Estrogenicity of Metabolites of Benzophenone Derivatives Examined by a Yeast Two-Hybrid Assay

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The estrogenic activities of S-9 metabolites of benzophenone derivatives (benzophenone, 2-hydroxy-4-methoxybenzophenone, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-octyloxybenzophenone, 2,4-dihydroxybenzophenone and 2,3,4-trihydroxybenzophenone) and benzhydrol were examined with a yeast two-hybrid screening system. After chemicals were incubated in an S-9 mix at 37°C for 4 hr prior to their incubation with the yeast strain, the S-9 mix containing metabolites was assayed for the estrogenic activity by the yeast two-hybrid assay. Benzophenone, 2-hydroxy-4-methoxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone exhibited estrogenic activities after incubation with the S-9 mix. The estrogenic metabolites of 2-hydroxy-4-methoxybenzophenone were fractionated by high-performance liquid chromatography, one of which was identified as 2,4-dihydroxybenzophenone. This assay will be a useful tool for detecting proestrogens.

Key words —— yeast two-hybrid assay, benzophenone, estrogenic metabolite

INTRODUCTION

Benzophenone is listed among the "chemicals suspected of having endocrine disrupting effects" by the World Wildlife Fund, the National Institute of Environmental Health Sciences in the U.S.A., and the Ministry of Environment in Japan. However, benzophenone is an important compound in everyday life because of its ability to absorb and dissipate ultra violet (UV) light.[1] Its twelve derivatives, designated as benzophenone-1 through benzophenone-12, are used in cosmetics and sunscreens to protect human skin and hair from UV irradiation. 2-Hydroxy-4-methoxybenzophenone (benzophenone-3, BZ-3) is one of the most widely used UV absorbers for sunscreens on the market. Orally or topically administered BZ-3 is converted to at least three metabolites, 2,4-dihydroxybenzophenone (benzophenone-1, BZ-1), 2,3,4-trihydroxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenone (benzophenone-8, BZ-8).[2–5] BZ-1 and 2,3,4-trihydroxybenzophenone exhibited estrogenic activities in an *in vitro* assay system using MCF-7 cells.[6] Benzophenone is converted to an estrogenic metabolite, *p*-hydroxybenzophenone.[7] Thus, benzophenone derivatives can be categorized as proestrogens. However, the estrogenic activities of the metabolites of benzophenone derivatives have not been fully elucidated.

We have developed a novel assay procedure for detecting the hormonal activities of chemicals using a yeast two-hybrid system.[8] We tested the estrogenic activities of various chemicals, and found that a phenol with a hydrophobic moiety at the para-position is the key structural moeity of estrogenic chemicals.[9] The phenyl or phenylether residues of lipophilic chemicals can be converted to a phenol residue by drug metabolizing enzymes. These facts imply that some chemicals exert their estrogenic activities by metabolic activation *in vivo*. The endocrine activities of pesticides and natural products can be affected by metabolism.[10,11] For example, methoxychlor (MXC) is metabolized to 2,2-*bis*(hydroxyphenyl)-1,1,1-trichloroethane to exert estrogenic activity.[12] The EDSTAC final report recommends that the evaluation of chemicals using *in*

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vitro high throughput prescreens should be performed in the presence and absence of metabolically active extracts to detect proestrogens.[13] There are few reports of assay procedures that are able to evaluate the estrogenic activity of metabolites.[14–16] In mutagenicity testing, incubation with an S-9 extract mixture has been the standard method for *in vitro* metabolic activation.[17] Here we apply a yeast two-hybrid assay for detection of estrogenic activity after metabolic activation by incubation with an S-9 extract mix (S-9 mix), and examine the estrogenic activities of metabolites of benzophenone derivatives.

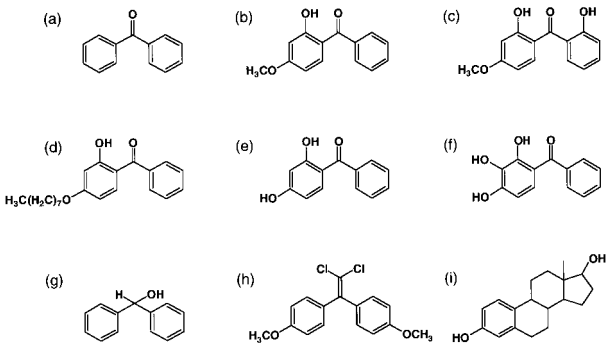

Fig. 1. The Structure of Chemicals Examined in This Assay

a), benzophenone; b), BZ-3; c), BZ-8; d), 2-hydroxy-4-octyloxybenzophenone (BZ-12); e), 2,4-dihydroxybenzophenone (BZ-1); f), 2,3,4-trihydroxybenzophenone; g), benzhydrol; h), MXC, i), E_2.

