JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Uptake and Transformation of Pesticide Metabolites by Duckweed (*Lemna gibba*)

TAKUO FUJISAWA,* MOTOHIRO KUROSAWA, AND TOSHIYUKI KATAGI

Environmental Health Science Laboratory, Sumitomo Chemical Company, Ltd., 2-1, Takatsukasa 4-Chome, Takarazuka, Hyogo 665-8555, Japan

Uptake and transformation of ^{14}C-labeled metabolites from several pesticides, 3-methyl-4-nitrophenol (**1**), 3,5-dichloroaniline (**2**), 3-phenoxybenzoic acid (**3**), (*R*,*S*)-2-(4-chlorophenyl)-3-methylbutanoic acid (**4**), and (1*RS*)-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (**5**), were examined by using duckweed (*Lemna gibba*) in Hoagland's medium. More uptake into duckweed from the exposure water at pH 7.0 was observed for non-ionized **1** and **2** than for **3**−**5** in an ionized form, and their hydrophobicity accounted for these differences. While carboxylic acids **4** and **5** were scarcely transformed in duckweed, **1**−**3** mainly underwent phase II conjugation with glucose for **1** and **2**, malic acid for **3**, glutamic acid for **2**, and malonylglucose for **3**, the chemical identities of which were confirmed by various spectrometric analyses (LC−MS, LC−MS/MS, and NMR) and/or HPLC cochromatography with reference synthetic standards.

KEYWORDS: Uptake and metabolism in duckweed (*Lemna gibba*); metabolite of pesticide; phase II conjugation

INTRODUCTION

Pesticides and their degradates reach various water bodies mainly through direct application, spray drift, foliar washing by precipitation, erosion, and runoff from agricultural land. A critical aspect of environmental assessment is to determine their distribution, transformation, and accumulation in water, sediment, and biota in relation to ecotoxicological impacts. Although the fate of a pesticide and its metabolites has been extensively investigated in a water−sediment system (*1*), neither their effects on aquatic species nor their metabolic profiles therein have been extensively examined. Aquatic plants are known to be habitat and food for many species, and hence, the distribution and metabolism of pesticides in them become important in studying their effects on an aquatic ecosystem. Several investigators have studied the toxicity and/or metabolism of polychlorinated phenols or DDT using aquatic plants such as duckweed and elodea (*2*−*6*). Information about phase I metabolism in aquatic plants is very limited. It is reported that, as in higher plants, oxidation, hydrolysis, and decarboxylation proceed in duckweeds with no mineralization of chlorinated phenols or aniline, and phase II metabolism (conjugation) seems to occur rapidly to form glucose, malonylglucose, and aspartic acid conjugates as common pathways. However, information about its metabolic profile is still scarce for phenolic, anilinic, and carboxylic compounds which are main partial structures of pesticides and have been reported to be primary degradates and metabolites in the environment via photolysis, hydrolysis, and microbial degradation. The possible toxicity of transformation products

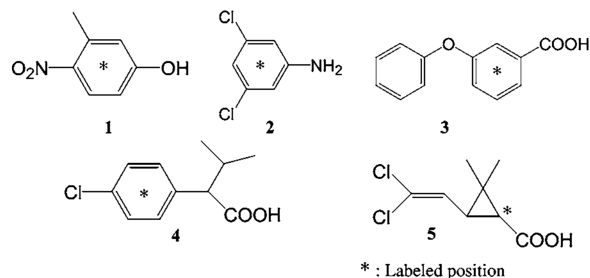

Figure 1. Chemical structures of ^{14}C-labeled **1**–**5**.

from these metabolites is a concern with respect to not only aquatic plants themselves but also aquatic species via food chains.

To collect the basic information about biotic transformations of pesticide metabolites, the aquatic angiosperm *Lemna gibba* (duckweed) was chosen as a model species for this study. Duckweeds are adapted to a wide variety of geographic and climatic zones and can be found in all but waterless deserts and permanently frozen polar regions (*7*). Members of the Lemnaceae family are ubiquitous, ecologically important, easily grown, and readily and reliably manipulated. Also, its value as a test species for toxicity assessment has been discussed worldwide, and it has been adopted as a test material in guidelines used for registration of agrochemicals (*8*). Given the wide distribution and ecological importance of duckweed, it is indispensable for aquatic risk assessment in determining its uptake and metabolic response to typical pesticide metabolites. However, there has not, to our knowledge, been any comparative study on pesticide metabolites in duckweed, simultaneously. From this standpoint, we have conducted an uptake and

* Corresponding author. E-mail: fujisawat1@sc.sumitomo-chem.co.jp.

10.1021/jf061301g CCC: $33.50 © 2006 American Chemical Society
Published on Web 08/02/2006

metabolism study in *L. gibba* using one phenolic, one anilinic, and three carboxylic compounds which are the primary degradates from various kinds of agrochemicals (organophosphate, pyrethroid, carbamate, etc.).

MATERIALS AND METHODS

Chemicals. The ^{14}C-labeled test substances (**Figure 1**), 3-methyl-4-nitrophenol (**1**), 3,5-dichloroaniline (**2**), 3-phenoxybenzoic acid (**3**), (*R*,*S*)-2-(4-chlorophenyl)-3-methylbutanoic acid (**4**), and (1*RS*)-*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (**5**), were prepared in our laboratory according to the reported methods (*9*−*12*). The chemical structures of radiolabeled compounds **1**−**5** were confirmed by LC−MS and by comparing their HPLC retention times with those of the non-radiolabeled reference standards. The specific activities of **1**−**4** uniformly labeled at the phenyl ring ([*phenyl*-^{14}C]**1**, -**2**, and -**3** and [*phenoxyphenyl*-^{14}C]**4**) and **5** labeled at position 1 of the cyclopropane ring ([*cyclopropane*-1-^{14}C]**5**) were ca. 5.0 and 2.0 GBq/mmol, respectively. Their radiochemical purity was more than 97% as determined by HPLC. The non-radiolabeled authentic standards, **1**−**3**, were purchased from Aldrich (Milwaukee, WI). **4** and **5** were synthesized in similar manners to the corresponding radiolabels. Glucose conjugates of **1** (**6**) and **2** (**7**) were synthesized by modifying the procedure reported by Sinnott et al. and Mitts et al., respectively (*13*, *14*). The acid chloride of **3**, prepared by treatment with oxalyl chloride, was reacted in tetrahydrofuran with dibenzyl 2-hydroxysuccinate, prepared from DL-malic acid, to produce the malate conjugate of **3** (**8**) with its two carboxylic acids protected by benzyl groups. The catalytic reduction of this derivative by 10% Pd-C under a hydrogen atmosphere yielded the authentic standard of **8**. All intermediates and **8** were purified by silica gel column chromatography. The chemical structures of **6**−**8** were confirmed by ^{1}H NMR (δ_H vs TMSP, parts per million) and LC−ESI−MS (*m/z*) spectrometries as follows. **6**: ^{1}H NMR δ 3.61 (s, 3H, aromatic-CH_3), 3.40−5.10 (m, 7H, sugar-H), 7.05−8.01 (d, 3H, aromatic-H); MS *m/z* 316 ([M + H]$^+$). **7**: ^{1}H NMR δ 3.30−4.60 (m, 7H, sugar-H), 6.75−6.80 (s, 3H, aromatic-H); MS *m/z* 324 ([M + H]$^+$). **8**: ^{1}H NMR δ 2.97 (dd, 2H, CHC*H*$_2$), 5.54 (t, 1H, C*H*CH$_2$), 7.05−7.86 (m, *9*H, aromatic-H); MS *m/z* 329 ([M − H]$^-$).

