Estrogenic Activity of Styrene Oligomers after Metabolic Activation by Rat Liver Microsomes

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In this study we examined estrogenic activity of styrene oligomers after metabolic activation by rat liver microsomes. *trans*-1,2-Diphenylcyclobutane (TCB), *cis*-1,2-diphenylcyclobutane (CCB), 1,3-diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene, and 1α-phenyl-4β-(1′-phenylethyl)tetralin were negative in the yeast estrogen screening assay and estrogen reporter assay using estrogen-responsive human breast cancer cell line MCF-7. However, TCB exhibited estrogenic activity after incubation with liver microsomes of phenobarbital-treated rats in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Minor activity was observed when liver microsomes of untreated or 3-methylcholanthrene–treated rats were used instead of those from phenobarbital-treated rats. CCB, 1,3-diphenylpropane, and 2,4-diphenyl-1-butene also exhibited estrogenic activity after metabolic activation by liver microsomes, but the activity was lower than that of TCB. 2,4,6-Triphenyl-1-hexene and 1α-phenyl-4β-(1′-phenylethyl)tetralin did not show estrogenic activity after such incubation. When TCB was incubated with liver microsomes of phenobarbital-treated rats in the presence of NADPH, three metabolites were detected by high-performance liquid chromatography (HPLC). One metabolite isolated by HPLC exhibited a significant estrogenic activity. The active metabolite was identified as *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane by mass and nuclear magnetic resonance spectral analysis. These results suggest that the estrogenic activity of TCB was caused by the formation of the 4-hydroxylated metabolite. *Key words: trans*-1,2-diphenylcyclobutane, endocrine disruption, estrogenic activity, human breast cancer cell line MCF-7, metabolic activation, rat liver microsomes, styrene oligomer, yeast estrogen screening assay. *Environ Health Perspect* 111:329–334 (2003). doi:10.1289/ehp.5723 available via *http://dx.doi.org/* [Online 1 November 2002]

Styrene oligomers—such as *trans*-1,2-diphenylcyclobutane (TCB), *cis*-1,2-diphenylcyclobutane (CCB), 1,3-diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene, and 1α-phenyl-4β-(1′-phenylethyl)tetralin—are incorporated into polystyrene resin as impurities in the course of manufacture and may have a variety of biologic actions, including hormonal activity (Kawamura et al. 1998b, 1998c). Polystyrene has been used to manufacture food containers for takeout, such as coffee cups, meat trays, salad boxes, and soup bowls, as well as containers in which instant foods such as noodles, pasta, and rice are cooked by adding hot water. Some reports indicate that styrene oligomers migrate from these containers into the food contents (Kawamura et al. 1998b; Sakamoto et al. 2000). Kawamura et al. (1998b) reported that the quantity of migrated styrene trimers was higher than that of bisphenol A transferred from the lacquer coating of vegetable cans to the food contents, as reported by Brotons et al. (1995).

Some manmade compounds mimic the effect of estradiol; they include chlorinated organics, such as the insecticides kepone, *o,p′*-DDT (*o,p′*-dichlorodiphenyltrichloroethane), *p,p′*-DDD (*p,p′*-dichlorodiphenyldichloroethane), and dieldrin and some polychlorinated biphenyl congeners, as well as nonchlorinated compounds such as the plasticizer bisphenol A and the surfactant breakdown product nonylphenol (Colborn 1995; Andersen et al. 1999). Such compounds can accumulate in our environment, and these so-called environmental estrogens may play a role in the increasing incidence of breast cancer, testicular cancer, and other problems of the reproductive system. Quantitative structure–activity relationship work on the structural features of estrogen receptor ligands shows that an unhindered hydroxyl group on an aryl ring and a hydrophobic group attached *para* to the hydroxyl group are essential (Anstead et al. 1997; Fang et al. 2000; Shi et al. 2001; Hong et al. 2002). The ligand binding assay and studies in a reporter/transcriptional system for the estrogen receptor support the requirement for these structural features (Nishihara et al. 2000; Blair et al. 2000; Branham et al. 2002). Recently, we showed that *trans*-stilbene, the parent compound of diethylstilbestrol, was not estrogenic but exhibited a potent estrogenic activity after metabolic activation by a liver microsomal oxidation system (Sugihara et al. 2000). In that report, we suggested that the estrogenic activity of *trans*-stilbene was caused by hydroxylated metabolites. TCB, a styrene dimer, may also be converted to an estrogen by metabolic activation, based on the similarity of its structure to that of *trans*-stilbene.

For this report, we examined the estrogenic activities of styrene oligomers in the presence or absence of a liver microsomal metabolic system using a yeast estrogen screening (YES) assay and an estrogen-responsive element (ERE)-luciferase reporter assay in the estrogen-responsive human breast cancer cell line MCF-7. We examined TCB, CCB, 1,3-diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene, and 1α-phenyl-4β-(1′-phenylethyl)tetralin in this study (Figure 1). We found that some styrene oligomers, especially TCB, exhibit significant estrogenic activities after activation by rat liver microsomal mixed function oxidase.

Materials and Methods

Chemicals. TCB (99.3%), CCB (99.9%), 1,3-diphenylpropane (98.1%), 2,4-diphenyl-1-butene (96.1%), 2,4,6-triphenyl-1-hexene (99.4%), and 1α-phenyl-4β-(1′-phenylethyl)tetralin (99.1%) were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan), and 17β-estradiol (E_2; > 98%) and tamoxifen (> 99%) from Sigma Chemical Co. (St. Louis, MO, USA). Chlorophenol red β-D-galactopyranoside (CPRG) was obtained from Roche Diagnostics (Mannheim, Germany). We obtained *trans*-1,2-bis-(4-hydroxyphenyl)cyclobutane (96%) by isomerization of *cis*-1,2-bis-(4-hydroxyphenyl)cyclobutane by the method of Williard and Fryhle (1980), and synthesized *cis*-1,2-bis-(4-hydroxyphenyl)cyclobutane according to the method of Brown (1968).