MATERIALS AND METHODS

Chemicals —— 17-β-Estradiol (E_2, > 97.0%), MXC (> 97.0%), benzophenone (> 98.0%), 2,4-dihydroxybenzophenone (> 98.0%), 2,3,4-trihydroxybenzophenone (> 98.0%), and benzhydrol (> 98.0%) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2-Hydroxy-4-methoxybenzophenone (> 98.0%), 2,2′-dihydroxy-4-methoxybenzophenone (> 98.0%) and 2-hydroxy-4-octyloxybenzophenone (> 98.0%) were purchased from Aldrich Chem. Co. (Milwaulkee, WI, U.S.A.). All other chemicals were reagent grade, obtained from commercial sources, and used without further purification.

Activation by an S-9 Fraction —— S-9 extracts (rat liver 9000 × *g* supernatant fraction induced with phenobarbital and 5,6-benzoflavone) and glucose-6-phosphate dehydrogenase (G6PDH) were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). To a tube containing 990 μl of the S-9 mix (S-9 mix: 20 μl S-9, 0.8 μmol NADPH, 0.8 μmol NADH, 1.0 μmol glucose-6-phosphate, 0.4 U G6PDH, 20 μmol Na_2HPO_4, 20 μmol NaH_2PO_4, 6.6 μmol KCl and 1.6 μmol $MgCl_2$) 10 μl of each test chemical dissolved in dimethyl sulfoxide (DMSO) was added and then incubated at 37°C for 4 hr. The chemicals, after incubation with the S-9 mix, were stored at –80°C until their application to the yeast two-hybrid strain. The heat-inactivated S-9 extract was prepared by incubation at 95°C for 5 min, and used for the negative control experiments. The structures of chemicals examined in this paper are shown in Fig. 1.

Yeast Two-Hybrid Assay for Detecting Estrogenic Activity after Metabolic Activation —— In this study, we used the yeast two-hybrid system with the estrogen receptor, estrogen recepter α (ERα), and the coactivator, transcriptional intermediary factor 2 (TIF2), as previously described.[8,9] Yeast cells carrying the pGBT9-estrogen receptor ligand binding domain (pGBT9-ERLBD) and pGAD424-TIF2 plasmids were grown overnight at 30°C with vigorous shaking in selective medium (S.D. medium lacking tryptophan and leucine). The yeast cells, resuspended in 2 × S.D. medium made up at twice the usual concentration, were mixed at a 1 : 1 (v/v) ratio with the test chemicals, which had been treated with the S-9 mix, and then incubated at 30°C for 4 hr. Aliquots of cells were withdrawn and washed by centrifugation. The cell density was determined by measurement of the absorbance at 595 nm. A lysate was prepared by enzymatic digestion of the cells with 1 mg/ml Zymolyase 20T at 37°C for 15 min. The lysate (200 μl) was mixed with 4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (40 μl) and incubated at 30°C for 30 min. The enzymatic reaction was stopped by the addition of 1 M Na_2CO_3 (100 μl). β-Galactosidase activity was calculated as described previously.[8] Estrogenic activity was also tested by MCF-7 proliferation assay[18] and a reporter gene assay using HeLa cells.[19]

HPLC Analysis of BZ-3 Metabolites —— BZ-3 (1.0×10^{-4} M) was incubated with the S-9 mix as mentioned above. The metabolites after S-9 activation (1.5 ml) were extracted twice with 3.0 ml ethylacetate. The extracts were dried under an N_2 stream and dissolved in 30 μl methanol. The extracts (15 μl) were applied to a reverse phase HPLC column (Cadenza CD-C18, 4.6 × 250 mm, 3 μm; Imtakt, Kyoto, Japan). LC-10AD pumps were used with a DGU-14A degassing unit and C-R7A integrator (Shimadzu, Kyoto, Japan). The HPLC column was eluted with a 75% methanol/water at a flow rate of

1.0 ml/min. The eluate was monitored at 230 nm with an SPD-10AV detector (Shimadzu). Each 0.5 ml fraction was collected and dried under an N_2 stream, and dissolved in 10 μl DMSO to be applied to the yeast two-hybrid strain for testing estrogenic activity.

