products of aqueous chlorination of bisphenol A and their estrogenic activity

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To assess the estrogenic activity potentially stemming from bisphenol A (BPA) in drinking water, APCI/LC/MS and NMR were used to identify the products of its aqueous chlorination under the following conditions: 500 μg/L bisphenol A and 1.46 mg/L sodium hypochlorite (pH 7.5) at 25 °C. The 13 products (4-chloro-BPA; 2,6'-dichloro-BPA; 2,6-dichloro-BPA; 2,2',6'-trichloro-BPA; 2,2',6,6'-tetrachloro-BPA; trichlorophenol; 4-isopropyl-2'-hydroxyphenol; and six kinds of polychlorinated phenoxophenols (PCPPs)) were found in the chlorinated BPA solution. Three main pathways are proposed: (1) chlorine-substitution reactions on the aromatic ring, followed by dehydration to form the chlorine-substituted BPA, (2) chlorine substitution reactions following by cleavage of the α-C on the isopropyl moiety with positive partial charge and β-C on the benzene moiety with a negative partial charge to form trichlorophenol and 4-isopropyl-2'-hydroxyphenol, and (3) the formation of PCPPs. Especially for pathway 2, the reaction mechanism was clarified based on semiempirical quantum mechanical calculations. The reaction proceeded by attack of the OH and Cl (from HOCl) on the α-C on the isopropyl moiety with a positive partial charge and on the β-C with a negative partial charge on the benzene moiety. The activation energies for the HOCl/4-chloro-BPA and 2,2',6,6'-tetrachloro-BPA reactions were 0.14 and 0.15 kcal/mol, respectively. Finally, the estrogenic activity of the aqueous chlorinated BPA solution was assessed by an estrogen receptor binding assay and a yeast two-hybrid system. It was found that the binding affinity of the chlorinated aqueous BPA at 60 min was 24 times that before chlorination. The transcriptional activation-induced byproducts were detected by a yeast two-hybrid system based on the ligand-dependent interaction of two proteins, a human ER and a coactivator, suggesting that the chlorinated BPA solution elicits an ability to mimic the effect of the estrogen hormone.

Introduction

A number of papers have highlighted the potentially detrimental reproductive effects on wildlife and humans of some anthropogenic compounds. There is increasing evidence that these compounds can alter endocrine function and may disrupt growth, development, and reproduction by interfering with the production, release, transport, metabolism, binding, action for the elimination of endo- and exogenesis, and regulation of developmental processes (1, 2). The wide variety of pollutants which have been reported to disrupt normal pathways in animals includes pesticides, polycyclic aromatic hydrocarbons, phthalate plasticizers, certain polychlorinated biphenyls, dioxins, furans, alkylphenols, and synthetic steroids (3).

Of particular concern is bisphenol A (BPA). BPA was reported to cause reproductive toxicity and to affect cellular development in rats and mice (4, 5) and to be not merely an estrogen mimic in several in vivo and in vitro tests (6–8) but to be likely to interact with ERα in a unique manner to produce its own spectrum of activity (9). This concern arises in part from the fact that BPA is a monomer used in the manufacture of epoxy resins, polycarbonate, and polyester-styrene resins. Such resins are widely used in canned food, beverage packing, and dental resins leading to potential human exposure to BPA (10, 11). Specific migration of BPA has been assessed both in foods in contact with polymeric materials and in substitutive simulants. For canned vegetables, the recommended simulants are distilled water or 3% acetic acid in water, depending on the pH of the preserved vegetables (12). In view of the low estrogenic potency of BPA released from resins, it seems logical to think that the human environmental exposure to these compounds is negligible. However, there is concern that BPA might reach biologically significant levels in humans exposed to low environmental levels as a consequence of slow clearance.

Another human exposure routine to BPA is from drinking water. BPA in drinking water was reported to stem from epoxy and polyester-styrene resins used in lacquer coatings of concrete tanks and the lining of steel pipes in water supply systems. The BPA released from the lacquer coatings of concrete tanks was found to be 5.6 μg/mL, and that from the lining of steel pipe was 2.7 μg/mL at 23°C and 16 h of contact time (13). While mineral water was used as a substitutive simulant of drinking water, the in the aforesaid experiment, the effects of residual hypochlorite in drinking water on releasing BPA from resins were not under consideration. It is well-known that phenolic compounds are reactive with hypochlorite (14). Thus, knowledge of the fate of BPA after chlorination is needed to allow for the effective assessment of the endocrine disruption potential of residual BPA in drinking water. On the other hand, BPA is prevalent in surface water. According to the survey in Japan (1998), BPA was detected at 88 sites among 130 sites surveyed, and the maximum concentration was reported to be 0.94 μg/L (15).

