— Research Letter —

Transesterification in the Microbial Degradation of Phthalate Esters

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(Received January 12, 2011; Accepted March 17, 2011; Published online March 25, 2011)

Our previous study revealed that phthalate esters (PEs), a group of suspected endocrine-disrupting chemicals, acquire estrogenic activities by ring 4-hydroxylation. In addition, the estrogenic activities are modified depending on alkyl chain structures (chain length and branching), which can be altered in the environmental conditions such as microbial degradation. Therefore, it is important to determine the environmental fate of these alkyl chains to evaluate the biological impact of PEs on humans and wildlife. PEs are known to undergo biodegradation via sequential hydrolysis, resulting in the formation of monoester and dicarboxylic acid forms. In this study, dipropyl phthalate chosen as one of PEs was cultivated with *Acinetobacter lwoffii*, a known PE-degrading bacterium, in the presence of a limited amount of CH_3OH as a PE-solvent. As a result, several unknown biotransformation products were detected. The products were characterized as methyl propyl phthalate, dimethyl phthalate, and monomethyl phthalate, suggesting that environmental PEs are processed through novel biotransformation pathways. The products can be produced both by esterification of monoester forms and transesterification of diester forms. However, when monobutyl phthalate—a monoester of dibutyl phthalate—was used as a substrate, esterified products were not detected, indicating phthalate methyl esters were formed via transesterification. A stable-isotope tracer experiment using CD_3OH instead of CH_3OH revealed the production of phthalate methyl esters, the molecular ions of which shifted by 3 or 6 atomic mass units. These results revealed that PE was bacterially transformed via transesterification in the presence of alcohol. We demonstrated that PEs are transformed in the environment via more diverse ways than expected, although the environmental concentration of alcohols is very low. It would be worthwhile to perform a systematic assessment on the possibility that transesterification products may be associated with the potential adverse effects of PEs in the environment.

Key words —— plasticizer, biotransformation, bacterium, environmental pollutant, stable isotope

INTRODUCTION

Phthalate esters (PEs), a group of suspected endocrine-disrupting chemicals, are manufactured in large quantities and mainly used as plasticizers in polyvinyl chloride. One of the most abundant PEs is di(2-ethylhexyl) phthalate (dEHP), the worldwide production of which are over 2 million tons per year.[1)] PEs have been reported to show several adverse effects on humans and wildlife. In several epidemiological studies, the correlation of urinary phthalate concentration with sperm motility/concentration, anogenital distance in male infant, gestational age, head circumference of newborn children, serum glucose concentration, and obesity incidence have been reported.[2–6)] Our recent study revealed that the biological effects of PEs varied depending on the structure of their alkyl chains. For example, differences in alkyl chain lengths and branching are associated with drastic differences in the estrogen receptor (ER)-mediated effects of ring-hydroxylated PEs, which are active metabolites/photo-oxidation products of PEs and are bound to ERs.[7–10)] The ER-binding potential of ring-hydroxylated PEs increases with increasing alkyl chain length and branching. A yeast reporter gene assay revealed that PEs with short alkyl chains (*e.g.*, ethyl or propyl esters) promoted ER-dependent reporter gene expression, whereas PEs with long alkyl chains (*e.g.*, octyl or 2-ethylhexyl esters) did not, even though the latter exhibited extremely high ER-binding affinities. Peroxisome proliferator-activated receptors (PPARs) have also been reported to mediate PE-induced adverse effects.[11,12)] PPAR-activation potential of PEs is influenced by the alkyl chain structure.[13)]

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Since PEs are not covalently bound to plastic polymers, they leach from matrices. Therefore, PEs are widely distributed in the atmosphere[14] and hydrosphere.[15–17] In our previous study, we measured the concentrations (0.1–10 μg/l) of dEHP and dibutyl phthalate (dBP, the second-most abundant PE) in several water samples (river water, tap water, well water, and mineral water).[15] In the Tama River, Tokyo, Japan, the concentrations of dEHP and dBP were found to be 0.013–3.60 and 0.010–0.54 μg/l, respectively; moreover, the concentrations were higher in the downstream areas than in upstream areas.[17]