Other reagents were of the purest grade commercially available.

Spectroscopy. ^{1}H NMR and H−H COSY spectra were measured with a Varian Unity 400 FT-NMR spectrometer operating at 400.45 MHz with a 5 mm PFG ATB probe, using trimethylsilylpropionate-2,2,3,3-d_4 (TMSP) as an internal standard (δ = 0.0 ppm). Liquid chromatography−atmospheric chemical ionization−mass spectrometry (LC−APCI−MS) and liquid chromatography−electrospray ionization−mass spectrometry (LC−ESI−MS) in positive and negative ion modes were simultaneously performed using a Waters Micromass ZQ spectrometer equipped with Waters separation module model 2695 and photodiode array detector model 2996 as a liquid chromatograph. Liquid chromatography−electrospray ionization−tandem mass spectrometry (LC−ESI−MS/MS) in positive and negative ion modes with a collision energy of 10−40 V was conducted using a ThermoFinnigan TSQuantum instrument attached with an Agilent 1100 series liquid chromatograph. Samples dissolved in methanol were manually injected into an ionization source through a Sumipax ODS A-212 column (150 mm × 6 mm inside diameter, 5 μm, SCAS Co., Ltd.) with a flow rate of 1.0 mL/min using a gradient system with acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). The composition of the mobile phase was changed stepwise as follows: 10% A and 90% B at 0 min to 90% A and 10% B at 40 min (method A).

Radioassay. Radioactivity in the plant extracts and exposure water was determined by liquid scintillation counting (LSC). An aliquot of the sample was mixed with 10 mL of Packard Scintillator Plus and counted on a Packard model 1600TR and 2000CA liquid scintillation counter equipped with an automatic external standard. The average background of the LSC instrument was 30 dpm which was subtracted from the measured sample disintegrations per minute. Radioactivity in the unextractable residues from the treated plants was measured by using a Packard model 306 sample oxidizer. Unextractable plant residues were air-dried at room temperature overnight and weighed with a Mettler model AE240 scale. An aliquot of each sample was subjected to combustion. The $^{14}CO_2$ that was produced was absorbed into 9 mL of Packard Carb-CO$_2$ absorber, mixed with 15 mL of Packard Permafluor scintillator, and the radioactivity was quantified by LSC. The efficiency of combustion was determined to be greater than 90%.

Chromatography. High-performance liquid chromatography (HPLC) was carried out using a Shimadzu LC-20AT pump linked in series with an SPD-20A UV−vis detector and a Perkin-Elmer Radiomatic610TR radiodetector equipped with a 500 μL liquid cell. Ultima-Flo AP (Packard) was utilized as a scintillator. A Sumipax ODS A-212 column was employed for both analytical and preparative purposes with a flow rate of 1.0 mL/min. The following gradient systems were used for typical analysis, separation, and purification of the metabolites: 0.01% trifluoroacetic acid (solvent A) and acetonitrile (solvent B), 90% A and 10% B at 0 min, 10% A and 90% B at 40 min, 0% A and 100% B at 40.1 min, and 0% A and 100% B at 50 min (method B); 1/33 M phosphate buffer (solvent A) and acetonitrile (solvent B), 90% A and 10% B at 0 min and 10% A and 90% B at 40 min (method C). Retention times of **1**−**8**, the glutamate conjugate of **2** (**9**), and the malonylglucose conjugate of **3** (**10**) in method B are 17.4, 31.5, 29.1, 29.8, 32.0, 9.5, 18.2, 25.2, 22.9, and 22.3 min, respectively.

Thin-layer chromatography (TLC) was conducted using silica gel 60 F$_{254}$ thin-layer chromato plates (20 cm × 20 cm, 0.25 mm thick, E. Merck). The cochromatography between **8** isolated from duckweeds and its reference standard was carried out with a solvent system of toluene, ethyl formate, and formic acid (5:7:1, v/v/v). An autoradiogram was prepared by exposing the TLC plate to a BAS-IIIs Fuji Imaging Plate for several hours. The radioactivity on the imaging plate was detected by using a Typhoon (Amersham Bioscience Co., Ltd.), and the non-radiolabeled reference standard was detected by exposing the chromato plate to ultraviolet light. The typical R_f value of **8** was 0.35.

Plant Material, Maintenance, and Treatment. Duckweed (*L. gibba*) was obtained from a paddy field located at the Kasai experimental farm of Sumitomo Chemical Co., Ltd. (Hyogo, Japan). The duckweed plants were maintained in pots filled with a water/sediment system collected from the paddy field. The plants were grown in a greenhouse equipped with a quartz glass ceiling, and the temperature was kept at 25 °C. The plants were appropriately grown using Hyponex liquid fertilizer with an N:P:K ratio of 20:20:20 (Hyponex Japan). Prior to the experiments, sediment was removed from roots under running tap water for 10 min and sterilized with 0.5% sodium hypochlorite for 1 min (*15*), and the sodium hypochlorite was washed off the plants by dipping them into 100 mL of sterilized water. Hoagland's medium [pH adjusted to 7.0 with 1 N NaOH (*16*)] was sterilized by autoclaving the medium for 20 min at 120 °C with an SS-325 autoclave (Tomy). ^{14}C test substance was then dissolved in the medium, and the plants were added. The treated plants were grown in glass beakers without any closing caps in a greenhouse at 25 °C.

For investigation of the uptake of a chemical into duckweed, 0.1 μg/mL aqueous solutions (100 mL) of [^{14}C]**1**−**5** were individually prepared in 200 mL glass beakers. **1**−**5** were isotopically diluted with corresponding non-radiolabeled reference standards to give a total radioactivity of ca. 83 kBq (5 000 000 dpm) in exposure water. A 3 g sample of duckweed was exposed to each chemical, and sampling of plants and exposure water was conducted on days 1, 2, and 4. During the incubation, the mass of duckweed increased finally to 4.3−5.5 g.

To investigate metabolic profiles of each metabolite, 100 mL of aqueous solutions of [^{14}C]**1**−**5** at the exaggerated concentration of 1 μg/mL were each prepared in a 200 mL beaker. The total radioactivity in exposure water was set to be equal to those used for the plant uptake experiments by isotopically diluting ^{14}C labels with the corresponding reference standards. Sampling was conducted during the fourth day of exposure.

