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Multiple Metabolic Pathways of 2,4,6-Tribromophenol in Rice Plants

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Abstract

Bromophenols occur naturally and are used globally as man-made additives in various industrial products. They are decomposition products of many emerging organic pollutants, such as tetrabromobisphenol A, polybrominated dibenzo-*p*-dioxin (PBDD), polybrominated diphenyl ethers (PBDE), and others. To characterize their biotransformation pathways, bromophenol congener 2,4,6-tribromophenol, being used most frequently in the synthesis of brominated flame retardants and having the greatest environmental abundance, was selected to hydroponically expose rice plants. After exposure for 5 days, 99.2% of 2,4,6-tribromophenol was metabolized by rice. Because of the lack of relative reference standards, an effective screening strategy was used to screen for potential metabolites that were further qualitatively identified by gas and liquid chromatography combined with high-resolution mass spectrometry. Forty transformation products were confirmed or tentatively identified at different confidence levels, including 9 phase I and 31 phase II metabolites. A large number of metabolites (39) were found in rice root, and 10 of them could be translocated and detected in rice stems or leaves. Many transformation pathways were proposed, including debromination, hydroxylation, methylation, coupling reactions, sulfation, and glycosylation. It was remarkable that a total of seven hydrophobic, persistent, and toxic OH-PBDEs and PBDD/Fs were found, indicating the biotic dimeric reactions of 2,4,6-tribromophenol that occurred in the rice plants. These results improve our understanding of the transformation and environmental fates of bromophenols, and they indicate new potential sources for OH-PBDEs and PBDD/Fs in the environment, especially in food chains.

Graphical Abstract

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The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b01514.

Texts S1–S4, Tables S1 and S2, and Figures S1–S4, including additional details on the descriptions of rice plant cultivation and exposure experiments, sampling and sample pretreatment, and the parameters of UHPLC and GC (PDF)

INTRODUCTION

Bromophenols (BPs) make up an important group of phenolic compounds that can be naturally produced in great abundance in the marine environment.^{1,2} However, some of the bromophenol congeners in the environment are of anthropogenic origin. For instance, 4-bromophenol, 2,4-dibromophenol, and 2,4,6-tribromophenol are extensively applied as wood preservatives and industrial intermediates in the synthesis of brominated flame retardants.³ Bromophenols have emerged during chlorination of bromine-containing wastewater and are the decomposition products of other emerging man-made brominated contaminants (i.e., polybrominated diphenyl ethers).^{4–7} As a result of extensive usage and formation from various types of processes, bromophenols are frequently detected in aquatic environments and food webs.^{4,8} Particularly for 2,4,6-tribromophenol, a wide range of concentrations from 0.3 to 3690 $\mu\text{g}/\text{kg}$ is found in surface water, landfill leachates, and sediment,³ where some extremely high levels occurred. Bromophenols can cause odor problems in drinking water⁹ and are potent competitors binding to transthyretin and disrupting thyroid hormone (TH) homeostasis in human cells.^{10,11} Because of their high toxicity and potential ecological risks to aquatic organisms, some bromophenols have been listed as “priority pollutants” and “Chemicals of Emerging Arctic Concern”.¹²

Several studies have demonstrated that bromophenols were precursors to the formation of more hydrophobic, persistent, and toxic dimeric products, e.g., hydroxylated brominated diphenyl ethers (OH-BDEs) and polybrominated dibenzo-*p*-dioxins or dibenzofurans (PBDD/Fs), in abiotic simulation experiments under photocatalysis or high-temperature oxidation conditions.^{13–19} Biotransformation studies showed that 25–30% of 2,4,6-tribromophenol was methylated in zebra fish (*Danio rerio*) after exposure for several weeks.²⁰ Additionally, 2,4-dibromophenol was metabolized to form saccharide and amino acid conjugates in carrots.²¹ Only limited types of metabolites and a hint of the environmental fates of bromophenols have been reported. Thus, a systematic search for the biotransformation products and the discovery of new metabolism pathways for bromophenols are critically needed.

Due to complex matrix effects, the search for unknown metabolites, especially those without reference standards, is often difficult. Recent developments in high-resolution mass spectrometry (HRMS) provide high resolving power and mass accuracy for quasimolecular ions and specific fragments, thus making it possible to screen and identify suspected metabolites without standards.^{22–24} A suspect screening strategy has been successfully used to investigate the transformation of emerging contaminants in several recent studies. For example, some new metabolites of amitriptyline and carbamazepine were successfully discovered in gilt-head bream (*Sparus aurata*) and *Pleurotus ostreatus* fungus.^{25,26}

The scarcity of freshwater supplies makes reused water from municipalities that is increasingly utilized for agricultural irrigation.²⁷ Bromophenols in the reused water and natural irrigation water could accumulate in and be metabolized by crops and vegetables, ultimately entering human food and causing unintended health risks.^{5,28} The fate of bromophenols in crops requires special focus. Rice is one of the most popular staple foods in Asia, providing sufficient calories for human activity. Rice plants (Nipponbare) are naturally cultivated in aqueous environments and, therefore, can grow well in hydroponic cultivation. Rice is an important model agricultural plant for investigating the fates of emerging contaminants in the laboratory. To facilitate the identification of metabolites, the most environmentally abundant congener, 2,4,6-tribromophenol, was selected and used to hydroponically expose rice plants at relatively high environmental concentrations (4.00 mg/L). A suspect screening strategy was used to discover its extensive number of biotransformation products. Forty phase I and phase II metabolites were identified using both gas chromatography (GC) and liquid chromatography (LC) combined with HRMS. These identified metabolites and proposed transformation pathways provided systematic insight into the fate and environmental health risks of bromophenols.