Animals. Male Sprague-Dawley rats (190–220 g) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed at 22°C with a 12-hr light/dark cycle, with free access to tap water and a standard

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We thank J. Sumpter (Brunel University, UK), who provided the recombinant yeast for estrogenicity assay, and S. Aoki for helpful advice in carrying out this study.

This work was supported by a Grant-in-Aid for Scientific Research on a Priority Area (13027256) from the Japanese Ministry of Education, Science, Sports and Culture, and a Grant-in-Aid for Scientific Research (C13672343) from the Japan Society for the Promotion of Science.

Received 16 April 2002; accepted 12 August 2002.

pellet diet (MM-3; Funabashi Farm, Funabashi, Japan). In some experiments, rats were given intraperitoneal administration of phenobarbital (80 mg/kg) or 3-methylcholanthrene (25 mg/kg) daily for 3 days before use.

Preparation of liver microsomes. The livers were excised from exsanguinated rats and immediately perfused with 1.15% KCl. The livers were homogenized in four volumes of the KCl solution using a Potter-Elvehjem homogenizer (Iwaki Glass Co., Ltd., Tokyo, Japan). The microsomal fraction was obtained from the homogenate by successive centrifugation at 9,000 × *g* for 20 min and 105,000 × *g* for 60 min. The fraction was washed by resuspension in the KCl solution and resedimentation. The pellets of microsomes were resuspended in the solution to make 1 mL equivalent to 1 g of liver. Protein contents in the liver microsomal preparation of untreated, phenobarbital-treated, and 3-methylcholanthrene–treated rats were 13.7–17.6, 13.9–15.4, and 6.18–8.16 mg protein/mL, respectively, as determined by the method of Lowry et al. (1951).

Cell culture. MCF-7 cell lines were maintained in minimum essential medium (MEM; Sigma Chemical Co.) containing penicillin and streptomycin with 5% fetal bovine serum (Life Technologies, Rockville, MD, USA).

Assay of estrogenic activities of styrene oligomers in recombinant yeast. For the YES assay, a recombinant yeast strain transfected with the human estrogen receptor (ERα) gene and expression plasmids carrying the reporter gene *lac-Z* preceded by ERE were kindly provided by J. Sumpter (Brunel University, Uxbridge, Middlesex, UK). The yeast estrogenicity assay was conducted as described by Routledge and Sumpter (1996, 1997), with some minor modifications (Yoshihara et al. 2001). In brief, a styrene oligomer in yeast assay medium containing recombinant yeast and CPRG, a chromogenic substrate of the β-galactosidase reporter enzyme, was dispensed into 96-well plastic microtiter plates. The plates were incubated at 32°C. After 24–48 hr, the absorbance of the red color due to the hydrolysis product of CPRG was read using a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA) at 540 nm. The data were corrected for turbidity based on the absorbance at 620 nm (optical density, OD_{620}), and the values were calculated as follows: Net OD_{540} = (OD_{540} for test – OD_{620} for test) – (OD_{540} for blank – OD_{620} for blank). For the assay of the metabolites produced from styrene oligomers, substrates (0.1 μmol) were incubated with 0.1 mL of rat liver microsomes in the presence of 1 μmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for 30 min in a final volume of 1 mL of 0.1 M phosphate buffer. After the incubation, the mixture was extracted with 5 mL of ethyl acetate and the organic solution was evaporated to dryness. The residue was dissolved with 1 mL of ethanol and an aliquot was used for the estrogenic activity assay. The total concentration of the substrate and its metabolites was calculated from the original amount of the substrate. In some experiments, tamoxifen was added at the concentration of 1×10^{-8} M to wells as an antiestrogen.

Assay of estrogenic activities of styrene oligomers in MCF-7 cells. For ERE–luciferase reporter assay using MCF-7 cells, the culture medium was changed to phenol red–free MEM (Sigma Chemical Co.) containing penicillin, streptomycin, and dextran–charcoal-treated fetal bovine serum for 2–3 days. Transient transfections of MCF-7 cells were performed with Transfast (Promega Co., Madison, WI, USA), using the manufacturer's protocol. Transfections were done in 12-well plates at 1×10^5 cells/well with 1.9 μg $p(ERE)_3$-SV40-luc and 0.1 μg pRL/CMV (Promega Co.) as an internal standard. Twenty-four hours after addition of the sample (final concentration, 10^{-4} to 10^{-9} M) dissolved in 10 μL ethanol, the assay was performed with a Dual Luciferase assay kit (Promega Co.) according to the manufacturer's protocol. For assay after incubation of chemicals with the rat liver microsomal enzyme system, a sample from the incubation mixture was subjected to the assay as described above.

Isolation of TCB metabolites from the incubation mixture of rat liver microsomes. The incubation mixture consisted of 12 μmol TCB, 30 μmol NADPH, and 1.2 mL liver microsomes equivalent to 1.2 g liver (wet weight) in a final volume of 20 mL of 0.1 M phosphate buffer (pH 7.4). The incubation was performed at 37°C for 30 min. Then, the mixture was extracted twice with two volumes of ethyl acetate. The combined extract was dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. A metabolite of TCB with estrogenic activity was purified by preparative high-performance liquid chromatography (HPLC).

Assay of TCB oxidase activity. An incubation mixture consisted of 0.1 μmol TCB, 0.5 μmol NADPH, and 0.02 mL liver microsomes equivalent to 20 mg liver wet weight (0.2–0.3 mg protein) in a final volume of 1 mL 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was performed at 37°C for 5 min. After incubation, 0.1 μmol *trans*-stilbene oxide as an internal standard was added, and the mixture was extracted with 5 mL ethyl acetate. The extract was evaporated to dryness, the residue was dissolved in 0.1 mL methanol, and an aliquot (5 μL) was analyzed by HPLC.

