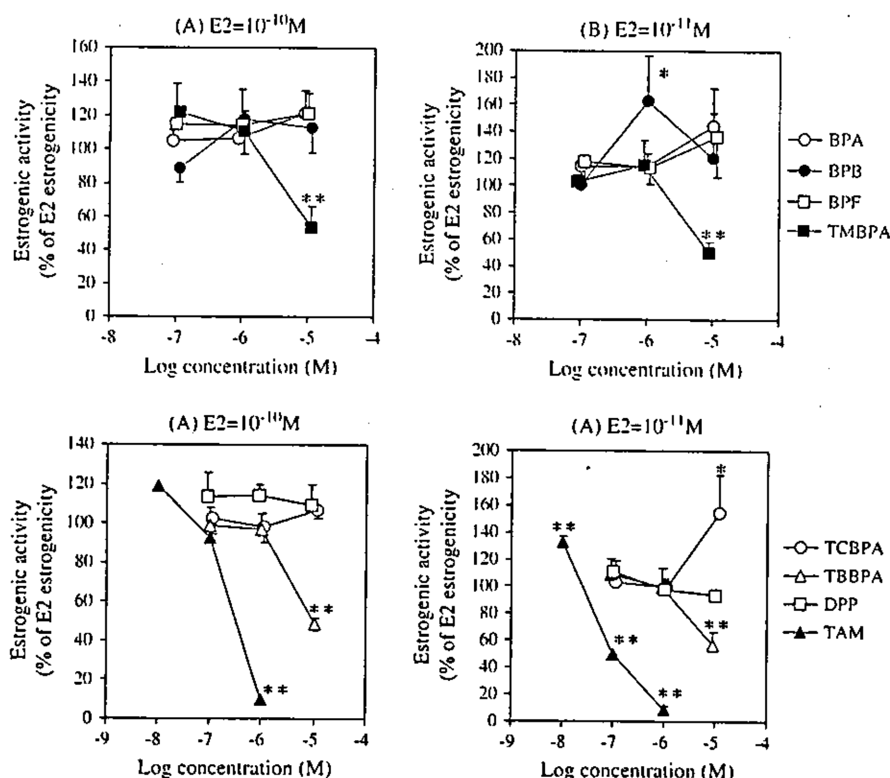

FIG. 3. Anti-estrogenic activity of bisphenol A (BPA) and related compounds against estrogenic activity of 17-β-estradiol (E2) in ERE-luciferase reporter assay with MCF-7 cells. Each bar represents the mean ± SD of four experiments. Anti-estrogenic activity was expressed as a relative activity with respect to the E2 activity using MCF-7 cells. One-hundred percent at 10^{-11} M and 10^{-10} M corresponds to 9.1- and 32-fold induction (see Fig. 2). BPB; bisphenol B, BPF; bisphenol F, TMBPA; tetramethylbisphenol A, TBBPA; tetrabromobisphenol A, TCBPA; tetrachlorobisphenol A, DPP; 2,2-diphenylpropane, TAM; tamoxifen. *$p < 0.05$, **$p < 0.01$ compared with control.

in the case of TCBPA) – 1×10^{-4} M. BPA and HDM also showed estrogenic activity at higher concentrations. However, DPP and DPM, which lack a hydroxyl group, were inactive (Fig. 2). DMBPA, TMBPA, BPF, BPAD, BPA catechol, BPA ol, BPA carboxylic acid, and TBBPA also showed estrogenic activity at $1 \times 10^{-6} - 1 \times 10^{-4}$ M. When ICI 182,780, a pure estrogen receptor antagonist, was added at the concentration of 1×10^{-8} M, the estrogenic activities of these compounds were markedly inhibited (data not shown). The EC50 values of estrogenic activity of the positive compounds are shown in Table 1. TCBPA showed the highest activity, followed by BPAF, BPB, HPP, BPCH, HDM, DMBPA, BPA, TMBPA, BPAD, and BPF. When the propane bridge of BPA was substituted with a hydrophilic group, the estrogenic activities of these compounds were markedly inhibited, showing the specific nature of this response (compare BPA ol and BPA carboxylic acid with BPA). On the contrary, the activity was increased by substitution with a hydrophobic group (compare BPA, BPAF and BPB with BPF). BPA catechol, 3-hydroxyl derivative of BPA, exhibited little estrogenic activity. DPP, the dehydroxylated derivative of BPA, and DPM were negative in the estrogen screening assay. IPP, lacking the B-phenyl ring, showed no activity. Thus, at least one 4-hydroxyl group of BPA derivatives is essential for estrogenic activity. The second phenyl group attached the 2-position of propane is also necessary.

Anti-estrogenic Activity of BPA and Related Compounds

Anti-estrogenic activity of these BPA derivatives was also examined by the addition of these compounds to the E2 assay system in MCF-7 cells. Inhibitory effects of TMBPA and TBBPA on the estrogenic activity of 1×10^{-10} and 1×10^{-11} M E2 were observed at the concentration of 1×10^{-5} M. However, little effect on the estrogenic activity of E2 was observed with BPA, BPB, DPP, TCBPA, and BPF in the concentration range of $1 \times 10^{-7} - 1 \times 10^{-5}$ M (Fig. 3). Other BPA derivatives did not inhibit the estrogenic activity of E2 (data not shown). Thus, BPA and related compounds, except TMBPA and TBBPA, lack significant anti-estrogenic activity, at least in the MCF-7 reporter assay.

Estrogenic Activity of BPA Derivatives in Vivo

Estrogenic potential of TCBPA, TBBPA, and BPA *in vivo* was further investigated by use of the uterotrophic assay with ovariectomized mice. The body weight of rats administered

TABLE 2
Estrogenic Activity of Bisphenol Derivatives *in Vivo*

Group	Uterus/body weight (mg/kg)	% of control
Control	435 ± 30	100
E_2		
50 μg/kg	2157 ± 518**	496
TBBPA		
20 mg/kg	538 ± 85*	124
100 mg/kg	594 ± 67**	137
300 mg/kg	716 ± 56**	164
500 mg/kg	538 ± 88*	123
TCBPA		
20 mg/kg	514 ± 84*	118
100 mg/kg	582 ± 52**	134
300 mg/kg	716 ± 56**	164
500 mg/kg	713 ± 82**	164
BPA		
20 mg/kg	640 ± 48**	147
100 mg/kg	705 ± 75**	162
300 mg/kg	806 ± 70**	185
500 mg/kg	765 ± 131**	176

Note. The uterotrophic assay of bisphenol derivatives. Weights of the uteri of rats are shown. Ovariectomized female B6C3F1 mice were treated with 17β-estradiol (E2; 50 μg/kg/day), 2,2-bis(3,5-dibromo-4-hydroxyphenyl)propane (TBBPA; 20, 100, 300, 500 μg/kg/day), 2,2-bis(3,5-dichloro-4-hydroxyphenyl)propane (TCBPA; 20, 100, 300, 500 mg/kg/day), or 2,2-bis(4-hydroxyphenyl)propane (BPA; 20, 100, 300, 500 mg/kg/day) for three days. Each group consisted of five animals. Uterus/body weight (mg/kg) indicates mean ± SEM of five mice.

** $p < 0.01$, * $p < 0.05$ indicate significant differences from control (OVX mice) experiments.

TCBPA, TBBPA, or BPA was not decreased compared with other groups. The uterine weight in the groups given these compounds was increased compared with the group given vehicle only. BPA was most effective, followed by TBBPA and TCBPA. BPA gave a 147% increase in uterus weight over the castration control at 20 mg/kg, while 124 and 118% increases were noted in the TBBPA and TCBPA groups, respectively. Weight increase was dependent on the administered dose between 0 and 300 mg/kg for each compound, albeit changes were moderate when compared with the response to E2 (Table 2). Thus, the estrogenic effect of these bisphenol derivatives was confirmed *in vivo*.

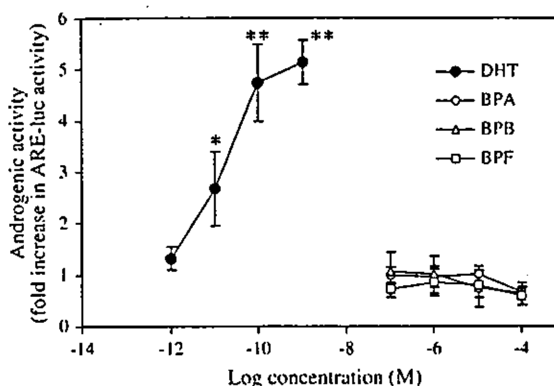

FIG. 4. Androgenic activity of bisphenol A (BPA) and related compounds in ARE-luciferase reporter assay with NIH3T3 cells. Each bar represents the mean ± SD of four experiments. Androgenic activity was expressed as a relative activity with respect to the control using NIH3T3 cells. BPB; bisphenol B, BPF; bisphenol F, DHT; dihydrotestosterone. * $p < 0.05$, ** $p < 0.01$ compared with control.

Androgenic Activity of BPA and Related Compounds

Androgenic activity of BPA and related compounds was examined using NIH3T3 cells transfected with an AR responsive luciferase reporter gene. DHT exhibited markedly the androgenic activity toward NIH3T3 cells at 1×10^{-11}–1×10^{-9} M. However, no androgenic activity of BPA, BPB, or BPF was observed in the concentration range of 10^{-7}–10^{-4} M (Fig. 4). Other BPA derivatives did not show androgenic activity (data not shown).

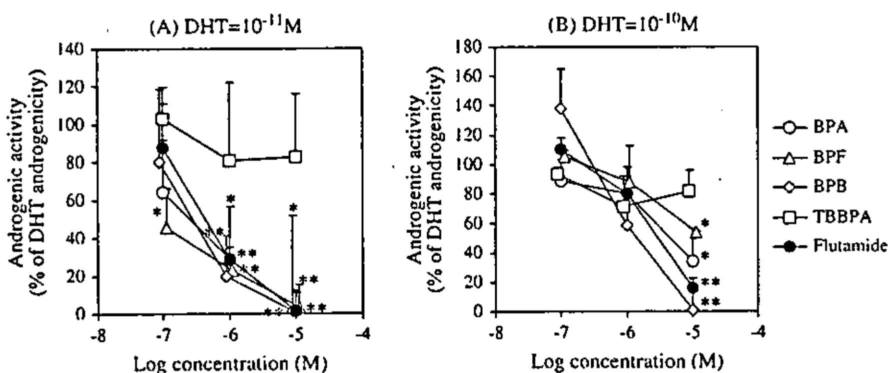

FIG. 5. Anti-androgenic activity of bisphenol A (BPA) and related compounds against androgenic activity of dihydrotestosterone (DHT) in ERE-luciferase reporter assay with NIH3T3 cells. Each bar represents the mean ± SD of four experiments. Antiandrogenic activity was expressed as a relative activity with respect to DHT using NIH3T3 cells. One-hundred percent at 10^{-11} M and 10^{-10} M corresponds to 2.6- and 4.7-fold induction (see Fig. 4). BPB; bisphenol B, BPF; bisphenol F, TBBPA; tetrabromobisphenol A. * $p < 0.05$, ** $p < 0.01$ compared with control.

TABLE 3
IC50 Values of Bisphenol Derivatives for Androgen Activity of Dihydrotestosterone in NIH3T3 Luciferase Reporter Assay

	IC50 (μM)
TMBPA	0.29
BPAF	1.3
BPAD	1.4
BPB	1.7
DMBPA	2.0
HDM	3.9
HPP	4.2
BPA	4.3
IPP	6.2
BPCH	7.9
BPD	7.9
BPF	12
BPA catechol	14
BPS	17
DPP	370
TCBPA	870
TBBPA	>1000
DPM	>1000
BPA ol	>1000
BPA acid	>1000
Flutamide	2.5

Note. EC50 for DHT: 1.1×10^{-11}.

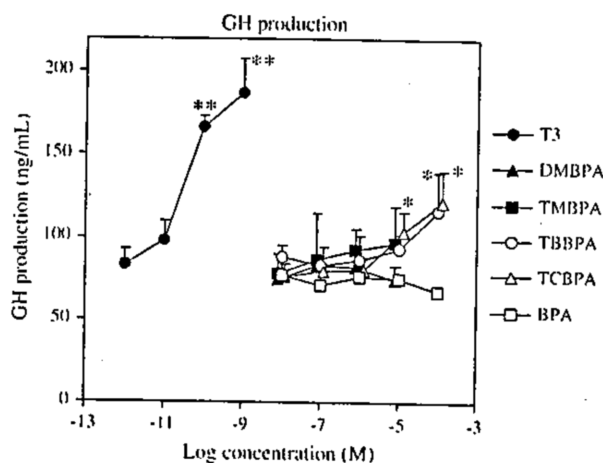

FIG. 6. Thyroid hormonal activity of bisphenol A (BPA), tetrabromobisphenol A (TBBPA), tetrachlorobisphenol A (TCBPA), tetramethylbisphenol A (TMBPA), and 3,3′-dimethylbisphenol A (DMBPA) in terms of GH production in GH3 cells. Each bar represents the mean ± SD of four experiments. Thyroid hormonal activity was expressed as a relative activity with respect to the control using GH3 cells. T3; L-3,5,3′-triiodothyronine. $*p < 0.05$, $**p < 0.01$ compared with control.

Anti-androgenic Activity of BPA and Related Compounds

When BPA, BPF, or BPB was added to the DHT assay system in the concentration range of 1×10^{-7}–1×10^{-5} M, the activity of 1×10^{-11} M DHT was inhibited concentration-dependently. The anti-androgenic effects of these compounds were also observed at 1×10^{-10} M DHT. However, no effect of TBBPA on the androgenic activity of DHT was observed (Fig. 5). These anti-androgenic activities were in the same order as that of flutamide. Anti-androgenic activities of BPS, HPP, and DPP were also observed at 1×10^{-6}–1×10^{-4} M (data not shown). Table 3 summarizes the IC50 values of these compounds against the androgenic activity of 1×10^{-10} M DHT. The highest activity among the test compounds was that TMBPA, followed by BPAF, BPAD, BPB, DMBPA, HDM, and HPP. IPP, without a phenyl group at the 2-position of the propane moiety, was also active. 3-Hydroxylated BPA, BPA catechol, showed weak activity. DPP and DPM, the dehydroxylated compounds of BPA and BPF, showed little or no antiandrogenic activity, and TBBPA, BPA ol, and BPA carboxylic acid were negative in the assay. These results show that at least one 4-hydroxyl group of BPA derivatives is essential for the activity. 3,5-Substituents markedly influenced anti-androgenic activity. TMBPA showed the highest activity, but TBBPA and TCBPA did not show any significant anti-androgenic effect in the concentration range of 1×10^{-7}–1×10^{-4} M. These findings indicate that some BPA derivatives are anti-androgenic as well as estrogenic.