MATERIALS AND METHODS

Standards and Reagents

2,4,6-Tribromophenol (98.7%) was used for hydroponic exposure, and the 2,4-dibromophenol (98.0%), 2,4-dibromoanisole (98.0%), and 2,4,6-tribromoanisole (98.0%) standards were obtained from Tokyo Chemical Industry (Shanghai, China) and J&K Scientific (Shanghai, China). 2,4,6-Tribromophenol (99.2%) used for quantitative analysis and the surrogate standard of [¹³C₆]-2,4,6-tribromophenol (>98.0%) were purchased from Wellington Laboratories (Guelph, ON). The negative and positive Pierce ion calibration solution kits were purchased from Thermo Fisher Scientific (Waltham, MA). Methanol (HPLC grade) and *n*-hexane (pesticide grade) were supplied by J. T. Baker (Phillipsburg, NJ). Ammonium acetate (HPLC grade) was obtained from DikmaPure (LakeForest, CA). Ultrapure water was produced using a Milli-Q system (Millipore, Billerica, MA).

Exposure Experiments

Viable rice (*Oryza sativa* Japonica cv. Nipponbare) seeds were obtained from Nanjing Agricultural University (Nanjing, China) in 2018. The plant cultivation procedures are given in detail in Text S1 of the Supporting Information. The uniform rice plants at 2 weeks of age were selected for exposure experiments when the rice plants grew to a height of 15 cm.

Hydroponic cultivation and soil–water cultivation are alternative systems for investigating the metabolism of the organic pollutants under laboratory conditions. For the soil–water system, the bioavailability of contaminants is largely affected by the natural organic matter in the soil. The corresponding growth conditions for rice plants are closer to the real world in comparison with the hydroponic system. However, the complex microorganism communities that exist in soil may cause interference in the determination of the phytometabolism of 2,4,6-tribromophenol. Therefore, the hydroponic system was chosen for rice plant exposure. The volume of the hydroponic solution for 2,4,6-tribromophenol exposure was 50 mL with an initial concentration of 4.00 mg/ L, and the level of the solvent spiked into the solution did not exceed 0.1% (v/v). The initial concentration was close to the environmental concentration of 2,4,6-tribromophenol and did not cause visible toxic effects or damage to rice plants during the exposure period. Details about the planted exposure group (E1) and the untreated and treated control groups (E2–E8) are described in Text S1 and Figure S1.

Sample Collection and Extraction

Solution samples and rice plant tissues (roots, stems, and leaves) were collected after exposure for 5 days. Root samples were rinsed with deionized (DI) water, and the rinsewater was combined with the exposure solution for analysis. All of the plant samples were vacuum freeze-dried in a lyophilizer at $-50\text{ }^{\circ}\text{C}$ for 2 days (Boyikang Instrument Ltd., Beijing, China) and stored at $-20\text{ }^{\circ}\text{C}$ before further pretreatment.

The solution sample was divided into two fractions and pretreated immediately after sampling. One fraction was extracted with ethyl acetate, and another was diluted with methanol for instrumental analysis. The rice plant samples were extracted twice with methanol, and then the samples were further cleaned up in the HLB (200 mg, Waters, Milford, MA) cartridge. Details about sample pretreatment are given in Text S2.

Instrumental Analysis

Parent and daughter bromophenols were assessed with the UltiMate 3000 BioRS ultra-highperformance liquid chromatograph (UPLC, Thermo Fisher Scientific) coupled with a Triple Quad 5500 MS/MS system (AB SCIEX Inc., Framingham, MA) using multiple-reaction monitoring (MRM) mode.²⁹ The chromatographic conditions are described in Text S3. The MRM parameters of LC–MS/ MS are given in Table S1.

Other hydrophilic metabolites were qualitatively analyzed by the UPLC–Orbitrap Fusion MS system (Thermo Fisher Scientific) using both negative and positive electrospray ionization (ESI) sources. The chromatographic conditions were the same as those described above for UPLC–MS/MS (Text S3). For MS detection, full scan mode was used in the range of m/z 70–1000 with a resolution of 120000. When the typical parent ions of suspected metabolites were found, their characteristic daughter ions were further studied in high-resolution MS fragmentation mode. To take full advantage of the isotopic properties of bromine in metabolite structure elucidation, a relatively wide window ($m/z = 9$) was selected for precursor ions. The fragmentation occurred with higherenergy collisional dissociation (HCD) (5–50%) with a resolution of 30000.

Hydrophobic metabolites were analyzed with an Agilent 7200 gas chromatograph coupled with an accurate mass QTOF/MS system (Agilent, Santa Clara, CA). The detailed chromatographic conditions are described in Text S3. Suspected metabolites were screened by full scan mode (MS^1) using EI mode in the range of m/z 70–800. Additionally, the mass information about characteristic peaks (precursor and daughter ions) was further investigated with respect to a wide MS^1 resource with 200 ms scan time. The collision energy ranged from 10 to 30 eV.

