

Full Paper

Natural Abundance ^{14}C Content of Dibutyl Phthalate (DBP) from Three Marine Algae

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Abstract: Analysis of the natural abundance ^{14}C content of dibutyl phthalate (DBP) from two edible brown algae, *Undaria pinnatifida* and *Laminaria japonica*, and a green alga, *Ulva* sp., revealed that the DBP was naturally produced. The natural abundance ^{14}C content of di-(2-ethylhexyl) phthalate (DEHP) obtained from the same algae was about 50-80% of the standard sample and the ^{14}C content of the petrochemical (industrial) products of DBP and DEHP were below the detection limit.

Keywords: brown alga; green alga.; ^{14}C natural abundance; dibutyl phthalate; di-(2-ethylhexyl) phthalate.

1. Introduction

Dialkyl phthalates, such as di-(2-ethylhexyl) phthalate (DEHP), diisononyl phthalate (DINP), diisodecyl phthalate (DIDP) and dibutyl phthalate (DBP), are petrochemicals used as plasticizers or solvents in a variety of industrial products. These chemicals have been thought to be environmental pollutants and detected in soils, sediments, terrestrial and marine waters, and also living organisms.

DEHP has been isolated from terrestrial and marine organisms including plants [1], marine algae [2-5], and fungal and bacterial culture broths [6,7]. However, it is difficult to determine whether DEHP was produced by these organisms, the sample was contaminated during the separation process, or DEHP accumulated in the organisms, because DEHP has been used abundantly in petrochemical phthalate esters. More than 65% of all the phthalate esters used in Japan in 2003 were DEHPs followed by DINPs (>26%), DIDPs (about 3%), and DBPs (about 1%), and this relative ratio has changed little in recent years.

Interestingly, there has been no report of the isolation of DINP and DIDP from organisms, whereas DBP has been isolated from plants, marine algae, bacteria, and fungi [2, 8-11]. Diethyl phthalate (DEP), a less common petrochemical product, was obtained as a chemotactic factor from the culture medium of *Helicobacter pylori* [12] and also detected in the broth of *Streptomyces* sp. isolated from Antarctic sediment [13]. Moreover, di-(2-methylheptyl) phthalate, which is not a petrochemical used in the chemical industry, was isolated from a plant *Hypericum hyssopifolium* (Guttiferae) [14]. Chen reported the incorporation of ^{14}C into DBP and DEHP by culturing the filamentous phase of a red alga, *Bangia atropurpurea*, with ^{14}C -enriched NaHCO_3 [2]. DBP was enriched by about 171 times the background level and DEHP, by 5.7 times.

These reports led us to investigate the origin of the phthalate esters more closely. Recently, Teuten et al. demonstrated that polybrominated diphenyl esters isolated from a True's beaked whale (*Mesoplodon mirus*) were naturally produced compounds and not derived from artificial products by analyzing the natural abundance ^{14}C content of the isolated compounds and industrial products [15,16].

Therefore, we intended to investigate if DBP and DEHP obtained from marine algae are natural products or contaminants of petrochemical products using a similar method [17,18].

In this report, we suggest that DBP isolated from the CH_2Cl_2 -methanol extracts of *Undaria pinnatifida* (Harvey) Suringar (Alariaceae, Phaeophyceae), *Laminaria japonica* Areschoug (Laminariaceae, Phaeophyceae) and *Ulva* sp. (Ulvaceae, Chlorophyceae) was a natural product based on an analysis of the natural abundance ^{14}C content of the isolates.

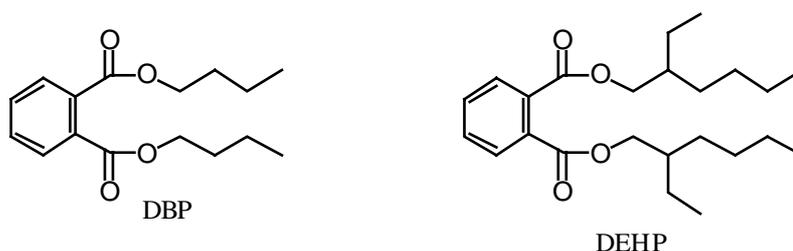


Figure 1. Structures of dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP).

2. Results and Discussion

Industrial products of DBP and DEHP contain very small amounts of ^{14}C which cannot be detected even by the most sensitive method, accelerator mass spectrometry (AMS), because these compounds are made from fossil oils. If these phthalate esters are produced by living organisms, natural abundance ^{14}C will be detected in the same amounts as modern carbon.

The isolation of DBP and DEHP was first examined using *Ulva* sp. collected in May 2005 to determine solvents suitable for the extraction process and procedures for concentrating lipophilic compounds. The conditions for HPLC and GC were established using authentic samples of DBP and DEHP.