HPLC and thin-layer chromatography. HPLC was performed in a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with a 150 × 4.6 mm Inertsil ODS-3 column (GL Science, Tokyo, Japan). The mobile phase was acetonitrile–water (6:4, vol/vol). The chromatograph was operated at a flow rate of 0.5 mL/min at a wavelength of 240 nm. The elution times of *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane, TCB, CCB, and *trans*-stilbene oxide (an internal standard) were 14.6, 68.0, 55.3, and 25.2 min, respectively. For the detection of *trans*-1,2-bis-(4-hydroxyphenyl)cyclobutane, the mobile phase was acetonitrile–water (3:7, vol/vol). The chromatograph was operated at a flow rate of 0.5 mL/min at a wavelength of 240 nm. The elution time of *trans*-1,2-bis-(4-hydroxyphenyl)cyclobutane was 12.2 min.

Silica gel plates (Kieselgel 60 GF254, 0.1 mm thick; Merck, Darmstadt, Germany) were developed in *n*-hexane–ethyl acetate (9:1, vol/vol). Spots were visualized under ultraviolet light (254 nm). The R_f (rate of

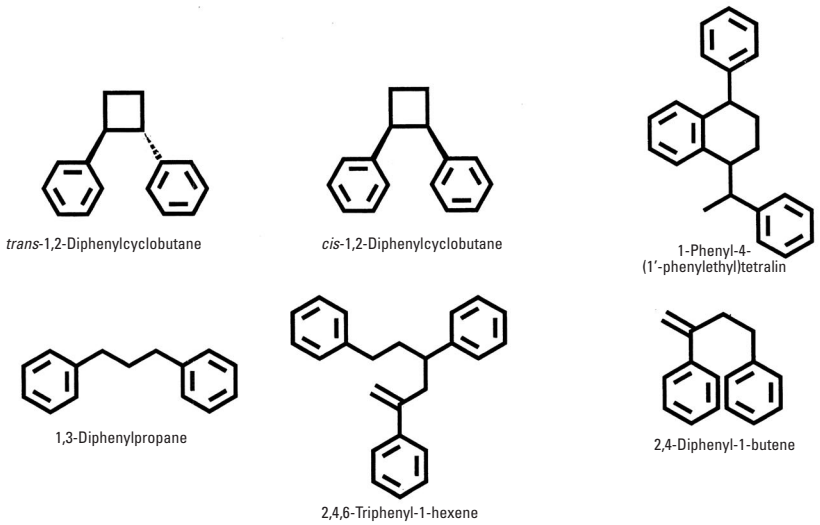

Figure 1. Structures of styrene oligomers.

flow) values of TCB, *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane, and *trans*-1,2-bis-(4-hydroxyphenyl)cyclobutane were 0.78, 0.44, and 0.17, respectively.

Mass and nuclear magnetic resonance spectra. A Japan Electron Optics Laboratory HX-100 mass spectrometer (JEOL, Tokyo, Japan) in electron impact mode was used for structure analysis of the metabolites. Mass spectroscopy was carried out at a collision energy of 70 eV and an acceleration voltage of 10 kV.

Results

Estrogenic activity of TCB and CCB with or without a microsomal activation system. Estrogenic activity of TCB and CCB in the presence or absence of a rat liver microsomal oxidation system was examined in the YES assay. These compounds did not show estrogenic activity in this assay. However, when TCB was incubated with liver microsomes of phenobarbital-treated rats in the presence of NADPH, the extract of the incubation mixture exhibited an estrogenic effect on the cells in the range of 10^{-5} to 10^{-6} M. In contrast, little effect was obtained when liver microsomes of untreated or 3-methylcholanthrene–treated rats were used instead of those from phenobarbital-treated rats. CCB showed estrogenic activity after incubation with the liver microsomes of 3-methylcholanthrene–treated rats, but the activity was much lower than that of TCB (Figure 2). These estrogenic activities were inhibited by the addition of tamoxifen at the concentration of 1×10^{-6} M (data not shown).

Estrogenic activity of 1,3-diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene, and 1α-phenyl-4β-(1′-phenylethyl)tetralin with or without a microsomal activation system. 1,3-Diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene, and 1α-phenyl-4β-(1′-phenylethyl)tetralin were negative in the YES assay, except for marginal activity at higher concentrations. However, 1,3-diphenylpropane exhibited estrogenic activity after incubation with rat liver in the presence of microsomes of untreated and 3-methylcholanthrene–treated rats in the presence of NADPH. Lower activity was observed after incubation with liver microsomes of phenobarbital-treated rats. 2,4-Diphenyl-1-butene showed estrogenic activity after incubation with liver microsomes of 3-methylcholanthrene–treated rats. Little effect was obtained when liver microsomes of untreated or phenobarbital-treated rats were used instead of those from 3-methylcholanthrene–treated rats. In contrast, 2,4,6-triphenyl-1-hexene and 1α-phenyl-4β-(1′-phenylethyl)tetralin did not show estrogenic activity after similar incubation (Figure 3).

These facts suggest that some styrene oligomers, especially TCB, exhibit estrogenic activity after metabolic activation by rat liver microsomes.

Metabolism of TCB by rat liver microsomes. TCB was incubated with liver microsomes of phenobarbital-treated rats in the presence of NADPH for the detection of the metabolites as described in "Materials and Methods." Three peaks were detected in an HPLC chromatogram of the extract of the TCB incubation mixture (Figure 4). These peaks were not detected in the control, which was incubated without the substrate. The metabolites of TCB detected at 7.2, 10.9, and 14.6 min were designated TCB metabolites 1, 2, and 3 (TD-M1, -M2, and -M3), respectively.

Identification of estrogenic active metabolites of TCB generated by rat liver microsomes. After the incubation of TCB with liver microsomes of phenobarbital-treated rats in the presence of NADPH, HPLC fractions were collected for the identification of the active metabolites. The fraction corresponding to TD-M3 exhibited estrogenic activity (Figure 4). The mass spectrum of TD-M3 showed *m/z* 196 (M^+), suggesting that TD-M3 is a monohydroxylated metabolite of TCB. The nuclear magnetic resonance spectrum of TD-M3 showed signals at δ 6.75 (doublet, 2H, J = 8 *Hz*, phenyl groups), 7.09 (doublet, 2H, J = 8 *Hz*, phenyl groups), 7.24 (multiplet, 5H, phenyl groups), and 8.00 (singlet, 1H, OH). The further oxidized metabolite, *trans*-1,2-bis-(4-hydroxyphenyl)cyclobutane, could not be detected by HPLC or thin-layer chromatography as a metabolite of TCB. These facts suggest that TCB is mainly metabolized to *trans*-1-(4-hydroxyphenyl)-2-phenylcylobutane by rat liver microsomes.