Extraction and Isolation of Metabolites. The harvested samples were first divided into duckweed and exposure water by filtering them through a glass wool plug. Sampled duckweeds and exposure water were stored in a freezer (less than −20 °C) and a refrigerator (<4 °C), respectively, until analysis was conducted. Duckweeds were cut into small pieces by using scissors and then extracted using a homogenizer (Nissei, AM-8) at 10 000 rpm for 10 min with an acetone/water mixture (4:1, v/v) at a ratio of 5 mL/g of plant. The mixture was filtered through a filter paper (pore size, 7 μm), and the residue was extracted two

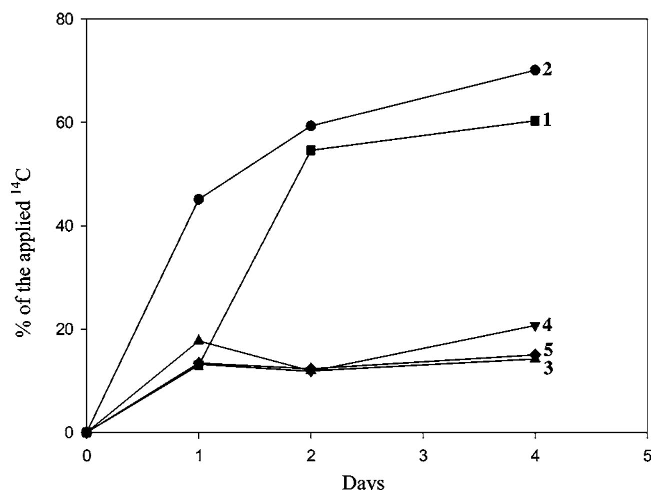

Figure 2. Radioactivity taken up by duckweed.

additional times in the same way. Aliquots of exposure water and plant extracts were individually radioassayed by LSC. Plant extract from the uptake experiment of **3** and plant extracts and exposure water from the metabolic experiments of **1**–**5** were analyzed by HPLC cochromatography with authentic standards. Plant residues were air-dried in open vessels at room temperature for 1 week, and subsamples of the dried residues were subjected to combustion analysis to determine the remaining radioactivity.

For the purpose of isolating unknown metabolites for spectroscopic analysis, plant extracts at the exaggerated concentration were subjected to preparative purification by the solid-phase absorbent Waters Sep-Pack C18 cartridge. First, the concentrated extracts were reconstituted in 5 mL of water, and the mixture was loaded onto the Sep-Pack cartridge. The cartridge was successively washed with 30 mL of water followed by 30 mL of methanol, and the water/methanol wash was repeated three times. From LSC and HPLC analysis, most of the unknown metabolites were eluted in the methanol fraction. Further purification was conducted with HPLC using method B.

RESULTS AND DISCUSSION

Recovery of Radioactivity. From the results of uptake experiments, a good recovery of [^{14}C]**1**–**5** from the test system was observed during the incubation for up to 1 day: 90.0% (**1**), 119.8% (**2**), 92.0% (**3**), 94.4% (**4**), and 92.4% (**5**) of the applied ^{14}C. However, it appears that the recovery of radioactivity decreased as the exposure period was extended. The ^{14}C recovery of [^{14}C]**1**–**5** after 4 days dropped to 78.0% (**1**), 80.8% (**2**), 76.1% (**3**), 80.7% (**4**), and 86.5% (**5**) of the applied ^{14}C, possibly due to azeotropic vaporization. The insignificant differences in the loss of ^{14}C were observed, although Henry's law constants of **1**–**5** estimated by EPI-Suite (*17*) greatly varied by a factor of 500: 1.61×10^{-8} (**1**), 8.11×10^{-6} (**2**), 5.27×10^{-8} (**3**), 3.81×10^{-7} (**4**), and 4.20×10^{-6} atm m^3 mol^{-1} (**5**).

Uptake of Test Compounds into Plants. The radioactivity taken up by duckweed was estimated by combining extractable and unextractable ^{14}C, the latter of which was found to be less than <1% of the total radioactive residues (TRR) in any case. The characteristics of uptake of radioactivity into duckweed are shown in **Figure 2**. A significant uptake of ^{14}C was observed for **1** and **2** (60.3% and 70.1% of the applied ^{14}C, respectively) and was close to reaching a plateau after 4 days. In contrast, much less but similar amounts of carboxylic derivatives **3**–**5** with very different chemical structures were shown to be taken up by duckweed, reaching a plateau after 1 day (14.2–20.7% of the applied ^{14}C). Phenoxyacetic and benzoic acid derivatives have been reported to be taken up by *Lemna minor* at pH 5.1, where the acids were partly undissociated to an extent that was

Table 1. Physicochemical Properties of **1**–**5**

compound	log P^a	pK_a^{a}	Henry's law constant^b
1	2.48	8.33	1.61×10^{-8}
2	2.69	2.37	8.11×10^{-6}
3	3.91	3.95	5.27×10^{-8}
4	3.38	4.65	3.81×10^{-7}
5	0.5 (pH 7)	5.02	4.20×10^{-6}

[a] Experimental values from refs *32–36.* [b] Calculated values using EPI-Suite, in atmospheres per cubic meter per mole.

Table 2. Radioactivity and Metabolite Distribution of **3**

	% of the applied ^{14}C		
	day 1	day 2	day 4
duckweed ^{14}C			
extractable	17.7	11.9	14.2
3	5.6	3.0	4.4
8	7.7	6.4	8.6
10	3.0	2.4	1.2
unextractable	1.0	0.6	1.0
exposure water ^{14}C	73.3	78.0	60.9
recovered ^{14}C	92.0	90.5	76.1

dependent on substituents at the phenyl ring (*18*). The physicochemical properties of each compound tested such as dissociation constant (pK_a) and octa-1-nol/water partition coefficient (log *P*) were likely to account for these profiles. Since the pH of the incubation medium was adjusted to 7.0, **1** and **2** existed completely in a non-ionized form as determined by their pK_a values (**Table 1**), while **3**–**5** would be mostly dissociated as carboxylate ions. Therefore, the ionized species were considered to be taken up much less by duckweeds, which indicated that the uptake is sensitive to the chemical structure. The efficiency of translocation of a chemical to shoots from root has been explained by Shone and Wood (*19*), Briggs et al. (*20*, *21*), and Hsu et al. (*22*), using a concept of TSCF (transpiration stream concentration factor) defined by (concentration in transpiration stream)/(concentration in exposure water). Briggs et al. (*20*, *21*) have suggested that there is an optimum lipid/water distribution for translocation, more polar or more lipophilic compounds being less well translocated when TSCF is investigated. These papers describe measurements of TSCF of barley and soybean plants hydroponically grown, using 18 and 12 non-ionized compounds, respectively, with a wide range of lipophilicity as measured by their log *P* values. The translocation from root to shoot versus log *P* plot showed a Gaussian curve with a maximum at a log *P* of 1.8 for barley (*21*) and a log *P* of 3.1 for soybean (*22*). If this relationship can be applied to the uptake of a chemical from root to tallus in duckweed, the pesticide metabolite with its log *P* value of 2–3 would have a great advantage in being taken up (high values of TSCF). These TSCF concepts seem to account for the uptake profiles of **1**–**5** in duckweed. Under the test condition at pH 7.0, **1** and **2** were efficiently taken up as determined by their log *P* values of non-ionized forms (2.48 and 2.69, respectively). The pK_a values of **3**–**5** (3.9–5.0) indicate that they are almost completely ionized in the incubation medium at pH 7.0. For the ionized species, the log *D* value instead of log *P* is used for evaluation of its partitioning ability. Log *D* is defined as the ratio of the equilibrium concentrations of all the species (non-ionized and ionized) of a molecule in octanol to that of the same species in the water phase at a given temperature, usually 25 °C. Generally, the log *D* value of