The extraction and separation of *U. pinnatifida* were performed according to the procedures used for small amounts of *Ulva* sp. The aqueous residue obtained after the evaporation of organic solvents from the CH₂Cl₂-MeOH extract of the alga, was dissolved in MeOH and adsorbed on Hyflo Super-cell[®]. The adsorbent was placed in a glass column and lipophilic compounds were eluted with CH₂Cl₂. The lipophilic fraction was adsorbed on Hyflo Super-cell and applied to a silica gel column with hexane. The silica gel column was eluted successively with hexane and hexane-EtOAc (gradient). The eluate was monitored by TLC (hexane-EtOAc = 4:1) and three fractions containing DBP and/or DEHP were obtained. The first fraction (Fr. 1) contained DEHP and other compounds and the second (Fr. 2) and third (Fr. 3) fractions contained DBP, DEHP and other lipophilic compounds. DBP (1.24 mg) was separated by HPLC (ODS) with 80% MeOH-H₂O from Frs. 2 and 3, and DEHP was recovered by changing the solvent to 100% MeOH. This DEHP fraction was combined with Fr. 1 and DEHP (2.14 mg) was isolated by HPLC with 100% MeOH.

Since the particles of Hyflo Super-cell were too small, dispersion and formation of masses occurred during the evaporation of solvents when large amounts of extract or lipophilic fraction were adsorbed. Therefore, silica gel was used in lieu of Hyflo Super-cell for the separation from *L. japonica* and *Ulva* sp. The other separation procedures for these algae were the same as those for *U. pinnatifida*.

DBP and DEHP obtained from three algae were identified and analyzed the purity by ¹H NMR and GC-mass spectra. The ¹H NMR spectra of DBP and DEHP from *U. pinnatifida* were clean and only a very few faint signals due to contaminants were detected. On the other hand, pigments and lipophilic compounds disturbed the separation of DBP and especially DEHP from *L. japonica* and *Ulva* sp. DEHP fractions obtained by the first HPLC separation from *L. japonica* and *Ulva* sp. contained a large quantity of contaminants, which were most probably unsaturated fatty acids. These fractions were separated repeatedly by HPLC. The ¹H NMR spectra of the final fraction of DBP and DEHP from these two algae still showed the signals due to the contaminants. It is unclear if the difference in the amounts/species of contaminants between *U. pinnatifida* and the other two algae is due to the nature of these algae or the season when they were collected. *L. japonica* and *Ulva* sp. were harvested in the winter (cold water), when they usually contain more unsaturated fatty acids.

The concentration (w/w%) of DBP and DEHP in the final samples, analyzed by GC-MS utilizing the selected ion monitoring (SIM) method, was 60% (DBP) and 70% (DEHP) from *L. japonica* and 75% (DBP) and 60% (DEHP) from *Ulva* sp. It was not feasible to conduct further purification since the separation of contaminant(s) is difficult and would significantly reduce the amounts of DEHP. We thought that the measurement of ¹⁴C with these samples would give decipherable results because petrochemical compounds contain no measurable ¹⁴C and biogenetic products contain modern levels of ¹⁴C. Therefore, we submitted DBP and DEHP samples obtained from *L. japonica* and *Ulva* sp. without further separation, together with DBP and DEHP from *U. pinnatifida*.

Natural abundance ¹⁴C content was analyzed at the Tandetron AMS ¹⁴C Dating Laboratory, Center for Chronological Research at Nagoya University by the AMS method. The results are summarized in Table 1. The ¹⁴C concentration is shown as percent modern carbon (pMC) [19]. This unit represents a

percentage value of the ratio of sample ^{14}C concentration to the absolute ^{14}C concentration, which is defined as corresponding to AD1950.

Table 1. Natural abundance ^{14}C content of dibutyl phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP) of marine algal and industrial origins.

compound	source ^a	^{14}C content (pMC) ^b	compound	source ^a	^{14}C content (pMC) ^b
DBP	<i>U. pinnatifida</i>	249.4 ± 0.6	DEHP	<i>U. pinnatifida</i>	49.8 ± 0.2
DBP	<i>L. japonica</i>	281.2 ± 0.6	DEHP	<i>L. japonica</i>	85.6 ± 0.2
DBP	<i>Ulva</i> sp.	138.9 ± 0.4	DEHP	<i>Ulva</i> sp.	87.2 ± 0.2
DBP	industrial	0.21 ± 0.02	DEHP	industrial	0.11 ± 0.02

^a*U. pinnatifida*: *Undaria pinnatifida*; *L. japonica*: *Laminaria japonica*; industrial: petrochemical product.

^bPercent Modern Carbon: relative ratio (%) of the ^{14}C concentration of the sample to that of the standard.