Oxidase activities of rat liver microsomes toward TCB. The oxidase activity of rat liver microsomes transforming TCB to TD-M3 was examined using liver microsomes of phenobarbital-treated rats. The time course of formation of the hydroxylated metabolite was essentially linear for 10 min (Figure 5A). The oxidase activity of the liver microsomes increased linearly with increasing amount of liver microsomes up to 0.5 mg of protein (Figure 5B). Liver microsomes of phenobarbital-treated rats exhibited the highest oxidase activity in the presence of NADPH. The NADPH-linked activity was inhibited by the addition of SKF 525-A (1×10^{-4} M). Little oxidase activity was observed when liver microsomes of untreated or 3-methylcholanthrene–treated rats were used instead of those of phenobarbital-treated rats (Figure 5C). Only marginal activity was observed with reduced nicotinamide adenine dinucleotide (NADH) instead of NADPH (data not shown). This suggests that TCB was mainly metabolized to *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane by cytochrome P450 2B1 in rats.

Estrogenic activity of styrene oligomers in estrogen reporter assay using MCF-7 cells. Estrogenic activity of styrene oligomers in the presence or absence of a rat liver microsomal oxidation system was also examined by ERE–luciferase reporter assay using MCF-7 cells. TCB, CCB, 1,3-diphenylpropane, and 2,4-diphenyl-1-butene were negative in this estrogen screening test, except for a marginal effect at 1×10^{-5} M. When TCB was incubated with liver microsomes of phenobarbital-treated rats in the presence of NADPH, the extract of the incubation mixture exhibited estrogenic activity at the concentration of 1×10^{-5} M, as in the YES assay. Lower activity was obtained in this assay when liver microsomes of untreated or 3-methylcholanthrene–treated rats were used instead of those from phenobarbital-treated rats. In contrast, CCB showed estrogenic activity after incubation with the liver microsomes of untreated, phenobarbital-treated, and 3-methylcholanthrene–treated rats, but these activities were lower than that of TCB. 1,3-Diphenylpropane exhibited estrogenic activity after incubation with liver microsomes of untreated and 3-methylcholanthrene–treated rats in the presence of NADPH. 2,4-Diphenyl-1-butene

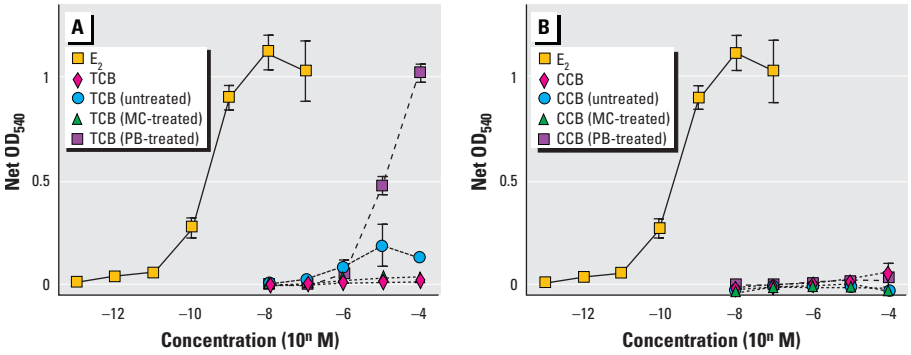

Figure 2. Estrogenic activity of TCB and CCB with or without a rat liver microsomal enzyme system in the YES assay. Abbreviations: E_2, 17β-estradiol; PB, phenobarbital; MC, 3-methylcholanthrene. (*A*) Estrogenic activity of TCB. (*B*) Estrogenic activity of CCB. Each bar represents the mean ± SD of four experiments. TCB or CCB was incubated with liver microsomes in the presence of NADPH, and the extract of the incubation mixture was subjected to the screening test.

showed estrogenic activity after similar incubation with liver microsomes of 3-methylcholanthrene–treated rats (Figure 6). These estrogenic activities were completely inhibited by the addition of tamoxifen at the concentration of 1×10^{-6} M (data not shown).

Thus, the results of the estrogen screening test with MCF-7 cells confirm that some styrene oligomers exhibit estrogenic activity after metabolic activation by rat liver microsomes.

Discussion

Styrene oligomers such as TCB are by-products in the manufacture of polystyrene and may later be released from the resin (Kawamura et al. 1998b; Sakamoto et al. 2000). Nobuhara et al. (1999) reported that styrene oligomers did not induce the proliferation of MCF-7 cells. We also showed in this study that these compounds do not exhibit estrogenic activity in the YES assay or estrogen screening assay using MCF-7 cells. However, some of them yield estrogenic metabolites after metabolic activation. Ohyama et al. (2001) reported that some styrene dimers and trimers were estrogenic without metabolic activation in a cell proliferation assay with estrogen-responsive MCF-7 cells. They reported that TCB, CCB, 1,3-diphenylpropane, and 2,4-diphenyl-1-butene were positive without metabolic activation. We cannot explain the difference from our present results. However, one possibility is that because the substrates were in contact with MCF-7 cells for a long time in their assay, estrogenic metabolites were generated in the cells.