Quality Assurance (QA) and Quality Control (QC)

Blank solvents used in the experiment and the procedural blanks were prepared following the same extraction and cleanup processes that were used for the samples. No bromophenols or metabolites of concern were detected. The accuracy of the determination was assessed by spiking recoveries of isotope-labeled standards using blank matrices. The recoveries of isotope-labeled bromophenols ranged from 78.8% to 97.0% in different plant tissues (roots, stems, and leaves). To ensure the accuracy of the molecular mass during UPLC–HRMS analysis, negative and positive ion calibration solutions (Thermo Fisher Scientific) were periodically injected.

Suspect Screening Strategy and Data Analysis

The metabolites without reference standards were screened and identified using the suspect screening strategy.^{25,30} Briefly, Metworks 1.3 SP4 software (Thermo Fisher Scientific) was used to predict the potential metabolites of 2,4,6-tribromophenol based on the known biotic and even abiotic transformation pathways of other phenolic xenobiotics. A diversity of metabolic reactions, including phase I (i.e., debromination, hydroxylation, and coupling reactions) and phase II reactions (i.e., methylation, acetylation, sulfation, and glycosylation), were proposed for the suspect screening strategy. Lists of suspected metabolites, their molecular formulas, and their precursor ions for suspected screening are listed in Table S2. The centroid raw MS data of GC–HRMS and UPLC–HRMS were processed using an Agilent MassHunter (Agilent) and Xcalibur version 2.2 (Thermo Fisher Scientific), respectively, to screen the pseudomolecular ions of suspected metabolites. The pseudomolecular ions were then manually verified with a mass tolerance of 10 ppm. The screened possible metabolites were further identified by an MS^2 product ion scan. The isotopic pattern of bromide also helped to confirm the formula of metabolites via software.

The distributions of metabolites that have reference standards were evaluated according to their quantitative analysis in rice tissues, while the distributions of metabolites that lacked reference standards were roughly estimated by their relative abundances in different plant tissues. The relative abundances of metabolites were calculated as the proportions of the sum of the peak areas in each rice tissue sample. The formula was

$$R = A_{\text{tissue}} / A_{\text{total}}$$

where A_{tissue} is the peak area of a metabolite in rice tissue and A_{total} is the sum of all of the peak areas of metabolites in the rice tissue samples. Statistical analyses were performed

using SPSS statistics, and variance post hoc tests ($p = 0.05$; analysis of variance, Tukey's test) were conducted to evaluate the differences between data.

RESULTS AND DISCUSSION

2,4,6-Tribromophenol in the Exposure and Control Systems

The spiked amount of 2,4,6-tribromophenol in the solution was 0.20 mg. Total recoveries of 2,4,6-tribromophenol in the treated systems were determined via the percentages of total mass in both solutions and rice plants with each treatment. The average recovery of the planted exposure group (E1) was only 0.8%, and 2,4,6-tribromophenol could accumulate in the rice plant. The level of 2,4,6-tribromophenol decreased in the following order: roots > leaves > stems. This indicated acropetal translocation of 2,4,6-tribromophenol in the rice plant. No 2,4,6-tribromophenol was detected in plant tissues or solutions of the planted blank control group (E2) after exposure for 5 days, confirming that there was neither interference from laboratory background contamination nor memory effects between experiments. As described in detail in Text S4, the recoveries of 2,4,6-tribromophenol in unplanted treated controls (E3–E8, 85.6–96.1%) were significantly higher than that of the planted exposure group [E1, 0.8%; $P < 0.01$, two-tailed unpaired Student's *t* test (Figure S2)]. These results illustrated that no obvious sorption, volatilization, and transformation losses unrelated to the rice plant occurred in the exposure group. In addition, the ingredients of root exudates and the microorganisms that may exist in root exudates or were introduced during the exposure process had no significant effect on the degradation of 2,4,6-tribromophenol. Actually, because of its antifungal property,³ 2,4,6-tribromophenol could inhibit the activities of microorganisms as well as their biotic degradation in the treated systems. All of these results suggest that the huge amount of 2,4,6-tribromophenol lost from the planted exposure system was indeed biologically metabolized by rice plants.

Identification of Metabolites

Metabolism of xenobiotics in plants is well-known to be orchestrated in three phases according to enzymatic metabolic pathways.^{31–34} Phase I consists of activation; phase II is conjugation, and phase III is sequestration into the cell wall or vacuoles.³⁵ Using the suspect screening strategy, a diversity of transformation pathways (i.e., debromination, hydroxylation, methylation, coupling reactions, sulfation, and glycosylation) was observed. Forty bromine-containing transformation products were found in the planted exposure group, including 9 phase I and 31 phase II metabolites, some of which were isomers because the MS peaks at different retention times showed the same precursor ion. The chromatograms and mass spectra of those metabolites are shown in Figures S3 and S4. No metabolites of 2,4,6-tribromophenol were detected in the planted blank control, treated water control, or the treated root exudate controls, further verifying that the metabolites found in rice plants indeed formed during biological processes within the rice plant.