The industrial samples of DBP and DEHP did not give measurable ^{14}C concentration as they are petrochemical products. The DBP samples from three algae contained modern ^{14}C , which indicated that DBP was synthesized naturally and not of industrial origin. The higher ^{14}C content in DBP can be ascribed to the enrichment of the ^{14}C level caused by the testing of nuclear bombs during the 1950s and 1960s [20]. The enrichment of the atmospheric ^{14}C is now 15% of the level before testing [21,22]. The ^{14}C may have accumulated in marine sediments.

Significant amounts of ^{14}C were detected in the DEHP samples from three algae. In the most reliable (highly purified) sample obtained from *U. pinnatifida*, about 50% of the ^{14}C concentration was modern carbon. The DEHP samples from *L. japonica* and *Ulva* sp. showed even higher percentages. The contaminant(s) (unsaturated fatty acid(s)) in the former two samples would raise the ^{14}C concentration. It may be that remarkable amounts of industrial DEHP were incorporated into the algae and/or contaminated the sample during the isolation procedures and that some amount of DEHP was biosynthesized to give the significant ^{14}C content.

This study revealed that DBP obtained from two edible brown algae, *U. pinnatifida* and *L. japonica*, and a green alga, *Ulva* sp., was a natural product since modern ^{14}C was detected in the isolated DBP samples.

DEHP may also be produced naturally, but the results of this study were not conclusive. The culture with ^{14}C -enriched NaHCO_3 gave no clear result for DEHP either [2]. Therefore, further studies will be required to confirm whether this compound is biosynthesized by these algae.

Di-(2-methylheptyl) phthalate isolated from *H. hyssopifolium* must be a biogenetic product of this plant [14], because it has not been synthesized. DBP and DEP detected in the culture media of microorganisms would also be produced by microorganisms [8-13]. These phthalate esters were in the environment long before their commercial manufacture and release.

Roles of phthalate esters are not known. It may be true that they are stored in the cell membrane and affect the flexibility of the cell [2]. The physiological functions of phthalate esters are interesting subjects for future studies.

3. Experimental Section

3.1. General experimental procedures

^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured on a JEOL AL-400 spectrometer in CDCl_3 . GC-MS data were obtained with a Shimadzu GCMS-QP5050A spectrometer using Class-5000 software and a TC-70 column (0.25 μm x 30 m): carrier gas, He (0.4 ml/min); interface and ion source temperatures, 300 °C. For a typical elution, the column temperature was first set at 100 °C for 5 min, raised to 300 °C at 2 °C/min, and kept at 300 °C for 20 min. DBP and DEHP were eluted at about 47 and 60 min, respectively. HPLC was carried out with a Hitachi L-2130 Pump and L-2400 UV Detector (280 nm) equipped with a D-2500 data processor using a Senshu Pack Pegasil-ODS column (10 mm x 250 mm) at a flow rate of 3 ml/min. DBP was eluted at about 20 min with 80% MeOH- H_2O . DEHP was eluted at about 10 min with 100% MeOH. Standard samples (petrochemical products) of DBP and DEHP were provided by SHOWA ETHER CO., LTD. and CG ESTER CORPORATION, respectively. All other solvents and reagents were of the highest grade commercially available and used without further purification.

3.2. Algal material and extraction

U. pinnatifida was collected at Kanazawahakkei, Yokohama, Japan in April 2005. The alga (about 13.5 kg) was cut into pieces and extracted four times with CH_2Cl_2 -MeOH (2:1, each 10.5 l). The organic extract was evaporated to give the aqueous residue (772.6 g). *L. japonica* was collected at Hachinohe, Aomori Prefecture, Japan in January 2006. The alga (about 9.3 kg) was cut into pieces and extracted four times with CH_2Cl_2 -MeOH (2:1, each 12 l). The organic extract was evaporated to give the aqueous residue (780.2 g). *Ulva* sp. was collected at Yokohama Marine Park, Yokohama, Japan in May and December 2005. The alga collected in December (about 13.5 kg) was cut into pieces and extracted four times with CH_2Cl_2 -MeOH (2:1, each 12 l). The organic extract was evaporated to give the aqueous residue (1.23 kg).

