounds were subjected to YES assay. BPB metabolites 1a and 1b showed similar activity, being more than 100-fold more potent than BPB (Fig. 6A). The DMBPA metabolites were unstable, so the whole amounts obtained were dissolved and serially diluted for testing. Compounds 2a and 2b showed estrogenic activities, but compound 2d was inactive (Fig. 6B). TMBPA metabolite 3a exhibited estrogenic activity as strong as that of TMBPA, while compound 3c was inactive (Fig. 6C).
Discussion

We have reported that BPA is metabolically activated by rat liver S9 fraction in the presence of a NADPH generating system (Yoshihara et al., 2001; Yoshihara et al., 2004), and the active metabolite, MBP, showed in vivo estrogenic activity about 500-fold more potent than that of BPA in uterotrophic assay using ovariectomized rats (Okuda et al., 2010). MBP is produced by recombination of the one-electron oxidation product of carbon-phenyl bond cleavage of BPA. Here, similar metabolic activation was demonstrated for various other BPA-related compounds (Fig. 2). The putative structures of the active metabolites were confirmed by synthesis (Table 2).

Fig. 2 shows the effects of metabolism by rat liver S9 fraction on the estrogenic activities of BPA analogues. In the cases of BPB, DMBPA and TMBPA, as well as BPA, formation of active metabolite(s), which are more estrogenic than the parent compounds, was suspected because the total estrogenic activity was increased. CP has a deoxy-BPA structure at the 4-position of the benzene ring. If oxidative metabolism occurred at the 4-position of the benzene ring, CP would be converted to more estrogenic BPA. However, since its estrogenic activity was rather decreased after metabolism, such metabolism does not appear to occur, at least under our conditions. As shown in Table 1, BPA analogues afford two types of metabolites: catechols and dimer-type metabolites which are eluted at later retention times than the parent compounds on reversed-phase HPLC. All of the BPA analogues, except for TMBPA, showed catechol formation. Since both o-positions of the hydroxyl group are methyl-substituted, TMPBA can not be metabolized to a catechol. It has been reported that 3-hydroxy-BPA (BPA catechol) has fairly weak estrogenic activity compared to BPA (Kitamura et al., 2005). Thus, metabolism to catechol is likely to reduce the estrogenicity of BPA analogues. Interestingly, no peak of CP or BPA was found in the case of S9-incubated DP, suggesting that the mono-hydroxylated metabolite at the 4-position, that is CP, formed from DP was immediately hydroxylated. Also it appeared that hydroxylation of the 4′-position did not proceed after the 4-hydroxylation, because neither BPA nor BPA catechol was observed as a metabolite of CP. The synthesized compounds corresponding to the putative dimer-type metabolites of BPA analogues were
active in YES assay (Figs. 2 and 6). The potent activity of compounds 1a and 1b might explain why the extract from the incubation system with rat liver S9 fraction in the presence of a NADPH-generating system showed stronger activity than that in the absence of a NADPH generating system. Comparison of 3a and 3c suggests that a flexible structure of the bridging moiety might be essential to bind estrogen receptor, since 3c, which has a fixed structure, was inactive. Because only limited amounts of 2a, 2b and 2d were available, their estrogenic activities could not be precisely determined, but the structure-activity relationship seemed consistent with the requirement of a flexible bridging moiety for activity.

As shown in Fig. 7, hydroxyl groups on both aryl rings (BPA, CP and DP) are essential for generation of the dimer-type metabolite, presumably serving to stabilize the radical and promote carbon-aryl bond cleavage. A quaternary central carbon is also required for this reaction.

The metabolic activation of BPA required the coexistence of both microsomal and cytosolic fractions, namely S9 fraction. This was also the case for the compounds tested here (data not shown). We have confirmed the involvement of cytochrome P450 (CYP) as a microsomal factor by means of inhibition studies using anti-CYP antibodies. Both anti-CYP3A2 and anti-CYP2C11 antibodies strongly inhibited the formation of MBP (our unpublished data). We suggested that these CYP isoforms and certain cytosolic component(s) might take charge of part of generating dimer type metabolite, that is, radical generation, oxidative cleavage and dimerization. Unfortunately, however, the exact function of cytosol is still unclear. On the other hand, glucuronic acid/sulfate conjugates are the predominant metabolites of BPA, and these conjugates are rapidly excreted in urine (Knaak and Sullivan, 1966; Pottenger et al., 2000; Snyder et al., 2000; Völkel et al., 2002). Thus, metabolic activation to MBP may not be significant, at least under usual circumstances, and this might be one of the reasons why an increased risk to humans from BPA at the current levels in the environment was not identified in the recently update of the EU risk assessment report (EU, 2010). However, metabolic activation to MBP might occur under circumstances where glucuronidation is unable to work efficiently as a detoxification pathway of BPA. It is well known that rat and human fetal livers...
show little or no glucuronidation activity (Coughtrie et al., 1988; Pacifici et al., 1993). Indeed, similar levels of unconjugated BPA, but not the glucuronide, have been detected in fetal plasma of rats (Miyakoda et al., 2000; Takahashi and Oishi, 2000) and humans (Ikezuki et al., 2002; Schönfelder et al., 2002). Blood concentration of BPA in newborn was predicted to be 3 times greater than that in adult (Mielke and Gundert-Remy, 2009). Although the activity of CYP-dependent xenobiotic metabolism in the rodent fetal liver is limited compared with the adult liver, some forms of CYP are only expressed in late gestation (Cresteil et al., 1986; Raucy and Carpenter, 1993). Further, in contrast to rodents, human fetal liver exhibits a significant metabolizing capacity for xenobiotics (Hakkola et al., 1998; Ring et al., 1999) and expresses certain isoforms of CYP, such as CYP3A7 (Kitada et al., 1987). Therefore, metabolic activation of BPA might occur in vivo, especially in the fetus, which is one of the most important targets of EDCs. Other BPA-related compounds might behave similarly.