Thyroid Hormonal Activity of BPA and Related Compounds Evaluated by Growth Hormone Production Assay of GH3 Cells

The thyroid hormonal activities of BPA and related compounds were examined by measuring the ability of these compounds to induce the thyroid hormone-dependent production of growth hormone by GH3 cells. Growth hormone-releasing activity was observed with T_3 in the concentration range of 1×10^{-12}–1×10^{-9} M. An increase of growth hormone release from GH3 cells was observed after the addition of TBBPA or TCBPA in the concentration range of 1×10^{-6} to 1×10^{-4} M. TMBPA also weakly induced growth hormone release, but DMBPA was ineffective. The effects of DMBPA and TMBPA at 1×10^{-4} M could not be judged due to the cytotoxicity of these compounds (Fig. 6). BPA, BPF, BPS, BPAF, BPAD, and BPB also showed no activity (data not shown). TBBPA had the highest activity, followed by TCBPA and TMBPA. These results indicate that some BPA derivatives show thyroid hormone-like activity, and that a 4-hydroxyl group is essential for this activity, as well as for estrogenic and anti-androgenic activities. Bulky 3- and 5-substituents play an important role in the activity. However, other BPA derivatives were negative in the assay. In contrast, when the inhibitory effects of TBBPA, TCBPA, TMBPA, DMBPA, BPA, and BPB on the hormonal activity of T3 towards GH3 cells were examined, these compounds at 1×10^{-5} and 1×10^{-4} M showed no antagonistic action towards growth hormone production induced by the thyroid hormone (data not shown).

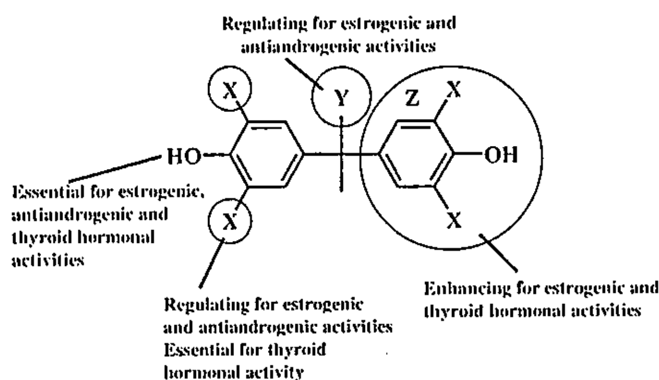

FIG. 7. Structural requirement of bisphenol A and related compounds for endocrine-disrupting activity.

These results suggest that TBBPA, TCBPA, TMBPA, and DMBPA act as thyroid hormone agonists, but not antagonists.

DISCUSSION

In this study, we examined the relationship between the structure of BPA derivatives and endocrine-disrupting activity, i.e., estrogenic, anti-estrogenic, anti-androgenic, and thyroid hormonal activities. Minimum structural requirements for estrogenic activity of BPA derivatives seems to be a 4-hydroxyl group on the A-phenyl ring and a hydrophobic moiety at the 2-position of the propane moiety, judging from the activity estimated in this study. However, a 2-hydroxyl or 3-hydroxyl group attached to a phenyl ring of biphenyl or benzophenone has been reported to be effective for estrogenic activity, and the order of the activity is 4-hydroxyl>3-hydroxyl>2-hydroxyl in both cases (Blair *et al.*, 2000; Kawamura *et al.*, 2003; Kitamura *et al.*, 2003b; Paris *et al.*, 2002; Soto *et al.*, 1997). In our preliminary study using MCF-7 reporter assay, 2-hydroxydiphenylmethane and 3-hydroxydiphenylamine showed activity, with EC50 values of 32.4 μM (0.32 μM for 4-hydroxydiphenylmethane) and 4.2 μM (2.0 μM for 4-hydroxydiphenylamine), respectively. It is possible that a 2- or 3-hydroxyl group also contributes to the estrogen receptor affinity of bisphenol derivatives. Moreover, substituents at the 3,5-positions of the A-phenyl ring and at the methylene bridge markedly influence the estrogenic activity. TCBPA, TMBPA, and DMBPA showed high activity, but TBBPA had lower activity. Estrogenic activity of TCBPA has already been reported (Fukazawa *et al.*, 2002; Kuruto-Niwa *et al.*, 2002; Olsen *et al.*, 2003). Hydrophobic substituents in place of the 1-methyl group of the propane moiety increased the hormonal activity, as seen in BPAF and BPB, but a hydrophilic group, such as a hydroxymethyl or carboxylic acid group, decreased the activity. Hydrophobicity at the methylene bridge of BPA derivatives is an important factor for the estrogenic activity (Fig. 7).

For estrogen receptor ligand activity, xenobiotics require an unhindered hydroxyl group on an aryl ring and a hydrophobic group attached *para* to the hydroxyl group (Blair *et al.*, 2000; Elsby *et al.*, 2000; Fang *et al.*, 2000; Hong *et al.*, 2002; Nishihara *et al.*, 2000). The key structural requirement for estrogenic activity of bisphenol derivatives is the phenolic hydroxyl group. It is reported that 3-hydroxyl group of E2 interacts with Glu353 and Arg394 at the binding pocket of human estrogen receptor α via hydrogen bonding, and the 17β-hydroxyl group interacts with His524, based on a crystallographic analysis of estrogen receptor α bound with E2 (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). Perhaps the 4-hydroxyl group of BPA also interacts with these amino acids. TCBPA and TMBPA exhibited higher estrogenic activity than did BPA. 3,5-Chloro and methyl substituents of BPA may assist tight fitting of the ligand into the ligand-binding pocket. However, a lower activity was observed in the case of TBBPA. This may be due to steric hindrance by the bulky bromo substituent. Regarding the estrogenic activity of TBBPA, Christiansen *et al.* (2000) reported that the vitellogenin level of male rainbow trout did not increase after ip injection of TBBPA. Olsen *et al.* (2003) reported that the estrogenic activity of TBBPA is lower than that of BPA. The activity of 3-hydroxy-BPA (BPA catechol) was lower than that of BPA. The reason may be steric hindrance, because the 3-hydroxyl group should be effective for estrogenic activity. The hydrophobic moiety of the protein, consisting of Met323, Ala350, Leu346, Phe404, Leu428, etc., is also important for the interaction with the hydrophobic B, C, and D rings of E2 (Brzozowski *et al.*, 1997; Shiau *et al.*, 1998). The hydrophobic propane and B-ring moieties of BPA may bind tightly with the hydrophobic binding site of the estrogen receptor. Attachment of hydrophilic substituents at the methylene bridge markedly decreased the estrogenic activity. This may be due to a decrease in the stability of the interaction with the hydrophobic protein site. In contrast, we observed antagonistic activity of TMBPA and TBBPA against the estrogenic activity of E2, but other BPA derivatives lacked this activity. TMBPA showed very high estrogenic activity among the test compounds. TMBPA and TBBPA acted as both agonist and antagonist at the estrogen receptor. The structural requirements for antagonistic action need further study.

In the present study, MCF-7 was primarily used for examining estrogenic activity. Although this cell line has been widely used to screen estrogenic activity in environmental chemicals (Soto *et al.*, 1995), assay data from a single cell line may contain both false negative and false positive results which are related to certain cross-talk pathways in the cell. Therefore, we also utilized other cell lines, including a rat pituitary cell line expressing a high level of ER α, MtT/E-2 and a mouse fibroblast cell line, NIH3T3, transiently transfected with ER α or β (Fujimoto *et al.*, 2004; Maruyama *et al.*, 1999). We generally confirmed the estrogenic activity of compounds with one of these cell lines after screening in MCF-7. It is noteworthy that ERE-dependent transcriptional activation of BPB and BPA reached more than 200% of the maximal E2

FIG. 8. Postulated metabolic pathways and the effect of metabolic modification on the estrogenic and anti-androgenic activities of bisphenol A.

effect. Such "supramaximal" effects have been reported with genistein and other phytoestrogens, although the mechanism involved is not clear (Kuiper *et al.*, 1998).

It is reasonable that estrogenic activity was decreased by transformation to further oxidized metabolites, or conjugates with glucuronic acid or sulfuric acid at the hydroxyl group (Elsby *et al.*, 2001; Matthews *et al.*, 2001; Nakagawa and Suzuki, 2001; Pottenger *et al.*, 2000; Snyder *et al.*, 2000). In this study, BPA catechol, BPA ol, and BPA carboxylic acid, candidates for BPA metabolites formed by liver microsomal enzymes, showed decreased activity. However, Yoshihara *et al.* (2001, 2004) reported that when BPA was incubated with liver microsomes and cytosol together, the native estrogenic activity was enhanced. The enhanced activity might be due to a dimerized type of metabolite, 4-methyl-2,4-bis(*p*-hydroxyphenyl)pent-1-ene, which is a potent estrogen (Fig. 8). Further study is necessary to establish the effect of metabolic modification on the activity of BPA. The *in vivo* estrogenic activity of BPA has been reported (Ashby *et al.*, 2000; Kim *et al.*, 2001; Matthews *et al.*, 2001; Tinwell *et al.*, 2000): the weight of the ovary in ovariectomized rats dosed with BPA was increased compared to that in rats dosed with vehicle alone. The estrogenic activity of BPA in rats *in vivo* seems to be due to both BPA itself and its metabolites. However, there are some reports indicating that BPA does not show estrogenic activity *in vivo* (Coldham *et al.*, 1997; Gould *et al.*, 1998). In the present study, we examined the estrogenic activity of BPA, TCBPA, and TBBPA *in vivo* by means of uterotrophic assay in ovariectomized mice. These compounds were positive in this study. However, the activity of TCBPA, which showed the highest activity among the bisphenol derivatives tested in the reporter assay conducted in this study, was lower than that of BPA. Perhaps, this lower activity of TCBPA *in vivo* is due to greater metabolic inactivation as compared with BPA. Alternatively, metabolic activation as in the case of BPA may not occur with TCBPA. In contrast, TBBPA showed a significant estrogenic activity *in vivo* in ovariectomized mice, in spite of having little activity in *in vitro* assay. TBBPA might be resistant to metabolic inactivation by glucuronidation or sulfation at the 4,4′-dihydroxyl groups due to steric hindrance. It is clearly necessary to consider the activity of metabolites produced from the parent compounds in assessing the hormonal toxicity of environmental contaminants, including bisphenol derivatives.

In this study, we found that BPA, BPB, and BPS are potent anti-androgens. However, BPA catechol, BPA ol, BPA carboxylic acid, DPM, and TBBPA were inactive. BPA is reported to have an inhibitory effect on the androgenic activity of DHT in a yeast-based assay (Sohoni and Sumpter, 1998). BPA also has binding affinity for androgen receptor in PALM cells (Paris *et al.*, 2002). The hydroxyl group on the A-phenyl ring of BPA is essential for the anti-androgenic activity as well as estrogenic activity. In this study, anti-androgenic activity of bisphenol derivatives was restricted to 4-hydroxyl derivatives. However, a 2-hydroxyl or 3-hydroxyl group has been reported to be effective in phenylphenols and hydroxybenzophenones (Kitamura *et al.*, 2004; Paris *et al.*, 2002). The activity of phenylphenols was in the order of 3-hydroxyl>4-hydroxyl>2-hydroxyl,

while that of hydroxybenzophenones was in the order of 3-hydroxyl>2-hydroxyl>4-hydroxyl. Substitution at the 3,5-positions markedly altered anti-androgenic activity. TMBPA showed the highest activity among the test compounds, but TBBPA and TCBPA showed little activity (Fig. 7). The crystal structures of DHT bound to rat androgen receptor (Sack *et al.*, 2001) and of R1881 bound to human androgen receptor (Matias *et al.*, 2000) showed that the 3-carbonyl group of DHT interacts with Arg752, and the 17β-hydroxyl group interacts with Asn705 and Thr877 at the ligand binding site consisting of 18 amino acids of the receptor, and the hydrophobic moiety of the protein fixes the B, C, and D rings of DHT. The 3-carbonyl and 17β-hydroxyl groups of DHT are essential for agonistic action. BPA derivatives may act as antagonistic agents by interfering with the interaction of DHT with Arg752 of the receptor owing to the presence of the 4-hydroxyl group and hydrophobic moiety of BPA. The 4'-hydroxyl group may also interfere with the binding of the 17β-hydroxyl group of DHT to the androgen receptor. Hydrophilic substituents at the methylene bridge of BPA may disturb the interaction with a hydrophobic moiety of the protein in the same way as with the estrogenic receptor. Unexpectedly, *p*-isopropylphenol, which lacks the B-phenyl ring of BPA, exhibited anti-androgenic activity. Clearly the B-phenyl ring is not essential for this activity, unlike estrogenic activity. Substitution by a bulky group at the 3,5-positions of the A-ring seems not to hinder the interaction with androgen receptor.