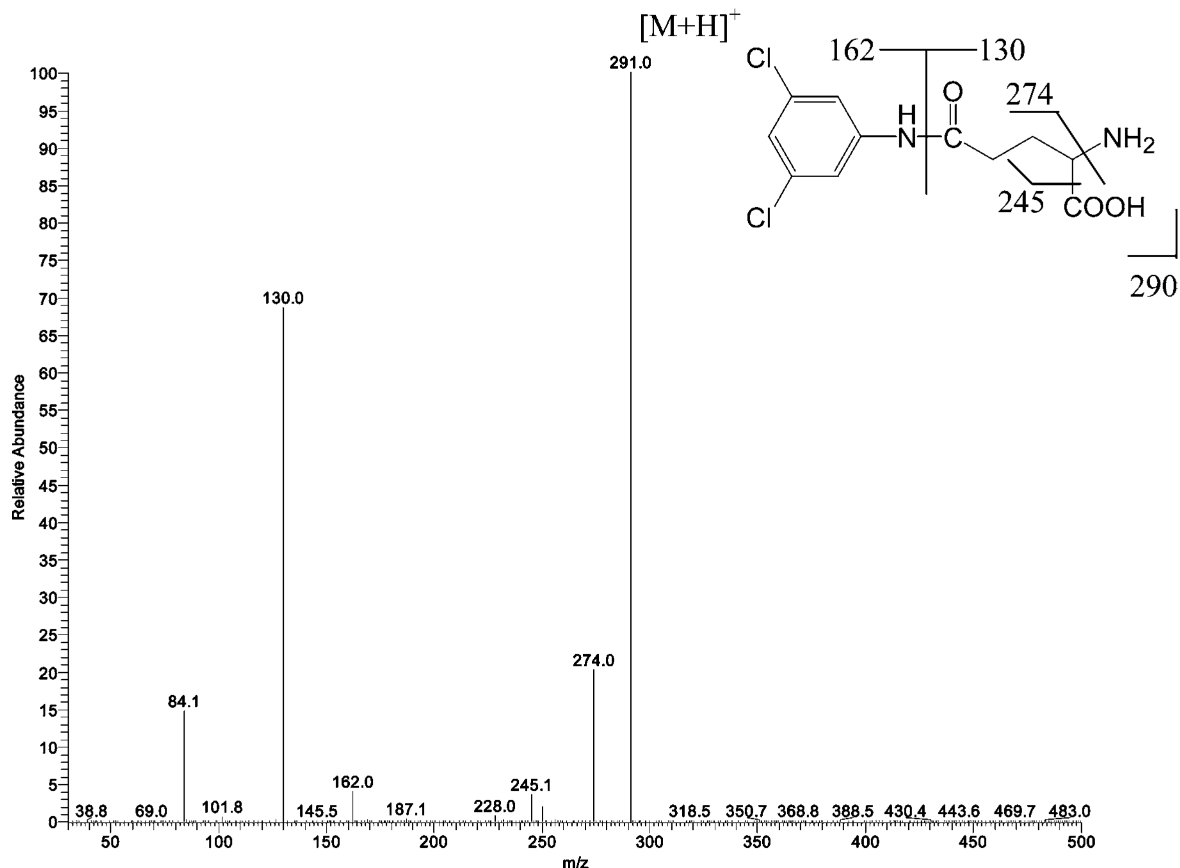

Figure 3. LC–ESI–MS/MS spectrum of **9**.

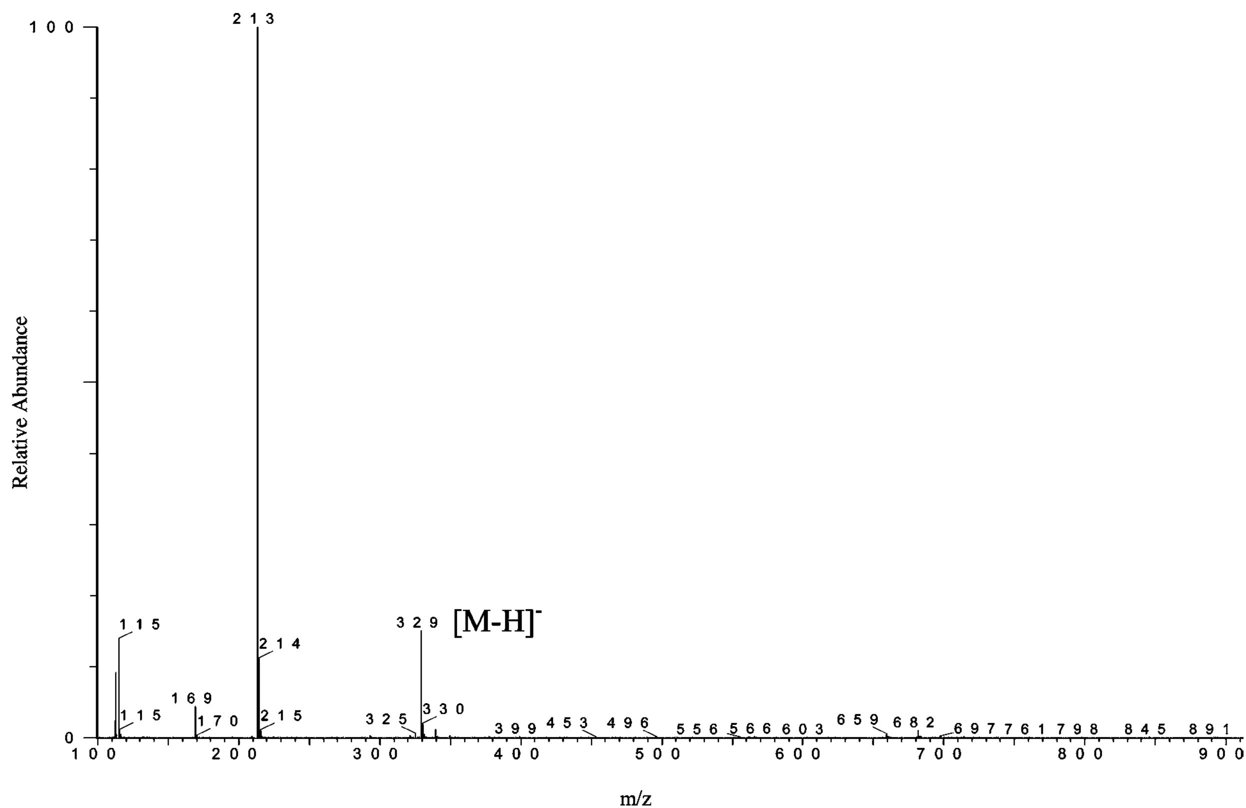

Figure 4. LC–ESI–MS spectrum of **8**.

carboxylate ion has been reported to be ~4.1 log units lower than the log *P* value of the corresponding undissociated form (*23*). Therefore, the fact that the log *D* values of **3**−**5** are likely to be in the range of −0.7 to 0.5 implies less favorable uptake of these compounds into duckweed, which is in accordance with the observation.

