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Identification of Triclosan Intermediates Produced by Oxidative Degradation Using TiO_2 in Pure Water and Their Endocrine Disrupting Activities

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Abstract The photodegradation pathways of 2-(2,4-dichlorophenoxy)-5-chlorophenol (triclosan) in water were studied. The main purposes were to identify structures of intermediates derived by radical reaction using TiO_2 advanced oxidation processes and to evaluate the endocrine disrupting activities in treated triclosan during oxidative reactions. Intermediates such as dichlorophenols, 2,8-dibenzo-*p*-dioxin, tetrachlorinated diphenyl ether (tetraclosan) and hydroxylated triclosan were produced by photoreaction. The estrogen, thyroid hormone and retinoid X receptor activities of the treated triclosan were measured with the yeast two-hybrid assay. It was found that tetraclosan and 2,4-dichlorophenol have stronger thyroid hormone activities than triclosan in the presence of S9.

Keywords Triclosan · Photodegradation · Frontier electron density · Thyroid hormone activity

Triclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy)phenol), which is found in pharmaceuticals and personal care products (PPCPs), has been used as a bactericidal agent. TCS has been interfused to numerous commercial products including antimicrobial soap, medical skin cream, dental powder, deodorant and textiles (Canosa et al. 2005; Aranami and Readman 2007) resulting in an increase in their use for personal hygiene. However, current studies show that ordinary municipal wastewater treatment plants have insufficient capacity to remove TCS completely from sewage water, and TCS has been detected in river water (Fair et al. 2009). A concern related to public health and the ecosystem is the aftereffect of TCS in the water environment (Adolfsson-Erici et al. 2002). Ishibashi et al. (2004) reported that TCS has high toxicity on the early life stages of Japanese medaka, and that the metabolite of TCS may be a weak estrogenic compound with the potential to induce vitellogenin in male medaka. Also TCS potentially could transform to byproducts of dichloro-dibenzo-*p*-dioxins: DCDDs (Mezcua et al. 2004; Aranami and Readman 2007). In addition, some recent reports have shown strong evidence that secondarily produced organohalogenated phenolic compounds, such as hydroxylated polychlorinated biphenyls (OH-PCBs) and hydroxylated polybrominated diphenyl ethers (OH-PBDEs) disrupt the thyroid hormone (TH) homeostasis because of their structural similarity to thyroxin (Arulmozhiraja et al. 2005; Li et al. 2010). Also it has been proven that these compounds exhibit hormone-like activities to estrogen receptors (Arulmozhiraja et al. 2005; Nomiyama et al. 2005). The literature implies that TCS degraded products can exhibit stronger adverse effects than TCS. Although some reports have referred to estrogenic and TH activities of TCS (Crofton et al. 2007; Foran et al. 2000; Ishibashi et al. 2004), adverse effects of TCS in oxidative degradation process are still not understood.

The aim in the present study was to contribute to the investigation on comprehensive risk assessment of TCS, including its potential intermediates. Advanced oxidation processes (AOP) by the photoreactor that equip TiO_2 were employed to decompose TCS rapidly. Identification of

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degraded substances was carried out by GC/MS after derivatization of polar compounds. Moreover, we investigated the human estrogen receptor (hERα), TH receptor (hTRα and β), retinoid X receptor (hRXRα and β) and Japanese medaka fish (*Oryzias latipes*) ER (medERα) agonist activities by degradation of TCS. These agonist activities were evaluated for cases of both −S9 and +S9 by using a constructed yeast two-hybrid assay system (Arulmozhiraja et al. 2005). In particular, this study focused on the TH activity of intermediates of the TCS. The intermediates of TCS oxidation will be expected with a range of features that might provide structural information on affinity for TH receptor and give some insight into the toxicological implications of TCS oxidation.