We have already reported the agonistic activity of TBBPA and TCBPA on the thyroid hormonal activity of T_3 (Kitamura *et al.*, 2002). In the present study, we found that DMBPA and TMBPA, in addition to TBBPA and TCBPA, show thyroid hormonal activity, though other BPA derivatives do not. Moriyama *et al.* (2002) reported that BPA acts as an antagonist of the thyroid hormone action of T_3. We could not observe agonistic or antagonistic action of BPA. Ishihara *et al.* (2003) also did not observe affinity of BPA for thyroid hormone receptors. Perhaps the thyroid hormonal potency of TBBPA, TCBPA, DMBPA, and TMBPA found in this study is due to their structural resemblance to thyroid hormones. The rat pituitary cell line GH3, isolated from a rat pituitary tumor, has been widely used as a standard pituitary cell model, in which the growth hormone secretion depends markedly on thyroid hormones, but little on estrogen (Kitagawa *et al.*, 1987). In the present study using GH3 cells, we observed agonistic activities of TBBPA, TCBPA, DMBPA, and TMBPA toward thyroid hormone, but we could not detect anti-thyroid hormonal action of these compounds. A 4-hydroxyl group and double substitution by halogen or methyl group at 3,5-positions of the A-phenyl group were essential for thyroid hormonal activity in this study. Relatively large substituents at the 3,5-positions of the phenyl ring, besides the 4-hydroxyl group, seem to be necessary for thyroid hormonal activity (Fig. 7). Thyroid hormone receptor shows a rigid substrate specificity compared with estrogenic and androgenic receptors, because of the relatively smaller size of the active site. Wagner *et al.* (1995, 2001) conducted crystallographic analyses of rat thyroid hormone receptor α1 and its ligands, and reported that the 4'-hydroxyl group of thyroid hormones interacts with His381 of thyroid hormone receptor via hydrogen-bounds, and the 3'-iodo group interacts with the hydrophobic pocket formed by Gly290, Gly291, Phe215, and Met388. The thyroid hormonal activity decreased in the order of bromine, chlorine, and methyl substituents at the 3,5-positions of BPA. Weaker binding ability, due to these smaller-size substituents compared with iodine, with the hydrophobic pocket in the active site may be a reason for the decreased activity. Various polybrominated biphenyls, polybrominated diphenyl ethers or their metabolites with such structural features may compete with thyroid hormones for binding to the thyroid hormone receptor. Thyroid hormone disruption could occur through interaction with serum transport proteins, such as transthyretin and thyroid-binding globulin. Some PCBs, for instance, have been reported to bind to transthyretin with very high affinity. Although we previously found that both TCBPA and TBBPA are able to bind thyroid hormone receptor, the interactions of these compounds with serum transport proteins and other metabolic components should be examined in the future.

The 3,5-substituents of BPA markedly influenced the endocrine-disrupting activity. TCBPA exhibited the highest estrogenic activity among the test compounds. In contrast, TBBPA exhibited the highest thyroid hormonal activity. Some 3,5-substituted BPAs showed combined endocrine-disrupting activity. TCBPA, TMBPA, and TBBPA showed both estrogenic and thyroid hormonal activities. TMBPA and TBBPA showed both estrogenic and anti-estrogenic activities. TMBPA and DMBPA showed higher anti-androgenic activity. However, anti-androgenic activity was not observed with TBBPA or TCBPA. As 3,5-substituted BPA derivatives are widely used as industrial materials or found as environmental contaminants, it is necessary to consider other possible toxicities. In contrast, BPA, BPB, BPAF, HPP, and BPF exhibited both high estrogenic and anti-androgenic activities. The endocrine-disrupting activity of BPA derivatives *in vivo* may be based on combinations of endocrine-disrupting actions, as observed *in vitro*. Hydroxy-PCBs were also reported to show both estrogenic and anti-thyroid hormonal activities (Cheek *et al.*, 1999; Connor *et al.*, 1997; Korach *et al.*, 1988). The possibility of multiple endocrine-disrupting activities in animals *in vivo* should be taken into consideration in assessing the toxicity of environmental contaminants, including BPA derivatives.

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REFERENCES

Andersen, H. R., Andersson, A.-M., Arnold, S. F., Autrup, H., Barfoed, M., Beresford, N. A., Bjerregaard, P., Christiansen, L. B., Gissel, B., Hummel, R., Jørgensen, E. B., Korsgaard, B., Le Guevel, R., Leffers, H., McLachlan, J., Møller, A., Nielsen, J. B., Olea, N., Oles-Karasko, A., Pakdel, F., Pedersen, K., Perez, P., Skakkebaek, N. E., Sonnenschein, C., Soto, A. M., Sumpter, J. P., Thorpe, S. M., and Grandjean, P. (1999). Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. *Environ. Health Perspect.* 107(Suppl.), 89–108.

Ashby, J., and Tinwell, H. (1998). Uterotrophic activity of bisphenol A in the immature rat. *Environ. Health Perspect.* **106**, 719.

Ashby, J., Odum, J., Paton, D., Lefevre, P. A., Beresford, N., and Sumpter, J. P. (2000). Re-evaluation of the first synthetic estrogen, 1-keto-1,2,3,4-tetrahydrophenanthrene, and bisphenol A, using both the ovariectomised rat model used in 1933 and additional assays. *Toxicol. Lett.* **115**, 231–238.

Atkinson, A., and Roy, D. (1995). In vitro conversion of environmental estrogenic bisphenol A to DNA binding metabolite(s). *Biochem. Biophys. Res. Commun.* **210**, 424–433.

Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., Tong, W., Shi, L., Perkins, R., and Sheehan, D. M. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. *Toxicol. Sci.* **54**, 138–153.

Brotons, J. A., Olea-Serrano, M. F., Villalobos, M., Pedraza, V., and Olea, N. (1995). Xenoestrogens released from lacquer coatings in food cans. *Environ. Health Perspect.* **103**, 608–612.

Brzozowski, A.M., Pike, A.W., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* **389**, 753–759.

Cheek, A. O., Know, K., Chen, J., and McLachlan, J. A. (1999). Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. *Environ. Health Perspect.* **107**, 273–278.

Christiansen, L. B., Pedersen, S. N., Korsgaard, B., and Bjerregaard, P. (2000). *In vivo* comparison of xenoestrogens using rainbow trout vitellogenin induction as a screening system. *Environ. Toxicol. Chem.* **19**, 1867–1874.

Coldham, N. G., Dave, M., Sivapathasundaram, S., McDonnell, D. P., Connor, C., and Sauer, M. J. (1997). Evaluation of a recombinant yeast cell estrogen screening assay. *Environ. Health Perspect.* **105**, 70–76.

Connor, K., Ramamoorthy, M., Moore, M., Mustain, M., Chen, I., Safe, S., Zacharewski, T., Gillesby, B., Joyeux, A., and Balague, P. (1997). Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: Structure-activity relationships. *Toxicol. Appl. Pharmacol.* **145**, 111–123.

Elsby, R., Ashby, J., Sumpter, J. P., Brooks, N., Pennie, W. D., Maggs, J. L., Lefevre, P. A., Odum, J., Beresford, N., Paton, D., and Park, B. K. (2000). Obstacles to the prediction of estrogenicity from chemical structure: Assay-mediated metabolic transformation and the apparent promiscuous nature of the estrogen receptor. *Biochem. Pharmacol.* **60**, 1519–1530.

Elsby, R., Maggs, J. L., Ashby, J., and Park, B. K. (2001). Comparison of the modulatory effects of human and rat liver microsomal metabolism on the estrogenicity of bisphenol A: Implications for extrapolation to humans. *J. Pharmacol. Exp. Ther.* **297**, 103–113.

Fang, H., Tong, W., Perkins, R., Soto, A. M., Prechtl, N. V., and Sheehan, D. M. (2000). Quantitative comparisons of *in vitro* assay for estrogenic activities. *Environ. Health Perspect.* **1081**, 723–729.

Fukazawa, H., Hoshino, K., Shiozawa, T., Matsushita, H., and Terao, Y. (2001). Identification and quantification of chlorinated bisphenol A in wastewater from wastepaper recycling plants. *Chemosphere* **44**, 973–979.

Fukazawa, H., Watanabe, M., Shiraishi, F., Shiraishi, H., Shiozawa, T., Matsushita, H., and Terao, Y. (2002). Formation of chlorinated derivatives of bisphenol A in waste paper recycling plants and their estrogenic activities. *J. Health Sci.* **48**, 242–249.

Fujimoto, N., Honda, H., and Kitamura, S. (2004). Effects of environmental estrogenic chemicals on AP1 mediated transcription with estrogen receptors alpha and beta. *J. Steroid Biochem. Mol. Biol.* **88**, 53–59.

Gaido, K. W., Leonard, L. S., Lovell, S., Gould, J. C., Babai, D., Portier, C. J., and Mcdonell, D. P. (1997). Evaluation of chemicals with endocrine modulating activity in yeast-based steroid hormone receptor gene transcription assay. *Toxicol. Appl. Pharmacol.* **143**, 205–212.

Gray, L. E., Jr., Ostby, J., Manosson, E., and Kelce, W. R. (1999). Environmental antiandrogens: Low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. *Toxicol. Ind. Health* **15**, 48–64.

Gould, J. C., Leonard, L. S., Maness, S. C., Wagner, B. L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D. P., and Gaido, K. W. (1998). Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Mol. Cell Endocrinol.* **142**, 203–214.

Hashimoto, Y., Moriguchi, Y., Oshima, H., Kawaguchi, M., Miyazaki, K., and Nakamura, M. (2001). Measurement of estrogenic activity of chemicals for the development of new dental polymers. *Toxicol. in Vitro* **15**, 421–425.

Helleday, T., Tuominen, K.-L., Bergman, A., and Jenssen, D. (1999). Brominated flame retardants induce intragenic recombination in mammalian cells. *Mutation Res.* **439**, 137–147.

Hong, H., Tong, W., Fang, H., Shi, L., Xie, Q., Wu, J., Perkins, R., Walker, J. D., Branham, W., and Sheehan, D. M. (2002). Prediction of estrogen receptor binding for 58,000 chemicals using an integrated system of a tree-based model with structural alerts. *Environ. Health Perspect.* **110**, 29–36.

Ishihara, A., Sawatsubashi, S., and Yamauchi, K. (2003). Endocrine disrupting chemicals: interference of thyroid hormone binding to transthyretins and to thyroid hormone receptors. *Mol. Cell. Endocrinol.* **199**, 105–117.

Kawamura, Y., Ogawa, Y., Nishimura, T., Kikuchi, Y., Nishikawa, J., Nishihara, T., and Tanamoto, K. (2003). Estrogenic activities of UV stabilizers used in food contact plastics and benzophenone derivatives tested by the yeast two-hybrid assay. *J. Health Sci.* **49**, 205–212.

Kelce, W. R., Stone, C. R., Laws, S. C., Gray, L. E., Kemppainen, J. A., and Wilson, E. M. (1995). Persistent DDT metabolite *p,p'*-DDE is a potent androgen receptor antagonist. *Nature* **375**, 581–585.

Kim, H. S., Han, S.-Y., Yoo, S. D., Lee, B. M., and Park, K. L. (2001). Potential estrogenic effects of bisphenol-A estimated by *in vitro* and *in vivo* combination assays. *J. Toxicol. Sci.* **26**, 111–118.

Kitagawa, S., Obata, T., Willingham, M. C., and Cheng, S.-Y. (1987). Thyroid hormone action: Induction of morphological changes and stimulation of cell growth in rat pituitary tumor GH3 cells. *Endocrinology* **120**, 2591–2596.

Kitamura, S., Jinno, N., Ohta, S., Kuroki, H., and Fujimoto, N. (2002). Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. *Biochem. Biophys. Res. Commun.* **293**, 554–559.

Kitamura, S., Ohmegi, M., Sanoh, S., Sugihara, K., Yoshihara, S., Fujimoto, N., and Ohta, S. (2003a). Estrogenic activity of styrene oligomers after metabolic activation by rat liver microsomes. *Environ. Health Perspect.* **111**, 329–334.

Kitamura, S., Sanoh, S., Kohta, R., Suzuki, T., Sugihara, K., Fujimoto, N., and Ohta, S. (2003b). Metabolic activation of proestrogenic diphenyl and related compounds by rat liver microsomes. *J. Health Sci.* **49**, 298–310.

Kitamura, S., Suzuki, T., Ohta, S., and Fujimoto, N. (2003c). Antiandrogenic activity and metabolism of the organophosphorus pesticide fenthion and related compounds. *Environ. Health Perspect.* **111**, 503–508.

Kojima, H., Iida, M., Katsura, E., Kanetoshi, A., Hori, Y., and Kobayashi, K. (2003). Effect of a diphenyl ether-type herbicide, chlornitrofen, and its

amino derivative on androgen and estrogen receptor activities. *Environ. Health Perspect.* **111**, 497–502.

Korach, K. S., Sarver, P., Chae, K., McLachlan, J. A., and McKinney, J. D. (1988). Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: Conformationally restricted structural probes. *Mol. Pharmacol.* **33**, 120–126.

Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L., and Feldman, D. (1993). Bisphenol A: An estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132**, 2279–2286.

Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van-der Saag P. T., van-der Burg, B., and Gustafsson, J. A.(1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139**, 4252–4263.

Kuruto-Niwa, R., Terao, Y., and Nozawa, R. (2002). Identification of estrogenic activity of chlorinated bisphenol A using a GFP expression system. *Environ. Toxicol. Pharmacol.* **12**, 27–35.

Lans, M. C., Spiertz, C., Brouwer, A., and Koeman J. H. (1994). Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs, PCDFs. *Eur. J. Pharmacol.* **270**, 129–136.

Maruyama, S., Fujimoto, N., Yin, H., and Ito, A. (1999). Growth stimulation of a rat pituitary cell line MtT/E-2 by environmental estrogens *in vitro* and *in vivo*. *Endocr.J.* **46**, 513–520.

Matias, P. M., Donner, P., and Coelho, R. (2000). Structural evidence for ligand specificity in the binding domain of the human androgen receptor: Implications for pathogenic gene mutations. *J. Biol. Chem.* **275**, 26164–26171.

Matthews, J. B., Twomey, K., and Zacharewski, T. R.(2001). In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors α and β. *Chem. Res. Toxicol.* **14**, 149–157.

Moriyama, K., Tagami, T., Akamizu, T., Usui, T., Saijo, M., Kanamoto, N., Hataya, Y., Shimatsu, A., Kuzuya, H., and Nakao, K. (2002). Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J. Clin. Endocrinol. Metab.* **87**, 5185–5190.

Nakagawa, Y., and Suzuki, T. (2001). Metabolism of bisphenol A in isolated rat hepatocytes and oestrogenic activity of a hydroxylated metabolite in MCF-7 human breast cancer cells. *Xenobiotica* **31**, 113–123.

Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S., and Utsumi, H. (2000). Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J. Health Sci.* **46**, 282–298.

Olsen, C. M., Meussen-Elholm, E. T. M., Samuelsen, M., Holme, J. A., and Hongslo, J. K. (2003). Effects of the environmental oestrogens bisphenol A, tetrachlorobisphenol A, tetrabromobisphenol A, 4-hydroxybiphenyl and 4,4′-dihydroxybiphenyl on oestrogen receptor binding, cell proliferation and regulation of oestrogen sensitive proteins in the human breast cancer cell line MCF-7. *Pharmacol. Toxicol.* **92**, 180–188.