Biodegradation of PEs involves the sequential hydrolysis of each ester-alkyl chain, which is accompanied by the formation of phthalic acid and certain monoesters.[18] However, it is uncertain whether additional transformation products contribute to the toxicity of PEs. Considering the dynamic changes in the biological effects of PEs with changes in the structure of the alkyl chains of the esters, it is important to determine the environmental fate of these alkyl chains in order to perform human and environmental risk assessments. In our previous study, we found that biotransformation of dBP resulted in the formation of dimethyl phthalate (dMP), monomethyl phthalate (mMP), monobutyl phthalate (mBP; a typical dBP-degradation product), and an unknown diester.[15] This suggests the existence of novel biotransformation pathways, and the above-mentioned transformation products may contribute to the potential adverse effects of PEs. In the present study, we identified new diesters produced during the biotransformation of dipropyl phthalate (dPP) and elucidated alternative microbial transformation pathways for environmental PEs on the basis of direct evidences.

MATERIALS AND METHODS

Materials —— PEs used in this study are shown in Fig. 1. dMP, diethyl phthalate (dEP), and dPP were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Deuterated methanol (CD_3OH) and deuterated dimethyl sulfoxide (DMSO-d^6) were obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, U.S.A.). Phthalic anhydride and deuterated ethanol (C_2D_5OD) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). mMP, monopropyl phthalate (mPP), and methyl propyl phthalate (MPP) were synthesized in our laboratory as described below.

	R1	R2	PE	Abbreviation
Monoesters	CH_3	H	Monomethyl phthalate	mMP
	C_3H_7	H	Monopropyl phthalate	mPP
	C_4H_9	H	Monobutyl phthalate	mBP
Diesters	CH_3	CH_3	Dimethyl phthalate	dMP
	C_2H_5	C_2H_5	Diethyl phthalate	dEP
	C_3H_7	C_3H_7	Dipropyl phthalate	dPP
Asymmetrical esters	CH_3	C_3H_7	Methyl propyl phthalate	MPP
	C_2H_5	C_3H_7	Ethyl propyl phthalate	EPP

Fig. 1. Structures and Abbreviations of PEs Used in this Study

Synthesis of Phthalate Monoesters and Asymmetrical Diesters —— Two phthalate monoesters (mMP and mPP) were synthesized from phthalic anhydride with methanol (CH_3OH) and 1-propanol (C_3H_7OH), respectively. Phthalic anhydride was refluxed with the alcohol, and the residual alcohol was subsequently removed under vacuum. Next, the reaction mixture was purified by TLC, silica gel chromatography, and HPLC using a Wakosil 5C18 column [internal diameter (i.d.), 10×250 mm; Wako Pure Chemical Industries Ltd.]. MPP was synthesized from mPP by methylation with diazomethane.[19] The methylated products were purified with the same purification procedures used for phthalate monoesters. The structure of synthesized phthalates was confirmed by electron impact ionization-MS (JMS-700; Jeol Ltd., Tokyo, Japan) and ^{1}H-NMR (A-400 or ECP-500; Jeol Ltd.) analyses.

Biotransformation of PEs —— In our previous report, the R-3 strain of *Acinetobacter lwoffii* (*A. lwoffii*) showed the highest rate of dBP biodegradation among 8 bacterial strains isolated from water samples collected from Tempaku River water in Nagoya, Japan.[15] Therefore, we chose the R-3 strain for the biotransformation of PE in this study. The substrate (1 mg/ml dPP or mBP in CH_3OH, CD_3OH, C_2D_5OD, or DMSO) was added to 200 ml 1/10 nutrient broth (NB, 0.8 g/l NB, pH 7.0) at a final concentration of 20 μg/ml. Next, 4 ml cell suspension (optical density at 610 nm, 0.2) was added to the medium. The cells were cultivated for 7 d with shaking at 25°C in the dark. The culture-containing medium (100 μl) was sampled at specific time periods, treated with 900 μl acetonitrile (CH_3CN), and centrifuged at $9000 \times g$ for 10 min.