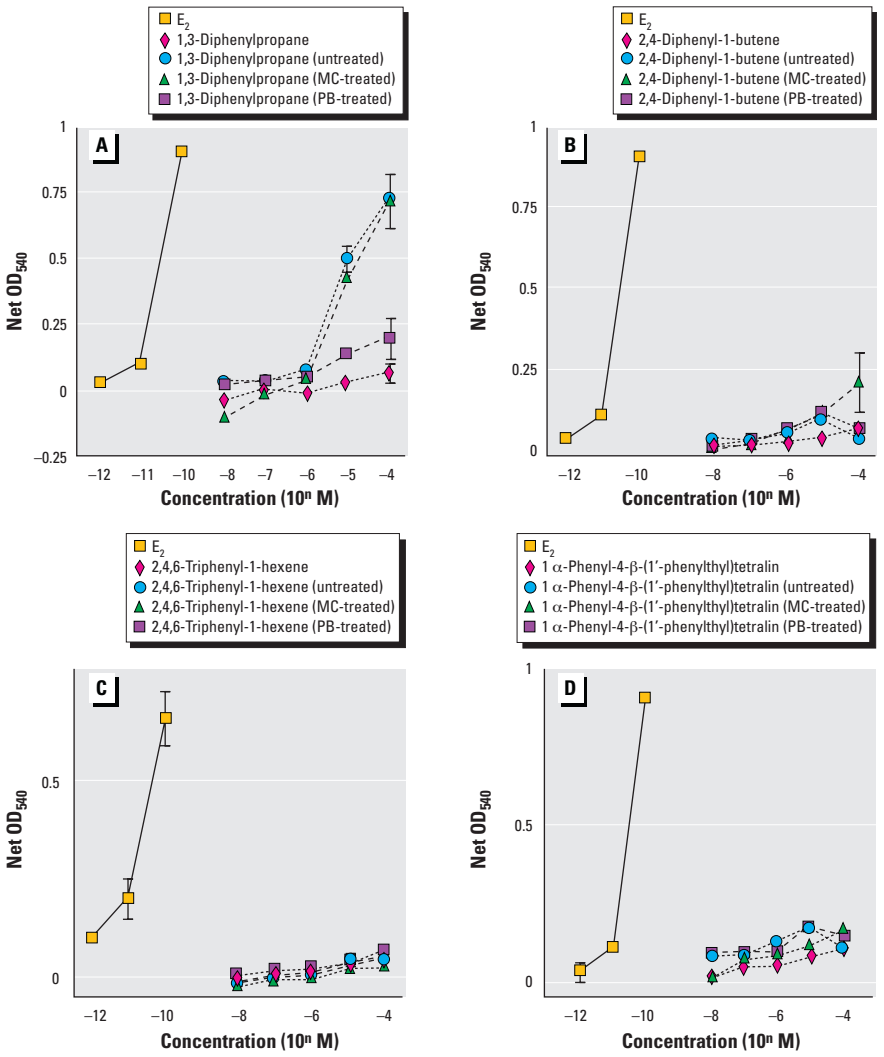

Figure 3. Estrogenic activity of 1,3-diphenylpropane, 2,4-diphenyl-1-butene, 2,4,6-triphenyl-1-hexene, and 1α-phenyl-4β-(1′-phenylethyl)tetralin with or without a rat liver microsomal enzyme system in the YES assay. Abbreviations: E_2, 17β-estradiol; PB, phenobarbital; MC, 3-methylcholanthrene. (*A*) Estrogenic activity of 1,3-diphenylpropane. (*B*) Activity of 2,4-diphenyl-1-butene. (*C*) Activity of 2,4,6-triphenyl-1-hexene. (*D*) Activity of 1α-phenyl-4β-(1′-phenylethyl)tetralin. Each bar represents the mean ± SD of four experiments. A styrene oligomer was incubated with liver microsomes in the presence of NADPH, and the extract of the incubation mixture was subjected to the screening test.

We present here the first evidence that TCB, CCB, 1,3-diphenylpropane, and 2,4-diphenyl-1-butene are converted to metabolites with estrogenic activity by liver microsomal enzymes. It has been reported that some styrene oligomers do not give a positive response in uterotrophic assay (Bachmann et al. 1998; Fail et al. 1998; Prinsen and Gouko 2001). However, the levels of active metabolites in the body would be an important factor determining the effects of the oligomers *in vivo*. The apparent affinities of such xenoestrogens for the estrogen receptor are lower than those of endogenous estradiol or phytoestrogens. Nevertheless, endogenous estrogens are tightly regulated in the body. We speculate that phytoestrogens, which are present in foods that have been consumed from the earliest times, may also be subject to regulation. This may not be the case for xenoestrogens, which may act directly on the endocrine organs. Further, xenoestrogens may be accumulated in the body, their intake amounts vary considerably among consumers, and the amounts released from polystyrene resin are variable. Styrene oligomers may exhibit estrogenic activity in combination with other xenoestrogens. Further work is necessary to assess the *in vivo* endocrine-disrupting action of styrene oligomers, taking into account the activities of the metabolites produced from the parent compounds.

We showed in previous reports that *trans*-stilbene and *trans*-stilbene oxide were oxidized to hydroxylated derivatives by rat liver microsomes (Sugihara et al. 2000; Sanoh et al. 2002). In those studies, we obtained evidence that *trans*-stilbene is oxidized to the 4-hydroxy and 4,4′-dihydroxy derivatives by cytochrome P450 1A1/2 in rat liver microsomes and is thereby activated to exhibit estrogenic activity. In contrast, *cis*-stilbene and *cis*-stilbene oxide exhibited only weak estrogenic activity even after metabolic activation. TCB structurally resembles *trans*-stilbene and *trans*-stilbene oxide, and this may be the reason why its metabolites exhibited the highest activity among the compounds tested in this study. As in the case of *cis*-stilbene and *cis*-stilbene oxide, the activities of the *cis*-isomers were much lower than those of the *trans*-compounds (Sugihara et al. 2000). The activity of CCB after activation is also lower than that of the *trans*-isomer.