Olea, N., Pulgar, R., Perrez, P., Olea-Serrano, F., Rivas, A., Novillo-Fertrell, A., Pedraza, V., Soto, A. M., and Sonnenschein, C. (1996). Estrogenicity of resin-based composites and sealants used in dentistry. *Environ. Health Perspect.* **104**, 298–305.

Paris, F., Balaguer, P., Terouanne, B., Servant, N., Lacoste, C., Cravedi, J.-P., Nicolas, J.-C., and Sultan, C. (2002). Phenylphenols, biphenols, bisphenol-A and 4-*tert*-octylphenol exhibit α and β estrogen activities and antiandrogen activity in reporter cell lines. *Mol. Cell. Endocrin.* **193**, 43–49.

Pottenger, L. H., Domoradzki, J. Y., Markham, D. A., Hansen, S., Cagen, S. Z., and Waechter, J. M., Jr. (2000). The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. *Toxicol. Sci.* **54**, 3–18.

Sack, J. S., Kish, K. F., and Wang, C. (2001). Crystallographic structures of the ligand-binding domains of the androgen receptor and its T877A mutant complexed with the natural agonist dihydrotestosterone. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4904–4909.

Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* **95**, 927–937.

Sellström, U., and Jansson, B. (1995). Analysis of tetrabromobisphenol A in a product and environmental samples. *Chemosphere* **31**, 3085–3092.

Sjödin, A., Carlsson, H., Thuresson, K., Sjölin, S., Bergman, A., and Östman, C. (2001). Flame retardants in indoor air at an electronics recycling plant and at other work environments. *Environ. Sci. Technol.* **35**, 448–454.

Snyder, R. W., Maness, S. C., Gaido, K. W., Welsch, F., Sumner, S. C. J., and Fennell, T. R. (2000). Metabolism and disposition of bisphenol A in female rats. *Toxicol. Appl. Pharmacol.* **168**, 225–234.

Sohoni, P., and Sumpter, J. P. (1998). Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **158**, 327–339.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., and Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. *Environ. Health Perspect.* **103**(Suppl. 7), 113–122.

Soto, A. M., Fernandez, M. F., Luizzi, M. F., Karasko, A. S. O., and Sonnenschein, C. (1997). Developing a marker of exposure to xenoestrogen mixtures in human serum. *Environ. Health Perspect.* **105**(Suppl. 3), 647–654.

Spivack, J., Leib, T. K., and Lobos, J. H. (1994). Novel pathway for bacterial metabolism of bisphenol A. *J. Biol. Chem.* **269**, 7323–7329.

Sugihara, K., Kitamura, S., Sanoh, S., Ohta, S., Fujimoto, N., Maruyama, S., and Ito, A. (2000). Metabolic activation of the proestrogens *trans*-stilbene and *trans*-stilbene oxide by rat liver microsomes. *Toxicol. Appl. Pharmacol.* **167**, 46–54.

Tamura, H., Maness, S. C., Reischmann, K., Dorman, D. C., Gray, L. E., and Gaido, K. W. (2001). Androgen receptor antagonism by the organophosphate insecticide fenitrothion. *Toxicol. Sci.* **60**, 56–62.

Thomsen, C., Janak, K., Lundanes, E., and Becher, G. (2001). Determination of phenolic flame-retardants in human plasma using solid-phase extraction and gas chromatography-electron-capture mass spectrometry. *J. Chromatography B* **750**, 1–11.

Tinwell, H., Joiner, R., Pate, I., Soames, A., Foster, J., and Ashby, J. (2000). Uterotrophic activity of bisphenol A in the immature mouse. *Reg. Toxicol. Pharmacol.* **32**, 118–126.

Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B. L., Baxter, J. D., and Fletterick, R. J. (1995). A structural role for hormone in the thyroid hormone receptor. *Nature* **378**, 690–697.

Wagner, R. L., Huber, B. R., Shiau, A. K., Kelly, A., Lima, S. T. C., Scanlan, T. S., Apriletti, J. W., Baxter, J. D., West, B. L., and Fletterick, R. J. (2001). Hormone selectivity in thyroid hormone receptors. *Mol. Endocrinol.* **15**, 398–410.

Watanabe, I., Kashimoto, T., and Tatsukawa, R. (1983). Identification of the flame retardant tetrabromobisphenol-A in the river sediment and the mussel collected in Osaka. *Bull. Environ. Contam. Toxicol.* **31**, 48–52.

Yoshihara, S., Makishima, M., Suzuki, N., and Ohta, S. (2001). Metabolic activation of bisphenol A by rat liver S9 fraction. *Toxicol. Sci.* **62**, 221–227.

Yoshihara, S., Mizutare, T., Makishima, M., Suzuki, N., Fujimoto, N., Igarashi, K., and Ohta, S. (2004). Potent estrogenic metabolites of bisphenol A and bisphenol B formed by rat liver S9 fraction: Their structures and estrogenic potency. *Toxicol. Sci.* **78**, 50–59.

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Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens

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Abstract

Estrogenic and antiandrogenic activities of benzophenone and 16 of its derivatives, which are used as UV stabilizers, were comparatively examined with hormone-responsive reporter assay in various cell lines. Hydroxylated benzophenones exhibited estrogenic activity in human breast cancer cell line MCF-7, but their activities varied markedly. The highest activity was observed with 2,4,4′-trihydroxybenzophenone (2.4.4′-triOH-BP), followed by 2,3′,4,4′-tetrahydroxybenzophenone, 4,4′-dihydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 4-hydroxybenzophenone and 2,4-dihydroxybenzophenone. Benzophenone itself showed little activity in the assay. In contrast, benzophenone and some related compounds showed significant inhibitory effects on the androgenic activity of dihydrotestosterone in rat fibroblast cell line NIH3T3. The highest activity was observed with 2,4,4′-triOH-BP, followed by 2,3′,4,4′-tetrahydroxybenzophenone, 2,2′,4,4′-tetrahydroxybenzophenone, 3-hydroxybenzophenone and 2,2′-dihydroxybenzophenone. However, 2,3,4,4′-tetrahydroxybenzophenone and 2,3,4-trihydroxybenzophenone showed little activity. 2,4-Dihydroxybenzophenone, 2,4,4′-triOH-BP and benzophenone gave positive responses in uterotrophic assay using ovariectomized rats, and 2,4,4′-triOH-BP was positive in the Hershberger assay using castrated rats. These results suggest that a 4-hydroxyl group on the phenyl ring of benzophenone derivatives is essential for high hormonal activities, and the presence of other hydroxyl groups markedly alters these activities.
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Keywords: Benzophenones; Estrogenic activity; Antiandrogenic activity; Human breast cancer cell line MCF-7; Rat cancer cell line NIH3T3

Introduction

Hydroxybenzophenones such as 2,4-dihydroxybenzophenone (2,4-di-OH-BP; benzophenone-1) and 2,2′,4,4′-tetrahydroxybenzophenone (2,2′,4,4′-tetra-OH-BP; benzophenone-2) have the ability to absorb and to dissipate ultraviolet (UV) light A (400–315 nm) (Klein, 1992). Consequently, they are used as UV stabilizers in plastic surface coatings on food packaging to prevent polymer degradation and loss of quality of the packed food owing to UV light irradiation. These compounds may be transferred from the packaging to the contents, and subsequently ingested by humans. However, they are natural components of plants such as mango and muscat grape, and are also used as flavorings. It has been reported that some derivatives have cytotoxic effects (Matsumoto et al., 2003). Hydroxybenzo-

Abbreviations: (BP), Benzophenone; (2-OH-BP), 2-hydroxybenzophenone; (3-OH-BP), 3-hydroxybenzophenone; (4-OH-BP), 4-hydroxybenzophenone; (2,2′-diOH-BP), 2,2′-dihydroxybenzophenone; (2,4-diOH-BP), 2,4-dihydroxybenzophenone; (4,4′-diOH-BP), 4,4′-dihydroxybenzophenone; (2,3,4-triOH-BP), 2,3,4-trihydroxybenzophenone; (2,4,4′-triOH-BP), 2,4,4′-trihydroxybenzophenone; (2,2′,4,4′-tetraOH-BP), 2,2′,4,4′-tetrahydroxybenzophenone; (2,3,4,4′-tetraOH-BP), 2,3,4,4′-tetrahydroxybenzophenone; (2,3′,4,4′-tetraOH-BP), 2,3′,4,4′-tetrahydroxybenzophenone; (2-OH-4-MeO-BP), 2-hydroxy-4-methoxybenzophenone; (2,2′-diOH-4,4′-diMeO-BP), 2,2′-dihydroxy-4,4′-dimethoxybenzophenone; (4-OH-2′,4′-diMeO-BP), 4-hydroxy-2′,4′-dimethoxybenzophenone; (2-OH-4-MeO-BP-5-sulfonic acid), 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid; (2,2′-diOH-4-MeO-BP), 2,2′-dihydroxy-4-methoxybenzophenone; (DHT), dihydrotestosterone; (E2), 17β-estradiol.

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phenones and their derivatives are also used as sunscreens for humans. These compounds are reported to be absorbed through human skin, and bioaccumulation may occur in wildlife and humans (Felix et al., 1998; Hagedorn-Leweke and Lippold, 1995; Hayden et al., 1997; Jiang et al., 1999). In spite of some toxic effects of benzophenone, such as hepatotoxicity, hydroxylated benzophenones are safe for topical application to humans. Acute and subchronic systemic toxicity of these compounds after dermal application is considered to be rather low (Burdock et al., 1991; Dutta et al., 1993; Okereke et al., 1995). However, some problems have arisen with photoallergic reactions in patients with suspected clinical photosensitivity (Schauder and Ippen, 1997).

Endocrine-active chemicals arise from many different sources, including pesticides, industrial chemicals, pharmaceuticals, and phytochemicals. These chemicals are widely distributed in the environment and are able to mimic the biological activities of hormones. Chlorinated insecticides, such as kepone, *o,p′*-DDT, dieldrin and methoxychlor, and compounds used in the plastics and detergent industries, such as alkylphenols and bisphenol A, are known to have estrogenic activity (Andersen et al., 1999; Gaido et al., 1997; Soto et al., 1994). *p,p′*-DDE, a metabolite of p,p'-DDT, vinclozolin, an antifungal agent, and fenitrothion and fenthion, insecticides, are known to have anti-androgenic activity (Chen et al., 1997; Gray et al., 1999; Kelce et al., 1995; Kitamura et al., 2003; Kupfer and Bulger, 1987; Tamura et al., 2001). Interactions of estrogenic and anti-androgenic compounds with the respective hormone receptors have been demonstrated to account for the endocrine-disrupting actions of the compounds. These chemicals can alter reproductive development in mammals. Some hydroxylated benzophenones also show estrogenic activity in the yeast two hybrid system with estrogen receptor and coactivators (Kawamura et al., 2003). It was reported that 2-hydroxy-4-methoxybenzophenone (2-OH-4-MeO-BP) and 4-hydroxybenzophenone (4-OH-BP) are weakly positive in the uterotrophic assay using immature rats (Nakagawa and Tayama, 2001; Schlumpf et al., 2001). Androgenic and anti-androgenic properties of hydroxylated benzophenones are also an important problem. Yamasaki et al. (2003) reported that some hydroxybenzophenones do not act as antiandrogens in the Hershberger assay. Satoh et al. (2001) showed that benzophenone has no affinity for androgen receptor. Ma et al. (2003) reported that 2-OH-4-MeO-BP is a weak antagonist for androgen receptor. However, the activities in reporter assays have not been fully examined, and the relationship between the structure and activity of benzophenone derivatives is not yet understood.

In this report, endocrine-disrupting activity, that is, estrogenic, androgenic, and anti-androgenic activities, of hydroxybenzophenones and related compounds was examined using hormone-responsive reporter assays in human breast cancer cell-line MCF-7 for estrogenic activity and rat fibroblast cell line NIH3T3 for androgenic activity. Benzophenone derivatives tested in this study are listed in Fig. 1. We found that hydroxybenzophenones and some related compounds exhibit estrogenic and anti-androgenic activities. The structure–activity relationship of hydroxylated benzophenones is discussed.

Materials and methods

Chemicals. Benzophenone (BP), 2-hydroxybenzophenone (2-OH-BP), 3-hydroxybenzophenone (3-OH-BP), 4-OH-BP, 2,2′-dihydroxybenzophenone (2,2′-diOH-BP), 2,4-diOH-BP, 4,4′-dihydroxybenzophenone (4,4′-diOH-BP), 2,3,4-trihydroxybenzophenone (2,3,4-triOH-BP), 2,4,4′-trihydroxybenzophenone (2,4,4′-triOH-BP), 2,2′,4,4′-tetraOH-BP, 2,3,4,4′-tetrahydroxybenzophenone (2,3,4,4′-tetraOH-BP), 2,3′,4,4′-tetrahydroxybenzophenone (2,3′,4,4′-tetraOH-BP), 2-OH-4-MeO-BP, 2,2′-dihydroxy-4,4′-dimethoxybenzophenone (2,2′-diOH-4,4′-diMeO-BP), 4-hydroxy-2,′4′-dimethoxybenzophenone (4-OH-2′,4′-diMeO-BP), 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid (2-OH-4-MeO-BP-5-sulfonic acid), 2,2′-dihydroxy-4-methoxybenzophenone (2,2′-diOH-4-MeO-BP), 4-hydroxyacetophenone, 5α-dihydrotestosterone (DHT), and flutamide were obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), 17β-estradiol (E2) was from Sigma (St. Louis, MO).

Animals. Rats were housed at 22 °C and a relative humidity of 55% with a 12-h light/dark cycle, with free access to tap water and a standard pellet diet MM-3 (Funabashi Farm, Funabashi, Japan). All experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" of Hiroshima University.

Cell culture. NIH3T3 and MCF-7 cells were maintained in DMEM (Sigma) plus 5% calf serum (Gibco/Invitrogen Corp., Carlsbad, CA) and DMEM plus 5% fetal bovine serum (Gibco/Invitrogen), respectively, containing penicillin and streptomycin. For assay, the medium was changed to phenol red-free DMEM (Sigma) containing the same antibiotics along with dextran-charcoal-treated calf serum or fetal bovine serum for a week.