The chromatographic and mass information used to identify the metabolites is summarized in Table 1. The abundance ratios of characteristic MS isotopic peaks of bromine-containing metabolites found by GC and LC-HRMS were approximately 1:2:1, 1:3:3:1, 1:4:6:4:1, and 1:5:10:10:5:1, suggesting that these metabolites contained two to five bromine atoms. Thirty

metabolites, TP₂₅₀, TP₂₆₆, four TP₂₈₀ isomers (TP_{280a}, TP_{280b}, TP_{280c}, and TP_{280d}, numbered by their elution order), TP₄₀₈, four TP₄₂₈ isomers (TP_{428a}-TP_{428d}), TP₄₇₀, TP₄₉₀, TP₅₁₄, TP₅₃₂, TP₅₆₀, six TP₅₉₀ isomers (TP_{590a}-TP_{590f}), TP₅₉₅, TP₆₂₂, TP₆₃₂, TP₆₅₂, two TP₆₇₆ isomers (TP_{676a} and TP_{676b}), TP₆₉₄, and TP₇₃₈, were detected using LC systems, and all were eluted from the separation column before 2,4,6-tribromophenol, indicating that their polarities were higher than that of the parent bromophenol. Almost all of these metabolites could be detected in negative ESI mode, while only TP₅₉₅ ($M + NH_4^+$) could be detected in positive mode (Figure S3).

TP₂₅₀ was the debromination product, 2,4-dibromophenol, which was verified with level 1 confidence by comparing its LC retention time and MS data with those of a corresponding reference standard (Table 1). TP₂₆₆ was inferred as the hydroxylation metabolite by its characteristic $[M - H]^-$ ions (m/z 264.85013, 266.84805, and 268.84595, $C_6H_3Br_2O_2^-$). The confidence level was level 4 because characteristic daughter ions were not found.³⁶ The typical LC-HRMS chromatogram and mass spectrum of TP₂₈₀ (Figure S3) show the characteristic neutral loss of CH_3 based on the m/z differences between the precursor ion and product ion, and four TP₂₈₀ isomers were inferred to be dimethyl dibromophenol ($[M - H]^-$) metabolites, perhaps the methylation metabolites of TP₂₆₆. According to precursor ions and the characteristic isotopic mass spectrum, TP₃₀₉ was inferred to be the acetylation metabolite of TP₂₆₆.

All of the mass spectra of metabolites TP₄₀₈, TP₄₉₀, TP₅₃₂, TP₆₂₂, TP₆₅₂, and TP₆₉₄ showed four representative ions at m/z 326.76613, 328.764081, 330.76203, and 332.75999 that were the characteristic isotopic precursor ions ($[M - H]^-$) of 2,4,6-tribromophenol, indicating that these six metabolites all contained the same skeleton of 2,4,6-tribromophenol. They were inferred to be the conjugates of 2,4,6-tribromophenol. Among them, TP₄₀₈ showed a neutral loss of $[HSO_3]$ from the precursor ion to form the product ion, suggesting that it was a sulfate conjugation metabolite ($C_6H_2Br_3SO_4^-$). The precursor ions of TP₄₉₀ (Figure S3), TP₅₃₂, TP₆₂₂, TP₆₅₂, and TP₆₉₄ showed different neutral losses of monosaccharide ($C_6H_{11}O_5$ and $C_8H_{13}O_6$) and disaccharide ($C_{11}H_{19}O_9$, $C_{12}H_{21}O_{10}$, and $C_{14}H_{23}O_{11}$) and were inferred to be the glucose conjugates of 2,4,6-tribromophenol with confidence level 2b.³⁷ The fragmentation of TP₅₉₅ showed a characteristic fragment ion at m/z 266.08688 ($C_9H_{12}O_8 + NH_4^+$) and a neutral loss of $C_6H_3Br_3O$ in ESI positive mode. The precursor ion of TP₅₉₅ was a malonylated hexose sugar conjugated with TP₄₉₀, indicating that it was the malonylated product of TP₄₉₀.

Metabolites TP_{428a}, TP_{428b}, TP_{428c}, TP_{428d}, and TP₄₇₀ all showed the characteristic neutral loss of monosaccharide ($C_6H_{11}O_5$ or $C_8H_{13}O_6$), producing a product ion at m/z 266.84848, which was the precursor ion of dihydroxyl dibromophenol (TP₂₆₆, $[M - H]^-$). TP₅₁₄ produced the same precursor ions as TP₄₇₀ after a characteristic neutral loss of CO_2 , indicative of a malonylated hexose sugar.³⁸ The six TP₅₉₀ isomers and two TP₆₇₆ isomers all showed a characteristic neutral loss of glycone, resulting in product ions that were the same as the precursor ions of TP₄₂₈. This indicated that the isomers of TP₅₉₀ and TP₆₇₆ are disaccharide conjugates containing the skeletons of monosaccharides such as TP₄₂₈. According to the predicted precursor ions shown in the suspected list, TP₅₆₀, TP₆₃₂, and

TP₇₃₈ were inferred to be glucose conjugates. No fragment ion was determined for those three metabolites, and the confidence level was level 4.

Fourteen metabolites, TP₂₆₅, four TP₂₈₀ isomers (TP_{280a}, TP_{280b}, TP_{280c}, and TP_{280d}), TP₃₀₉, TP₃₄₁, TP₃₄₃, three TP₄₁₉ isomers (TP_{419a}, TP_{419b}, and TP_{419c}), two TP₄₉₉ isomers (TP_{499a} and TP_{499b}), and TP₅₇₉, were detected using GC-QTOF in EI mode. Because the widest m/z window in GC-QTOF was only 1.5 for MS¹ precursor ions, the characteristic isotopic spectrum of bromine could not simultaneously appear in the same window. Therefore, the most abundant m/z of the metabolite was selected for the MS¹ precursor in EI mode. TP₃₄₃ and TP₂₆₅ were the methylation metabolites of di- and tribromophenol (Figure S4), and they were further identified by their authentic standards. TP₂₈₀ (four isomers) and TP₃₀₉ detected by LC-HRMS above could also be detected by GC-QTOF, and the mass information (m/z) about those metabolites was further confirmed. TP₃₄₁, three TP₄₁₉ isomers, two TP₄₉₉ isomers, and TP₅₇₉ (Figure S4) were verified as coupling metabolites (OH-PBDEs and PBDD/Fs) by their precursor ions and the debromination fragment ions [m/z 260.9535 for TP₃₄₁ and m/z 419.7803 for TP₅₇₉ (Figure S4)]. Therefore, the confidence levels of those metabolites (TP₃₄₁, TP₄₁₉, TP₄₉₉, and TP₅₇₉) were from 3 to 4. The measured masses and fragment ions of those metabolites are also summarized in Table 1.