Table 1. Calculated Molecular and Fragment Ions of PEs

Parent ion structures

$(s+t)^{a)}$	0	1	2	3	4	5	6
$[M+H]^+$ (m/z)	167	181	195	209	223	237	251

Primary fragment ion structures

$s^{a)}$	0	1	2	3
Fragment ion (m/z)	149	163	177	191

a) s = 0–3, t = 0–3

The resultant supernatant was subjected to HPLC. Biotransformation products were characterized by ion-trap liquid chromatography (LC)/MS and ^{1}H-NMR analyses.

HPLC, LC/MS, and ^{1}H-NMR Analyses—— The products obtained during PE-biotransformation were analyzed by using an HPLC apparatus equipped with a UV/visible (VIS) detector (UV-2070; Jasco Co. Ltd., Tokyo, Japan). Data collection and analysis were performed using the Borwin software (Jasco Co. Ltd.). The products were purified on a Cosmosil column (5C18-MS-II; i.d., 4.6×250 mm; Nacalai Tesque Inc., Kyoto, Japan) under the following conditions: flow rate, 1 ml/min; column temperature, 40°C; mobile phase, CH_3CN/H_2O containing 0.05% trifluoroacetic acid (TFA); and wavelength, 225 nm. LC/MS analysis was performed on an LCQ-DECA-XP Plus ion-trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) operated in the MS/MS mode coupled with an HPLC system (Agilent 1100 series; Agilent Technologies, Palo Alto, CA, U.S.A.). Conditions for MS analysis were as follows: ion spray voltage, 5 kV; transfer tube temperature, 275°C; collision gas, helium; and normalized collision energy, 35%. The alkyl chain lengths of PEs were determined on the basis of the fragmentation patterns, according to a method described in a previous report.[20] ^{1}H-NMR spectra were obtained using an A-400 (Jeol Ltd.). Chemical shifts are expressed in terms of δ (ppm) and are based on the tetramethylsilane (TMS) signal used as an internal reference.

RESULTS

Identification of dPP Biotransformation Products

Typical primary fragment ions of phthalate diesters were observed in MS/MS experiments: these ions corresponded to a loss of alcohol or water (ROH) from the molecular ions $[M+H]^+$, as shown in Fig. 2. Thus, the alkyl chain length of phthalate diesters can be determined on the basis of the fragment ions produced, as described in a previous report.[20] The calculated and observed molecular/fragment ions of dPP-biotransformation products are shown in Tables 1 and 2, respectively.

Table 2. Observed Molecular and Fragment Ions of Biotransformation Products of dPP

Product	$[M+H]^+$	Fragment ion
m-1	223.1	190.7, 163.1
m-2	209.2	190.7, 149.1
m-3	195.2	163.0
m-1′	226.2	190.7, 166.1
m-3′	201.3	166.1
e-1	233.4	181.9
e-2	242.3	190.7, 182.0

The R-3 strain of *A. lwoffii* was cultivated in the presence of dPP/CH_3OH. HPLC analysis revealed the presence of 4 biotransformation products (m-1, m-2, m-3, and m-4) in the culture medium in which the strain was cultivated for 7 d (data not shown). Product m-1 was eluted at 6.2 min with 60% CH_3CN-0.05% TFA; the elution time was not consistent with the elution times of any commercially available PE. The MS/MS spectrum of m-1 showed fragment ions at m/z 190.7 and 163.1, which corresponded to a loss of CH_3OH (m/z 32) and C_3H_7OH (m/z 60) mass units, respectively, from the molecular ion mass units (m/z 223.1). The ^{1}H-NMR (DMSO-d^6) spectrum of m-1 showed sp^3 proton signals corresponding to alkyl moieties as follows: δ 4.26–4.29 (2 H, t, -$CH_2$$CH_2CH_3$), 3.91 (3 H, s, -CH_3), 1.73–1.80 (2 H, q, -$CH_2$$CH_2$$CH_3$), and 0.99–1.02 (3 H, t, -$CH_2CH_2$$CH_3$). These data are in good agreement with those of synthesized MPP. On HPLC analysis, products m-2 and m-3