Plasmids. The p(ARE)2-luc plasmid was constructed with pGL3-basic (Promega Co., Madison, WI) by inserting a synthetic double-strand oligo-DNA containing the consensus androgen-responsive element 5′-CATAGTACGT-GATGTTCTAGGCCTAGTACGTGATGTTCTCTATAC (with *SacI/Xho*I ends). For pSG5-hAR, human androgen receptor cDNA was amplified from human prostate cDNA by PCR with LA-Taq (Takara Bio Inc., Otsu, Japan) and introduced into pCR2.1 with a TA Cloning Kit (Invitrogen). The *Eco*RI fragment was then inserted into the *Eco*RI site in the pSG5 plasmid. The $p(ERE)_3$-SV40-luc plasmid, which contains three tandem copies of the

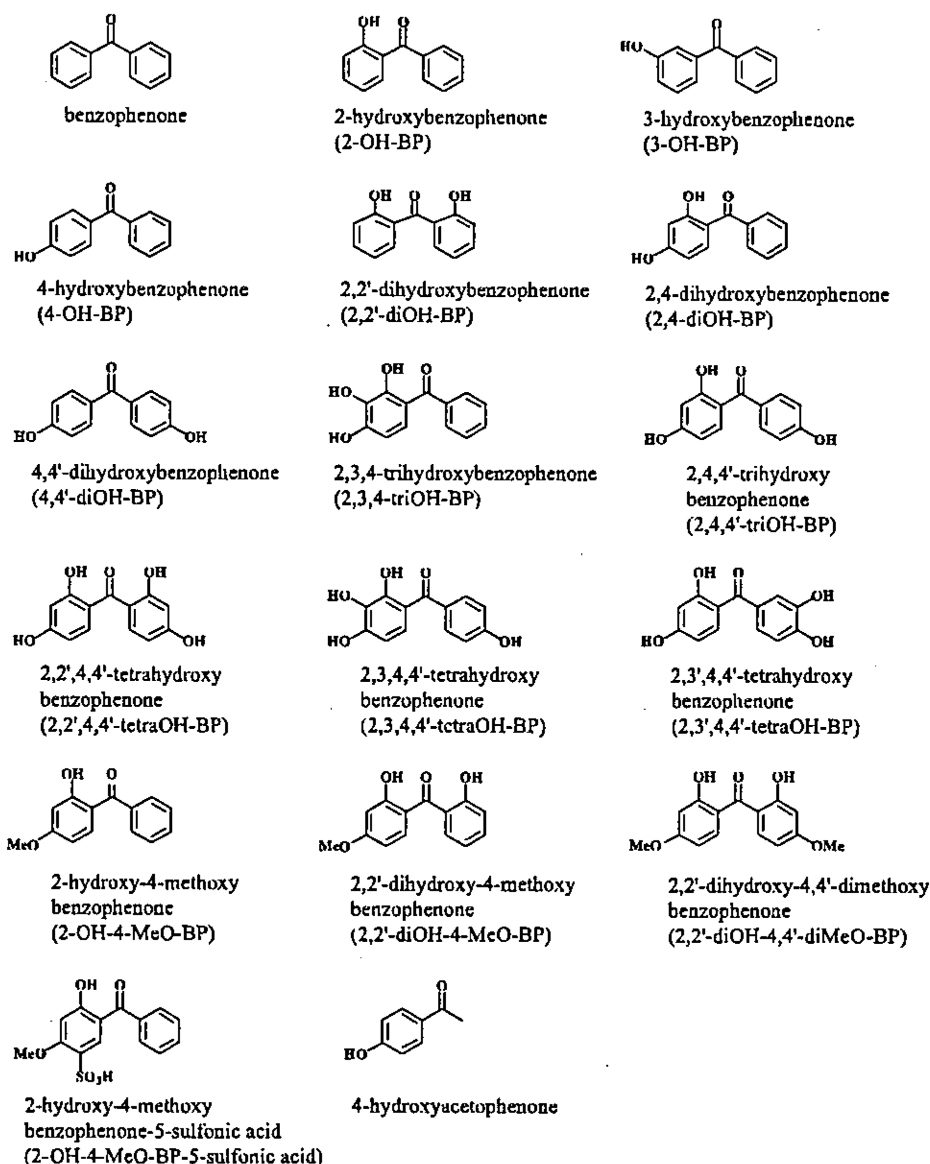


Fig. 1. Structures of benzophenone and related compounds tested in this study.

consensus estrogen-responsive element, was a gift from Dr. M. Kudoh, Yamanouchi Pharmaceutical Co., Tsukuba, Japan. PRL-CMV (Promega Co.) was used as the internal control.

Assay of estrogenic activity of benzophenone and related compounds. ERE-luciferase reporter assay using MCF-7 cells was performed according to the previously reported method (Sugihara et al., 2000). Briefly, transient transfections in MCF-7 cells were performed using Transfast (Promega Co.), according to the manufacturer's protocol. Transfections were done in 48-well plates at 2 10^4 cells/well with 0.3 μg of p(ERE)$_3$-SV40-luc and 2 ng of phRL/CMV (Promega Co.) as an internal standard. Twenty-four hours after addition of the sample (final concentration, 10^{4} to 10^{8} M), the assay was performed with a Dual Luciferase assay kit™ (Promega Co.).

Assay of androgenic and antiandrogenic activities of benzophenone and related compounds. Assay of androgens was performed by ARE-luciferase reporter assay using NIH3T3 cells according to the previously reported method (Kitamura et al., 2003). Briefly, the culture medium was changed to phenol red-free DMEM (Sigma) containing penicillin, streptomycin, and dextran-charcoal-treated fetal bovine serum for 2–3 days. Transient transfections of NIH3T3 cells were performed using Transfast™ (Promega Co.), according to the manufacturer's protocol. Transfections were done in 48-well plates at 2 10^4 cells/well with 0.3 μg of p(ERE)$_3$-SV40-luc and 2 ng of phRL/CMV (Promega Co.) as an internal standard. Twenty-four hours after addition of the sample (final concentration, 10^{5} to 10^{8} M) dissolved in 4 μl of ethanol, the assay was performed with a Dual Luciferase assay kit™ (Promega Co.) according to the manufacturer's protocol. For the assay of anti-

androgens, the inhibitory effect of benzophenone derivatives on the androgenic activity of DHT at the concentration of 1 10^{10} M was examined.

Assay of estrogenic activity in vivo (uterotrophic assay). F344 female rats obtained from Charles River Co. (Kanagawa, Japan) were used. They were surgically ovariectomized at 6 weeks old. At the age of 9 weeks, they were divided into eight groups each consisting of five animals. The rats were treated once a day for 3 days with intraperitoneal doses of 0.2 ml of vehicle (Panacete 810, Nippon Oils and Fats Co., Ltd., Tokyo, Japan), 17β-estradiol (50 μg/kg/day), 2,4,4′-triOH-BP, 2,4-diOH-BP, 2-OH-4-MeO-BP or BP (20–500 mg/kg/day). Animals were sacrificed under anesthesia and the uterus was dissected and weighed.

Assay of androgenic and antiandrogenic activities in vivo (Hershberger assay). F344 male rats were surgically castrated at 4 weeks old. At the age of 7 weeks, they were divided into five groups each consisting of 6 animals. The rats were treated once a day for 10 days with subcutaneous doses of 0.3 ml of vehicle (dimethyl sulfoxide), testosterone propionate (500 μg/kg/day), testosterone propionate plus flutamide (6 mg/kg/day) or testosterone plus 2,4,4′-triOH-BP (100 or 300 mg/kg/day). Animals were sacrificed under anesthesia and the prostate and seminal vesicles were dissected and weighed.

Results

Estrogenic activity of BP and related compounds

Estrogenic activity of benzophenone and its derivatives was examined using ERE-luciferase reporter assay in MCF-7 cells. Hydroxybenzophenones exhibited estrogenic activity in the estrogen screening assay, but their activities showed a wide range. Some tetra- and trihydroxybenzophenones showed significant estrogenic activity in the concentration range of 1 10^{7} – 1 10^{4} M. Di- and monohydroxybenzophenones showed estrogenic activity at 1 10^{6} – 1 10^{4} M. However, 2,3,4-trihydroxybenzophenones, methoxybenzophenones, and BP showed little activity. The active compounds were cytotoxic to MCF-7 cells at concentrations above 1 10^{5} M or 1 10^{4} M, so their apparent activities decreased above this concentration (Fig. 2). When ICI 182,780, a pure estrogen receptor antagonist, was added at the concentration of 1 10^{8} M, the estrogenic activities of the above compounds were markedly inhibited (data not shown). The EC_{50} values of the estrogenic activities are shown in Table 1. The highest activity was observed with 2,4,4′-triOH-BP, followed by 2,3′,4,4′-tetraOH-BP, 4,4′-diOH-BP, 2,2′,4,4′-tetraOH-BP, 4-OH-BP, 2,4-diOH-BP, 3-OH-BP, 2-OH-BP, BP, 2,3,4-triOH-BP, 2-OH-4-MeO-BP and 2,3,4,4′-tetraOH-BP. However, 4-hydroxyacetophenone, 2,2′-diOH-BP, 2-OH-4-MeO-BP-5-sulfonic acid, 2,2′-diOH-4-MeO-BP and 2,2′-diOH-4,4′-diMeO-BP were negative in the estrogen screening assay. Thus, 2,4,4′-trihydroxyl derivatives of benzophenone exhibited the highest activity. The activity was abolished by substitution with a methoxyl group. A 2,3,4-triol moiety on benzophenone markedly decreased the estrogenic activity. 4-Hydroxyacetophenone, lacking one phenyl ring of BP, showed little activity. Thus, hydroxyl group(s) on BP derivatives are essential for estrogenic activity. Both phenyl groups are also necessary.

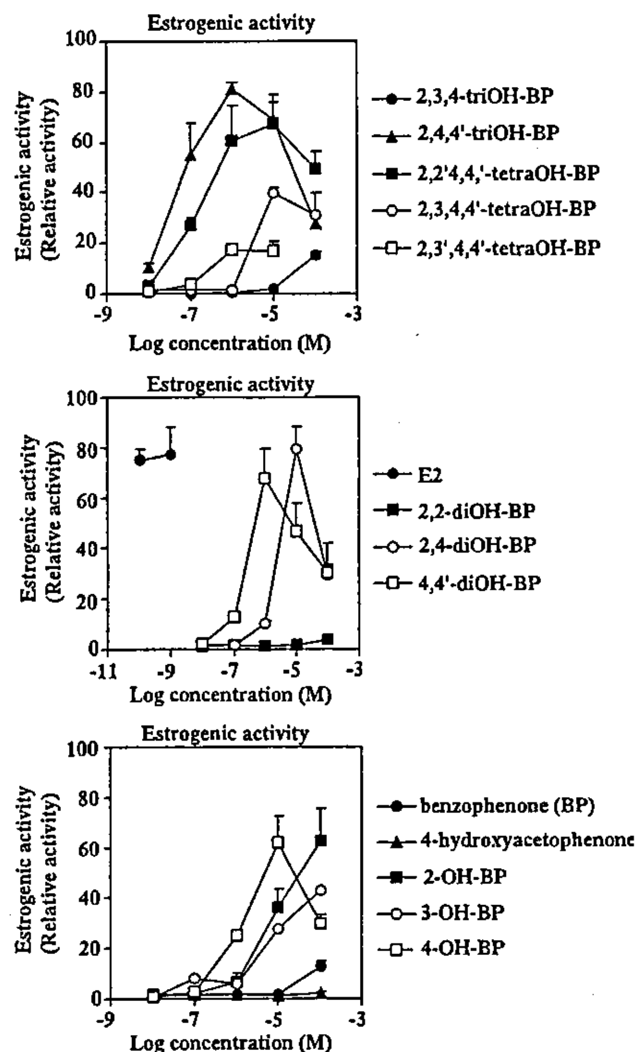
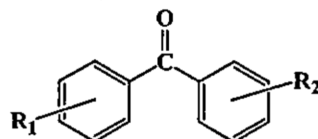


Fig. 2. Estrogenic activity of benzophenone (BP) and related compounds using ERE-luciferase reporter assay in MCF-7 cells. Each value represents the mean ± SD of four experiments. Estrogenic activity of benzophenones was expressed as a relative activity with respect to the control using MCF-7 cells. $*p < 0.05$, $**p < 0.01$ compared with control.

Androgenic and antiandrogenic activities of BP and related compounds

Androgenic activity of benzophenone and related compounds was examined using NIH3T3 cells transfected with

Table 1
Estrogenic activity (EC_{50}) and antiandrogenic activity (IC_{50}) of benzophenone derivatives



R1	R2	Estrogenic EC_{50} (μM)	Antiandrogenic IC_{50} (μM)
2,4-OH	4′-OH	0.10	0.30
2,4-OH	3′,4′-OH	0.14	0.97
4-OH	4′-OH	0.14	4.78
2,4-OH	2′,4′-OH	0.30	1.53
4-OH	H	0.72	5.01
2,4-OH	H	1.26	10.0
3-OH	H	1.54	3.02
2-OH	H	4.47	3.81
H	H	8.13	11.9
2,3,4-OH	H	11.8	>100
2-OH-4-MeO	H	19.5	>100
2,3,4-OH	4′-OH	31.3	26.5
4-hydroxyacetophenone		>100	>100
2-OH	2′-OH	>100	3.20
2-OH-4-MeO	2′-OH	>100	>100
2-OH-4-MeO	2′-OH-4′-MeO	>100	13.0
2-OH-4-MeO-5-SO_3H	H	>100	>100

a luciferase reporter gene. DHT exhibited marked androgenic activity toward NIH3T3 cells at 1 10^{11} – 1 10^{8} M. However, no androgenic activity of BP or the derivatives examined in this study was observed in the concentration range of 10^{7} to 10^{4} M (data not shown).