Proposed Transformation Pathways of 2,4,6-Tribromophenol in Rice Plants

According to the qualitative analysis presented above, hydroxylation, methylation, acylation, and conjugation metabolites were found in the exposed rice plants. The metabolic pathways of 2,4,6-tribromophenol were proposed and are summarized in Figures 1–3. As shown in Figure 1, the only detectable debromination metabolite was 2,4-dibromophenol (TP₂₅₀). This indicated that debromination of 2,4,6-tribromophenol occurred for bromine atoms at the *ortho* position. The debromination of concerning emerging contaminants occurs via typical biotransformation pathways in plants,³⁹ whereas selective debromination among pollution is less frequently reported. This was conducive to further transformation or conjugation of phenolic hydroxyl substitution through reduction of steric hindrance. Hydroxylation reactions are well-known metabolic pathways of plants, and hydroxylated metabolites have previously been observed for BDE-28 and BDE-47 in pumpkins in our laboratory.^{39,40} For the hydroxylation metabolism in this work, the bromine atom was replaced by a hydroxyl to form dihydroxyl dibromophenol (TP₂₆₆), similar to the metabolism of 2,4-dibromophenol in carrot cell cultures.²¹ Debromination and hydroxylation were generally considered to be representative of phase I metabolism.

Some of the xenobiotics and their phase I metabolites could act as substrates in phase II metabolism.^{27,28,37,38,41–43} In rice plants, the phenolic hydroxyl group of 2,4,6-tribromophenol was sulfated to form TP₄₀₈ (Figure 1). Six methylation products, TP₂₆₅, four TP₂₈₀ isomers, and TP₃₄₃, were formed from the parent 2,4,6-tribromophenol and its phase I metabolites (Figure 1). Methylation is a common metabolism pathway for phenolic micropollutants (i.e., hydroxylated polychlorinated biphenyls, triclosan, and tetrabromobisphenol A)^{39,42,44,45} and is generally considered to occur under the catalysis of glycine N-methyltransferase in various plants (e.g., pumpkin and rice plants).^{39,42} The

hydroxylation metabolite (TP₂₆₆) could further undergo acetylation in the plant (Figure 1). The acetylation products of TP₂₆₆ were more lipophilic than the parent compound.

2,4,6-Tribromophenol and its hydroxylation metabolites (TP₂₆₆) were able to form glycosylation metabolites through covalent linkages between hydroxyl groups and glucose (monosaccharide and disaccharide) under the catalysis of Oglucosyltransferase. Several glycosylation conjugates of 2,4-dibromophenol and triclosan were previously observed in carrot cell cultures.^{21,39} In comparison, the metabolites and metabolic pathways determined for 2,4-dibromophenol and triclosan in carrot cells are less diverse than those of 2,4,6-tribromophenol in rice plants. Twenty-three glycosylation products were found in rice plants (Figure 2). Because monosaccharide and disaccharide conjugates were simultaneously detected in the exposed rice plant, it was reasonable to hypothesize that a sugar substitution was added to the monosaccharide conjugates (TP₄₉₀ and TP₄₂₈) to form the disaccharide conjugates (TP₅₆₀, TP₅₉₀, TP₆₂₂, and TP₆₅₂) in sequence. The structures of malonyl esters, which have rarely been described for glycosylation metabolites,^{21,39} were also discovered in several metabolites (TP₅₁₄, TP₅₉₅, TP₆₇₆, and TP₇₃₈). They were inferred to form through the malonylation of glycosylation conjugates (TP₄₂₈, TP₄₉₀, TP₅₉₀, and TP₆₂₂). Esterification was considered as a signal for glycosylation conjugates translocating into the vacuole or apoplast.^{37,46} In addition, acetylation of hydroxyl groups of the saccharides was also observed in rice plants, leading to metabolites TP₄₇₀, TP₅₃₂, TP₆₃₂, and TP₆₉₄, similar to the hydroxylation of atrazine in rice plants.⁴⁷ Acetylation of both saccharide and disaccharide conjugates was less frequently reported in transformation processes, but it may play an important role in the growth and antistress physiology in plants.⁴⁸

For hydroxyl polybrominated diphenyl ethers (OH-PBDEs) (Figure 3) and polybrominated dibenzo-*p*-dioxin/dibenzofuran (PBDD/F) (TP₃₄₁, TP₄₁₉, TP₄₉₉, and TP₅₇₉), the coupling metabolites and formation pathways are proposed for the first time in rice plants. Apart from natural and anthropogenic sources, OH-PBDEs and PBDD/Fs are known to be produced as unintended transformation products by coupling of bromophenols in biocatalysis and photocatalysis processes.^{13–18} Remarkably, the bromophenols, including 2,4,6-tribromophenol and its debromination and hydroxylation metabolites (TP₂₅₀ and TP₂₆₆, respectively), comprised the biotic coupling reactions forming OH-PBDEs and PBDD/Fs. Compared to 2,4,6-tribromophenol, OH-PBDEs are more potent in endocrine disruption and neurotransmitter release.⁴⁹ Some studies have shown that the toxicities of PBDD/Fs are similar to those of the analogous PCDD/Fs.⁵⁰ Thus, those highly reactive biotransformation products have altered or enhanced toxicological effects.^{49–51} Our results indicate a novel source of those highly toxic brominated dimeric compounds, formation from naturally or anthropogenically derived bromophenols in rice plants. The coupling reaction poses a great risk for food safety. Thus, the potential risks of bromophenols have been greatly expanded.

