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# Microbial Metabolism of the Environmental Estrogen Bisphenol A

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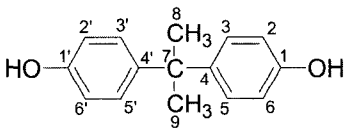
Preliminary microbial metabolism studies of bisphenol A (BPA) (**1**) on twenty six microorganisms have shown that *Aspergillus fumigatus* is capable of metabolizing BPA. Scale-up fermentation of **1** with *A. fumigatus* gave a metabolite (**2**) and its structure was established as bisphenol A-*O*-β-D-glucopyranoside (BPAG) based on spectroscopic analyses.

**Key words**: Microbial metabolism, Bisphenol A, Bisphenol A-*O*-β-D-glucopyranoside, *Aspergillus fumigatus*

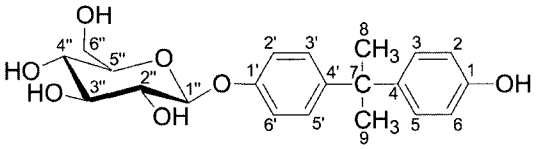
## INTRODUCTION

Bisphenol A [2,2-(4,4-dihydroxydiphenyl)propane, BPA] (**1**) is widely used as a starting material for the synthesis of polycarbonate plastics, epoxy resins, polyesters and coatings which have extensive applications in the food-packaging industry (Brotons *et al.*, 1995; Staples *et al.*, 1998). Since the estrogenic effects of BPA on human and animal reproductive organs were suspected (Dodds and Lawson, 1936), xenoestrogenic activity of BPA has been extensively investigated and established *in vivo* and *in vitro* (Nagel *et al.*, 1997; Farabollini *et al.*, 1999; Hiroi *et al.*, 1999; Howdeshell *et al.*, 1999; Welshons *et al.*, 1999; Markey *et al.*, 2001; Rubin *et al.*, 2001; Markey *et al.*, 2002; Kubo *et al.*, 2003).

Understanding of how BPA is metabolized is a necessary step in the assessment of its toxicologic effects and biochemical mechanism in human. Although metabolism studies have traditionally been performed using small laboratory animal models or *in vitro* enzyme systems, microorganisms, particularly fungi, have been used successfully as *in vitro* predictive models for mammalian metabolism of xenobiotics or drugs (Smith *et al.*, 1975; Smith *et al.*, 1977; Clark *et al.*, 1985; Clark and Hufford, 1991). This method often produces significant quantities of metabolites that would be difficult to obtain from either animal systems or *in vitro* enzyme systems, thus makes it feasible to isolate and identify the metabolites chemically.



bisphenol A (BPA) (**1**)



bisphenol A-*O*-β-D-glucopyranoside (BPAG) (**2**)

**Fig. 1.** Structures of bisphenol A (**1**) and bisphenol A-*O*-β-D-glucopyranoside (BPAG) (**2**)

As part of our microbial metabolism studies to better understand the fate of BPA, the preparative scale biotransformation of this compound by *Aspergillus fumigatus* KCTC 6145 afforded **2** as a major metabolite, which was produced as a result of microbial enzymatic reaction and was identified as BPA-*O*-β-D-glucopyranoside (Fig. 1) based on the spectroscopic analyses.

## MATERIALS AND METHODS

### Chemicals

Bisphenol A (97%) was purchased from Aldrich Chemical Company, Inc. and the chemical was of the highest purity

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commercially available.

### General experimental

$^1$H-(500 MHz) and $^{13}$C-(125 MHz) NMR spectra were obtained on a Varian Unity INOVA 500 spectrometer. Chemical shifts were expressed in parts per million (ppm) relative to TMS as the internal standard, and coupling constants (*J*) were given in hertz. MS were obtained on a VG Biotech platform spectrometer. UV spectra were recorded on a Jasco V-530 UV/Vis spectrophotometer. TLC was carried out on Merck Silica gel $F_{254}$-precoated glass plates and RP-18 $F_{254S}$ plates. MPLC was carried out with Silica gel 60 (230-400 mesh) and Lobar® Größe A (240-10) Lichroprep® RP-18 (40-63 μm) (Merck) prepacked columns.

### Microorganisms

The cultures were obtained from Korean Collection for Type Cultures (KCTC). The cultures used for preliminary screening of bisphenol A are as follows: *Absidia spinosa* 6588, *Aspergillus fumigatus* 6145, *Candida albicans* 7965, *Candida famata* 7000, *Candida solani* 7689, *Debaryomyces hansenii* var. *hansenii* 7645, *Debaryomyces occidentalis* var. *occidentalis* 7194, *Debaryomyces robertsiae* 7299, *Filobasidium capsuligenum* 7102, *Filobasidium neoformans* 7902, *Gliocladium deliquescens* 6173, *Kluyveromyces marxianus* 7155, *Metarhizium flavoviride* var. *minus* 6310, *Metschnikowia pulcherrima* 7605, *Microbacterium lacticum* 9230, *Mortierella ramanniana* var. *angulispora* 6137, *Mycobacterium phlei* 3037, *Penicillium chrysogenum* 6933, *Pichia pastoris* 7190, *Saccharomyces cerevisiae* 7904, *Saccharomycodes ludwigii* 7126, *Torulaspora delbrueckii* 7116, *Tremella mesenterica* 7131, *Trichoderma koningii* 6042, *Trigonopsis variabilis* 7263, *Zygosaccharomyces rouxii* 7191.