When BP and its derivatives were added to the DHT assay system in the concentration range of 1 10^{8} to 1 10^{5} M, the activity of 1 10^{10} M DHT was inhibited concentration-dependently (Fig. 3). Table 1 shows the IC_{50} values of these compounds against the androgenic activity of 1 10^{10} M DHT. The highest inhibitory effect among the test compounds was observed with 2,4,4′-triOH-BP, followed by 2,3′,4,4′-tetraOH-BP, 2,2′,4,4′-tetraOH-BP, 3-OH-BP, 2,2′-diOH-BP, 2-OH-BP, 4,4′-diOH-BP, 4-OH-BP, 2,4-diOH-BP, 2,3,4,4′-tetraOH-BP, BP and 2,2′-diOH-4,4′-diMeO-BP. However, 2,3,4-triOH-BP, 2,2′-diOH-4-MeO-BP, 2-OH-4-MeO-BP, 2-OH-4-MeO-BP-5-sulfonic acid and 4-hydroxyacetophenone showed little activity. These results suggest that some benzophenone derivatives have anti-androgenic as well as estrogenic activity, and hydroxyl groups are essential for this activity.

Estrogenic and antiandrogenic activities of BP derivatives in vivo

Estrogenic potential of hydroxylated benzophenones in vivo was further investigated in the uterotrophic assay using ovariectomized rats. Body weight of the rats administered 2,4,4′-triOH-BP, 2,4-diOH-BP, 2-OH-4-MeO-BP, or BP was not decreased compared with other groups. The uterine weight in the group dosed 2,4,4′-triOH-BP, 2,4-diOH-BP or BP was weakly increased compared with the group given vehicle only, but that in the 2-OH-4-MeO-BP group was not changed (Fig. 4A). Thus, the estrogenic effect of hydroxylated benzophenones was confirmed in vivo.

The in vivo antiandrogenic potential of 2,4,4′-triOH-BP, which showed the highest antiandrogenic activity in the in vitro reporter assay, was also investigated by the Hershberger assay using F344 rats (Hershberger et al., 1953). Body weight in the group administered 2,4,4′-triOH-BP (300 mg/kg/day) was decreased by 9% compared with the group given testosterone, while there were no significant differences among other groups. Testosterone treatment significantly increased the weights of the prostate and seminal vesicles, and these increases were completely blocked by flutamide administration. 2,4,4′-triOH-BP also significantly suppressed the effect of testosterone on the weight of both glands (Fig. 4B). Thus, the antiandrogenic effect of 2,4,4′-triOH-BP was confirmed in vivo.

Discussion

Hydroxylated benzophenones are used in sunscreens as skin-protecting agents and as UV stabilizers for plastic packaging. In this study, we examined the relationship between the structure of BP derivatives and their estrogenic and antiandrogenic activities. For estrogen receptor ligand activity, xenobiotics require an unhindered hydroxyl group on an aryl ring and a hydrophobic group attached *para* to the hydroxyl group (Blair et al., 2002; Fang et al., 2000;

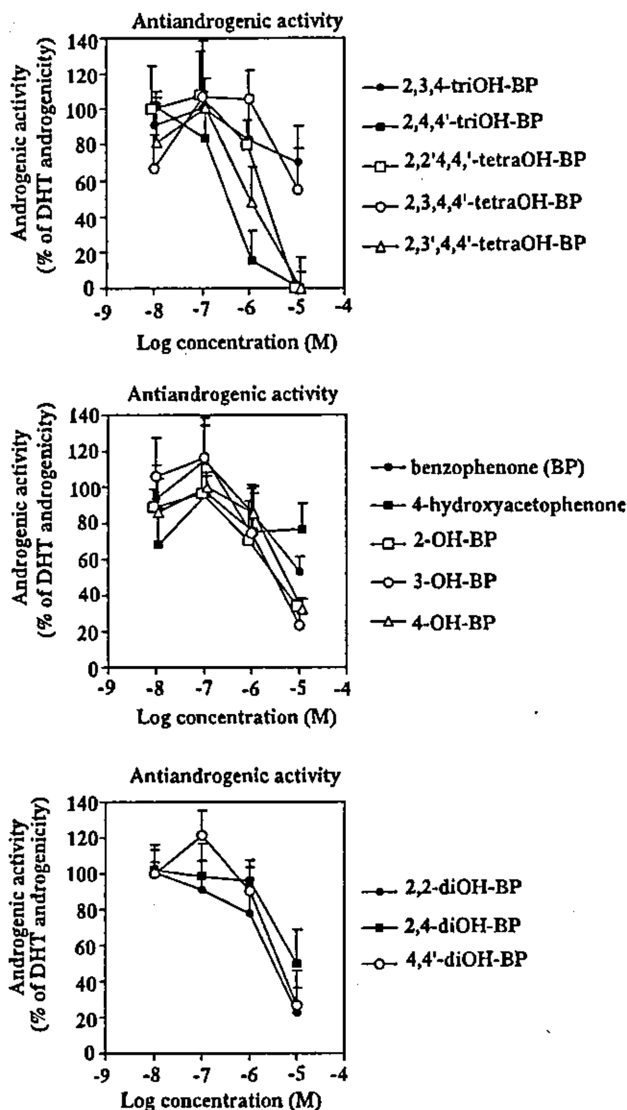


Fig. 3. Antiandrogenic activity of benzophenone (BP) and its derivatives against the androgenic activity of dihydrotestosterone (DHT) using ARE-luciferase reporter assay in NIH3T3 cells. Antiandrogenic activity of benzophenones was expressed as percent values of ARE-luciferase activity relative to the level induced by DHT (1 10 ^{10} M) using NIH3T3 cells. Each value represents the mean ± SD of four experiments. $*p < 0.05$ and $**p < 0.01$ indicate significant differences from control experiments in which only DHT was added.

Hong et al., 2002). The key structural requirement for estrogenic activity of BP derivatives is a phenolic hydroxyl group. The number and position of hydroxyl substituents markedly influences the activity. High activity was observed with tetra- and trihydroxybenzophenones. Benzophenones hydroxylated at the 2-, 4- and 4′-positions showed the highest activity. The 4-hydroxyl group is presumably necessary for binding with the estrogen receptor, like 3-OH in 17β-estradiol, and the 4′-hydroxyl group is also required as an H-bond donor. The 2-hydroxyl group forms a hydrogen bond with the carbonyl group, contributing to the hydrophobicity. Monohydroxybenzophenones exhibited activity, in the order of 4-hydroxyl>3-hydroxyl>2-hydroxyl. Schultz et al. (2000) also reported weak estrogenic activities of 4-OH-BP and 3-OH-BP, but detected no activity of 2-OH-BP. Benzophenones with three adjacent hydroxyl groups, such as 2,3,4,4′-tetraOH-BP and 2,3,4-triOH-BP, showed very low activity, presumably because of steric hindrance. Nakagawa and Suzuki (2002) reported that 2,4-diOH-BP and 2,3,4-triOH-BP showed estrogenic activity in proliferation assay using MCF-7 cells, and the activity of 2,3,4-triOH-BP was lower than that of 2,4-diOH-BP. Kawamura et al. (2003) reported that symmetrically hydroxylated benzophenones showed lower activity than asymmetrical derivatives in the yeast two-hybrid assay. This tendency was not observed in our study using reporter assay in MCF-7 cells.

Yamasaki et al. (2003) reported that some hydroxylated benzophenones did not show antiandrogenic activity in Hershberger assay. In this study, we found that 2,4,4′-triOH-BP was positive in the Hershberger assay. This positive result may have been due to subcutaneous dosing of 2,4,4′-triOH-BP; Yamasaki et al. used oral administration. Furthermore, we demonstrated that benzophenone and some of its derivatives are potent in vitro antiandrogens, though the activity varies markedly depending on the structure. The reasons for this are not clear, but hydroxyl groups may be a factor for the antiandrogenic activity, as in the case of estrogenic activity. Tri- and tetrahydroxyl substitution on the phenyl rings seems to increase the activity. Benzophenones substituted with hydroxyl groups at the 2,4,4′-positions showed the highest activity, as found for estrogenic activity. The order of activity is also the same, that is, 2,4,4′-triOH-BP>2,3′,4,4′-tetraOH-BP>2,2′,4,4′-tetra-BP. Benzophenones substituted with 2,3,4-trihydroxyl groups showed little inhibitory effect on the androgenic activity of DHT. Further study is needed to understand the relationship of structure to anti-androgenic activity of environmental chemicals, including BP derivatives.

The in vivo estrogenic activity of 2,4,4′-triOH-BP, 2,4-diOH-BP and BP was confirmed in uterotrophic assay, that is, the weight of the uterus in ovariectomized rats dosed with these BP derivatives was increased compared to that in rats dosed with vehicle alone. Yamasaki et al. (2003) also examined the estrogenic activities of some hydroxylated benzophenones in uterotrophic assay using immature rats. 2,2′,4,4′-TetraOH-BP and 2,4,4′-triOH-BP increased the weight of the uterus, while 4-OH-BP and 4,4′-diOH-BP showed weak activity. The activity of hydroxylated benzophenones may be decreased by reduction of the carbonyl moiety or conjugation with glucuronic acid or sulfuric acid at a hydroxyl group. However, Okereke et al. (1993) reported that 2-OH-4-MeO-BP is mainly converted to the desmethylated metabolite in rats in vivo. Thus, desmethylated metabolites of methoxybenzophenone derivatives, which are commonly used in sunscreens, may contribute to estrogenic activity. Metabolic activation of this group is possible, as observed with methoxychlor (Stresser and Kupfer, 1998). In contrast, Nakagawa and Tayama (2001)

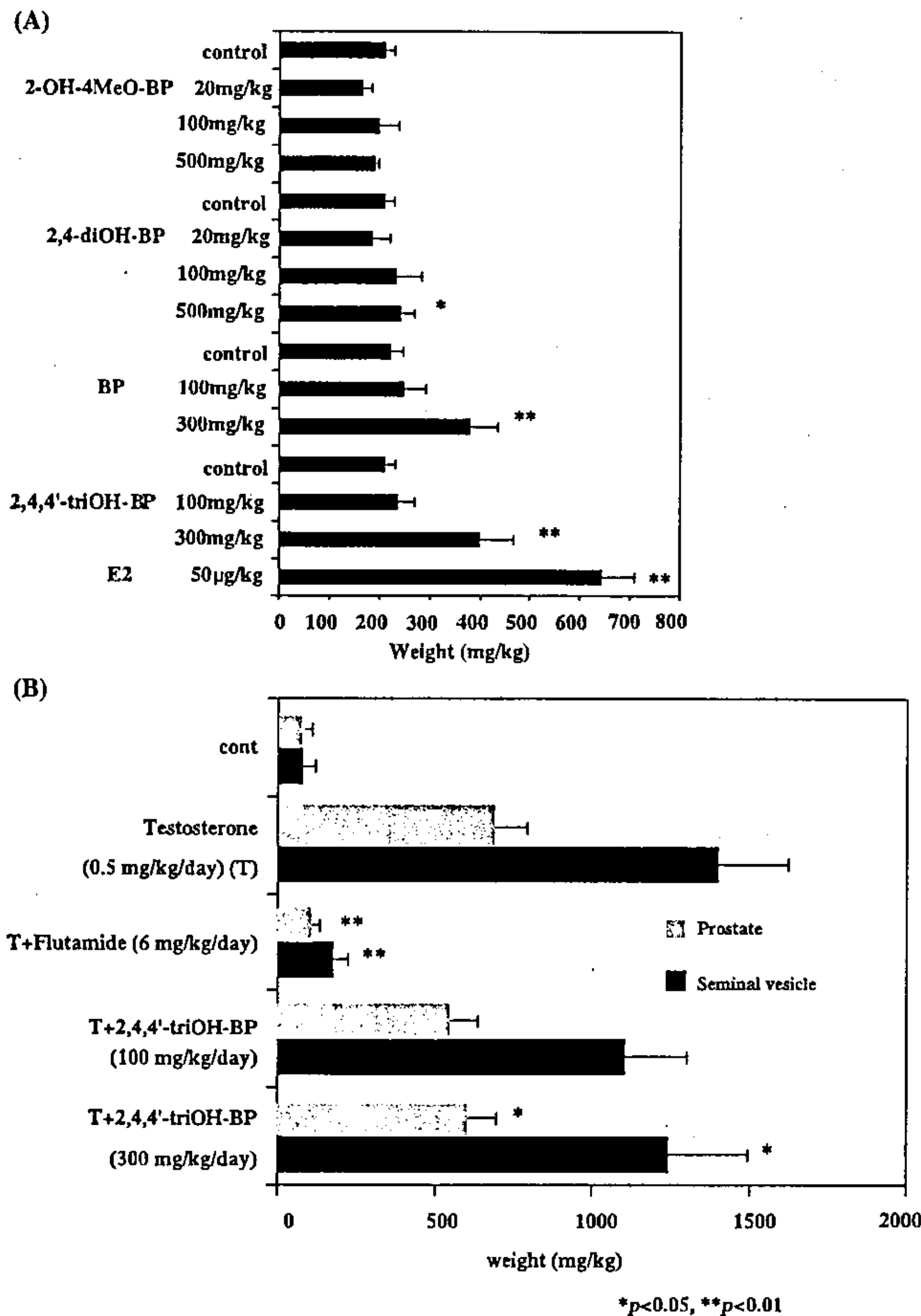


Fig. 4. Estrogenic and antiandrogenic activities of benzophenone derivatives in vivo. (A) The uterotrophic assay of benzophenone derivatives. Weights of the uteri of rats are shown. Ovariectomized female F344 rats were treated with 17β-estradiol (E2; 50 mg/kg/day), 2-OH-4-MeO-BP (20, 100, or 500 mg/kg/day), 2,4,4′-triOH-BP (100 or 300 mg/kg/day), benzophenone (100 or 300 mg/kg/day) or 2,4-diOH (20, 100, or 500 mg/kg/day) for a week. Each group consisted of six animals. (B) The Hershberger assay of benzophenone derivatives. Weights of the prostate gland and seminal vesicle of rats are shown. Castrated male F344 rats were treated with testosterone propionate (T; 500 mg/kg/day) and/or 2,4,4′-triOH-BP (100 or 300 mg/kg/day) or flutamide (6 mg/kg/day) for a week. Each group consisted of six animals. Each bar indicates mean ± SD of six rats. Asterisks indicate significant differences from testosterone-dosed rats at $*p < 0.05$ and $**p < 0.01$.

reported that 4-OH-BP, a metabolite of benzophenone, showed a positive result in uterotrophic assay using juvenile female rats, but benzophenone did not show such in vivo estrogenic activity. We observed estrogenic activity of benzophenone in vivo in this study. Benzophenone can be activated to the 4-hydroxyl derivative in the body, and so the estrogenic activity might have been due to the active metabolite. However, we did not find estrogenic activity of 2-OH-4-MeO-BP in vivo. However, Schlumpf et al. (2001) observed a weak activity of the compound in uterotrophic assay. Further study is necessary to evaluate the influence of metabolic modification of hydroxybenzophenones and methoxybenzophenones on the hormonal toxicity.