Distributions of the Metabolites in the Exposure System

Only debromination, sulfation, and glycosylation metabolites (2,4-dibromophenol, TP₄₀₈, and TP₄₉₀, respectively) were identified in a hydroponic solution after exposure for 5 days. Because the root exudate did not metabolize 2,4,6-tribromophenol, minor amounts of 2,4-dibromophenol, TP₄₀₈, and TP₄₉₀ were released from rice roots. For the exposed plants,

large numbers of metabolites (39) were formed and accumulated in rice roots. Some metabolites (2,4-dibromoanisole, 2,4,6-tribromoanisole, TP_{280a}, TP_{280b}, TP_{280c}, TP_{280d}, TP₄₀₈, TP_{419a}, TP_{419b}, and TP_{419c}) could be translocated and detected in rice stems or leaves. The metabolite TP_{419a} could be found in only rice leaves. The distributions of metabolites (those with upward translocation) were evaluated by their concentrations (with a reference standard) or estimated by relative abundance (without a reference standard). As shown in Figure 4, the level of sulfation metabolites (TP₄₀₈) decreased in the following order: roots (50.7%) > leaves (44.0%) > stems (5.3%). Methylation and one of the coupling metabolites (TP_{280a}-TP_{280d} and TP₃₄₃ and TP_{419c}) mainly (>83.1%) accumulated in rice roots; only a minor portion was identified in stems and leaves. However, the most abundant metabolites of methylation TP₂₆₅ (58.48%) and coupling metabolites TP_{419a} (78.42%) and TP_{419b} (79.44%) were observed in rice leaves. These metabolites formed preferentially at the top of the rice plant.

Environmental Implications

We undertook a systematic investigation of the biotransformation of an important emerging contaminant, 2,4,6-tribromophenol, in rice plants. As many as 40 metabolites were found using the suspect screening strategy. A diversity of biotransformation pathways was proposed for 2,4,6-tribromophenol in rice, including debromination, hydroxylation, methylation, acylation, sulfation, and glycosylation. Despite the growing recognition that toxic effects of some chemicals resulted from their phase I reactions,³⁹ the toxicological consequences of newly identified phase I metabolites of 2,4,6-tribromophenol must be characterized in future research. A large number of phase II conjugate metabolites, including sulfation and 23 glycosylation metabolites, were formed in the exposed rice plant. Conjugations are generally important detoxification mechanisms when plants take up organic pollutants. At the same time, some high-risk products were also formed through the hydroxylation pathway of common concern. More importantly, we first discovered that 2,4,6-tribromophenol undergoes a coupling metabolic pathway to form more lipophilic, persistent, and toxic OH-PBDEs and PBDD/Fs in rice plants. This is a new potential environmental source of OH-PBDEs and PBDD/Fs from agricultural plants. Although those highly toxic metabolites were only intermediates during overall metabolism, the health risks caused by such toxic metabolites and the parent bromophenols are of serious concern considering the role that rice plays in the human food chain. In addition, those toxic metabolites were able to enter the food chain through meat and milk after the animals and domestic animals ate the 2,4,6-TBP and its metabolites contained in straw. Thus, further research on the long-term exposure and accumulation of emerging contaminants and their metabolites in edible parts of agricultural plants is required. The future environmental risk assessments of emerging contaminants must take into account these new bioactive transformation products in agricultural plants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Debromination, hydroxylation, methylation, acetylation, sulfation, and glycosylation of 2,4,6-tribromophenol in rice plants. The details of glycosylation conjugates are described in the legend of Figure 2. Blue and red arrows represent phase I and phase II metabolism reactions, respectively. Level of confidence in the structures identified: black, confirmed structures (level 1); violet, probable structures (level 2); green, tentative candidates (level 3); and pink, equivocal molecular formula (level 4).

Figure 2.

Proposed formation pathways of glycosylated conjugates of (A) 2,4,6-tribromophenol and (B) its hydroxylation metabolite in rice plants. Red arrows represent phase II metabolism reactions. Level of confidence in the structures identified: black, confirmed structures (level 1); violet, probable structures (level 2); green, tentative candidates (level 3); and pink, equivocal molecular formula (level 4)

Figure 3.

Coupling reactions of 2,4,6-tribromophenol and its metabolites in rice plants. Blue arrows represent phase I metabolism reactions. Level of confidence in the structures identified: black, confirmed structures (level 1); green, tentative candidates (level 3); and pink, equivocal molecular formula (level 4).

Figure 4. Distribution of metabolites of 2,4,6-tribromophenol in rice tissues. TP₂₆₅ and TP₃₄₃ are identified as 2,4-dibromoanisole and 2,4,6-tribromoanisole, respectively, at confidence level 1, and quantitatively analyzed using corresponding reference standards. Other metabolites that have no standards were plotted by their relative abundances.