In this study, products of BPA chlorination were identified, and a reasonable pathway was proposed based on APCI/LC/MS, NMR methods, and quantum chemical modeling analysis. Finally, the estrogenic activity of aqueous chlorinated BPA products was investigated using a human estrogen receptor binding assay and a yeast two-hybrid system.

Materials and Methods

Computational Chemistry. MOPAC (version 6) was used as adapted by CAChe Scientific Inc. (Oxford, U.K.) and carried out on an IBM 600E computer. The AM1 parameter (14) served to optimize stable and transition-state (TS) structures. The program was used to obtain optimum and transition-state geometries, atom partial charges, vibrational spectra, and the intrinsic reaction coordinates (IRCs).

Chlorination Procedures. The experiments were carried out in a glass reactor which was placed in a water bath to...
maintain the reaction temperature at 25 °C. Synthetic raw water was prepared by dissolving 3.5 mg of a standard BPA (Kanto Chemical Co., Tokyo; purity 99%) into 7 L of Mill-Q pure water (Mill-Q SP VOC, Millipore Co., Bedford, MA), adjusted to pH 7.5 by phosphoric acid (Kanto Chemical Co., Tokyo). One liter was removed for the determination of the estrogen receptor binding affinity before sodium hypochlorite (1.46 mg/L) (ca. 5% available Cl; Kanto Chemical Co., Tokyo) was added to the remaining solution. Samples (1 L) were taken out at 10, 30, 60, 180, 360, and 1440 min. After decomposition of the residual sodium hypochlorite by the addition of Na2S2O3 (Wako, Osaka), the BPA and its byproducts were extracted by solid-phase extraction (SPE). The cartridges filled with polystyrene/divinylbenzene sorbent (500 mg) purchased from Yokokawa Analytical Systems Inc. (SPE-GLF, Tokyo, Japan). The cartridges were washed with 5 mL of acetonitrile followed by 5 mL of distilled water before use. A total of 500 mL samples with the pH adjusted to 3.5 by 1

FIGURE 1. APCI/LC/MS chromatogram of aqueous chlorinated bisphenol A solution (chlorination time, 10 min; for analytical conditions, see Materials and Methods section): mono-CBPA, monochlorobisphenol A; di-CBPA, dichlorobisphenol A; tri-CBPA, trichlorobisphenol A; tetra-CBPA, tetrachlorobisphenol A; TCP, trichlorophenol.

TABLE 1. Analytical Results by NMR

<table>
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<tr>
<th>H chemical shifts</th>
<th>Multiplicity (^a) (coupling constants)</th>
<th>H chemical shifts</th>
<th>Multiplicity (^b) (coupling constants)</th>
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<tbody>
<tr>
<td>1</td>
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<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
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<tr>
<td>3</td>
<td>7.181</td>
<td>3</td>
<td>7.116</td>
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<tr>
<td>4</td>
<td>dd(2.4)</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>dd(2.4, 8.5)</td>
<td>1(^\prime)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>d(8.5)</td>
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<td>3(^\prime)</td>
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<td>3(^\prime)</td>
<td>m(2.2, 8.9)</td>
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<td></td>
<td>CH(_3)</td>
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<td>4-OH</td>
<td>ca. 5.78</td>
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<tr>
<td></td>
<td></td>
<td>4(^\prime)-OH</td>
<td>ca. 5.94</td>
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</table>

\(^a\) s, singlet; d, doublet; m, multiplet; dd, double doublet. \(^b\) \(^1\)H–\(^1\)H coupling constants (Hz).
M HNO₃ were forced to pass through the cartridges at a flow rate of 10 mL/min by using a vacuum manifold (VAC ELUT SPS24, Varian, Palo Alto, CA). The most water was expelled by vacuum for 1 min after the sample was passed through the cartridge. The residual water was removed by passing a gentle nitrogen stream through the cartridges for about 10 min. The BPA and its byproducts were eluted by passing through the trap with 3 mL of methylene chloride. The effluents were dried under a gentle nitrogen stream, and the final volume was made to 0.1 mL (DMSO). Samples of 2 and 2.5 μL were used to detect estrogenic activity using an ER binding affinity assay and a yeast two-hybrid system and 2 μL to analyze the products by APCI/LC/MS (model M-1200H, Hitachi, Japan).