Materials and Methods

A 500 mL of TCS solution (1 mg L^{-1}) was introduced into the photoreactor and recirculated (100 mL min^{-1}) while being irradiated with UV light (Nomiyama et al. 2005). In this study, four different experiments were carried out separately. Throughout all experiments, water solutions were collected every 60 min (0, 60, 120, 180, 240 min). The sample solutions (5 mL) were collected in order to measure the photodegradation rate of TCS. Then, the pH of each portion of the sample solution was adjusted between 4 and 5 by addition of hydrochloric acid. The TCS was extracted two times with dichloromethane (1 mL) by shaking vigorously. The extracts were dehydrated with anhydrous sodium sulfate and were evaporated in the presence of a gentle stream of nitrogen. Subsequently, [*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide] (BSTFA) solution (0.5 mL) was added to the residue and heated at 70°C for 1 h. Finally, deuterium-labeled phenanthrene-d_{10} was spiked to the derivatized sample as an internal standard (IS), and it was analyzed with a gas chromatograph HP 6890 (Agilent Technologies, CA) equipped with a mass spectrometer JMS-700 (JEOL, Tokyo, Japan) (GC/MS).

Portions of sample solutions (400 mL) were taken periodically to determine any variation of the intermediates of TCS concentrations according to irradiation time. The sample solutions were evaporated to dryness with a rotary evaporator. Immediately, acetone (1 mL) was added to the residues and they were transferred to a vial. The solvents were evaporated to dryness in the presence of a gentle stream of nitrogen and were derivatized by BSTFA. The IS was added to the samples and analyzed with GC/MS to identify hourly photodegraded compounds of TCS.

The following section describes the analysis of dichlorophenols (DCPs) that were produced by degradation of TCS with the UV irradiation. Periodically sample solutions (400 mL) were collected from the photoreactor and acidified with hydrochloric acid. DCPs were extracted three times with dichloromethane. After a 0.1 M K_2CO_3 water solution (20 mL) was added to the dichloromethane extracts, the mixtures were shaken three times. After the water phases were combined, acetic anhydride (0.5 mL) was added to acetylate DCPs and hexane (5 mL) was added. The hexane phases including derivatized DCPs were dried in the presence of a gentle stream of nitrogen and dissolved in acetone with phenanthrene-d_{10}. The treated samples were analyzed with GC/MS.

Sample solutions (10 mL) were collected and were extracted for measurement of DCDDs. The samples were extracted with dichloromethane (1 mL). The extracts were dried in the presence of a gentle stream of nitrogen and were transferred to hexane. These were concentrated and passed through activated silica-gel column chromatography. DCDDs were eluted with 50 mL of hexane: benzene (9:1 v/v) and concentrated and $^{13}C_{12}$-labeled hexachlorinated polychlorinated biphenyl (CB157) was added as a syringe spike for GC/MS analysis.

The hERα, hTRα and β, hRXRα and β, and medERα agonist activities of identified intermediates and sample solutions from TCS treated by TiO_2 at 0, 60, 120, 180 and 240 min of irradiation time were measured by a yeast two-hybrid assay system using *Saccharomycerviseae* Y190, both with and without possible metabolic activation by rat liver S9 preparation (Kikkoman Company, Noda, Japan). These hormone-like activities were measured with chemiluminescent intensity of β-galactosidase. The activity was described as EC × 10 that was defined as the concentration of the tested solutions producing a chemiluminescent signal 10 times that of the blank control. A pretreatment method for this assay has been reported in a previous report (Nomiyama et al. 2007).

The molecular orbital package (MOPAC, version 2000) program in this study was provided by CAChe Scientific Inc. (Fujitsu, Corp., Japan). The PM5 level Hamiltonian parameter was used to obtain optimum geometries, frontier electron density and atom partial charges (Nomiyama et al. 2005). An initial position for a possible OH radical attack was estimated from calculations of frontier electron densities (FED) and partial electronic charges of all carbon atoms in the TCS structures.

Results and Discussion

Figure 1 shows the photodegradation curve of TCS at each irradiation time by using a UV lamp. Hardly TCS was decomposed by TiO_2 without UV irradiation. With the TiO_2 approximately 90% of the initial concentrations are decomposed within 120 min of UV irradiation. According to research performed under the equal conditions, the