Distribution of Metabolites. The formation and declines of metabolites were examined for [^{14}C]**3** in detail through the

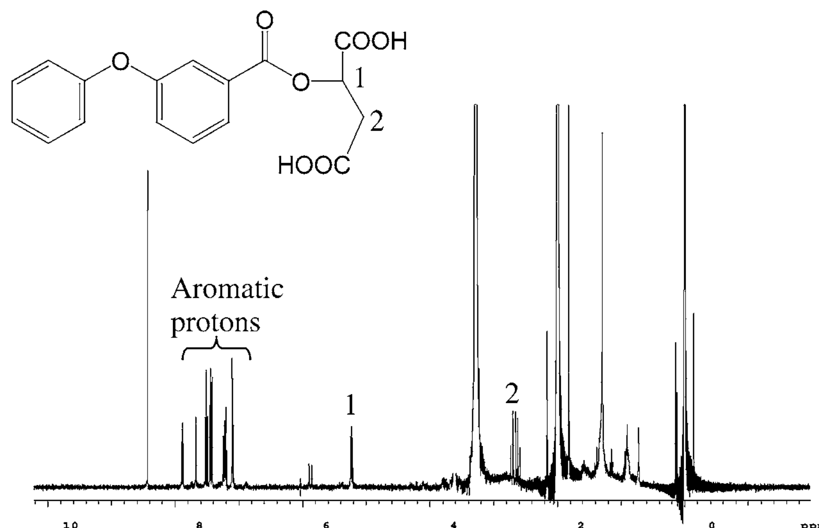

Figure 5. ^{1}H NMR spectrum of **8**.

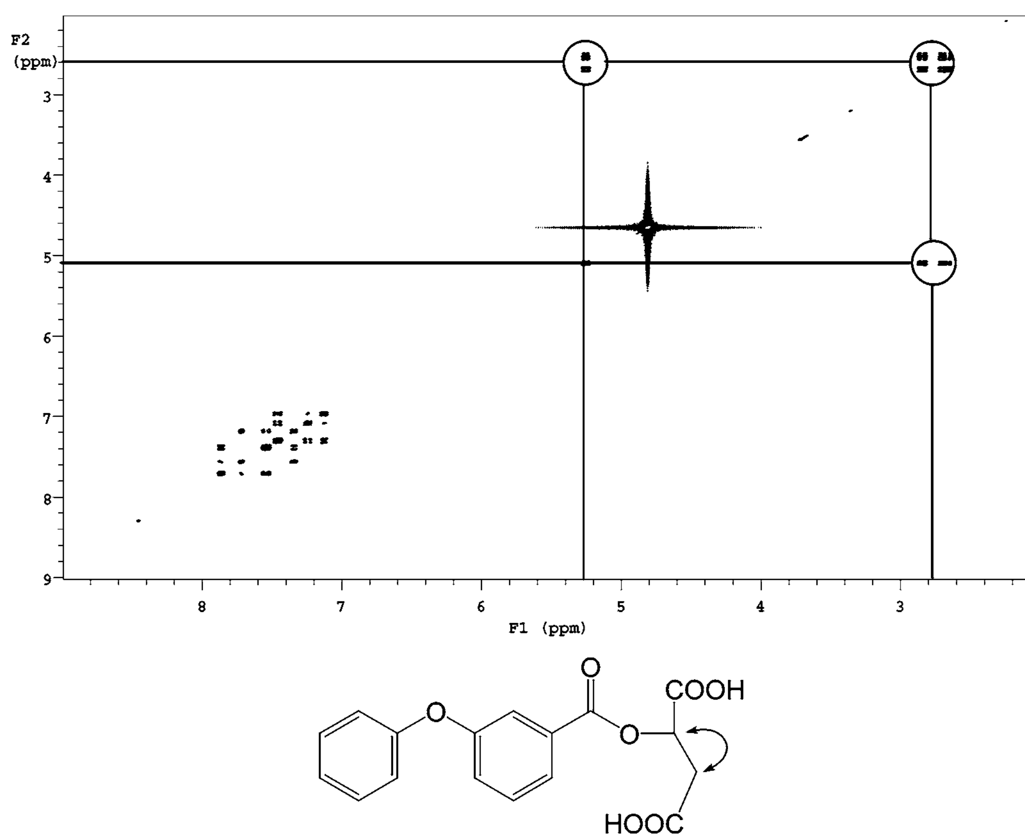

Figure 6. H–H COSY spectrum of **8**.

uptake experiment at 0.1 μg/mL. The product distribution is summarized in **Table 2**. After reaching a plateau at day 1, the amount of extractable fraction was almost constant during the incubation time. The amount of **3** remained almost constant around 30% of the extractable ^{14}C with metabolites **8** and **10**, and the amount of the latter gradually decreased with time. On the basis of this product distribution, the incubation period in the study examining metabolic profiles of [^{14}C]**1**−**5** at 1 μg/mL was taken to be 4 days after treatment of each chemical where uptake reached a plateau. The chemical identification of [^{14}C]**1**−**5** was confirmed by HPLC cochromatography with the corresponding authentic standard. With regard to phenolic compound **1**, **1** and **6** were the major components of the extractable ^{14}C after 4 days, amounting to 20.4% and 64.9% TRR, respectively. The remaining radioactivity (15% TRR) consisted of minor peaks, each of which was less than 4% TRR. Aniline derivative **2** was efficiently metabolized to **7** (51% TRR) and **9** (24% TRR), and **2** remained unchanged as 25% TRR. In the case of carboxylic acid derivatives **3**−**5**, clear differences were observed in the metabolic profiles. **3** existed as 29% TRR after a 4 day incubation period with metabolites **8** and **10** amounting to 57% and 8% TRR, respectively (**Table 2**), while **4** and **5** were scarcely metabolized (<4% TRR) in duckweed. Simultaneously, HPLC analysis of radioactivity in exposure water showed that any degradation of [^{14}C]**1**−**5** did not occur in the exposure water during the incubation period.

Identification of Metabolites. The chemical structure of metabolite **6** from **1** was confirmed to be an *O*-glucoside conjugate by HPLC cochromatography with the synthetic standard. This type of conjugation is well-known for the various

