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GLYCOSIDATION OF CHLOROPHENOLS BY *LEMNA MINOR*

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Abstract—Metabolic fate of xenobiotics in plant tissues has an important role in the ultimate fate of these compounds in natural and engineered systems. Chlorophenols are an important class of xenobiotics used in a variety of biocides and have been shown to be resistant to microbial degradation. Three chlorophenyl glycosides were extracted from tissues of *Lemna minor* exposed to 2,4-dichlorophenol (DCP). The products were identified as 2,4-dichlorophenyl-β-D-glucopyranoside (DCPG), 2,4-dichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside (DCPMG) and 2,4-dichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuranoside (DCPAG). Identification was based on reverse phase retention (C18), electrospray mass spectra collected in negative and positive mode (ESI-NEG and ESI-POS, respectively), and nuclear magnetic resonance (NMR) spectra comparisons to reference materials synthesized in the laboratory. Liquid chromatography-mass spectrometry (LC-MS) analysis of plants exposed to 2,4,5-trichlorophenol (TCP) formed analogous compounds: 2,4,5-trichlorophenyl-β-D-glucopyranoside (TCPG), 2,4,5-trichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside (TCPMG) and 2,4,5-trichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuranoside (TCPAG). Enzyme catalyzed hydrolysis with β-glucosidase was ineffective in releasing the β-glucosides with chemical modifications at C6. Presence of these glucoconjugates confirmed that *L. minor* was capable of xenobiotic uptake and transformation. Identification of these products suggested that chlorophenols were incorporated into vacuoles and cell walls of *L. minor*.

Keywords—Glycosides Phytoremediation Plant metabolism Xenobiotics

INTRODUCTION

Plants play an important role in the environment by providing habitat and food sources for a variety of ecosystems [1]. Xenobiotic accumulation in plant tissues may have significant impacts on the health of ecosystems and associated food webs. In addition, successful implementation of phytoremediation and natural attenuation strategies with aquatic plants requires positive identification of metabolites of plant origin to satisfy scientific and regulatory concerns.

Previous studies on plant metabolism of xenobiotics have described three types of cometabolic reactions, or phases, analogous to liver detoxification [2]. Phase 1 processes are characterized by chemical modification of xenobiotics, e.g., oxidation, reduction, or hydrolysis. Phase 1 products are usually hydrophilic and have reactive functional groups amenable to further modification. Phase 2 processes are condensations of xenobiotics or phase 1 products with common biomolecules such as carbohydrates, amino acids, or polypeptides. Phase 2 products have increased molecular weight and generally increased hydrophilicity [3]. Phase 3 processes result in excretion or sequestration of xenobiotics or metabolites into tissues that minimize toxic effects. Phase 3 processes in plants are dominated by sequestration [2].

Glycosidation, a typical phase 2 process, occurs in a variety of plant species. In one study, 28 of 31 plant species formed β-glycosides of 1,4-dihydroxybenzene or 1,3-dihydroxybenzene [4]. Also, a variety of herbicides have been shown to form β-O- and β-N-glycosides [5,6]. Glycosides of hydroxylated 2,4-dichlrophenoxyacetic acid (a phase 1 product) from soybean and wheat were identified by enzyme-catalyzed hydrolysis [7]. Similarly, β-glycosides of pentachlorophenol [7,8], trinitrotoluene [9], and trichloroethene [10] have also been reported using this technique. However, enzymatic hydrolysis has potential shortcomings associated with substrate specificity of enzyme preparations. In contrast, intact β-glycosides of phenol, 2,4-dichlorophenol, and 2,4,5-trichlorophenol have been extracted from axenic *Lemna gibba* [11,12].

Formation of malonate conjugates and malonate esterification of conjugates is widespread in plant studies with xenobiotics [5]. Malonyl addition to glucose is proposed to occur by esterification of malonyl-coenzyme A at C6. Malonate addition is hypothesized to block further metabolism by signaling sequestration of xenobiotics in vacuoles [13,14] or preventing incorporation of multiple carbohydrate units onto the glycoside [15]. Malonyl glycosides of pentachlorophenol [16] and DCP [17] previously have been reported with axenic plants and cell cultures.

The objectives of this study were to demonstrate that plant metabolism plays an important role in active xenobiotic removal in systems closely resembling a natural aquatic ecosystem with naturally occurring microbial populations and provide positive identification of metabolites in plant tissues. Toward this end, an experimental system was developed using *Lemna minor* because it is easily cultured and has a recognized role in toxicological investigations [18–20]. Both DCP and TCP were chosen as representative xenobiotics due to use of these compounds and their derivatives in a variety of biocides, e.g., 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, and triclosan.