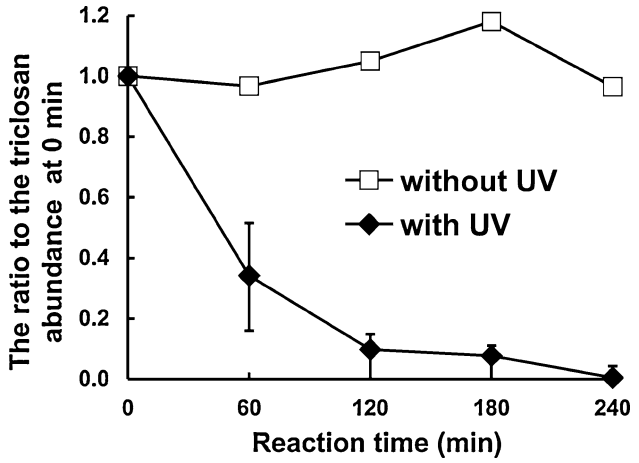

Fig. 1 Photodegradation time curve lines of triclosan using a UV lamp. Measured triclosan abundance at 0 min was considered the base line, and relative residual triclosan was shown by the ratio to the base line. Symbols represented the means plus minus standard deviation (n = 3)

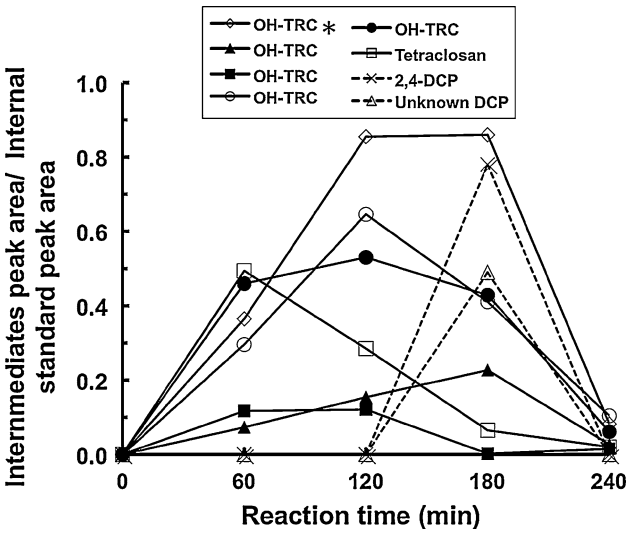

Fig. 2 Time courses of the intermediates produced by photodegradation of triclosan. *Hydroxylated triclosan

photoreactor adopted in this study decomposed approximately 90% of three tetra-chlorinated biphenyl isomers within 180 min (Nomiyama et al. 2005).

In this study, nine significant intermediates of TCS were found in the irradiated sample solutions. From mass spectra data, two intermediates were identified as tetraclosans: 4,5- and 5,6-dichloro-2-(2,4-dichlorophenoxy)phenol, which are mono-chlorinated derivatives of TCS. Also, two kinds of DCPs were found in the irradiated samples and one of them was identified as 2,4-DCP. The production of 2,4-DCP has also been reported by Canosa et al. (2005), who investigated the aquatic degradation of TCS in the presence of free chlorine. Although another detected DCP was not identified in this study, these results suggest that DCPs are one of the main intermediates in aquatic degradation of TCS. The mass spectrum of the other five intermediates consists of the molecular ions at *m/z* 448 (M^+), 450 ($M^+ + 2$) by TMS derivatization. Expected raw molecular weight of these phenolic compounds corresponded with hydroxylated TCS (*m/z* 304 (M^+)). Moreover, the production of 2,8-DCDD was found with GC/MS analysis in SIM mode. The photochemical production of this lower chlorinated dioxin has already been reported (Aranami and Readman 2007).

Figure 2 shows the relative intensity of intermediates as a factor of irradiation time. Changes in relative amounts in the intermediates are shown by ratio to intensity of IS. The primary photoreaction caused chlorination of TCS and consequently produced tetraclosan within 60 min of irradiation time and gradually decreased thereafter. In the oxidative degradation, the OH radicals directly attacked the two benzene rings of TCS, which were changed to hydroxylated TCS by the substitutional reaction of the hydroxyl group followed by dehydration. Two kinds of DCPs were produced at 180 min exponentially. However, at 180 min most TCS had been degraded (Fig. 1). This result implies degradation pathways of TCS; DCPs were derived from TCS via the hydroxylated TCS and tetraclosan (Fig. 3). Almost all significant intermediates were degraded after 240 min (Fig. 2). The quantified concentration of 2,8-DCDD increased at 60 min and it reached the maximum concentration of 0.25 μg L^{-1} at 120 min. Detectable 2,8-DCDD was not observed in the sample during the last 60 min (data not shown). The conversion rate from oxidation of TCS to 2,8-DCDD was less than 0.1%. It seems to be that oxidative reaction to 2,8-DCDD from TCS poses only a small risk. However, as the conversion rate of TCS to 2,8-DCDD depends on the matrices of ambient water (Aranami and Readman 2007), there is a possibility that the production of 2,8-DCDD and other intermediates are enhanced according to water conditions (e.g. pH, salinity, temperature).