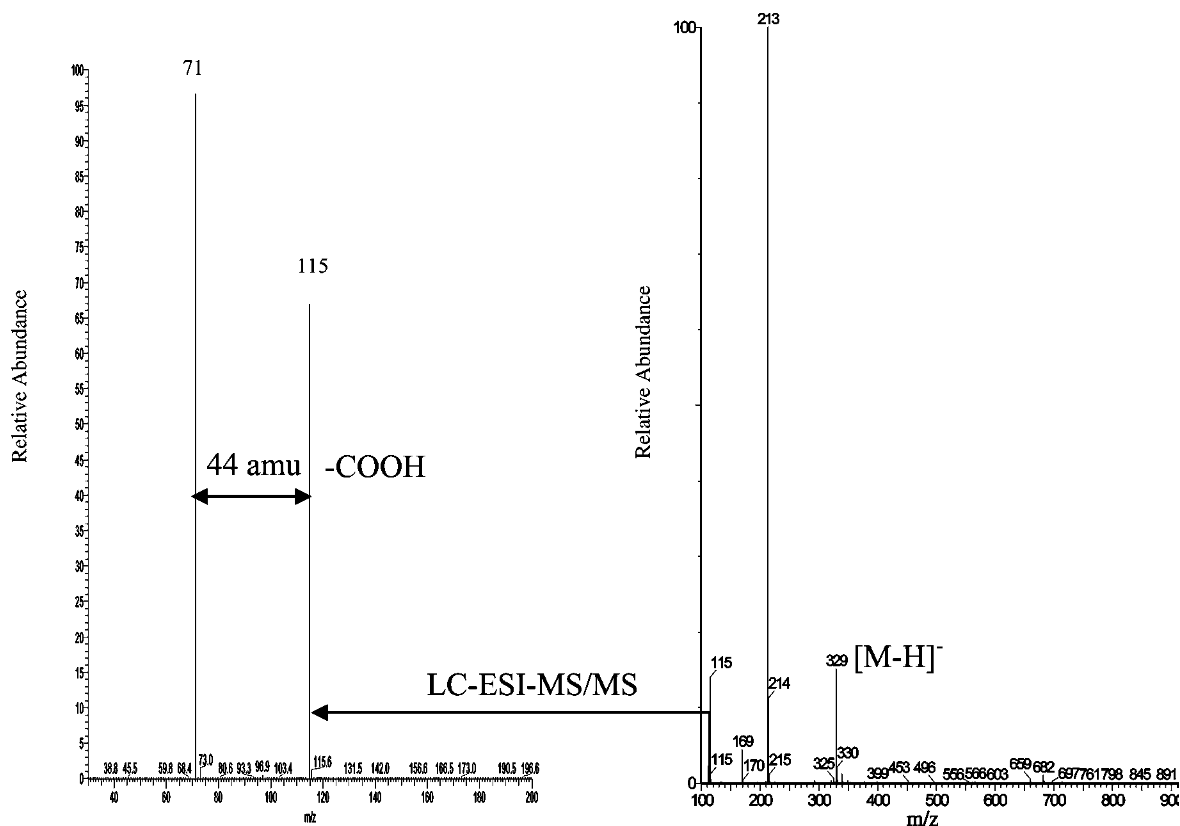

Figure 7. LC–MS/MS spectrum of the natural component portion constituting **8**.

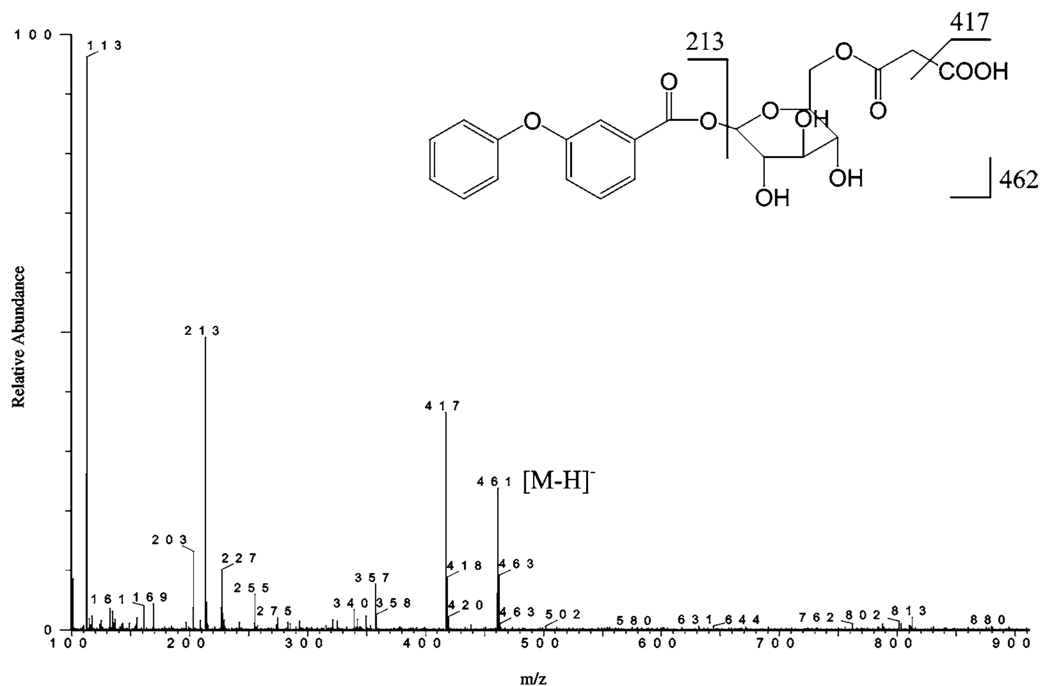

Figure 8. LC–ESI–MS spectrum of **10**.

types of phenols not only in terrestrial plants (*24*) but also in aquatic plants (*5*). In the case of **2**, *N*-glucoside **7** was formed as a major metabolite. The chemical structure of **7** was also confirmed by HPLC cochromatography with the synthetic standard. As an additional metabolic pathway of **2**, the conjugation with glutamic acid at the amino group to form metabolite **9** was confirmed. To examine the chemical identity of **9**, we first conducted LC−APCI−MS and LC−ESI−MS analyses in positive and negative ion modes. Metabolite **9** was shown to have a molecular weight of 291 ([M + H]$^+$) detected only by LC−ESI−MS in a positive ion mode. From an increase in the molecular weight of 129, some natural component was considered to attach to **2** and the chemical structure of **9** was assumed to be a glutamate conjugate of **2**. To further investigate the chemical nature of **9**, it was subjected to LC−ESI−MS/MS analysis in a positive ion mode, and a daughter ion pattern at *m/z* 291 was investigated (**Figure 3**). The mass fragment profile was totally consistent with the results reported by Mutlib et al. (*25*) for the glutamate conjugate of 1-[3-(aminomethyl)phenyl]-*N*-[3-fluoro-2-(methylsulfonyl)(1,1-biphenyl)-4-yl]-3-(trifluoro-

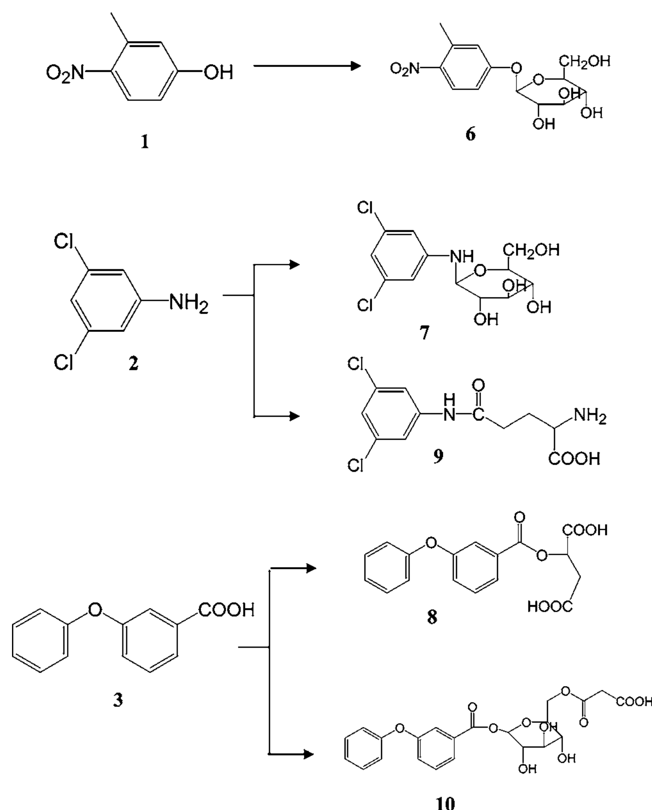

Figure 9. Proposed metabolic pathways of **1**–**3**.