MATERIALS AND METHODS

Materials

All chemical reagents were reagent grade or better. DCP, TCP, α-bromo-(2,3,4,6-tetra-O-acetyl)-D-glucopyranose, almond meal, deuterium oxide, malonic acid, sodium methoxide, benzoyl chloride, 1,2:3,5-di-O-isopropylidene-α-D-apiose, D-

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arabinose, β-bromo-(2,3,4-tri-O-acetyl)-L-arabinopyranose, and *tert*-butyl isocyanide were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN), acetic acid, sodium hydroxide, phosphoric acid, ethyl acetate, dichloromethane, and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Lemna minor was obtained from stock sources maintained at an outdoor, pilot-scale artificial wetland. A portion of the culture was acclimated to laboratory conditions and maintained in 60-L containers over several months. Each 60-L unit contained approximately 20 L of water, a 5 to 10 cm layer of commercial potting soil [21], and complete surface coverage by *L. minor*. Water was replenished on a weekly basis with house service deionized water, and potting soil was added every two to three months.

Media for exposure assessments was prepared by filtering (Whatman GF/D, Clifton, NJ, USA) stock culture media. A typical media preparation contained 213 ± 35 mg/L suspended solids and 30 ± 6 mg/L chemical oxygen demand. Desired initial concentrations of chlorophenols were prepared in single batches and divided among experimental units. Experimental units consisted of 250-mL Erlenmeyer flasks containing 100 mL of media and 3.5 to 4.0 g fresh wt (gFW) of *L. minor*. Experimental units were closed to the atmosphere with butyl-rubber stoppers. Reported values are averages and standard deviations of at least three experimental units unless otherwise noted.

Plant tissue samples were prepared by separating intact plants from media with a course screen (1–2 mm). Plants were air-dried for 30 min and blotted dry with a paper towel. Plant mass (gFW) obtained from an experimental unit was recorded prior to freezing (-80°C). Frozen plants were ground to powder with precooled (-80°C) mortars and pestles. Frozen tissues were extracted three times with 5-mL aliquots of 80% ACN per gFW. Solids were separated from each aliquot by centrifugation. Centrifugate was collected after each extraction and combined (crude extract). Analytical extracts were prepared by eluting 10-mL crude extract over a preconditioned 1 g × 6-mL solid phase extraction (SPE) tube (Supelco LC-18, Bellefonte, PA, USA). Tubes were preconditioned by eluting 5-mL ACN and, subsequently, 5-mL 5%-ACN. Tubes were flushed with an additional 3-mL ACN. Under these conditions, interfering compounds such as chlorophyll were retained on the SPE tube and target analytes were not retained. The 10-mL sample load and 3-mL flush were combined for analysis. Final volume of eluent was determined by recording mass and density of eluent. Resulting solutions were diluted 1:1 with water and analyzed by LC-MS. In some cases, extracts were further concentrated by evaporation under a stream of N_2. In these cases, phenolic concentrations were determined before evaporation of sample. Large-scale extracts for ^{1}H-NMR analysis were prepared in a similar manner with 10 g × 60-mL SPE tubes. Larger tubes were preconditioned with 50 mL ACN and 50 mL 10% ACN. Analytes were flushed with an additional 50-mL ACN. Large-scale extracts were concentrated by evaporation under a gentle stream of N_2. For experiments with inactivated plants, living plants were treated with two freeze-thaw cycles (-80–50°C) over 30 min, pretreated with 2 g/L NaN_3 for 24 to 48 h or macerated with a laboratory blender. Inactivated tissues were then treated in the same manner as active plants.