Figure 4 shows FED and partial electronic charges of TCS. The highest FED at C1- (0.203) and C1′- (0.212) show that TCS is expected to be cleaved at these positions. It is expected that 2,4-DCP is produced by the OH radicals attack to C1 atoms that bear the high electron density point (0.206) of TCS. The expectation was consistent with the experimental results, though rapid production of DCPs was not observed (Fig. 2). The reasons are hard to explain in this day. On the other hand, despite the fact that FED at a C3- (0.083) position was lower than that of C6-, C6′-, C5′- (0.095, 0.133 and 0.098) positions, the chlorination occurred selectively at C3- and C5-positions. There is more evidence that shows chlorination of TCS will occur at a C3-position (Buth et al. 2009). Similarly, FED in TCS

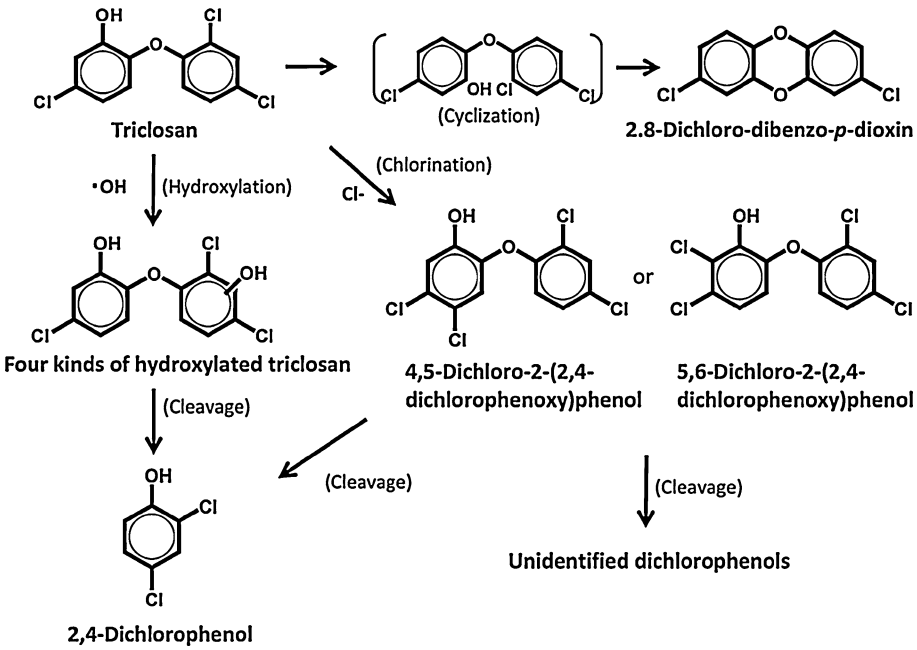

Fig. 3 Proposed degradation pathways of triclosan by UV irradiation

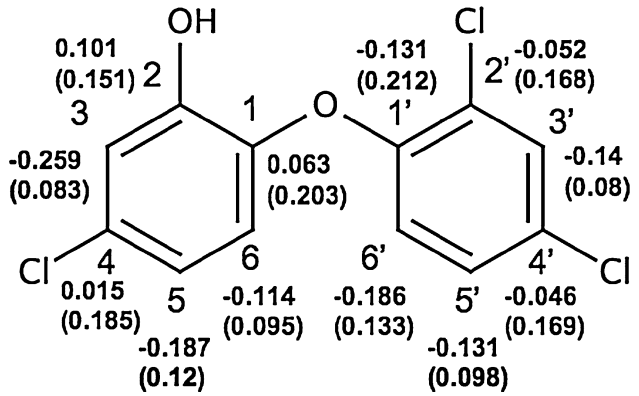

Fig. 4 Frontier electron densities (FED) and partial electronic charges on the carbon atom of triclosan by MOPAC calculation. The values in parentheses show the FED at each carbon atom