Liquid Chromatography/Mass Spectrometry Analysis of BZ-3 Metabolites —— Liquid chromatography/mass spectrometry (LC/MS) analysis was performed on an API3000 (Applied Biosystems, Foster City, CA, U.S.A.) equipped with an electrospray ionization (ESI) interface and an Agilent 1100 series HPLC from Agilent Technologies (Waldbronn, Germany). The HPLC system consisted of a G1312A HPLC binary pump, a G1367A autosampler and a G1379A degasser. The column used was a reverse phase HPLC column (Cadenza CD-C18, 2.0 × 100 mm, 3 μm; Imtakt). The mobile phases consisted of 100% acetonitrile (A) and 1% aqueous acetic acid (B). Elution was performed using a linear gradient from 30% A to 80% A during 30 min at 0.2 ml/min. The ESI interface was control by Analyst software (v.1.2). ESI-MS was operated in negative or positive ion mode. The heated capillary and voltage were maintained at 500°C with and –/+4.2 kV (negative/positive mode), respectively. Mass spectra were measured from *m/z* 50 up to *m/z* 300.

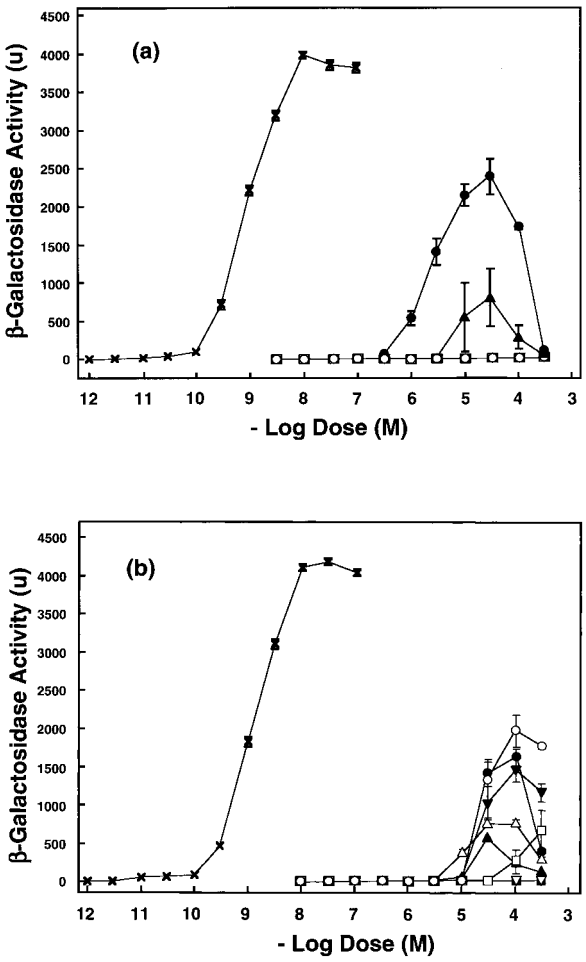

Fig. 2. Dose–Response Curve for Chemicals Incubated in Inactive S-9 Mix (a) and in Active S-9 Mix (b)

Values are means of three separate experiments (bars: S.D.). Benzophenone, (□); BZ-3, (○); BZ-8, (△); BZ-12, (▽); BZ-1,(●); 2,3,4-trihydroxybenzophenone, (▲); benzhydrol, (■); MXC, (▼); E2, (×).

RESULTS

Estrogenic Activity of Metabolites of Benzophenone Derivatives

The negative control experiments were performed using an S-9 mix containing inactive S-9 extracts (an inactive S-9 mix). Serial dilutions of E_2 were incubated with the inactive S-9 mix at 37°C for 4 hr, and then the reaction mixtures were applied to the yeast two-hybrid assay. Maximum β-galactosidase activity induced by incubation with E_2 was obtained at concentrations of 1.0×10^{-8} M and higher (Fig. 2a). The concentration of E_2 showing 10% of the 1.0×10^{-7} M activity (relative effective concentration, REC_{10}) was 1.7×10^{-10} M. Under these conditions BZ-1 and 2,3,4-trihydroxybenzophenone exhibited estrogenic activities. Their REC_{10} values were 6.5×10^{-7} and 6.8×10^{-6} M, respectively. Benzophenone, BZ-3, BZ-8, BZ-12, benzhydrol and MXC did not exhibit estrogenic activities.