Table 1.

Summary of the Identified Metabolites of 2,4,6-Tribromophenol in Rice Plants

metabolite	metabolic reaction	t_R (min)	instrument (ionization mode)	formula and exact mass	confidence level ^a	precursor ion for molecular formula	measured m/z (deviation) (ppm)	fragment ion (deviation) (ppm)
TP ₂₅₀	debromination	18.97	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₆ H ₄ Br ₂ O (248.85561, 250.85357, 252.85152)	level 1	[M – H] ⁺	248.85547 (–0.6), 250.85321 (–0.2), 252.85144 (–0.3)	169.73683 (–2.7), 171.93482 (–2.4)
TP ₂₆₅	debromination, methylation	10.32	GC-QTOF (M ⁺ , EI)	C ₇ H ₆ Br ₂ O (263.8780, 265.8759, 267.8739)	level 1	M ⁺	265.8758 (–0.4)	250.8522 (–1.1)
TP ₂₆₆	hydroxylation	8.40	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₆ H ₄ Br ₂ O ₂ (264.85053, 266.84848, 268.84643)	level 4	[M – H] ⁺	264.85013 (0.0), 266.84805 (–1.6), 268.84595 (–1.8)	ND
TP ₂₈₀ (a, b, c, d)	hydroxylation, methylation	16.77, 17.75, 20.79, 21.08	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₇ H ₆ Br ₂ O ₂ (278.86618, 280.86413, 282.86208)	level 3	[M – H] ⁺	278.86575 (–1.5), 280.86365 (–1.7), 282.86154 (–1.8)	263.84183 (–3.3), 265.83969 (–3.7), 267.83853 (–0.3)
TP ₃₀₉	hydroxylation, acetylation	15.92	GC-QTOF (M ⁺ , EI)	C ₉ H ₆ Br ₂ O ₃ (307.8678, 309.8658, 311.8637)	level 4	M ⁺	307.8671 (–2.3), 309.8665 (2.3), 311.8628 (–3.5)	ND
TP ₃₄₁	coupling reaction	30.75	GC-QTOF (M ⁺ , EI)	C ₁₂ H ₆ Br ₂ O ₂ (339.8729, 341.8709, 343.8688)	level 3	M ⁺	341.8709 (–0.0)	260.9535 (–4.2)
TP ₃₄₃	methylation	13.23	GC-QTOF (M ⁺ , EI)	C ₇ H ₄ Br ₂ O (341.7885, 343.7865, 345.7844, 347.7824)	level 1	M ⁺	343.7852 (–3.8)	328.7618 (–3.7)
TP ₄₀₈	sulfation	10.18	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₆ H ₃ Br ₃ SO ₄ (406.72294, 408.72089, 410.71885, 412.71680)	level 2b	[M – H] ⁺	406.72305 (0.3), 408.72095 (–0.2), 410.71887 (0.1), 412.71677 (–0.1)	326.76617 (0.1), 328.76410 (0.1), 330.76205 (0.1), 332.76004 (0.2)
TP ₄₁₉ (a, b, c)	coupling reaction	34.439, 34.730, 35.035	GC-QTOF (M ⁺ , EI)	C ₁₂ H ₇ Br ₃ O ₂ (417.7834, 419.7814, 421.7793, 423.7773)	level 4	M ⁺	419.7815 (0.2)	ND
TP ₄₂₈ (a, b, c, d)	hydroxylation, glycosylation	3.74, 4.41, 6.96, 11.56	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₂ H ₁₄ Br ₂ O ₇ (426.90335, 428.90130, 430.89926)	level 3	[M – H] ⁺	426.90274 (–1.4), 428.90067 (–1.5), 430.89862 (–1.5)	264.85214 (6.1), 266.84995 (5.5), 268.84805 (6.0)
TP ₄₇₀	hydroxylation, glycosylation, acetylation	2.35	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₄ H ₁₆ Br ₂ O ₈ (468.91392, 470.91187, 472.90982)	level 3	[M – H] ⁺	468.91382 (–0.2), 470.91177 (–0.2), 472.90976 (–0.1)	264.85046 (–0.3), 266.84805 (–1.6), 268.84595 (–1.8)
TP ₄₉₀	glycosylation	14.20	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₂ H ₁₃ Br ₃ O ₆ (488.81895, 490.81690, 492.81486, 494.81281)	level 2b	[M – H] ⁺	488.81836 (0.1), 490.81641 (0.1), 492.81439 (0.1), 494.81226 (0.2)	326.76678 (0.1), 328.76355 (0.1), 330.76154 (0.1), 332.75956 (0.2)