methyl)-1*H*-pyrazole-5-carboxamide detected in rat, using LC−MS/MS, ^{1}H NMR, ^{13}C NMR, and HMBC. In conclusion, metabolite **9** was identified as the glutamate conjugate of **2**. This type of conjugation has been reported for phenoxyacetic acid herbicides in the experiments using tissue culture (*24*) and indole-3-acetic acid (*26*). Recently, Mitsou et al. (*2*) have reported N-acetylation of 3,4-dichloroaniline in the metabolism of propanil in *L. minor*, but the corresponding metabolite could not be detected in our study. Either the substituted positions of chlorine atoms in the phenyl ring or species differences may yield the different metabolic patterns.

Benzoic acid derivative **3** was found to undergo two metabolic transformations. The major pathway was formation of metabolite **8** which was subjected to LC−APCI−MS and LC−ESI−MS analyses in positive and negative ion modes. The molecular weight of **8** isolated from the extracts was most likely to be 330, as demonstrated by the LC−ESI−MS peak in the negative ion mode. The $[M - H]^-$ peak was observed at *m*/*z* 329 together with the daughter ion corresponding to $[M - \text{malate} - H]^-$ at *m*/*z* 213 (**Figure 4**). The ^{1}H NMR spectrum of isolated **8** (**Figure 5**) showed the nine aromatic protons observed at 7.05−7.86 ppm very similar to those of **3**, indicating that no structural change occurred in the phenoxyphenyl moiety of **3**. The methine proton observed at 5.21 ppm (triplet) and methylene ones at 2.85 ppm (double doublet) were demonstrated to be correlated by the H−H COSY spectrum (**Figure 6**), indicating the presence of the CH−CH_2 structure in **8**. To investigate the chemical identity attached to **8** in more detail, the corresponding daughter ion to the natural component (115 amu) was subjected to LC−ESI−MS/MS analysis in a negative ion mode. The decrease of 44 amu from the parent ion strongly suggested the existence of a COOH group (**Figure 7**). Furthermore, the existence of a carboxyl group was indirectly shown when **8** was analyzed with HPLC method C; the corresponding peak to **8** moved to an earlier retention time. Taking into account all the spectrometric and chromatographic evidence together with information about conjugation forms of other pesticides (*27*−*29*), we proposed the chemical structure of **8** to be the malate conjugate of **3**. Finally, the chemical identity of **8** was definitely confirmed by both HPLC and TLC cochromatographies with the reference standard synthesized in our laboratory. Several researchers suggested the presence of malate conjugates in barley, plant cell cultures, and rat (*27*−*29*) on the basis of mass spectrometry. The study presented here gave the first concrete evidence of the malate conjugate by the definite identification using HPLC cochromatography with synthetic standards together with LC−ESI−MS and NMR analyses.

The minor pathway was malonylglucoside conjugation which has been reported for many phenol metabolites of pesticides (*24*). For identification of **10**, LC−APCI−MS and LC−ESI−MS analyses in positive and negative ion modes were conducted. The *m*/*z* value of 462 for **10** showed that the natural component with a high molecular weight was linked to **3** as a conjugate (**Figure 8**). The profiles of the daughter ions corresponding to $[M - COOH]^-$ at *m*/*z* 417 and $[M - \text{malonylglucose} - H]^-$ at *m*/*z* 213 were compared in detail with those of the phenol metabolite from tolclofos-methyl (*30*). From these results, **10** was identified as the malonylglucose conjugate of **3**.

Interestingly, although phase I metabolism such as oxidation and reduction (*3*, *31*) was not detected, **1**−**3** underwent a different phase II metabolism which was possibly dependent on their chemical structure and functional group. **1**−**3** were all conjugated by glucose, but differences were observed in the conjugation with small organic acid derivatives (**Figure 9**). In contrast, simple acids **4** and **5** were scarcely transformed in duckweed, and therefore, either reactivity or steric hindrance at the carboxyl group may result in these differences versus **3**. This phenomenon will be an interesting research area to be pursued.

LITERATURE CITED

(1) Katagi, T. Behavior of pesticides in water-sediment systems. *Rev. Environ. Contam. Toxicol.* **2006**, *187*, 133−252.

(2) Mitsuo, K.; Koulianou, A.; Lambropoulou, D.; Pappas, P.; Albanis, T.; Lekka, M. Growth rate effects, responses of antioxidant enzymes and metabolic fate of the herbicide Propanil in the aquatic plant *Lemna minor. Chemosphere* **2006**, *62*, 275−284.

(3) Gao, J.; Garrison, A. W.; Hoehamer, C.; Mazur, C. S.; Wolfe, N. L. Uptake and phytotransformation of *o,p*-DDT and *p,p*-DDT by axenically cultivated aquatic plants. *J. Agric. Food Chem.* **2000**, *48*, 6121−6127.

(4) Sharma, H. A.; Barber, J. T.; Ensley, H. E.; Polito, M. A. A comparison of the toxicity and metabolism of phenol and chlorinated phenols by *Lemna gibba*, with special reference to 2,4,5-trichlorophenol. *Environ. Toxicol. Chem.* **1997**, *16*, 346−350.

(5) Barber, J. T.; Sharma, H. A.; Ensley, H. E.; Polito, M. A.; Thomas, D. A. Detoxification of phenol by the aquatic angiosperm, *Lemna gibba.* Detoxification of phenol by the aquatic angiosperm, *Lemna gibba. Chemosphere* **1995**, *31*, 3567−3574.

(6) Ensley, H. E.; Barber, J. T.; Polito, M. A.; Oliver, A. I. Toxicity and metabolism of 2,4-dichlorophenol by the aquatic angiosperm *Lemna gibba. Environ. Toxicol. Chem.* **1994**, *13*, 325−331.

(7) Skillicom, P.; Spira, W.; Journey, W. Duckweed aquaculture. A new aquatic farming system for developing countries. http://www.p2pays.org/ref/09/08875.htm, accessed in 2006.

(8) Swanson, S. M.; Rickard, C. P.; Freemark, K. E.; Macquarrie, P. Testing for pesticide toxicity to aquatic plants: Recommendations for test species. In *Plants for Toxicity Assessment: Second Volume*; Gorsuch, J. W., Lower, W. R., Wang, W., Lews, M. A., Eds.; American Society of Testing and Materials: Philadelphia, 1991; pp 77−100.

(9) Yoshitake, A.; Kawahara, K.; Kamada, T.; Endo, M. Labeled organophosphorus pesticide I. Synthesis of carbon-14 labeled *O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate (Sumithion). *J. Labelled Compd. Radiopharm.* **1976**, *13*, 323−331.