We have shown here that *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane is a major active metabolite of TCB in a rat liver microsomal system. Furthermore, formation of the metabolite was stimulated by the pretreatment of rats with phenobarbital. This suggests that cytochrome P450 2B isoform is responsible for this activation. In a preliminary study, when TCB was incubated with microsomal preparations from cells expressing recombinant human cytochrome P450 2B6 and rat cytochrome P450 2B1 expressed in a

human B lymphoblastoid cell line (Gentest Corp., Woburn, MA, USA), cytochrome P450 2B6 exhibited a higher oxidase activity than did cytochrome P450 2B1. Significant oxidase activity was also observed in human liver microsomes (Gentest Corp.) (data not shown). Thus, cytochrome P450 isoforms other than cytochrome P450 2B may contribute to the metabolic activation in humans. In contrast, 2,4-diphenyl-1-butene was metabolically activated to an estrogenic derivative by liver microsomes of 3-methylcholanthrene–treated rats. In this case, cytochrome P450 1A may be mainly responsible for the activation. The oxidation of 2,4-diphenyl-1-butene was catalyzed by rat cytochrome P450 1A1/2. These isozymes are also known isoforms of cytochrome P450 in humans. It is thus possible that metabolic activation of these styrene oligomers to active estrogens occurs in humans. In contrast, activation of 1,3-diphenylpropane proceeded with liver microsomes of untreated and 3-methylcholanthrene–treated rats but not those of phenobarbital-treated rats. Possibly, the oxidative metabolism of 1,3-diphenylpropane by liver microsomes of phenobarbital-treated rats preferentially generated nonestrogenic metabolites. It is interesting that different styrene oligomers are activated by different cytochrome P450 isoforms, and the potencies of estrogenic activity of the metabolites formed are different.

A possible metabolic activation pathway of proestrogenic TCB and CCB with liver microsomes is shown in Figure 7. TCB is

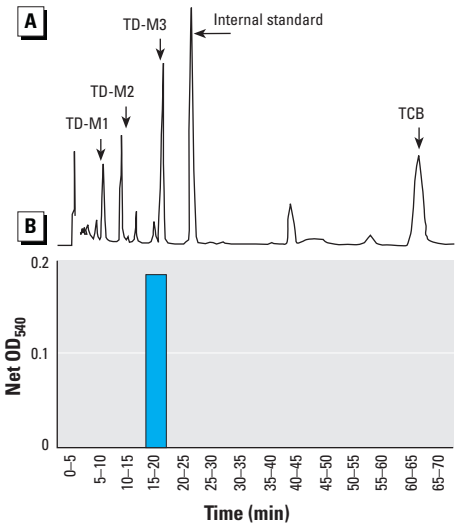

Figure 4. HPLC of the extract of an incubation mixture of TCB with rat liver microsomes, and estrogenic activity of each fraction in the YES assay. (*A*) HPLC chromatogram of the metabolites of TCB. (*B*) Estrogenic activity of each fraction in the YES assay. An incubation mixture consisting of 0.1 µmol of TCB, 0.5 µmol of NADPH, and 20 µL of liver microsomes was incubated for 10 min. The extract of the mixture with ethyl acetate was analyzed by HPLC, and the eluate was collected at 5-min intervals for the YES assay.

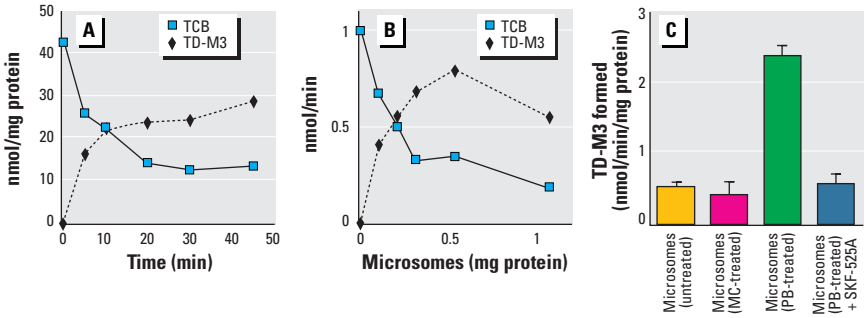

Figure 5. Oxidase activities of rat liver microsomes toward TCB. (*A*) Time course of the oxidase activity. (*B*) Liver microsome dependency of the reaction. (*C*) Oxidase activity of liver microsomes from untreated, phenobarbital (PB)-treated, and 3-methylcholanthrene (MC)–treated rats. Each value represents the mean ± SD of four rats. A mixture containing 0.1 µmol of TCB, 0.5 µmol of NADPH, and 20 µL of liver microsomes, except in (*B*), for which 0.1 M phosphate buffer (pH 7.4) was used, was incubated at 37°C for 20 min except in (*A*). Inhibitors were added at the concentration of 10^{-4} M. The *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane formed was determined using HPLC as described in "Materials and Methods."

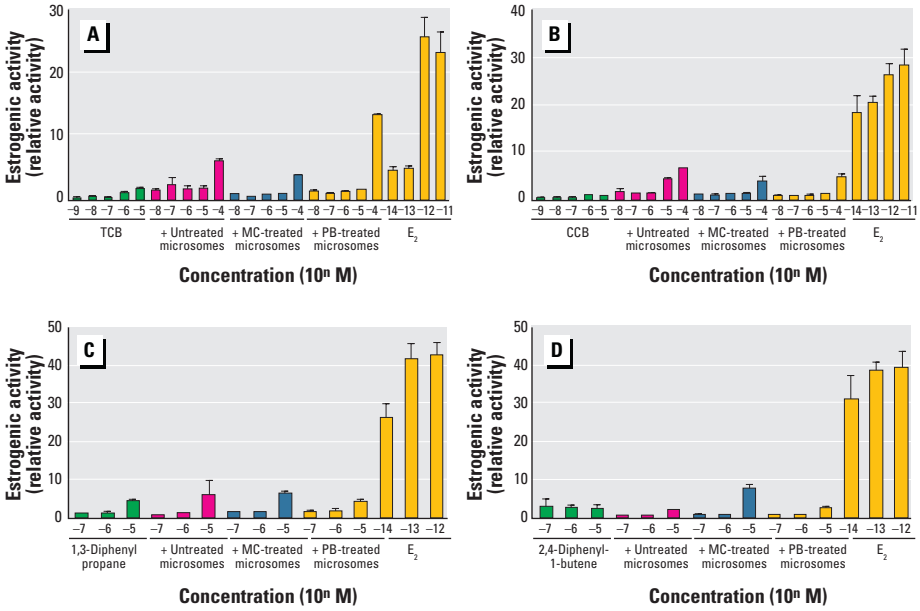

Figure 6. Estrogenic activity of styrene oligomers with or without a rat liver microsomal enzyme system using ERE–luciferase reporter assay in MCF-7 cells. Abbreviations: PB, phenobarbital; MC, 3-methylcholanthrene. (*A*) Estrogenic activity of TCB. (*B*) Activity of CCB. (*C*) Activity of 1,3-diphenylpropane. (*D*) Activity of 2,4-diphenyl-1-butene. Each bar represents the mean ± SD of four experiments. Estrogenic activity was expressed as a relative activity with respect to the control using MCF-7 cells.