metabolite	metabolic reaction	t_R^a (min)	instrument (ionization mode)	formula and exact mass	confidence level ^b	precursor ion for molecular formula	measured m/z (deviation) (ppm)	fragment ion (deviation) (ppm)
TP ₄₉₉ (a, b)	coupling reaction	32.727, 32.911	GC-QTOF (M ⁺ , EI)	C ₁₂ H ₄₄ Br ₅ O ₂ (495.6939, 497.6919, 499.6898, 501.6880, 503.6857)	level 4	M ⁺	499.6919 (4.2)	ND
TP ₅₁₄	hydroxylation, glycosylation, malonylation	2.35	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₅ H ₁₆ Br ₂ O ₁₀ (512.90375, 514.90170, 516.89965)	level 3	[M – H] [–]	512.90454 (1.5), 514.90216 (0.9), 516.89978 (0.3)	468.91522 (2.8), 470.91174 (–2.4), 472.91874 (1.9)
TP ₅₃₂	glycosylation, acetylation	7.81	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₄ H ₁₅ Br ₃ O ₇ (530.82951, 532.82747, 534.82542, 536.82337)	level 2b	[M – H] [–]	530.82825 (–2.4), 532.82654 (–1.7), 534.82538 (0.1), 536.82397 (–1.1)	326.76736 (3.8), 328.76541 (4.1), 330.76325 (3.7), 332.76102 (3.1)
TP ₅₆₀	hydroxylation, glycosylation	9.24	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₇ H ₂₂ Br ₂ O ₁₁ (558.94561, 560.94356, 562.94152)	level 4	[M – H] [–]	558.94360 (0.3), 560.94360 (–0.2), 562.94171 (–1.5)	ND
TP ₅₇₉	coupling reaction	35.15	GC-QTOF (M ⁺ , EI)	C ₁₂ H ₅ Br ₅ O ₂ (575.6201, 577.6181, 579.6160, 581.6140, 583.6119, 585.6099)	level 3	M ⁺	579.6123 (–6.4)	419.7803 (2.6)
TP ₅₉₀ (a, b, c, d, e, f)	hydroxylation, glycosylation	2.96, 3.53, 4.07, 7.67, 9.92, 11.00	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₈ H ₂₄ Br ₂ O ₁₂ (588.95617, 590.95413, 592.95208)	level 3	[M – H] [–]	588.95636 (0.3), 590.95404 (–0.2), 592.95117 (–1.5)	426.90314 (–0.5), 428.89972 (–3.7), 430.89841 (2.0)
TP ₅₉₅	glycosylation, malonylation	7.51	UPLC-HRMS (IM + NH ₄ ⁺ , negative, ESI)	C ₁₅ H ₁₅ Br ₂ O ₉ (593.86045, 595.85840, 597.85635, 599.85431)	level 2b	[M + NH ₄] ⁺	593.86047 (0.0), 595.85815 (–0.4), 597.85657 (0.4), 599.85510 (1.3)	266.08688 (–0.4)
TP ₆₂₂	glycosylation	10.71	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₇ H ₂₁ Br ₂ O ₁₀ (620.86121, 622.85916, 624.85711, 626.85507)	level 2b	[M – H] [–]	620.86151 (–0.5), 622.85901 (–0.3), 624.85742 (–0.5), 626.85529 (0.4)	326.76584 (–0.9), 328.76361 (–1.4), 330.76160 (–1.3), 332.75974 (–0.8)
TP ₆₃₂	hydroxylation, glycosylation, acetylation	2.39	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₂₀ H ₂₆ Br ₂ O ₁₃ (630.96674, 632.96469, 634.96265)	level 4	[M – H] [–]	630.96576 (–1.6), 632.96625 (2.5), 634.96497 (3.7)	ND
TP ₆₃₂	acetylation	13.53	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₁₈ H ₂₃ Br ₃ O ₁₁ (650.87177, 652.86973, 654.86768, 656.86563)	level 2b	[M – H] [–]	650.87134 (–0.7), 652.86963 (–0.2), 654.86786 (–0.3), 656.86572 (0.1)	326.76590 (–0.7), 328.76379 (–0.9), 330.76166 (–1.1), 332.75961 (–1.1)
TP ₆₇₆ (a, b)	hydroxylation, glycosylation, malonylation	1.88, 2.40	UPLC-HRMS (IM – H ⁺ , negative, ESI)	C ₂₁ H ₂₆ Br ₂ O ₁₅ (674.95657, 676.95452, 678.95248)	level 3	[M – H] [–]	674.95624 (–0.5), 676.95428 (–0.2), 678.95135 (–1.7)	426.90274 (–1.4), 428.90121 (–0.2), 430.89850 (–1.8), 468.91467 (1.6), 470.91235 (1.0), 472.91116 (2.8), 630.96606 (–1.1),

metabolite	metabolic reaction	t_R^a (min)	instrument (ionization mode)	formula and exact mass	confidence level ^b	precursor ion for molecular formula	measured m/z (deviation) (ppm)	fragment ion (deviation) (ppm)
TP ₆₉₄	glycosylation, acetylation	9.27	UPLC–HRMS ([M – H] [–] , negative, ESI)	C ₂₀ H ₂₅ Br ₃ O ₁₂ (692.88234, 694.88029, 696.87824, 698.87620)	level 2b	[M – H] [–]	692.88239 (1.4), 694.88080 (0.7), 696.87622 (–2.9), 698.87744 (1.8)	632.96417 (–0.8), 634.96295 (–0.5), 326.76584 (–0.9), 328.76361 (–1.4), 330.76160 (–1.3), 332.75974 (–0.8)
TP ₇₃₈	glycosylation, malonylation	9.27	UPLC–HRMS (negative, ESI)	C ₂₁ H ₂₅ Br ₃ O ₁₄ (736.87217, 738.87012, 740.86807, 742.86603)	level 4	[M – H] [–]	736.87366 (2.0), 738.87097 (1.3), 740.86914 (1.4), 742.86633 (0.1)	ND

^aThe retention time obtained from UPLC–HRMS and GC–QTOF analysis.

^bThe confidence values of metabolites were identified according to the method described by Schymanski et al.²⁴