indicated that OH radicals attack at C3-, C5-, C6-, C3′-, C5′- and C6′-positions to generate six hydroxides. However in this study, only five hydroxides were observed in GC/MS analysis. One reason for this discord may result from the instability of an undetected hydroxide. A lower chlorinated dioxin, 2,8-DCDD would be produced by cyclization at 6- and 6′- positions. Relatively-low FED at 6- and 6′- may explain the low amount of 2,8-DCDD produced in the irradiation.

To evaluate the potential endocrine disrupting effect induced by degradation of TCS in water environment, it is important to investigate the endocrine disrupting effect of these intermediates. Figure 5 shows observed endocrine disrupting effect of irradiated and non-irradiated TCS solutions in the absence of S9. Significant estrogenic agonist activity was observed at 120 min irradiated solution for hERα. Moreover, it was found that 0, 120 and 240 min irradiated TCS solutions exhibit estrogenic activities to medERα. Their estrogenic activities declined with the progression of the UV irradiation. Since the Medaka estrogen receptor is more sensitive than that of humans, this aquatic organism would be susceptible to TCS in ambient water (Nomiyama et al. 2007). The RXRβ and TRα agonist activities of the solutions were also measured. Although no sample solutions exhibited TH activity, the significant RXRβ activity was found at 0–60 min irradiated solutions. The RXR activity of the solutions decreased with the progression of the UV irradiation time. The results showed that the non-irradiated sample has 0.5% ligand activity compared to that of 9-*cis*-retinoic acid, the positive control. In the S9 treatment test, the weak estrogenic activity was found in decomposed TCS solutions at 0–60 min for medERα (data not shown). It is presumed that this estrogenic activity originates from TCS remained during 0–60 min of UV irradiation time (Ishibashi et al. 2004).

Figure 6 shows the TH activities of TCS, tetraclosan and 2,4-DCP standard references in the presence of S9. In the hTRα test, the agonist activity was detected in tetraclosan (Fig. 6). The TH activity of tetraclosan was 0.05% based on the relative activities to the positive control substance triiodthyronine (T3). In the hTRβ test, tetraclosan and 2,4-DCP showed significant TH activities. In these results, intermediates with higher activity than TCS were observed. A previous study showed that TCS disrupts the TH homeostasis in rats (Crofton et al. 2007). However, these results indicate that the oxidative degradation products hold an even stronger effect. Since TCS is a wide spread PPCP in the environment, the photolysis can proceed in the