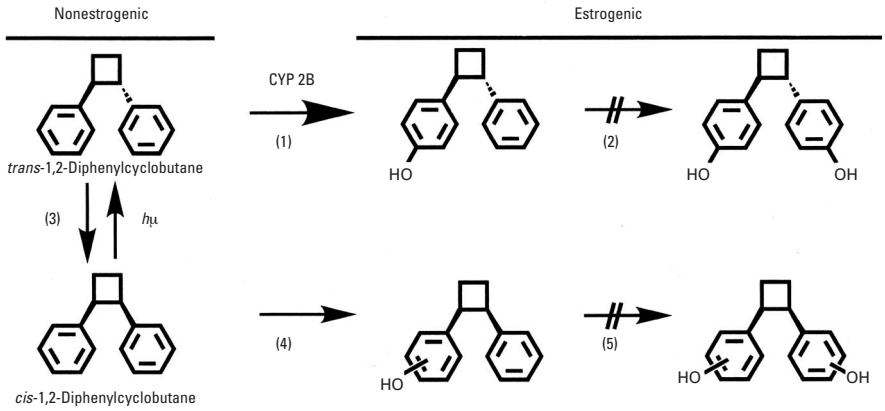

Figure 7. Proposed metabolic pathways for the activation of TCB and CCB in rats. CYP, cytochrome P450.

converted to hydroxylated derivatives by rat liver microsomes (pathways 1 and 2). In the microsomal system used in this study, the estrogenic activity of TCB is thought to be mainly exhibited by *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane, because pathway 2 does not proceed effectively in this system. The further oxidized metabolite of *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane, *trans*-1,2-bis-(4-hydroxyphenyl)cyclobutane, could not be detected. In contrast, CCB is thought to be metabolized to the corresponding hydroxylated metabolites with liver microsomes. However, the activity of CCB after metabolic activation was lower than that of the *trans*-isomer. *cis*-1-(4-Hydroxyphenyl)-2-phenylcyclobutane may exhibit lower estrogenic activity than the *trans*-isomer. The activity of CCB after metabolic activation may be due to *trans*-1-(4-hydroxyphenyl)-2-phenylcyclobutane formed after *cis–trans* isomerization (i.e., pathway 3). In our preliminary study, when CCB was incubated with liver microsomes without NADPH, a peak corresponding to TCB was detected by HPLC. The 2- or 3-hydroxylated metabolite of TCB may also be formed. However, relatively high activity was exhibited only by TD-M3 among the metabolites generated in the present oxidase system, as shown in Figure 4.

There are various other examples of metabolic activation to an estrogen, besides that of *trans*-stilbene. *p,p′*-DDT is metabolized to *p,p′*-DDD and *p,p′*-DDE (*p,p′*-dichlorodiphenyldichloroethylene) by reductive dechlorination and dehydrochlorination, respectively (Esaac and Matsumura 1980; Kitamura et al. 2002). *p,p′*-DDD shows estrogenic activity and *p,p′*-DDE shows antiandrogenic activity (Kelce et al. 1995; Chen et al. 1997). Further, methoxychlor is a proestrogen that requires demethylation by liver microsomal mixed function oxidase in animals before eliciting estrogenic activity (Kupfer and Bulger 1987; Stresser and Kupfer 1998). It is known that PCBs are converted to hydroxylated metabolites in animals (Koga et al. 1990). Some hydroxylated PCBs show estrogenic activity (Korach et al. 1988; Connor et al. 1997; Garner et al. 1999). *p*-Hydroxybenzophenone, which is formed from benzophenone, an antifungal agent, in rat hepatocytes, is also estrogenic (Nakagawa and Tayama 2001; Nakagawa and Suzuki 2002). Hydroxylated metabolites of benzo[*a*]pyrene also exhibit estrogenic activity (Charles et al. 2000; Fertuck et al. 2001). Elsby et al. (2000, 2001) predicted estrogenicity by a two-stage approach coupling human liver microsomes and a yeast estrogenicity assay. Methoxychlor, methoxybisphenol A, and 3,17β bisdesoxyestradiol were positive, but 6-hydroxytetralin was negative, in this screening system. Much further work is needed to identify potentially hazardous proestrogens in our environment.

References

Andersen HR, Andersson A-M, Arnold SF, Autrup H, Barfoed M, Beresford NA, et al. 1999. Comparison of short-term estrogenicity test for identification of hormone-disrupting chemicals. Environ Health Perspect 107(suppl 1):89–108.

Anstead GM, Carlson KE, Katzenellenbogen JA. 1997. The estradiol pharmacophore: ligand structure-estrogen receptor binding affinity relationships and a model for the receptor binding site. Steroids 62:268–303.

Bachmann S, Hellwig J, Jäckh R, Christin MS. 1998. Uterotrophic assay of two concentrations of migrates from each of 23 polystyrenes administered orally (by gavage) to immature female Wistar rats. Drug Chem Toxicol 21:1–30.

Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, et al. 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. Toxicol Sci 54:138–153.

Branham WS, Dial SL, Moland CL, Bruce LM, Hass BS, Blair RM, et al. 2002. Phytoestrogens and mycoestrogens bind to the rat uterine estrogen receptor. J Nutr 132:658–664.

Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N. 1995. Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect 103:608–612.

Brown WG. 1968. Cyclodimerization of styrene. J Am Chem Soc 90:1916–1917.