The concentrations of tetrachloro-BPA (Kanto Chemical Kogyo Co., purity 96%), 2,4,6-trichlorophenol (Tokyo Kasei Kogyo Co., purity 96%), and BPA at different steps of chlorination were determined by APCI/LC/MS.

**Characterization of Products.** The products in an aqueous chlorinated solution of BPA were characterized by APCI/LC/MS. Liquid chromatography was carried out on an HPLC apparatus equipped with a model L-6200 intelligent pump (Hitachi, Tokyo, Japan), a model L-4000 UV detector (Hitachi) with a model L-4000 high-pressure resistant cell, a model L-5025 column oven (Hitachi), an AS-4000 intelligent autosampler with a 200 μL loop, and a Rheodyne (Cotati, CA) model 7125 injector with a 50 μL loop at a flow rate of 1 mL/min. The analytes were chromatographed on a Capacell Pak C18 UG120S3 (Shiseido, Japan) silica packed LC column (4.6 mm i.d., 15 cm in length, 3 μm in particle diameter) at 25 °C. The injection volume of the sample was 2 μL. LC separation of the components of the aqueous chlorinated

**FIGURE 2.** (a) Mass spectra of peaks A–D. (b) Mass spectra of trichlorophenol.
BPA solution was performed by adding 0.1% acetic acid to the mobile phase to suppress the dissociation of products. The initial mobile phase composition was acetonitrile/water (20/80, v/v) which was increased linearly to 50/50 in 17 min, 55/45 after 55 min, and to 60/40 after 60 min.

Mass spectrometry was carried out in negative-ion mode on a model M-1200H LC/MS (Hitachi, Japan) quadrupole mass spectrometer equipped with an APCI interface. Mass spectra collected in full-scan mode were obtained by scanning over a range of 150–550 amu in 20.033 amu/s at a resolution of 48. The nebulizer and desolvator temperatures were set at 185 and 400 °C, respectively. The drift voltage was controlled at 80 V.

The 1H NMR, COSY, and NOESY spectra were measured on a VARIAN UNITY plus 500 model (1H, 500.2 MHz) instrument. Deuteriochloroform was used as the solvent. The chemical shifts (δ values) are given in ppm downfield from tetramethylsilane.

**Estrogenicity of Products.** A human receptor binding competition and yeast two-hybrid assays were applied to evaluate the estrogenicity of aqueous chlorinated BPA solution. For testing the binding affinity of compounds to ER, the assay was performed as previously described (16).

**Results and Discussion**

**Characterization of Products.** Figure 1 shows the LC/MS chromatogram of chlorinated BPA after 10 min of chlorination. BPA rapidly reacted with sodium hypochlorite, and several chlorinated products were formed. The molecular ion typically appears in an APCI/LC/MS mass spectrum as described in our previous paper (18). The molecular ions at m/z 261 (one chlorine atom), 295 (two chlorine atoms), 329 (three chlorine atoms), and 365 (four chlorine atoms) were observed on as four signals on the chromatogram at 21.62, 24.56, 28.47, and 34.53 min, respectively. Although no fragment ions were found, the products corresponding with these peaks were postulated to be monochloro-, dichloro-, trichloro-, and tetrachloro-BPA, on the basis of the molecular ion and the number of chlorine atoms. While the structures for monochloro, trichloro-, and tetrachloro-BPA were specified to be 2-chloro-BPA, 2,2'-6-trichloro-BPA, and 2,2',6,6'-tetrachloro-BPA from a chlorine-substitution mechanism, the structure of dichloro-BPA was speculated to be 2,6'-dichloro-BPA or 2,6-dichloro-BPA. An attempt to identify the structures of dichloro-BPAs using NMR combined with fractionation by HPLC was successful (Table 1) based on 1H NMR, COSY, and NOESY spectra. The ratio of 2,6'-dichloro-BPA/2,6-dichloro-BPA was determined to be 1/0.25.