The metabolic activation experiments were performed using an S-9 mix containing active S-9 extracts (an active S-9 mix). Maximum β-galactosidase activity induced by incubation with E_2 was obtained at concentrations of 1.0×10^{-8} M and higher (Fig. 2b). The REC_{10} of E_2 was 2.5×10^{-10} M. MXC, benzophenone, BZ-3 and BZ-8 exhibited estrogenic activities after incubation with the active S-9 mix. Their REC_{10} values were 2.5×10^{-5}, 1.5×10^{-4}, 1.4×10^{-5} and 1.0×10^{-5} M, respectively. The estrogenicities of BZ-1 and 2,3,4-trihydroxybenzophenone were reduced by incubation with the active S-9 mix. Their REC_{10} values were 1.5×10^{-4} and 2.1×10^{-5} M, respectively.

HPLC Analysis of the Metabolites of BZ-3

After incubation of 1.0×10^{-4} M BZ-3 with the active S-9 mix, the metabolites were extracted with ethylacetate and then fractionated by HPLC. Three

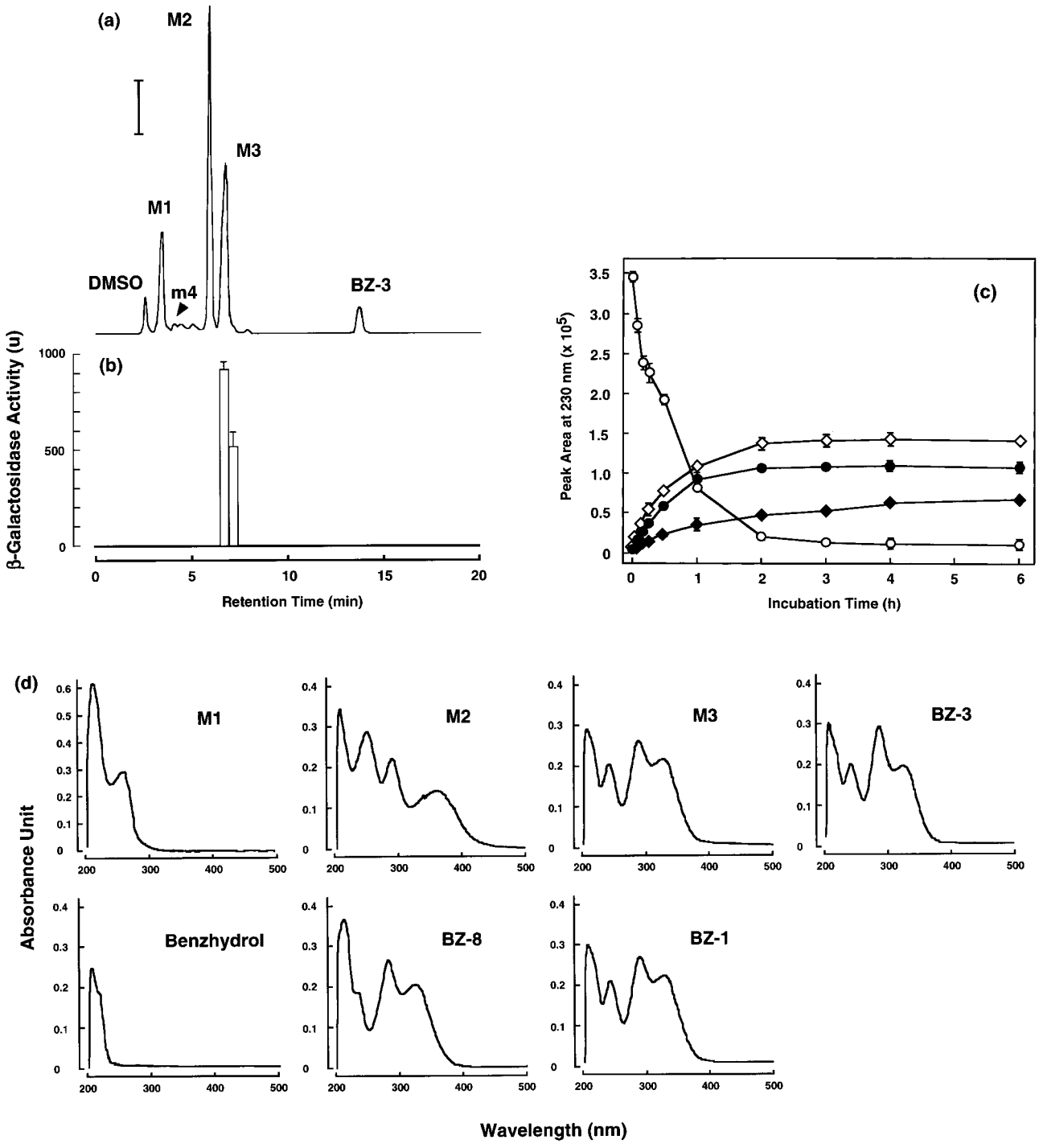

Fig. 3. The HPLC Analysis of the Metabolites of BZ-3 (a)

Bar expresses 0.2 auf. at 230 nm. The retention times were as follows (min): DMSO, 2.6; M1, 3.5; m4 (2,3,4-trihydroxybenzophenone), 4.1; M2, 5.9; M3 (BZ-1), 6.8; BZ-8, 8.1; BZ-3, 13.8. The estrogenic activities of the fractions (b). Values are means of three separate experiments (bars: S.D.). Changes in the levels of BZ-3 and its major metabolites, M1, M2 and M3 (c). Points express the peak area of recorded at 230 nm. Values are means of three separate experiments (bars: S.D.). BZ-3, (○); M1, (◆); M2, (◇); M3,(●). The concentration of BZ-3 at zero time was $9.5 \pm 0.1 \times 10^{-5}$ M. The UV spectra of M1, M2, M3, BZ-3, benzhydrol, BZ-8 and BZ-1 in methanol (d).

major metabolites (M1, M2 and M3) were detected (Fig. 3a). The retention time of a minor metabolite (m4) corresponded to that of 2,3,4-trihydroxybenzophenone. Each fraction was also examined for its estrogenic activity using the yeast assay system. The fractions containing M3 exhibited estrogenic activities (Fig. 3b). M1, M2 and M3 produced from BZ-3 concurrently (Fig. 3c). UV spectra of metabo-