(10) Yoshitake, A.; Kanamaru, H.; Shono, F.; Nakatsuka, I. Carbon-14-labeling of optically active fenvalelate, α-cyano-3-phenoxybenzyl (*S*)-2-(4-chlorophenyl)-3-methylbutyrate. I. *J. Labelled Compd. Radiopharm.* **1981**, *18*, 391−402.

(11) Kanamaru, H.; Kamada, T.; Shono, F.; Nakatsuka, I.; Mohri, Z.; Okumura, T.; Yoshitake, A. Carbon-14-labeling of optically active fenvalelate, (*S*)-α-cyano-3-phenoxybenzyl (*S*)-2-(4-chlorophenyl)-3-methylbutyrate. *J. Labelled Compd. Radiopharm.* **1981**, *18*, 1283−1293.

(12) Yoshitake, A.; Nakatsuka, I.; Tsunematsu, H. Synthesis of a ^{14}C-labelled fungicide, *N*-(3,5-dichlorophenyl)-1,2-dimethylcyclopropane-1,2-dicarboxyimide-(carbonyl-14C) (Sumilex). *Radioisotopes* **1977**, *26*, 547−549.

(13) Sinnot, M. L.; Souchard, I. J. L. The mechanism of action of β-galactosidase. Effect of aglycone nature and α-deuterium substitution on the hydrolysis of aryl galactosidase. *Biochem. J.* **1973**, *133*, 89−98.

(14) Mitts, E.; Hixon, R. M. The reaction of glucose with some amines. *J. Am. Chem. Soc.* **1944**, *66*, 483−486.

(15) Landolt, E.; Kandeln, R. In *The family of Lemnaceae: A monographic study*, *Vol.* 2, Geobotanischen Institutes der ETH, Stiftung Rubel: Zurich, Switzerland, 1987; pp 638.

(16) Cleland, C. F.; Briggs, W. R. Flowering response of the long-day plant *Lemna gibba* G3. *Plant Physiol.* **1967**, *42*, 1553−1561.

(17) Meylan, W. M.; Howard, P. H. Bond contribution method for estimating Henry's law constants. *Environ. Toxicol. Chem.* **1991**, *10*, 1283−1293.

(18) Kenney-Wallace, G.; Blackman, G. E. The uptake of growth substances. XIV. Patterns of uptake by *Lemna minor* of phenoxyacetic and benzoic acids following progressive chlorination. *J. Exp. Bot.* **1972**, *74*, 114−127.

(19) Shone, M. G.; Bartlett, B. O.; Wood, A. V. Comparison of the uptake and translocation of some organic herbicides and a systemic fungicide by barley. II. Relation between uptake by roots and translocation to shoots. *J. Exp. Bot.* **1974**, *25*, 401−409.

(20) Briggs, G. G.; Bromilow, R. H.; Evans, A. A. Relationships between lipophilicity and root uptake and translocation of non-ionised chemicals by barley. *Pestic. Sci.* **1982**, *13*, 495−504.

(21) Briggs, G. G.; Bromilow, R. H.; Evans, A. A.; Williams, M. R. Relationships between lipophilicity and the distribution of non-ionised chemicals in barley shoots following uptake by the root. *Pestic. Sci.* **1983**, *14*, 492−500.

(22) Hsu, F. C.; Marxmiller, R. L.; Yang, A. Y. S. Study of root uptake and xylem translocation of cinmethylin and related compounds in depotted soybean roots using a pressure chamber technique. *Plant Physiol.* **1990**, *93*, 1573−1578.

(23) Hansh, C.; Leo, A. In *Substituent constants for correlation analysis in chemistry and biology*, John Wiley & Sons, Ltd., New York, 1979; pp 352.

(24) Cole, D. J.; Edwards, R. Secondary metabolism of agrochemicals in plants. In *Metabolism of agrochemicals in plants*; Robert, T., Ed.; John Wiley & Sons, Ltd.: New York, 2000; Chapter 14, pp 107−154.

(25) Mutlib, A.; Shockcor, J.; Chen, S.; Espina, R.; Lin, J.; Graciani, N.; Prakash, S.; Gan, L. Formation of usual glutamate conjugate of 1-[3-(aminomethyl)phenyl]-*N*-[3-fluoro-2-(methylsulfonyl)-[1,1-biphenyl]-4-yl]-3-(trifluoromethyl)-1*H*-pyrazole-5-carboxiamide (DPC 423) and its analogs: The role of γ-glutamyltranspeptidase in the biotransformation of benzylamines. *Drug Metab. Dispos.* **2001**, *29*, 1296−1306.

(26) Cooke, T. J.; Poli, D. B.; Sztein, A. E.; Cohen, J. D. Evolutionary in auxin action. *Plant Mol. Biol.* **2002**, *49*, 319−338.

(27) Juneja, P. S.; Gholson, R. K. Acidic metabolites of benzyl alcohol in greenbug resistant barley. *Phytochemistry* **1976**, *15*, 647−649.

(28) Schlepphorst, R.; Barz, W. Metabolism of aromatic acid in plant cell suspension cultures. *J. Med. Plant Res.* **1979**, *36*, 333−342.

(29) Suzuki, Y.; Yamaguchi, I.; Murofushi, N.; Takahashi, N. Biological conversion of benzoic acid in *Lemna paucicostata* 151 and its relation to flower induction. *Plant Cell Physiol.* **1988**, *29*, 439−444.

(30) Ichise-Shibuya, K.; Fujisawa, T.; Katagi, T.; Takimoto, Y. Metabolism of Tolclofos-methyl in lettuce (*Lactuca sativa*). *J. Pestic. Sci.* **2004**, *29*, 322−327.

(31) Hughes, J. B.; Shanks, J.; Vanderford, M.; Lauritzen, J.; Bhadra, R. Transformation of TNT by aquatic plants and plant tissue cultures. *Environ. Sci. Technol.* **1997**, *31*, 266−271.

(32) Schwarzenbach, R. P.; Stierli, R.; Folsom, B. R.; Zeyer, J. Compound properties relevant for assessing the environmental partitioning of nitrophenol. *Environ. Sci. Technol.* **1988**, *22*, 83−92.

(33) Bintein, S.; Devillers, J. QSAR for organic chemical sorption in soils and sediment. *Chemosphere* **1994**, *28*, 1171−1188.

(34) Hsu, F. C.; Kleier, D. A.; Melander, W. R. Phloem mobility of xenobiotics. II. Bioassay testing of the unified mathematical model. *Plant Physiol.* **1988**, *86*, 811−816.

(35) Liansheng, W.; Liangji, X.; Liqing, T.; Zhang, Z. Simultaneous determination of acid dissociation constants and partition coefficients of fourteen substituted phenylacetic acids. *Environ. Chem.* **1988**, *7*, 1−9.

(36) Bjarnholt, N.; Svensmark, B.; Hansen, H. C. B. HPLC method with on-line SPE perconcentration for quantification of permethric acid sorption to goethite. *Int. J. Environ. Anal. Chem.* **2004**, *84*, 303−314.

Received for review May 8, 2006. Revised manuscript received June 22, 2006. Accepted June 26, 2006.

JF061301G