Charles GD, Bartels MJ, Zacharewski TR, Gollapudi BB, Freshour NL, Carney EW. 2000. Activity of benzo[*a*]pyrene and its hydroxylated metabolites in an estrogen receptor-α reporter gene assay. Toxicol Sci 55:320–326.

Chen CW, Hurd C, Vorojeikina DP, Arnold SF, Notides AC. 1997. Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. Biochem Pharmacol 53:1161–1172.

Colborn T. 1995. Environmental estrogens: Health implications for humans and wildlife. Environ Health Perspect 103:135–136.

Connor K, Ramamoorthy M, Moore M, Mustain M, Chen I, Safe S, et al. 1997. Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure-activity relationships. Toxicol Appl Pharmacol 145:111–123.

Elsby R, Ashby J, Sumpter JP, Brooks N, Pennie WD, Maggs JL, et al. 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 JL, Ashby J, Paton D, Sumpter JP, Park BK. 2001. Assessment of the effects of metabolism on the estrogenic activity of xenoestrogens: a two-stage approach coupling human liver microsomes and a yeast estrogenicity assay. J Pharmacol Exp Ther 296:329–337.

Esaac EG, Matsumura F. 1980. Metabolism of insecticides by reductive systems. Pharmacol Ther 9:1–26.

Fail PA, Hines JW, Zacharewski T, Wu ZF, Borodinsky L. 1998. Assessment of polystyrene extract for estrogenic activity in the rat uterotrophic model and an *in vitro* recombinant receptor reporter gene assay. Drug Chem Toxicol 21:101–121.

Fang H, Tong W, Perkins R, Soto AM, Prechtl NV, Sheehan DM. 2000. Quantitative comparisons of *in vitro* assay for estrogenic activities. Environ Health Perspect 1081:723–729.

Fertuck KC, Matthews JB, Zacharewski TR. 2001. Hydroxylated benzo[*a*]pyrene metabolites are responsible for *in vitro* estrogen receptor-mediated gene expression induced by benzo[*a*]pyrene, but do not elicit uterotrophic effects *in vivo*. Toxicol Sci 59:231–240.

Garner CE, Jefferson WN, Burka LT, Matthews HB, Newbold RR. 1999. *In vitro* estrogenicity of the catechol metabolites of selected polychlorinated biphenyls. Toxicol Appl Pharmacol 154:188–197.

Hong H, Tong W, Fang H, Shi L, Xie Q, Wu J, et al. 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.

Kawamura Y, Kawarura M, Takeda Y, Yamada T. 1998a. Determination of styrene dimers and trimers in food contact polystyrene. J Food Hyg Jpn 39:199–205.

Kawamura Y, Nishi K, Maeda T, Yamada T. 1998b. Migration of styrene dimers and trimers from polystyrene containers into foods. J Food Hyg Jpn 39:390–398.

Kawamura Y, Sugimoto N, Takeda Y, Yamada T. 1998c. Identification of unknown substances in food contact polystyrene. J Food Hyg Jpn 39:110–119.

Kelce WR, Stone CR, Laws SC, Gray LE, Kemppainen JA, Wilson EM. 1995. Persistent DDT metabolite *p,p′*-DDE is a potent androgen receptor antagonist. Nature 375:581–585.

Kitamura S, Shimizu Y, Shiraga Y, Yoshida M, Sugihara K, Ohta S. 2002. Reductive metabolism of *p,p′*-DDT and *o,p′*-DDT by rat liver cytochrome. Drug Metab Dispos 30:113–118.

Koga N, Beppu M, Yoshimura H. 1990. Metabolism in vivo of 3,4,5,3′,4′-pentachlorobiphenyl and toxicological assesment of the metabolism in rats. J Pharmacobiol Dyn 13:497–502.

Korach KS, Sarver P, Chae K, McLachlan JA, McKinney JD. 1988. Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls: conformationally restricted structural probes. Mol Pharmacol 33:120–126.

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

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

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

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

Nishihara T, Nishikawa J, Kanayama T, Dakeyama F, Saito K, Imagawa M, et al. 2000. Estrogenic activities of 517 chemicals by yeast two-hybrid assay. J Health Sci 46:282–298.

Nobuhara Y, Hirano S, Azuma Y, Date K, Ohno K, Tanaka K, et al. 1999. Biological evaluation of styrene oligomers for endocrine-disrupting effects. J Food Hyg Soc Jpn 40:36–45.

Ohyama K, Nagai F, Tsuchiya Y. 2001. Certain styrene oligomers have proliferative activity on MCF-7 human breast tumor cells and binding affinity for human estrogen receptor. Environ Health Perspect 109:699–703.

Prinsen MK, Gouko N. 2001. Determination of the oestrogenic (uterotrophic) activity of extracts of general purpose polystyrene (GPPS) using immature female rats. J Appl Toxicol 21:235–239.

Routledge EJ, Sumpter JP. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ Toxicol Chem 15:241–248.

Routledge EJ, Sumpter JP. 1997. Structural features of alkylphenolic chemicals associated with estrogenic activity. J Biol Chem 272:3280–3288.

Sakamoto H, Matsuzawa A, Itoh R, Tohyama Y. 2000. Quantitative analysis of styrene dimer and trimers migrated from disposable lunch boxes. J Food Hyg Jpn 41:200–205.

Sanoh S, Kitamura S, Sugihara K, Ohta S. 2002. Cytochrome P450 1A1/2 mediated metabolism of *trans*-stilbene in rats and humans. Biol Pharm Bull 25:397–400.

Shi LM, Fang H, Tong W, Wu J, Perkins R, Blair RM, et al. 2001. QSAR models using a large diverse set of estrogens. J Chem Inf Comput Sci 41:186–195.

Stresser DM, 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, et al. 2000. Metabolic activation of the proestrogens *trans*-stilbene and *trans*-stilbene oxide by rat liver microsomes. Toxicol Appl Pharmacol 167:46–54.

Williard PG, Fryhle CB. 1980. Boron trihalide-methyl sulfide complexes as convenient reagents for dealkylation of aryl ethers. Tetrahedron Lett 21:3731–3734.

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