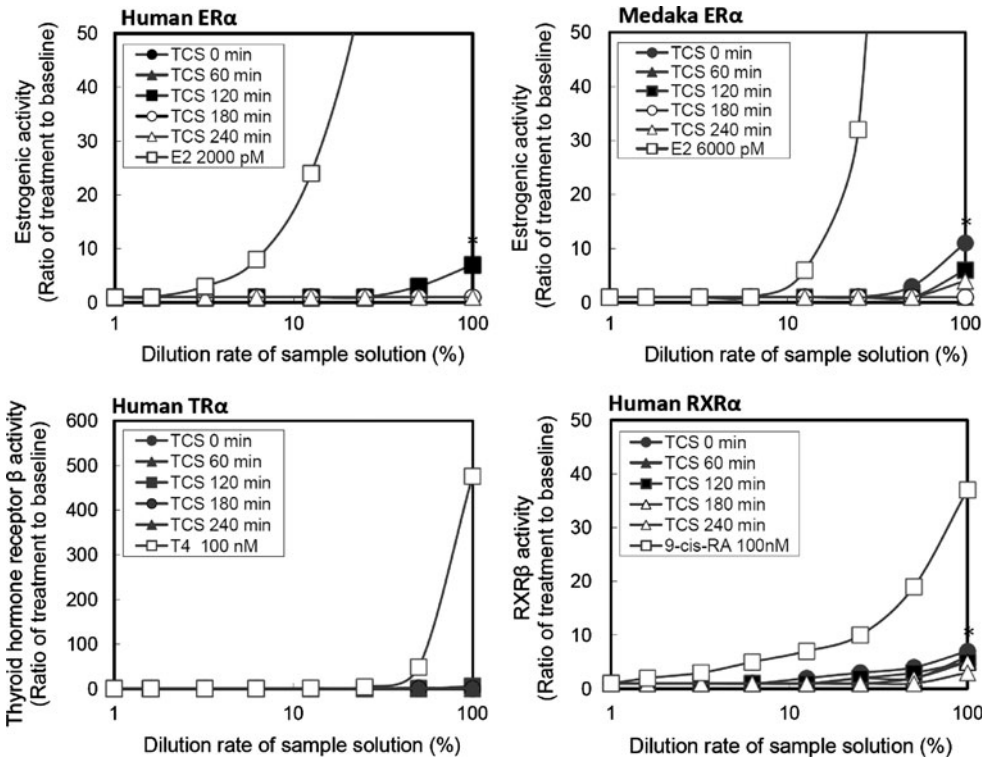

Fig. 5 Endocrine disrupting activities of the sample solutions in the two-hybrid assay in the absence of S9. *Y*-axis represents the ratio of a sample solution to a blank control (*baseline*) in chemiluminescence (CLN) intensity (T/B) of β-galactosidase. Each point represents the mean of three replicates. The *asterisk* symbols (*) of 120 min of sample solution for hERα, and 0, 120 and 240 min of sample solutions for medERα, and 0 and 60 min of sample solutions for hRXRβ denotes that significant differences were observed compared to the control baseline groups ($p < 0.01$, one-way analysis of variance (ANOVA)). *Standard errors* were smaller than the symbols used to represent the means and consequently are not shown

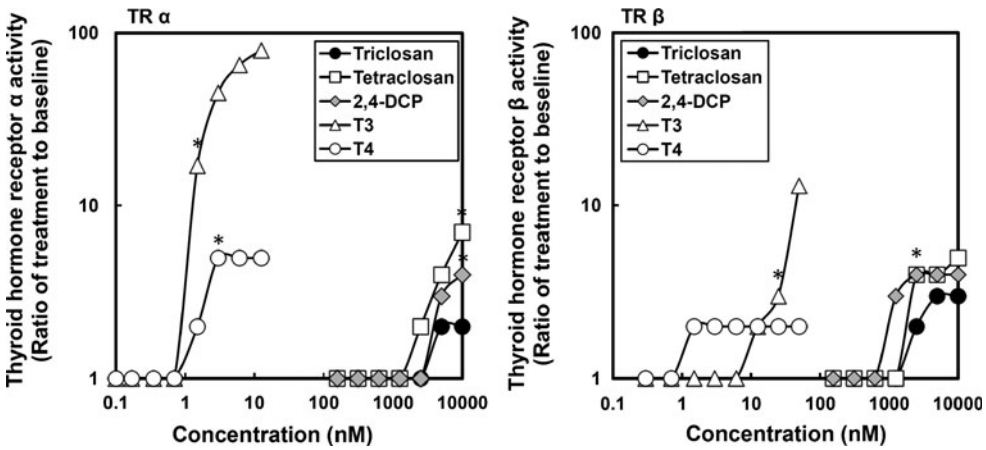

Fig. 6 The thyroid hormone like activities of TCS, tetraclosan and 2,4-DCP in the two-hybrid assay (hTRα and β) in the presence of S9. Each point represents the mean of three replicates. The *asterisk* symbols (*) of T3, T4, tetraclosan, and 2,4-DCP for TRα, and T3, tetraclosan and 2,4-DCP for TRβ denotes that significant differences were observed compared to the control baseline groups ($p < 0.01$, one-way analysis of variance (ANOVA)). *Standard errors* were smaller than the symbols used to represent the means and consequently are not shown

superficial layer of rivers and seas. Chronic exposure to those intermediates may result in a decrease in T4 level in serum and affect TH homeostasis in aquatic organisms (Crofton et al. 2007).

The present study emphasizes the importance of photochemical behavior of chemicals and their potential toxicities.

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