### Screening procedure

All preliminary screening and scale-up experiments were carried out on YM media at 25°C. The liquid cultures consisted of a basal medium of dextrose (10 g/L), peptone (5 g/L), yeast extract (3 g/L) and malt extract (3 g/L). Cultures were grown according to two-stage procedure. For fungal isolate 250 mL Erlenmeyer flasks, each containing 50 mL of the liquid medium, were inoculated from the actively growing edge of the appropriate isolate and incubated with gentle agitation at 25°C in a temperature-controlled shaking incubator. The ethanolic solution (10 mg/mL) of compound **1** was prepared and added to each flask 24 h after inoculation to give a final concentration of 2 μg/mL. Substrate controls consisted of BPA and sterile medium incubated under the same conditions without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without BPA addition. All fermentation cultures were generally sampled by removing 5 mL of entire culture at 24 and 48 h after addition of substrate. They were extracted with equal volumes of EtOAc, which were concentrated and developed on TLC plates with $CHCl_3$-MeOH (6:1).

### Preparative scale metabolism studies

Of a total of twenty six microbial cultures screened, *Aspergillus fumigatus* KCTC 6145 was found to be capable of metabolizing compound **1** based on TLC analyses. *A. fumigatus* was selected for preparative scale biotransformation reactions. *A. fumigatus* was grown on a shaking incubator at 25°C for 8 days in four 1 L Erlenmeyer flasks, each containing 500 mL of YM medium. The compound **1** dissolved in EtOH (60 mg/mL) was evenly distributed between flasks 1 day after incubation.

### Extraction and isolation

The liquid culture filtrate was extracted with EtOAc (4 L×2), and the organic layer was concentrated *in vacuo*. The EtOAc extract (976.1 mg) was chromatographed on a silica gel column (38.2 g) using a $CHCl_3$-MeOH (10:1 → 1:1) gradient system to give five fractions. Fraction 2 (23.4 mg) containing BPA metabolite was rechromatographed on a Lobar® Größe A (240-10) Lichroprep® RP-18 (40-63 μm) prepacked column using $H_2O$-acetone-MeOH (5:6:2) to give three subfractions. Subfraction 2 (16.8 mg) was rechromatographed by MPLC using a $CHCl_3$-MeOH (16:1 → 1:1) gradient system to provide the compound **2** (11.5 mg).

### Characterization of BPA-*O*-β-D-glucopyranoside

**BPA-*O*-β-D-glucopyranoside** (**2**, $C_{21}H_{26}O_7$)

Amorphous powder; UV (MeOH) $\lambda_{max}$ (log ε) 279 (3.37), 235 (3.48) nm; IR $\nu_{max}$ (KBr) 3380 (OH), 1625, 1511 (aromatic C=C), 1072 (glycosidic C-O) cm$^{-1}$; ESI-MS *m/z*: 389 [M-H]$^-$; $^1$H-NMR ($CD_3OD$) δ: 7.13 (2H, *d*, *J*=8.5, H-3' and 5'), 7.02 (2H, *d*, *J*=10.0, H-3 and 5), 6.98 (2H, *d*, *J*=8.5, H-2' and 6'), 6.67 (2H, *d*, *J*=10.0, H-2 and 6), 4.88 (1H, *d*, *J* = 7.0, H-1"), 3.88 (1H, *dd*, *J* = 12.0, 1.8, H-6"a), 3.69 (1H, *dd*, *J* = 12.0, 5.0, H-6"b), 3.44 (1H, *m*, H-2"), 3.43 (1H, *m*, H-3"), 3.40 (1H, *m*, H-5"), 3.38 (1H, *m*, H-4"), 1.59 (6H, *s*, H-8 and 9); $^{13}$C-NMR ($CD_3OD$) δ: 156.82 (C-1'), 156.06 (C-1), 146.50 (C-4'), 143.10 (C-4), 128.72 (C-3, 5, 3' and 5'), 117.14 (C-2' and 6'), 115.61 (C-2 and 6), 102.37 (C-1"), 78.07 (C-5"), 77.99 (C-3"), 74.94 (C-2"), 71.41 (C-4"), 62.51 (C-6"), 42.66 (C-7), 31.55 (C-8 and 9).