Human intake of benzophenones occurs from food and medicine, and these chemicals may accumulate in the body to some extent. Indeed, Hany and Nagel (1995) detected 2-OH-4-MeO-BP and octyl *p*-methoxycinnamate used in as sunscreen in human milk (total 16–417 ng/g fat) from Germans. This level corresponds to 10^{7} to 10^{6} M. The contents of 4-hydroxybenzophenones have not been reported. However, when 4-hydroxybenzophenones are applied to human skin as sunscreen (usually 1–2% content), the level may temporarily reach the level used in this study immediately after the dose. Subsequently, the level of 4-hydroxybenzophenones would decrease rapidly owing to metabolism and excretion. These considerations and the in vivo results obtained in this study suggest that benzophenones pose a relatively low risk to humans in terms of endocrine disrupting activity, in spite of the in vitro activity shown in this study. In contrast, some hydroxylated benzophenones, such as those hydroxylated at the 2- and 4-positions, showed both estrogenic and antiandrogenic activities. Thus, these compounds might exhibit endocrine-disrupting action via both mechanisms in animals. Their in vivo effects due to these combined activities should be further investigated.

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References

Andersen, H.R., Andersson, A.-M., Arnold, S.F., Autrup, H., Barfoed, M., Beresford, N.A., et al., 1999. Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environ. Health Perspect. 107 (1), 89–108.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R., Sheehan, D.M., 2002. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol. Sci. 54, 138–153.

Burdock, G.A., Pence, D.H., Ford, R.A., 1991. Safety evaluation of benzophenone. Food Chem. Toxicol. 11, 741–750.

Chen, C.W., Hurd, C., Vorojeikina, D.P., Arnold, S.F., Notides, A.C., 1997. Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. Biochem. Pharmacol. 53, 1161–1172.

Dutta, K., Das, M., Rahman, T., 1993. Toxicological impacts of benzophenone on the liver of guinea pig. Bull. Environ. Contam. Toxicol. 50, 282–285.

Fang, H., Tong, W., Perkins, R., Soto, A.M., Prechtl, N.V., Sheehan, D.M., 2000. Quantitative comparisons of in vitro assay for estrogenic activities. Environ. Health Perspect. 1081, 723–729.

Felix, T., Hall, B.J., Brodbelt, J.S., 1998. Determination of benzophenone-3 and metabolites in water and human urine by solid-phase microextraction and quadrupole ion trap GC-MS. Anal. Chim. Acta 371, 195–203.

Gaido, K.W., Leonard, L.S., Lovell, S., Gould, J.C., Babai, D., Portier, C.J., Mcdonell, D.P., 1997. Evaluation of chemicals with endocrine modulating activity in yeast-based steroid hormone receptor gene transcription assay. Toxicol. Appl. Pharmacol. 143, 205–212.

Gray Jr., L.E., Ostby, J., Manosson, E., Kelce, W.R., 1999. Environmental antiandrogens: low dose of the fungicide vinclozolin alter sexual differentiation of the male rat. Toxicol. Ind. Health 15, 48–64.

Hagedorn-Leweke, U., Lippold, B.C., 1995. Absorption of sunscreens and other compounds through human skin in vivo: derivation of a method to predict maximum fluxes. Pharmacol. Res. 12, 1354–1360.

Hany, J., Nagel, R., 1995. Detection of sunscreen agents in human breast milk. Deutsche Lebens.-Rund. 91, 341–345.

Hayden, C.G.J., Roberts, M.S., Benson, H.A.E., 1997. Systemic absorption of sunscreen after topical application. Lancet 350, 863–864.

Hershberger, L., Shipley, E., Meyer, R., 1953. Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. Proc. Soc. Exp. Biol. Med. 83, 175–180.

Hong, H., Tong, W., Fang, H., Shi, L., Xie, Q., Wu, J., Perkins, R., Walker, J.D., Branham, W., Sheehan, D.M., 2002. Prediction of estrogen receptor binding for 58,000 chemicals using an integrated system of a tree-based model with structural alerts. Environ. Health Perspect. 110, 29–36.

Jiang, R., Roberts, M.S., Collins, D.M., Benson, H.A.E., 1999. Absorption of sunscreens across human skin: an evaluation of commercial products for children and adults. Br. J. Clin. Pharmacol. 48, 635–637.

Kawamura, Y., Ogawa, Y., Nishimura, T., Kikuchi, Y., Nishikawa, J., Nishihara, T., et al., 2003. Estrogenic activities of UV stabilizers used in food contact plastics and benzophenone derivatives tested by the yeast two-hybrid assay. J. Health Sci. 49, 205–212.

Kelce, W.R., Stone, C.R., Laws, S.C., Gray, L.E., Kemppainen, J.A., Wilson, E.M., 1995. Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. Nature 375, 581–585.

Kitamura, S., Suzuki, T., Ohta, S., Fujimoto, N., 2003. Antiandrogenic activity and metabolism of the organophosphorus pesticide fenthion and related compounds. Environ. Health Perspect. 111, 503–508.

Klein, K., 1992. Encyclopedia of UV absorbers for sunscreen products. Cosmets. Toiletries 107, 45–64.

Kupfer, D., Bulger, W.H., 1987. Metabolic activation of pesticides with proestrogenic activity. Fed. Proc. 46, 1864–1869.

Ma, R., Cotton, B., Lichtensteiger, W., Schlumpf, M., 2003. UV filters with antagonistic action at androgen receptors in the MDA-kb2 cell transcriptional-activation assay. Toxicol. Sci. 74, 43–50.

Matsumoto, K., Akao, Y., Kobayashi, E., Ito, T., Ohguchi, K., Tanaka, T., Iinuma, M., Nozawa, Y., 2003. Cytotoxic benzophenone derivatives from Garcinia species display a strong apoptosis-inducing effect against human leukemia cell lines. Biol. Pharm. Bull. 26, 569–571.

Nakagawa, Y., Suzuki, T., 2002. Metabolism of 2-hydroxy-4-methoxybenzophenone in isolated rat hepatocytes and xenoestrogenic effects of its merabolites on MCF-7 human breast cancer cells. Chem. Biol. Inter. 139, 115–128.

Nakagawa, Y., Tayama, K., 2001. Estrogenic potency of benzophenone and its metabolites in juvenile female rats. Mol. Toxicol. 75, 74–79.

Okereke, C.S., Kadry, A.M., Abdel-Rahman, M.S., Davis, R.A., Friedman, M.A., 1993. Metabolism of benzophenone-3 in rats. Drug Metab. Dispos. 21, 788–791.

Okereke, C.S., Barat, S.A., Abdel-Rahman, M.S., 1995. Safety evaluation of benzophenone-3 after dermal administration in rats. Toxicol. Lett. 80, 61–67.

Satoh, K., Nagai, F., Aoki, N., 2001. Several environmental pollutants have binding affinities for both androgen receptor and estrogen receptor α. J. Health Sci. 47, 495–501.

Schauder, S., Ippen, H., 1997. Contact and photocontact sensitivity to sunscreens. Review of a 15 year experience and of the literature. Contact Dermatitis 37, 221–232.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. In vitro and in vivo estrogenicity of UV screens. Environ. Health Perspect. 109, 239–244.

Schultz, W.T., Seward, J.R., Sinks, G.D., 2000. Estrogenicity of benzophenones evaluated with a recombinant yeast assay: comparison of experimental and rules-based predicted activity. Environ. Toxicol. Chem. 19, 301–304.

Soto, A.M., Chung, K.L., Sonnenschein, C., 1994. The pesticides endosulfan, toxaphene and dieldrin have estrogenic effects on human estrogen-sensitive cells. Environ. Health Perspect. 102, 380–383.

Stresser, D.M., Kupfer, D., 1998. Human cytochrome P450-catalyzed conversion of the proestrogenic pesticide methoxychlor into an estrogen. Role of CYP2C19 and CYP1A2 in O-demethylation. Drug Metab. Dispos. 26, 868–874.

Sugihara, K., Kitamura, S., Sanoh, S., Ohta, S., Fujimoto, N., Maruyama, S., Ito, A., 2000. Metabolic activation of the proestrogens trans-stilbene and trans-stilbene oxide by rat liver microsomes. Toxicol. Appl. Pharmacol. 167, 46–54.

Tamura, H., Maness, S.C., Reischmann, K., Dorman, D.C., Gray, L.E., Gaido, K.W., 2001. Androgen receptor antagonism by the organophosphate insecticide fenitrothion. Toxicol. Sci. 60, 56–62.

Yamasaki, K., Takeyoshim, M., Sawaki, M., Imatanaka, N., Shinoda, K., Takatsuki, M., 2003. Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals. Toxicology 183, 93–115.

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COMPARATIVE METABOLISM OF POLYCHLORINATED BIPHENYLS AND TISSUE DISTRIBUTION OF PERSISTENT METABOLITES IN RATS, HAMSTERS, AND GUINEA PIGS

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ABSTRACT:

The present study was performed to compare the metabolite profiles of polychlorinated biphenyls (PCBs) in the liver and serum of rats, hamsters, and guinea pigs after exposure to a PCB mixture, Kanechlor 500 (100 mg/kg, i.p.). The percentage of contribution of major PCB residues in the liver 5 days after exposure indicated that nonplanar PCBs with 2,4- or 2,3,4-chlorine substitution were more abundant in the liver in the order rats (43% of total PCBs) > hamsters (20%) > guinea pigs (11%), whereas coplanar PCBs with 4-, 3,4-, or 3,4,5-chlorine substitution were predominant in guinea pigs (61%), followed by hamsters and rats (both 26%). The hepatic concentrations of methylsulfonyl metabolites ($MeSO_2$-CBs) were higher in the order guinea pigs > rats > hamsters. Whereas hamsters formed minute amounts of $MeSO_2$-CBs from 2,5-dichloro-substituted PCBs, guinea pigs formed higher levels of *meta*-$MeSO_2$-CBs derived from 2,3,6-trichloro-substituted PCBs. In contrast, the serum concentrations of phenolic PCBs were higher in the order hamsters > rats > guinea pigs. Metabolites were predominated by 4-OH-2,3,5,3 ,4 -pentaCB (89% contribution) for rats, 3-OH-2,4,5,2 ,4 -pentaCB (56%) for guinea pigs, and dihydroxylated metabolites (39%) for hamsters. The reduced elimination of coplanar PCBs and the specific distribution of $MeSO_2$- and phenolic PCBs may have implications for the differences in sensitivity to PCB toxicity among rats, guinea pigs, and hamsters.

Metabolism of PCBs proceeds via P450-mediated formation of arene oxide intermediates, which results in the distribution of both OH-PCBs and $MeSO_2$-CBs (Letcher et al., 2000). The ratios of formation of these metabolites are dependent on the degree of substrate chlorination (Kato et al., 1980; Haraguchi et al., 1997) as well as the metabolic capacity of the species (Safe, 1994; Koga and Yoshimura, 1996). Some OH-PCBs and $MeSO_2$-CBs are persistent and show specific retention in the blood or tissues of laboratory and wild animals (Bergman et al., 1994a,b; Oberg et al., 2002) as well as in humans (Sandau et al., 2000; Chu et al., 2003). Phenolic PCB metabolites cause alterations in thyroid hormone metabolism (Morse et al., 1996; Brouwer et al., 1998), inhibit estrogen sulfation and binding to estrogen receptors for estrogenic or antiestrogenic effects (Connor et al., 1997; Kester et al., 2002), and give rise to potentially cytotoxic dihydroxylated or quinoid PCB metabolites (Amaro et al., 1996). On the other hand, some $MeSO_2$-CBs induce expression of several P450 isozymes (Kato et al., 1997), reduce thyroid hormone levels in rats (Kato et al., 1999), and also show antiestrogenic effects (Letcher et al., 2002). These persistent metabolites may be responsible for the toxic effects of PCBs.

Guinea pigs are the most sensitive experimental animals to the toxicity of coplanar PCBs and polychlorinated dibenzo-*p*-dioxins (Kociba and Cabey, 1985), whereas hamsters are less sensitive (Olson et al., 1980; Wroblewski and Olson, 1988). These differences in sensitivity may be due to metabolic activation by the unique P450-dependent monooxygenase system and substrate specificity in these species (Koga et al., 1998). For example, in vitro studies have shown that 3,4,3 ,4 -tetraCB (CYP1A1) is hydroxylated by rats or hamsters but not by guinea pigs (Koga et al., 1995), whereas 2,4,5,2 ,4 ,5 -hexaCB (CYP2B18) is hydroxylated by guinea pigs but hardly by rats or hamsters (Ariyoshi et al., 1997). Therefore, the different metabolic capacities of PCBs may result in the differences in distribution profiles of metabolites in animals exposed to PCBs.

In the present study, we examined the species differences in tissue distribution of PCB residues and persistent PCB metabolites among rats, hamsters, and guinea pigs 5 days after exposure to a technical PCB mixture, Kanechlor 500. This article describes the 1) comparison of the residual PCB profiles (percentage of contribution) in the liver of these three species, 2) species differences in metabolite profiles of $MeSO_2$-CBs in the liver and of OH-PCBs in serum, and 3) GC/MS characterization of unknown metabolites retained in the serum of guinea pigs.

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ABBREVIATIONS: PCB, polychlorinated biphenyl; P450, cytochrome P450; OH, hydroxyl; CB, chlorobiphenyl; OH-PCB, hydroxylated PCB; $MeSO_2$-CB, methylsulfonyl PCB; GC/MS, gas chromatography-mass spectrometry; GC/ECD, gas chromatography-electron capture detection; MC, 3-methylcholanthrene.

TABLE 1

Residual patterns of PCBs in the livers of rats, hamsters, and guinea pigs dosed with Kanechlor 500 (100 mg/kg i.p.)

The 41 major isomers of a total of 61 were quantified and values represent the means for two animals.