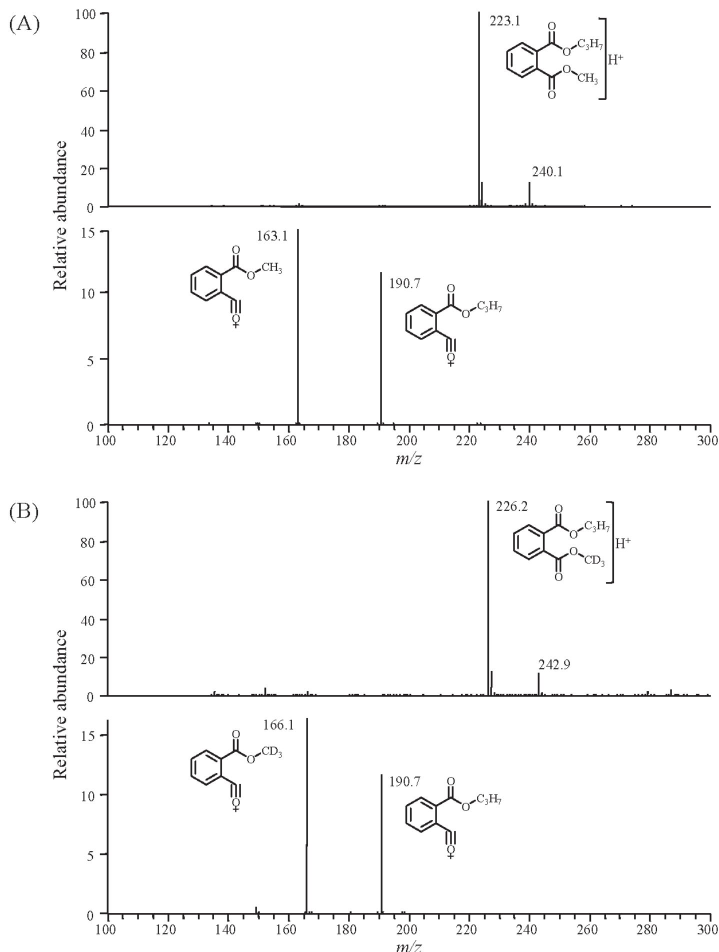

Fig. 2. Representative HPLC/electrospray Ionization/MS/MS Spectra of PEs: MPP (A) and MPP-d^3 (B, Methyl Group Deuterated)
The m/z value and proposed structure of each fragment ion are provided beside each signal.

were eluted at 6.9 and 7.8 min, respectively, with 40% CH_3CN-0.05% TFA. The observed retention time, and molecular ($[M + H]^+$, m/z 209.2) and fragment ion masses (m/z 190.7 and 149.1) of m-2 were consistent with those of mPP, which is a typical hydrolyzed product of dPP. The HPLC retention time, and molecular and fragment ion masses (m/z 195.2 and 163.0, respectively) of m-3 were similar to those of dMP. The HPLC retention time of m-4 (11.0 min with 20% CH_3CN-0.05% TFA) corresponded to that of mMP. These phthalate methyl esters (m-1, m-3, and m-4) were not detected when CH_3OH was replaced with DMSO as the PE solvent or when the bacterium was removed from the cultivation medium. Furthermore, dBP and dEP were also biotransformed to the asymmetrical forms (*e.g.*, butyl methyl phthalate and ethyl methyl phthalate, respectively) in the presence of CH_3OH, indicating that this biotransformation pathway would be universal for phthalate diesters.

Formation of Alcohol-dependent Biotransformation Products of PE

In order to confirm the origin of m-1, m-3, and m-4, the dPP biotransformation experiments were modified by replacing CH_3OH with CD_3OH. Four peaks (designated as m-1′, m-2′, m-3′, and m-4′) were observed on the HPLC profiles; the peaks were almost same as those obtained with CH_3OH. The retention times of these peaks corresponded to those of m-1, m-2, m-3, and m-4 (data not shown). A molecular ion of m-1′ was observed at m/z 226.2 with fragment ions at m/z 190.7 and 166.1. A molecular ion of m-3′ was observed at m/z 201.3 with a fragment ion at m/z 166.1. The molecular and fragment ion masses of m-1′ and m-3′ were 3 or 6 mass units larger than those of m-1 and m-3, respectively, depending on the number of methyl groups per molecule (MPP and dMP). Moreover, on ^{1}H-NMR analysis of m-1′, signals at δ 3.91 ppm corresponding to -COOCH_3 of MPP were not ob-