In addition to these four chlorine-substituted products of BPA, four peaks (A–D) were observed on the chromatogram. Figure 2a shows the mass spectrum of the product corresponding to peak A at a retention time of 12.7 min. On the basis of the molecular ion (M–H) at m/z 219 (two Cl atoms) and two fragment ions of m/z 201 (M–OH–H)– (two Cl atoms) and m/z 189 (M–2CH3–H)–, the possible structure was deduced to result from the breakdown between R–C and â=C on the benzene ring of the aforesaid four chlorinated BPAs, as shown in Figure 2a (peak B). The trichlorophenol observed at retention time 22 min (Figure 1) supported such a breakdown of the BPA molecule. In addition, the mass spectrum of the compound corresponding to peak B at 29.49 min provided a molecular ion at m/z 381 with four Cl atoms (a base ion) and a fragment ion (M–OH–H)– at m/z 363 as shown in Figure 2a (peak B). On the basis of the aforesaid structural information, a possible product, a polychlorinated phenoxyphenol (PCPP), was postulated to be formed by the reaction between product A and another product, trichlorophenol.

While the occurrence of PCPPs has been reported during the
chlorination of phenol or alkylphenol in water, the abundance is lower than that in this experiment (19, 20). The mass spectra of peaks C at 50.1 min and D at 59.07 min show that the two products contain four chlorine atoms with the same molecular ion (M – H)− of m/z 525 (six chlorine atoms), and fragment ions at m/z 489 (M – Cl – H)− and 363 (M – C6H4Cl2OH – H)− are indicative of the formation of PCPP between tetrachloro-BPA and trichlorophenol, with the structure shown in Figure 4. The molecular ion (M – H)− at m/z 491 and fragment ions at m/z 457 and 329 in the mass spectrum of peak E at 51.2 min indicate the formation of PCPP by the reaction between trichloro-BPA and trichlorophenol. Finally, the product corresponding with the peak F at 60.38 min, whose molecular ion (M – H)− was m/z 357 with five chlorine atoms, was speculated to be a PCPP, which results from the reaction between two trichlorophenol molecules.

**Chlorination Pathways of BPA.** Figure 5 shows the variation in levels of four chlorine substituted BPAs, trichlorophenol, and BPA with reaction time. It was found that BPA rapidly reacted with sodium hypochlorite and that ca. 80% of BPA had disappeared at the 10 min reaction time. While other products predominate at shorter contact times, trichlorophenol was the primary measured degradation product resulting from the chlorination of BPA for extended contact times of 360 min. It should be noted that the amount of trichlorophenol increased with the reaction time, even after no BPA could be detected. This suggests that trichlorophenol was formed by the reaction between chlorine-substituted BPAs and sodium hypochlorite. On the basis of the aforesaid results, pathways for the aqueous chlorination of BPA are proposed, as shown in Figure 6, which includes (1) chlorine substitution reactions followed by dehydration, (2) chlorine substitution reactions followed by cleavage of the R-C on isopropyl moiety and β-C on benzene moiety, and (3) the formation of PCPPs.

It should be noted that the three main pathways proposed previously were obtained under the experimental chlorination condition. In view of the fact that the formation of the byproducts will be affected by the factors such as pH, temperature, chlorine dose, reaction time, and TOC and so forth, the kinetics for aqueous chlorination of BPA is necessary to be further investigated.
Chlorination Mechanism of BPA. It is reported that the mechanism of the reaction between phenolic compounds and sodium hypochlorite proceeds through electrophilic attack of HOCl on the phenoxide ion and the formation of chlorinated derivatives. According to this mechanism, the presence of a negative charge on the nucleophilic substrate will facilitate the reaction. Thus, the chlorine substitution reaction followed by dehydration will primarily occur at C4, with a partial charge of $-0.194$, and 2-chloro-BPA will be formed (Figure 7). The 2-chloro-BPA subsequently reacts with sodium hypochlorite by chlorine substitution at C19, with a partial charge of $-0.193$, and results in the formation of 2,6-dichloro-BPA. Such a reaction will also occur at C2, with a partial charge of $-0.134$, and another dichloro-BPA (2,6-dichloro-BPA) will be formed, although the abundance will be lower than that of 2,6-dichloro-BPA due to the relatively low negative partial charge at C2. This is identical to the above NMR experimental results. Thus, the final chlorine derivative of BPA was tetrachloro-BPA, as shown in Figure 5.