## RESULTS AND DISCUSSION

A total of twenty six microorganisms were evaluated for their ability to biotransform compound **1** using the standard

two-stage screening procedure (Clark *et al.*, 1985; Clark and Hufford, 1991). TLC analyses of the culture extracts were used to identify microorganisms capable of metabolizing compound **1**. Substrate control and culture controls were utilized to ensure that the metabolite was produced as a result of enzymatic activity and not a consequence of degradation or other non-metabolic changes.

Based on TLC profiles, *A. fumigatus* KCTC 6145 was found to be capable of converting BPA to more polar metabolites. The EtOAc extract of *A. fumigatus* was subjected to successive column chromatography to afford a BPA metabolite (**2**).

The $^1$H-NMR spectrum of compound **2** was remarkably different from that of compound **1** in several aspects. The spectrum showed a number of characteristic signals typical with the sugar moiety, ranging from δ 3.38 to δ 3.88 which were not observed in case of its parent compound **1**. Anomeric proton of the sugar was observed in the downfield region at δ 4.88 (1H, *d, J* = 7.0 Hz, H-1") in the spectrum of **2**. Two *dd* (doublet of doublets) peaks at δ 3.69 (1H, *dd, J* = 12.0, 5.0 Hz, H-6"b) and δ 3.88 (1H, *dd, J* = 12.0, 1.8 Hz, H-6"a) were due to $^3J_{H\text{-}H}$ correlations between the H-5" and the H-6" signals. In addition, the aromatic proton signals at δ 6.98 (2H, *d, J* = 8.5 Hz, H-2' and 6') and δ 7.13 (2H, *d, J* = 8.5 Hz, H-3' and 5') were shifted downfield, when compared with that of **1** [δ 6.66 (4H, *d, J* = 10.0, H-2, 6, 2' and 6'), δ 7.02 (4H, *d, J*=10.0, H-3, 5, 3' and 5')], suggesting that metabolite **2** is an *O*-glycoside. From the analyses of the proton and carbon signals of the sugar moiety, the sugar in **2** was assigned to be β-D-glucopyranose.

Compared with compound **1**, the $^{13}$C-NMR spectrum of compound **2** exhibited eight new signals at δ 62.51 (C-6"), 71.41 (C-4"), 74.94 (C-2"), 77.99 (C-3"), 78.07 (C-5"), 102.37 (C-1"), 117.14 (C-2' and C-6'), 146.50 (C-4'), and 156.82 (C-1'). The chemical shift values of the glucose moiety were assigned as δ 62.51 (C-6"), 71.41 (C-4"), 74.94 (C-2"), 77.99 (C-3"), 78.07 (C-5"), and 102.37 (C-1") in compound **2**. Furthermore, the aromatic carbon signals shifted downfield at δ 117.14 (C-2' and 6'), 146.50 (C-4'), and 156.82 (C-1') indicated the presence of an *O*-glycosidic bond linked with β-D-glucose moiety. This notion was further supported by HSQC and HMBC experiments (Fig. 2). Based on these data, structure of the microbial metabolite (**2**) of BPA was established as BPA-*O*-β-D-glucopyranoside (BPAG).

Earlier traditional metabolism studies showed that bisphenol A glucuronide (GlcA-BPA) was the major metabolite of BPA in *in vivo* (Knaak and Sullivan, 1966; Pottenger *et al.*, 2000; Snyder *et al.*, 2000) as well as *in vitro* system (Yokota *et al.*, 1999; Nakagawa and Tayama, 2000; Elsby *et al.*, 2001; Nakagawa and Suzuki, 2001) including human liver microsomes (Elsby *et al.*, 2001).

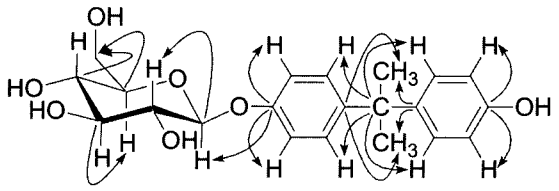


**Fig. 2.** Key HMBC correlations of BPAG (C→H)

However, recent *in vivo* metabolism studies of BPA showed that several glucosylated BPA metabolites including GlcA-BPAG, were produced in pregnant CD1 mice (Zalko *et al.*, 2003), suggesting that BPA metabolism is more complicated in mammals than previously thought.

To the best of our knowledge, this is the first report on the unambiguous NMR assignments of BPAG, although its presence was revealed from the culture of tobacco BY-2 plant cells with BPA (Nakajima *et al.*, 2002). Moreover, the production of BPAG by microbial metabolic transformation has never been yet reported. The previous microbial metabolism studies showed the bacterial degradation of BPA to 4-hydroxybenzoic acid and 4-hydroxyacetophenone by rearrangement and successive oxidation using Gram-negative aerobic bacterium, MV1 (Lobos *et al.*, 1992; Spivack *et al.*, 1994). To better understand the pharmacologic and toxicologic effects arising from human exposure to BPA, it is considered necessary that further metabolism studies should be thoroughly performed using various predictive models of human transformation.

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