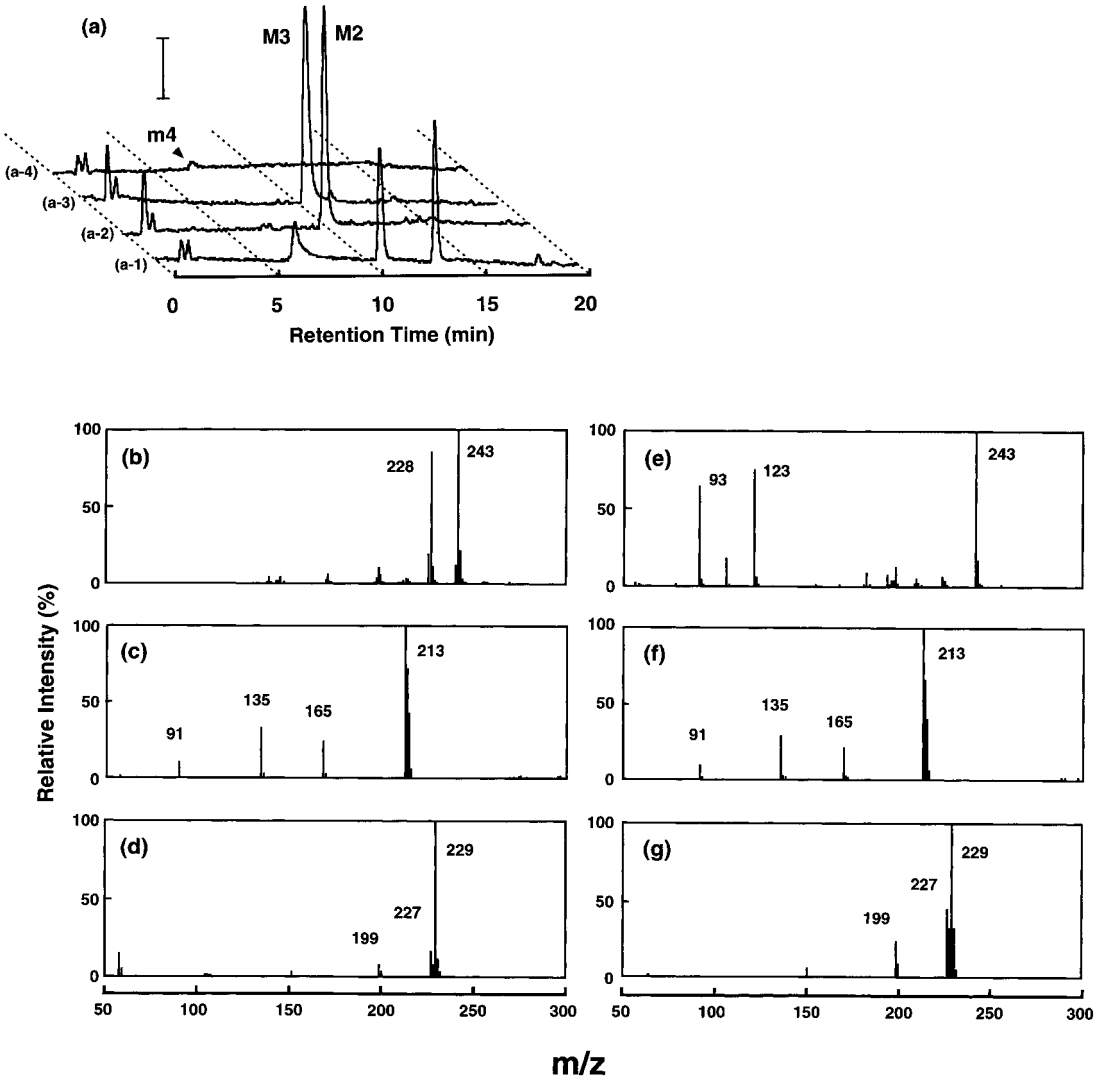

Fig. 4. Total Ion Chromatograms of BZ-3, BZ-8, BZ-1, 2,3,4-Trihydroxybenzophenone, M2, M3 and m4 (a)

Mixture of benzophenone derivatives containing 2.0×10^{-5} M BZ-3, BZ-8, BZ-1, and 2,3,4-trihydroxybenzophenone (a-1), M2 (a-2), M3 (a-3), m4 (a-4). Bar expresses the intensity of 1.0×10^{8} cps. The retention times were as follows (min): m4 (2,3,4-trihydroxybenzophenone), 6.7; M2, 10.0; M3 (BZ-1), 10.9; BZ-8, 13.5; BZ-3, 18.5. The mass spectra of M2 (b), M3 (c), m4 (d), BZ-8 (e), BZ-1 (f) and 2,3,4-trihydroxybenzophenone (g).

lites, BZ-3, benzhydrol, BZ-8 and BZ-1 in methanol were exhibited in Fig. 3d. Both benzhydrol and M1 did not have absorption above 280 nm. M2 absorbed from 200 to 440 nm with maxima at 290 and 360 nm and minima at 277 and 326 nm. M3 absorbed from 200 to 400 nm with maxima at 289 and 325 nm and minima at 262 and 312 nm. The profiles of UV spectrum and retention time in HPLC analysis of M3 were identical to those of BZ-1. After incubation of 1.0×10^{-4} M BZ-3 with the active S-9 mix for 4 hr, the concentrations of M3 (BZ-1) and BZ-3 were $3.3 \pm 0.1 \times 10^{-5}$ and $2.0 \pm 0.2 \times 10^{-6}$ M, respectively. To obtain further information about the metabolites, LC/MS analysis was also performed. The total ion chromatograms (negative ion mode) of M2, M3 and m4 were exhibited Fig. 4a. The mass spectra of M2, M3, m4, BZ-1 and 2,3,4-trihydroxybenzophenone were shown in Figs. 4b–4g, respectively. The base peak was detected at *m/z* 243 in the mass spectrum of M2. The mass spectra of M3 and m4 were identical to those of BZ-1 and 2,3,4-trihydroxybenzophenone, respectively. M1 did not detectable in both negative and positive ion mode.

DISCUSSION

P450 enzymes in the active S-9 mix are able to convert E_2 to its oxidative metabolites.[20] The dose–

response curves of E_2 after the incubation with the active S-9 mix were almost identical to that after the incubation with the inactive S-9 mix containing medium (Figs. 2a and b). Under these conditions, degradation of E_2 in the active S-9 mix containing medium was not observed (data not shown). The active S-9 mix fraction is able to mix with yeast cells suspended in 2 × S.D. medium without the need for additional measures.