Type	IUPAC	Structure	Relative Composition (%) of PCBs			
			Rat	Guinea Pig	Hamster	Kanechlor 500
Group A						
4-	28	2,4,4 -	0.08	1.03	0.15	0.86
	37	3,4,4 -	N.D.	6.01	0.04	0.15
	60	2,3,4,4 -	0.09	0.57	0.10	0.10
	74	2,4,5,4 -	0.72	4.33	0.74	0.54
	81	3,4,5,4 -	N.D.	0.19	0.02	0.007
	114	2,3,4,5,4 -	0.47	2.24	0.33	0.25
3,4-	77	3,4,3 ,4 -	0.02	1.82	0.06	0.03
	107	2,3,5,3 ,4 -	N.D.	2.46	N.D.	0.62
	118	2,4,5,3 ,4 -	17.6	32.2	18.6	7.68
	126	3,4,5,3 ,4 -	0.20	0.41	0.23	0.02
	156	2,3,4,5,3 ,4 -	4.15	5.87	3.33	1.25
3,4,5-	157	2,3,4,3 ,4 ,5 -	0.91	1.10	0.85	0.25
	167	2,4,5,3 ,4 ,5 -	1.26	2.22	1.74	0.45
		Sum of group A	25.5	60.5	26.2	12.2
Group B						
2,4-	47	2,4,2 ,4 -	0.18	0.003	N.D.	0.20
	49	2,4,2 ,5 -	0.11	0.04	0.05	0.83
	85	2,3,4,2 ,4 -	1.75	0.04	0.05	0.83
	99	2,4,5,2 ,4 -	12.6	2.44	3.45	3.22
	137	2,3,4,5,2 ,4 -	1.28	0.30	0.49	0.42
	147	2,3,5,6,2 ,4 -	0.27	0.02	0.02	0.11
2,3,4-	87	2,3,4,2 ,5 -	0.73	0.34	0.62	3.92
	105	2,3,4,3 ,4 -	5.16	3.92	4.44	2.99
	128	2,3,4,2 ,3 ,4 -	3.05	N.D.	N.D.	2.11
	132	2,3,4,2 ,3 ,6 -	0.17	N.D.	0.08	2.34
	138	2,3,4,2 ,4 ,5 -	13.7	2.22	8.81	5.56
	170	2,3,4,5,2 ,3 ,4 -	3.01	1.72	2.13	0.96
	177	2,3,4,2 ,3 ,5 ,6 -	0.71	0.04	0.08	0.23
	195	2,3,4,5,6,2 ,3 ,4 -	0.21	0.02	0.04	0.05
		Sum of group B	42.9	11.1	20.3	23.8
Group C						
2,4,5-	146	2,3,5,2 ,4 ,5 -	1.32	1.18	2.41	0.62
	153	2,4,5,2 ,4 ,5 -	12.1	10.3	17.0	5.36
	180	2,3,4,5,2 ,4 ,5 -	3.79	2.11	8.68	1.34
	183	2,3,4,6,2 ,4 ,5 -	1.30	0.35	1.33	0.41
	187	2,3,5,6,2 ,4 ,5 -	0.92	0.28	1.63	0.36
	203	2,3,4,5,6,2 ,4 ,5 -	0.51	0.05	1.21	0.15
2,3,4,5-	194	2,3,4,5,2 ,3 ,4 ,5 -	0.57	0.4	0.57	0.19
	201	2,3,4,5,2 ,3 ,5 ,6 -	0.32	0.27	0.94	0.10
		Sum of group C	20.8	15	33.8	8.53
Group D						
2,5-	52	2,5,2 ,5 -	0.08	0.36	1.40	5.56
	95	2,3,6,2 ,5 -	0.13	0.18	0.73	6.52
	101	2,4,5,2 ,5 -	1.35	2.15	4.68	10.0
	141	2,3,4,5,2 ,5 -	0.20	0.08	0.59	1.13
2,3,6-	110	2,3,6,3 ,4 -	0.35	0.16	0.41	7.44
	149	2,3,6,2 ,4 ,5 -	0.58	0.6	0.82	2.76
		Sum of group D	2.69	3.53	8.63	33.4
		Total %	91.9	90.0	88.9	77.9

N.D., not detected (0.002%).

Materials and Methods

Chemicals. The standard reference compounds, methoxy-PCBs and $MeSO_2$-CBs, were synthesized as described previously (Haraguchi et al., 1987; Bergman et al., 1995). Syntheses of veratrolic PCBs will be described elsewhere. The chemical names of $MeSO_2$-CBs and OH-PCBs were simplified on the basis of the IUPAC-derived numbering system of the parent PCBs. All solvents (*n*-hexane, acetone, ethanol, and dichloromethane) were of pesticide grade (Kanto Chemical Co., Tokyo, Japan).

Animals. Male Wistar rats (b.wt. 190–200 g), male Hartley guinea pigs (280–290 g), and male Golden Syrian hamsters (80–90 g) were housed three or four per cage in the laboratory with free access to commercial chow and tap water. Animals received a single i.p. injection of 100 mg/kg Kanechlor 500 dissolved in corn oil. Control animals received an equivalent volume of vehicle alone. The animals were sacrificed by decapitation 5 days after injection, and the liver, lungs, and serum were removed and stored at 20°C until analysis.

Isolation of Metabolites. Tissue samples were treated as described previously (Haraguchi et al., 1998). Briefly, tissues were homogenized and extracted with acetone/*n*-hexane (2:1, v/v). Two internal standards, 2,3,4,5,3 ,4 ,5 -heptaCB (70 ng) and 4-methyl-3-$MeSO_2$-5,2 ,3 ,4 ,5 -pentaCB (24 ng), were added to each extract, and the mixtures were applied to a gel-permeation column packed with Bio-Beads S-X3 (50 g; Bio-Rad, Hercules, CA). Dichloromethane/*n*-hexane (1:1) was used as a mobile phase at a flow rate of 4 ml/min. The metabolite fraction (120–200 ml) was divided into phenolic and neutral fractions by partitioning with potassium hydroxide (0.5 M in 30% ethanol) and *n*-hexane. The potassium hydroxide phase was acidified with hydrochloric acid (2 M, 10 ml) and extracted with *n*-hexane/diethyl ether (9:1), and then methylated with diazomethane for 30 min at 4°C. The neutral fraction (*n*-hexane layer) was further subjected to silica gel column chromatography (1 g; Wakogel S-1; Wako Pure Chemicals, Osaka, Japan) by elution with 10 ml of *n*-hexane for PCBs and successively with 10 ml of dichlorometh-

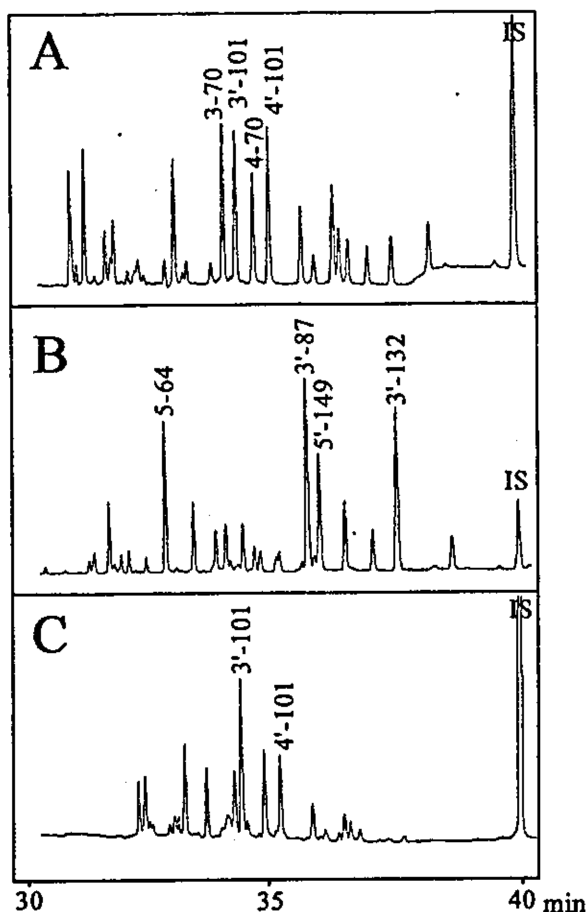

FIG. 1. GC/ECD chromatograms of methylsulfonyl PCB metabolites in the livers of rats (A), guinea pigs (B), and hamsters (C) after exposure to Kanechlor 500 (100 mg/kg i.p.).

ane for $MeSO_2$-CBs. Serum (0.1–1 ml) was spiked with an internal standard (2,3,4,5,3 ,4 ,5 -heptaCB), acidified with 4 volumes of 0.5 M HCl and extracted with *n*-hexane/diethyl ether (1:1). The phenolic PCB fraction was spiked with another internal standard (2,3,4,5,6,3 ,4 ,5 -octaCB) and methylated with diazomethane. Recoveries of 4 -hydroxy-2,3,4,5,3 ,5 -hexaCB added to control serum and liver samples prior to extraction were 89 to 97% (*n* 4) in the phenolic fraction.

Identification and Quantification. Identification of individual PCBs and phenolic PCB metabolites was performed on a GC/MS system (electron ionization-selected ion monitoring mode; QP-5000; Shimadzu, Kyoto, Japan) with a DB-5MS capillary column (60 m 0.25 mm i.d. and 0.25- m film thickness; J&W Scientific, Folsom, CA). The injection temperature was 250°C. The temperature program was initiated at 100°C (2 min) by 20°C/min to 250°C, and then by 2°C/min to 290°C (20 min). Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. The transfer line and quadrupole temperatures were 250 and 150°C, respectively. The mono- and dihydroxylated PCBs were monitored at molecular ion (M) and M 2 for methoxy derivatives of tetra-, penta-, and hexachlorinated congeners.

Quantification of PCBs, OH-PCBs, and $MeSO_2$-CBs was performed on a Shimadzu GC 14A equipped with an electron capture detector (^{63}Ni-ECD) and a split-splitless injector operated in the splitless mode. Nitrogen was used as a carrier and make-up gas. Column conditions were analogous to those described above for GC/MS. Individual PCBs and metabolites were quantified from the relative peak area to internal standards by comparison with authentic standards.

Results

Residual PCB Pattern in the Liver. Table 1 shows the percentages of composition of PCBs classified into four groups, A to D, by chlorination pattern in the livers of rats, hamsters, and guinea pigs. The original composition of major PCB congeners in Kanechlor 500 was 2,5,2 ,5 -tetraCB (5.6% of total PCBs), 2,3,6,2 ,5 -pentaCB (6.5%), 2,4,5,2 ,5 -pentaCB (10%); 2,3,6,3 ,4 -pentaCB (7.4%), 2,4,5,3 ,4 -pentaCB (7.7%), 2,3,4,2 ,4 ,5 -hexaCB (5.6%), and 2,4,5,2 ,4 ,5 -hexaCB (5.4%). After exposure of animals to Kanechlor 500, 2,5,2 ,5 -tetraCB, 2,3,6,2 ,5 -pentaCB, 2,4,5,2 ,5 -pentaCB, and 2,3,6,3 ,4 -pentaCB were eliminated rapidly from the livers of all species. Major PCB residues were 2,3,4,2 ,4 ,5 -hexaCB (14% of total PCBs) in rats, 2,4,5,2 ,4 ,5 -hexaCB (17%) in hamsters, and 2,4,5,3 ,4 -pentaCB (32%) in guinea pigs. The coplanar PCBs chlorinated at the 4-, 3,4-, or 3,4,5-position (group A, 12% of total PCBs) were detected in the liver in the following order: guinea pigs (61%) hamsters (26%) rats (26%). In contrast, the nonplanar PCBs chlorinated at the 2,4- or 2,3,4-position (group B, 24% of total PCBs) were more abundant in the liver in the following order: rats (43%) hamsters (20%) guinea pigs (11%). Highly chlorinated PCBs with 2,4,5-chlorine substitution (group C, 8.5% of total PCBs) were more

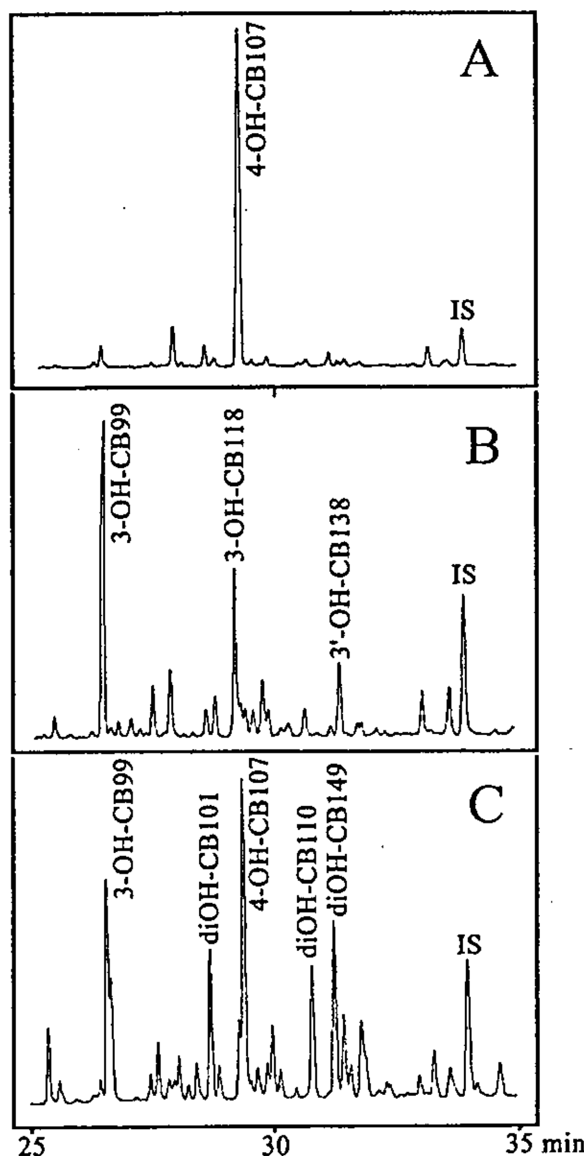

FIG. 2. GC/ECD chromatograms of phenolic PCBs (after methylation) in the serum of rats (A), guinea pigs (B), and hamsters (C) after exposure to Kanechlor 500 (100 mg/kg i.p.). The phenolic PCB fraction was spiked with 2,3,4,5,6,3 ,4 ,5 -octaCB (internal standard) after separation of PCBs and then methylated with diazomethane. See Table 2 for abbreviation on the peak.