Besides the aforesaid reactions, the breakdown of BPA was also followed by LC/MS. To clarify such a reaction mechanism, the transition state should be identified for the first step. A geometry-optimization search gave the structure of the transition state (TS) during HOCl/tetrachloro-BPA interaction (Figure 8) where the Cl atom with a positive partial charge attacked the C atom on benzene with a negative partial charge of $-0.094$ and the OH attacked the C atom on isopropyl moiety with positive partial charge of 0.067. Frequency analysis indicated that this structure had only one vibrational mode corresponding to an imaginary frequency, which is the direction of the vibrational mode corresponding to an imaginary frequency.
shown by the arrows as shown in Figure 8. The α-C on isopropyl moiety and β-C on benzene moiety bond length expanded to 9.83 Å, which means that the C–C bond between α-C on isopropyl moiety and β-C on benzene moiety is breaking in accordance with the reaction and that trichlorophenol and product A will be formed. Intrinsic reaction coordinate (IRC) calculations were performed for both the forward and reverse directions following the vibrational mode, and the activation energy was found to be 0.15 kcal/mol, which supports the reaction in which BPA breaks down to form trichlorophenol and 4-propyl-2'-hydroxylphenol. A similar calculation was performed for the HOCl/BPA reaction, with the activation energy being 0.14 kcal/mol.

Finally, no satisfactory results could be obtained from calculations to clarify the PCPP-forming mechanism, although the formation of PCPPs during chlorination of BPA was confirmed using LC/MS.

**Estrogenic Activity of Aqueous Chlorinated BPA Solution.** It was found that many byproducts were formed in the aqueous chlorination of BPA. The reports on their estrogenic activities, however, have been very limited, except for trichlorophenol which was reported to elicit very weak estrogenic activity (ca. 1/1000 of BPA activity) on the basis of the yeast bioassay (21). To evaluate the estrogenic activity potentially stemming from BPA in drinking water, it is necessary to investigate the estrogenic activity for their products. Figure 9 shows the variation of estrogen receptor binding affinity with chlorination time. It was found that the binding affinity increased with increasing chlorination time. Thus, the IC50 variation was: 116.10 (0 min), 29.92 (10 min), 10.94 (30 min), and 4.74 (60 min). The affinity at 60 min is 24 times that before chlorination, suggesting that the chlorinated BPA solution would elicit an ER-mediated response.

A yeast two-hybrid system based on the ligand-dependent interaction of two proteins, a human estrogen receptor and a coactivator, was also used to assess the estrogenic potency of the aforesaid chlorinated BPA solution by determining the ligand-induced transcriptional activation. The chlorinated BPA solutions at 10, 30, and 60 min induced β-galactosidase activity, as shown in Figure 10a. While the maximal β-galactosidase activities induced by the chlorinated solution at 30 and 60 min are lower than those at 10 min and before chlorination, their effective relative concentration showing half-maximal transcriptional response (EC50) which represents the concentration required to achieve half-maximal β-galactosidase activity, occurred at significantly lower times than that before chlorination.

Finally, the concentrations of tetrachloro-BPA, trichlorophenol, and BPA of which standards could be commercially obtained were determined (Table 2), and an attempt to interpret the activities of the chlorination products in BPA solutions is shown in Figure 10b. Because the concentration of trichlorophenol formed during chlorination is much lower than that showing transcriptional responses (> 10⁶ nM), Figure 10b only shows the concentration—response curves of single tetrachloro-BPA and single BPA together with those of chlorinated BPA solutions at 10, 30, and 60 min. It was found that tetrachloro-BPA elicited much weaker β-galactosidase activity in the concentration range from 10⁴ to 10⁶ nM. And, from the concentration—response curve (for BPA) of the chlorinated solution at 10 min, the β-galactosidase activity induced by the chlorinated solution is higher than the arithmetic sum of the single BPA and tetrachloro-BPA. The residual concentration of BPA was far below the range showing transcriptional responses (10⁴ to 10⁶ nM) after chlorination for 30 min. The concentration—response curves (for tetrachloro-BPA) at 30 and 60 min, however, indicate that the galactosidase activities induced by the two chlorinated solutions were much higher than that of the single tetrachloro-BPA. Thus, we can speculated that some other byproducts in chlorinated BPA solution elicited the ability to induce β-galactosidase activity through their additive and synergistic or antagonistic effects (22). To obtain more information on the endocrine effects of these byproducts, further fractionation of each byproduct will be performed.

**TABLE 2. Concentration of Trichlorophenol, Tetrachloro-BPA, and BPA in Chlorinated Solution**

<table>
<thead>
<tr>
<th>chlorinated solution</th>
<th>concentration (nM)</th>
<th>trichlorophenol</th>
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<tr>
<td>10 min</td>
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<tr>
<td>60 min</td>
<td>4564</td>
<td>39558</td>
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Acknowledgments

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Literature Cited


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