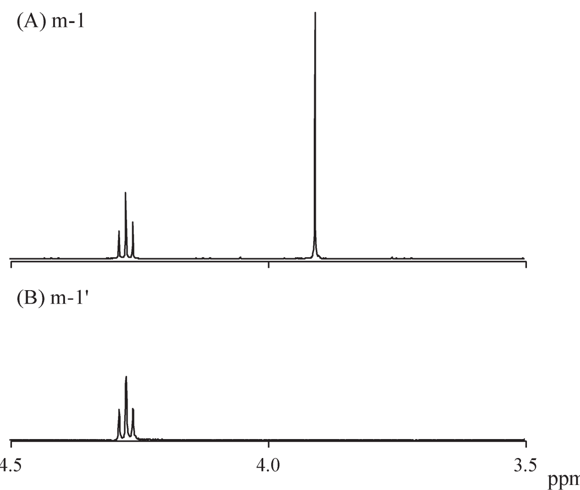

Fig. 3. ^{1}H-NMR Spectra of (A) m-1 and (B) m-1′

Products were isolated using a preparative HPLC, dissolved in DMSO-d^6 containing TMS, and then subjected to ^{1}H-NMR analysis. Chemical shifts are represented as δ (ppm) with TMS as an internal reference.

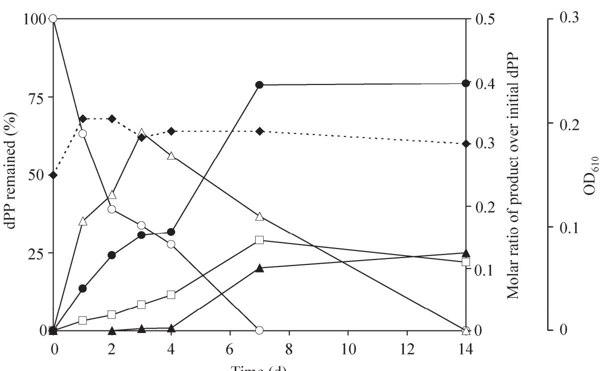

Fig. 4. Time Courses of Biotransformation Products of dPP

Products were quantified by measuring UV absorbance at 225 nm. The growth of bacterial cells was monitored at 610 nm. ○, dPP; △, MPP; □, dMP; ●, mPP; ▲, mMP; and ◆, bacterial cell density (OD_{610}).

served (Fig. 3), indicating that the methyl group of m-1′ is from CD_3OH. Furthermore, the use of C_2D_5OD produced 2 peaks (e-1 and e-2), which were eluted at 5.8 and 7.3 min, respectively, during HPLC (data not shown). A molecular ion of e-1 was observed at m/z 233.4 with a fragment ion at m/z 181.9. Although the retention time of e-1 was similar to that of dEP, the observed molecular and fragment ion masses of the former were 10 and 5 mass units larger than the calculated masses (*i.e.*, m/z 223 and 177), respectively. These results suggest that e-1 is dEP containing deuterated ethyl moieties. The retention time of e-2 differed from that of any commercially available PE. A molecular ion of e-2 was observed at m/z 242.3 with fragment ions at m/z 190.7 and 182.0. The fragment ion at m/z 190.7 was identical to a PE ion containing a propyl ester that was observed during the fragmentation of dPP, mPP, and MPP (Fig. 2). The fragment ion at m/z 182.0 was also observed during the fragmentation of e-1, as described above. Together with these results, e-2 was characterized as ethyl propyl phthalate (EPP) containing a deuterated ethyl group. Asymmetrical PEs such as MPP and EPP can be produced by esterification of carboxyl groups in monoester forms with a molecule of alcohol and transesterification of diester forms. However, the cultivation of mBP in the presence of CH_3OH did not result in the formation of butyl methyl phthalate, which is the corresponding asymmetrical form of mBP. This strongly suggests that asymmetrical PEs are produced by transesterification of diester forms and not by esterification of monoester forms.