High-performance liquid chromatography-MS

Liquid chromatography-MS was performed with an Agilent (Palo Alto, CA, USA) Model 1100 equipped with a XDB C-8 guard column (2.1 × 12.5 mm, 5 μm, Agilent) and SB-C18 analytical column (2.1 × 150 mm, 5 μm, Agilent). Reverse-phase separation of native glycosides was accomplished with linear gradients. Eluent flow rate was 0.3 mL/min with a 10-μL injection volume. Solvent A contained 0.02% glacial acetic acid and 5% ACN in water. Solvent B was ACN. Eluent was held at 5% B for 4 min, raised from 5 to 38% B at 25 min, raised from 38 to 100% B at 33 min, and held at 100% B until 50 min. All separations were conducted at 35°C. Glycosides and phenols were detected by ultraviolet absorbance (λ = 210 nm, bandwidth = 16 nm) with a diode array detector and a single quadropole mass selective detector via an electrospray interface (ESI) in positive (ESI-POS) or negative (ESI-NEG) mode. The drying gas, N_2, was delivered 10 L/min at 350°C and a nebulizer pressure of 2.07 × 10^5 Pa (30 psig). Capillary and fragmentor voltages were maintained at 4000 V and 80 V, respectively. The mass spectrometer was operated in scan mode from *m*/*z* 50–1000. Aqueous and extracted samples were amended with 0.1-mL of 1 M acetate buffer (pH 5.0) per mL sample and filtered through a 0.2 μm perfluorotetraethylene filter prior to analysis.

Perbenzoylated carbohydrates were also separated on the Agilent 1100 system with eluents identical to chlorophenol analyses. The gradient began at 65% B, increased to 88% B in 30 min, and immediately stepped to 100% B for 10 min. Separations were carried out at 35°C. Perbenzoylated carbohydrates were monitored at 230 nm diode array detector and a mass selective detector equipped with an atmospheric pressure chemical ionization interface (APCI). The APCI data were collected in positive mode with corona current of 5 μA, capillary voltage of 2500 V, N_2 flow rate of 4 L/min at 350°C, vaporizer temperature at 325°C, and nebulizer pressure of 4.14 × 10^5 Pa (60 psig). Retention was recorded relative to excess benzoic acid in final product mixture.

Preparative chromatography

Preparative liquid chromatography was performed on an Agilent 1050 system equipped with a SB-C18 guard column (4.5 × 12 mm, 5 μm, Agilent) and SB-C18 analytical column (4.5 × 250 mm, 5 μm, Agilent). Eluents were identical to the analytical system. Preparative separations were performed with an injection volume of 0.1 mL. Preparative samples were eluted with a linear gradient, 0 to 65% B over 40 min. Eluent composition was immediately switched to 100% B for an additional 10 min. Separations were performed at 35°C. Fractions were collected from LC effluent with a Foxy Jr. fraction collector (ISCO, Lincoln, NE, USA).

Metabolite synthesis

Both 2,4-dichlorophenyl-β-D-glucopyranoside (DCPG) and 2,4,5-trichlorophenyl-β-D-glucopyranoside (TCPG) were synthesized by adaptation of the Koenigs-Knorr method [12]. Approximately 9 mmol of parent phenol (DCP or TCP) and 5 to 6 mmol of α-bromo-(2,3,4,6-tetra-O-acetyl)-D-glucopyranose were dissolved in 10 mL 1 M NaOH and 15 mL of acetone. Reaction mixtures were stirred overnight in closed flasks. Acetone was removed by evaporation under a stream of N_2 leaving slurries of water and precipitate. Slurries were diluted to 75 mL with water and extracted three times with

30-mL aliquots of dichloromethane. Aqueous layers were discarded. Organic layers were extracted three times with 30-mL aliquots of 1 M NaOH. Organic fractions were evaporated to dryness under a stream of N_2. Resulting solids were redissolved in 30 mL warm methanol containing 16 μM sodium methoxide (~ 35°C). Mixtures were heated until solutions became homogenous. After 1.5 h, 0.4 mL 2 M HCl was added to quench the reaction. Solutions were evaporated to dryness under N_2. Further purification of the material was accomplished using the preparative chromatographic method. Typical yields of purified products were approximately 45%.

Malonyl glycosides were synthesized by adapting the method of Roscher et al. [22]. One hundred mg of parent glycoside (DCPG or TCPG) were combined with three molar equivalents of malonic acid. Contents were dissolved in 10 mL of previously dried ACN. Acetonitrile was dried by saturation with anhydrous sodium sulfate and allowed to stand at least 1 h prior to use. Reactions were initiated by adding 1.5 molar equivalents of *tert*-butyl-isocyanide to solutions. Mixtures were immediately closed and heated to 50°C for 1 h. Mixtures were removed from heat and stirred overnight. Solvent was removed by evaporation under a stream of N_2. Reaction resulted in approximately 50% conversion to several isomers. Desired products were separated from reaction mixtures by preparative chromatography. Typical yields of desired products were approximately 12%.