3.3. Isolation of DBP and DEHP

The aqueous residue obtained from the organic extract of *U. pinnatifida* was dissolved in MeOH and the solution was mixed with Hyflo Super-cell[®] (1 kg). MeOH and water were evaporated and the dried adsorbent was placed in a glass column (diameter, 70 mm; length, 600 mm). The column was eluted with CH_2Cl_2 (4 l) to give a lipophilic fraction (32.5 g). The lipophilic fraction was dissolved in CH_2Cl_2 , and the solution was mixed with 100 g of Hyflo Super-cell. After evaporation of the organic solvent, the dried adsorbent was mixed with hexane and poured on to a silica gel column (700 g) made up with hexane. The column was eluted with 2 l of hexane followed by hexane-EtOAc = 9:1 (2 l) and

4:1 (2 l). The eluates were monitored by TLC (hexane-EtOAc = 4:1) and divided into three fractions (Frs. 1-3). Fr. 1 contained DEHP and other compounds. DBP and DEHP were detected in two fractions (Frs. 2 and 3). Fr. 2 (120.2 mg) was separated by HPLC (ODS) with 80% MeOH-H₂O to obtain a DBP fraction and then a DEHP fraction was recovered with 100% MeOH. Fr. 3 (12.1 mg) was subjected to a similar HPLC separation to give DBP and DEHP fractions. Two DBP fractions were combined and separated by HPLC (80% MeOH-H₂O) to afford 1.24 mg of DBP. The DEHP fractions obtained from Frs. 2 and 3 were combined with Fr. 1, and DEHP (2.14 mg) was isolated by HPLC separation (100% MeOH).

The aqueous residue from *Ulva* sp. was dissolved in MeOH and the solution was mixed with silica gel (1.22 kg). MeOH and water were evaporated and the dried silica gel was placed in a glass column (diameter, 70 mm; length, 600 mm). The column was eluted with CH₂Cl₂ (3 l) to give a lipophilic fraction (216.8 g). The CH₂Cl₂ solution of the lipophilic fraction was mixed with 250 g of silica gel, and, after evaporation of the organic solvent, the adsorbed silica gel was applied to a silica gel column (800 g) with hexane. The column was eluted successively with 1 l each of hexane and hexane-CH₂Cl₂ = 9:1, 7:3, 1:1, 3:7, and 1:4. The eluates were monitored by TLC (hexane-EtOAc = 4:1) and divided into five fractions. DBP and DEHP were contained into two fractions (Frs. 3 and 4). Fr. 3 (127.1 mg) was separated by HPLC (ODS) with 80% MeOH-H₂O to obtain a DBP fraction and then a DEHP fraction was recovered with 100% MeOH. Fr. 4 (240.7 mg) was subjected to a similar HPLC separation to give DBP and DEHP fractions. The combined DBP fraction (12.8 mg) was further separated by repeated HPLC (80% MeOH-H₂O) to afford 1.78 mg of DBP. DEHP (3.87 mg) was isolated from the DEHP fraction (135.7 mg) by repeated HPLC separation (100% MeOH).

DBP (1.93 mg) and DEHP (2.47 mg) were isolated from the aqueous residue obtained from the organic extract of *L. japonica* by similar procedures.

DBP and DEHP isolated from these algae were identified based on the ¹H NMR and GC-mass spectra with the standard samples of DBP and DEHP.

3.4. Quantitative analysis of DBP and DEHP

DBP and DEHP isolated from *L. japonica* and *Ulva* sp. were analyzed by GC-MS using the selected ion monitor (SIM) method. The base peak at *m/z* 149 was selected for monitoring. A calibration table was created by injecting standard solutions containing 10, 5, 2, 1, 0.5, and 0.1 µg of the authentic DBP or DEHP. The calibration curve was calculated by linear-least squares regression. The samples in the same solvent were injected and the chromatogram was integrated in terms of concentration. The quantitative analysis of samples was performed between 0.5 and 2 µg/injection.

The GC column temperature used for the analysis of DBP was first set at 100 °C for 6 min, raised to 300 °C at 2 °C/min, and held at 300 °C for 20 min. DBP was eluted at around 50 min. The amounts of DBP isolated from *L. japonica* and *Ulva* sp. were about 60 and 75 (w/w)%, respectively.

The column temperature for DEHP was set at 100 °C for 6 min and raised to 300 °C at 4 °C/min, and DEHP was eluted at around 38 min. The amounts of DEHP isolated from *L. japonica* and *Ulva* sp. were about 70 and 60 (w/w)%, respectively.

3.5. Analysis of natural abundance ¹⁴C content

Natural abundance ^{14}C content was measured by the following three steps at the Tandetron AMS ^{14}C Dating Laboratory, Center for Chronological Research, Nagoya University utilizing a model 4130-AMS system [18,23]: (1) each organic compound was first burned into CO_2 , (2) the CO_2 was reduced to graphite, and (3) the graphite was analyzed by accelerator mass spectrometry (AMS) (Kitagawa, 1993; Nakamura et al., 2004). The results (Table 1) were expressed as percent modern carbon (pMC), a percentage value of the ratio of sample ^{14}C concentrations to the absolute ^{14}C concentration defined as corresponding to AD 1950 [17,19].

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