In conclusion, we have identified novel dimer-type metabolites of BPA-related compounds produced by a unique mechanism. Some of these metabolites show more potent estrogenic activity than the parent compounds. Further studies are necessary to establish in detail the mechanisms of cleavage and dimerization, and to identify the microsomal and cytosolic enzymes involved.
Acknowledgments

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Authorship Contributions

Participated in research design: Okuda, Takiguchi, Yoshihara

Conducted experiments: Okuda, Fukuuchi

Performed data analysis: Okuda, Fukuuchi

Wrote or contributed to the writing of the manuscript: Okuda, Takiguchi, Yoshihara
References


Footnotes

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Figure legends

Fig. 1. Chemical structures of BPA and BPA analogues tested.

Fig. 2. Effects of metabolism of BPA-related compounds by rat liver S9 fraction on estrogenic activity.

Each substrate (0.1 mM) was incubated with native rat liver S9 fraction for 1 h at 37°C in the presence of a NADPH-generating system, as described in Materials and Methods. As a control, the incubation was carried out with boiled S9 fraction. The estrogenicity of solid-phase extracts from the incubation mixtures was assayed using YES assay. Data represent the mean of two experiments.

Fig. 3. Mass chromatograms and spectra of BPB metabolites and authentic standards.

BPB (0.1 mM) was incubated with rat liver S9 fraction for 1 h at 37°C in the presence of a NADPH-generating system, as described in Materials and Methods. As a control, the incubation was carried out in the absence of a NADPH-generating system. The extracted ion chromatograms at m/z 147.1 were used to detect the dimer-type product (left panels). The mass spectra at the same retention time were compared between metabolite and authentic standard (right panels).

Fig. 4. Mass chromatograms and spectra of DMBPA metabolites and authentic standards.

DMBPA (0.1 mM) was incubated with rat liver S9 fraction for 1 h at 37°C in the presence of a NADPH-generating system, as described in Materials and Methods. As a control, the incubation was carried out in the absence of a NADPH-generating system. The extracted ion chromatograms at m/z 295.2 were used to detect the dimer-type product (left panels). The mass spectra at the same retention time were compared between metabolite and authentic standard (right panels).

Fig. 5. Mass chromatograms and spectra of TMBPA metabolites and authentic standards.

TMBPA (0.1 mM) was incubated with rat liver S9 fraction for 1 h at 37°C in the presence of a
NADPH-generating system, as described in Materials and Methods. As a control, the incubation was carried out in the absence of a NADPH-generating system. The extracted ion chromatograms at m/z 323.2 were used to detect the dimer-type product (left panels). The mass spectra at the same retention time were compared between metabolite and authentic standard (right panels).

Fig. 6. Estrogenic activity of authentic standards in YES assay.

Results of YES assay of BPB and its metabolites (A), DMBPA metabolites (B), TMBPA and its metabolites (C). Each value represents the mean ± SD of three experiments (A and C) or the mean of two experiments (B).

Fig. 7. Metabolic pathways of BPA analogues by rat liver S9 fraction.
Table 1

Table 1. LC/MS data of metabolites of BPA analogues, and the proposed structures

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<th>Substrate</th>
<th>RT (min)</th>
<th>(m/z)</th>
<th>Metabolites</th>
<th>RT (min)</th>
<th>(m/z)</th>
<th>Proposed structure</th>
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Table 2 Chemically synthesized BPA-analog products

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<tr>
<td>TMBPA</td>
<td><img src="image" alt="3a structure" /></td>
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</table>
Fig. 2
Fig. 6

A

B

C

- BPB
- Comp. 1a
- Comp. 1b

- Comp. 2a
- Comp. 2b
- Comp. 2d

- TMBPA
- Comp. 3a
- Comp. 3c

Net OD₅₄₀ vs. Concentration (log₁₀ M)

Net OD₅₄₀ vs. Dillution times

Net OD₅₄₀ vs. Concentration (log₁₀ M)
Fig. 7

Chemical reactions involving BPA, BPB, DMBPA, and TMBPA, with the confirmation of pathways A, B, and C for their metabolic processes.

- **BPA**: $R_1: H$, $R_2: H$, $R_3: H$  
  Confirmed pathway: A
- **BPB**: $R_1: H$, $R_2: H$, $R_3: CH_3$  
  Confirmed pathway: A
- **DMBPA**: $R_1: CH_3$, $R_2: H$, $R_3: H$  
  Confirmed pathway: A, B, C
- **TMBPA**: $R_1: CH_3$, $R_2: CH_3$, $R_3: H$  
  Confirmed pathway: A, C