Microbial Transformation Pathways of PEs Involving Transesterification

The time course of dPP biotransformation was investigated in order to evaluate the transformation pathways (Fig. 4). dPP disappeared within 7 d. MPP predominantly increased, leveled off on day 3 when its amount exceeded 30% of the initial dPP level, and then gradually decreased until it disappeared on day 14. The levels of dMP and mMP (hydrolyzed product of dMP) increased as the MPP level decreased. In the hydrolyzed product of dPP and MPP, mPP was the most abundant product after day 7. During the period of days 7–14, the amounts of mPP and mMP remained almost unchanged, whereas MPP and dMP were degraded further at different rates. This finding suggests that the R-3 bacterial strain preferentially degrades diester forms rather than monoester forms.

DISCUSSION

In general, during the biotransformation of PEs, each ester-alkyl chain undergoes sequential hydrolysis, which leads to the formation of monoesters and phthalic acid.[18] In the present study, we demonstrated the biotransformation of PEs via an alternative pathway; this pathway results in the production of unique asymmetrical PEs, such as MPP and EPP in dPP biotransformation. To the best of our knowledge, only few reports have described the formation of asymmetrical PEs via an alternative biotransformation pathway; however, the suggested mecha-

Fig. 5. Proposed Biotransformation Pathways of PEs in the Presence or Absence of Methanol

In the presence of methanol. Upper pathway, PEs undergo both ester hydrolysis and transesterification. In the absence of methanol. Lower pathway, PEs undergo transformation only via the sequential hydrolysis of esters. The carboxylic acid moiety of monoester forms did not undergo esterification with methanol. Under our experimental conditions (broken line). Further degradation of phthalic acid would occur in the presence of certain bacteria that can utilize phthalic acid as a carbon source (open arrows). For example: R_1 = propyl, R_2 = methyl. 1, dPP; 2, MPP; 3, dMP; 4, mMP; 5, mPP.

nisms in these reports differ from the one described in the present study. Cartwright *et al.* reported that soil bacteria degrade dEP to ethyl methyl phthalate in the presence of CH_3OH.[21] They concluded without performing additional experiments that ethyl methyl phthalate was produced by the demethylation of an ethyl group of dEP. Kim *et al.* also reported the formation of butyl methyl phthalate during the biodegradation of benzyl butyl phthalate by fungal cutinase and yeast esterase in the presence of CH_3OH.[22] In this study, by using a stable-isotope tracer technique, we clearly demonstrated that asymmetrical PEs were formed via transesterification with alcohols. Bacterial enzymes such as non-specific esterases could catalyze this reaction, because the crude enzyme extract from the bacterial strain R-3 also catalyzed the transesterification of PEs, as previously reported.[15]

The proposed new biotransformation pathways of PEs are shown in Fig. 5. PEs are hydrolyzed into monoester forms and phthalic acid (lower pathway in Fig. 5); however, in the presence of alcohol, a part of the PEs is transesterified into asymmetrical PEs and subsequently into diester and monoester forms (upper pathway in Fig. 5). This transesterification may occur in many PEs, because the transesterified products were detected during the biotransformation of dEP and dBP.[15] In the natural environment, PEs might have sufficient opportunities to undergo transesterification, because alcohols are generated through biodegradation, particularly under anaerobic conditions, *e.g.*, in sediments where PEs tend to accumulate. Moreover, the hydrosphere and geosphere are contaminated with alcohols produced industrially via household effluents and bioethanol-blended fuel. Although the hazardous properties of asymmetrical diesters are unknown, changes in ester-alkyl chain length and branching would certainly affect the toxicity of PEs. Further investigation into the environmental concentrations and biological effects of asymmetrical PEs will help understand the impact of PEs on humans and wildlife.

Acknowledgements This research was supported in part by the Academic Research Institute of Meijo University and by the Academic Frontier Project for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, 2007–2011.

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