The hydroxyl group of E_2 at the 3-position plays an important role in activating the ER.[21] Without the S-9 mix activation, benzophenone derivatives with a hydroxyl group at the 4-position, BZ-1 and 2,3,4-trihydroxybenzophenone, exhibited estrogenic activities. Some compounds containing phenol residues, such as *p*-alkylphenols, parabens and bisphenol A, exhibit estrogenic activities.[9,22–24] The phenol residues of these compounds are believed to participate in mimicking E_2 at the ER ligand-binding domain.[9] The phenol residue in benzophenone derivatives would also play such a role in activating ER. MXC, benzophenone, BZ-3 and BZ-8 exhibited estrogenic activities after incubation with the active S-9 mix. MXC is designated as a proestrogen for its conversion to an estrogenic metabolite, 2,2-*bis*(hydroxyphenyl)-1,1,1-trichloroethane.[12] It was also confirmed by us that benzophenone after metabolic activation showed estrogenic activity as well in the proliferation assay and the reporter gene assay using cultured cells (data not shown). This assay system is applicable for the detection of the proestrogens. We demonstrated that BZ-3 was converted to an estrogenic metabolite BZ-1 by incubation with the active S-9 mix. BZ-8 has an additional hydroxyl group at 2′-position of BZ-3. The estrogenic activities of its metabolites would depend on 2,2′,4-trihydroxybenzophenone and/or metabolites with hydroxyl group at a 4′-position. With both incubation with the inactive and the active S-9 mix, BZ-12 exhibited no estrogenic activity. The bulky octyloxy group moiety may prevent hydroxylation and/or ER activation.

BZ-3 is metabolized to BZ-1, BZ-8 and 2,3,4-trihydroxybenzophenone *in vivo*.[2–5] In the assay system, BZ-3 was metabolized to M1, M2, M3 (BZ-1) and m4 (2,3,4-trihydroxybenzophenone). The structures of M1 and M2, non-estrogenic metabolites, were inferred by examination of their UV spectra and LC/MS analysis. M1 was assumed to be a benzhydrol derivative for its lack of absorption above 280 nm. Benzhydrol could be one of the major metabolites of benzophenone in hepatocytes,[2–5] and had no absorption above 250 nm in methanol. Instead of formation of BZ-8, an unknown metabolite, M2, was detected. Compared to BZ-3, the UV spectrum of M2 was shifted to a longer wavelength. The mass spectrum suggested a molecular weight (M.W.) for M2 of 244. The M.W. of BZ-3 is 228.2. The UV and mass spectra of M2 were quite different from those of BZ-8 (Figs. 3d, 4b and 4e). M2 would be formed by the hydroxylation in the aromatic ring with methoxy and hydroxyl groups of BZ-3.

BZ-3 is used in many cosmetics and sunscreens as a UV-absorber.[1] The compound can be absorbed topically and converted to an estrogenic metabolite, BZ-1.[25,26] We demonstrated that BZ-3 was converted to the estrogenic metabolite, BZ-1, in a 33% yield by incubation with S-9 mix for 4 hr. At the same condition, 2,3,4-trihydroxybenzophenone was produced in a less than 1% yield (data not shown). Thus, the yield of non-estrogenic metabolites including M1 and M2 based on BZ-3 was approximately 60%. From 1 to 10% of BZ-3 in cosmetic products penetrates human skin.[25,26] These facts suggest that BZ-1 is produced *in vivo* by those applying a sunscreen or a cosmetic containing BZ-3. UV absorbers are increasingly used as a result of growing concern about UV irradiation and skin cancer. Schlumpf, *et al*. reported that other UV absorbers, such as 4-methyl-benzylidene camphor and octyl-methoxycinnamate, also exhibit estrogenic activity.[27] Studies of the effects on endocrine systems by UV absorbers should be performed more extensively, because of their use in children.

This assay system was able to detect the conversion of BZ-3 to an estrogenic metabolite in a minimum number of steps. This yeast two-hybrid system is able to evaluate the effects of chemicals on thyroid hormone receptors and androgen receptors by changing pairs of the receptors and coactivators to the relevant pairs.[8] Studies of the thyromimetic and anti-thyromimetic activities of metabolites of chemicals are ongoing in our laboratory. This assay system will be a useful tool for the detection of prohormonal activities of chemicals.

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