Perbenzoylation

Carbohydrate-containing samples were hydrolyzed in 1.2 M HCl at 100°C for 30 min prior to derivatization [23]. Perbenzoylation of hydrolysates was carried out by a modification of the method of Oehlke et al. [24]. A 100-μL sample of hydrosylate was combined with 240 μL 8 M NaOH and vortexed. Perbenzoylation was initiated by adding 40-μL benzoyl chloride. Reaction mixtures were vortexed 5 min. Immediately reaction mixtures were acidified with 40 μL 1.4 M H_3PO_4 and extracted with a 400-μL aliquot of ethyl acetate (vortexed 1 min). Three hundred μL of ethyl acetate extract were pipetted off the mixture and evaporated to dryness under N_2, redissolved in 60% ACN, and analyzed by LC-APCI-MS. Reference chromatograms were prepared from hydrolysates of D-arabinose, D-galactose, D-xylose, D-glucose, 1,2:3,5-di-O-isopropylidene-α-D-apiose (yielded free apiose), and β-bromo-(2,3,4-tri-O-acetyl)-L-arabinopyranose (yielded L-arabinose). Identifications were based on relative retention to benzoic acid, product distribution, and APCI mass spectra.

Benedict's test

Benedict's solution was prepared by addition of 1.7 g cupric sulfate, 17.3 g sodium citrate, and 11.7 g sodium carbonate to 70 mL water. Reactions were carried out at 50°C. One mL of Benedict's reagent was added to 2 mL of sample (~ 10 mM in analyte). After 1 h, reducing sugars produced a red precipitant, Cu_2O [25]. Glucose was used as a positive control and sucrose was used as a negative control.

Linkage analysis

A sample of DCPA was submitted to the Complex Carbohydrate Research Center (CCRC), University of Georgia (Athens, GA, USA) for glycoside linkage analysis. Samples were dissolved in dry dimethyl sulfoxide saturated with NaOH. Two sequential additions of methyl iodide were added to methylate free hydroxyl groups [26]. Samples were then hydrolyzed for 3 hr in 2 M trifluoroacetic acid at 100°C. Partially methylated residues were reduced to alditols with deuterated sodium borohydride and acetylated with pyridine/acetic anhydride. Partially methylated alditol acetates were analyzed by GC-MS (Hewlett-Packard 5970, Palo Alto, CA, USA) with electron impact ionization. Products were separated on a Supelco 2330 fused-silica capillary column (30 m) with a thermal gradient: 80°C for 2 min, raised to 170°C at 30°C/min, raised to 240°C at 40°C/min and held at 240°C for an additional 5 min [27].

^{1}H-NMR

All samples were prepared from the purest solutions available. Synthetic and extracted samples (2–5 mg of dry product) were dissolved in deuterium oxide (HOD, 99.9%). Correlation spectroscopy (COSY) and proton, ^{1}H, spectra were obtained on a Bruker DRX 500 (Billerica, MA, USA). All shifts were normalized to the residual deuterated water (HOD) signal, $\delta = 4.5$.

Hydrolysis

Enzymatic hydrolysis was accomplished with β-glucosidase from almond meal. Enzymatic hydrolysis was carried out in 0.1 M acetate buffer (pH 5.0). β-Glucosidase was dissolved at 1 to 3 mg/mL almond meal in pH 5.0 acetate buffer and filtered (0.2 μm) to remove particulate matter. Reactions were initiated by adding 0.1 mL of almond meal solution to 1.0 mL of aqueous test solutions. Dilute acid hydrolysis was carried out in 0.02 M HCl at room temperature. Acid hydrolysis was carried out in 1.2 M HCl at 55°C.

RESULTS AND DISCUSSION

To examine xenobiotic fate in plant controls, the extraction procedure was tested for DCP with inactivated *L. minor* with initial concentrations of 5–100 μM DCP. *Lemna minor* inactivated by freeze-thaw cycles rapidly sorbed approximately 20% of DCP from media reaching equilibrium in less than 6 h. Average recovery of DCP from inactivated systems was 98.9 ± 11.9% (5 levels, 3 replicates each). The TCP also rapidly reached equilibrium with inactivated tissue under similar experimental conditions. Plants pre-exposed to 2 g/L NaN_3 exhibited similar sorptive capacity to thermally inactivated tissues. In neither case were any transformation products observed in media or plant tissues. In a further effort to separate plant metabolism from microbial processes, active plants were macerated and immediately exposed to chlorophenols for periods up to 48 h. Again, these systems exhibited no evidence of the metabolites found in tissues of active plants.

Like systems containing inactive plants, no metabolites were detected in media of systems containing active plants. However, active *L. minor* removed chlorophenols from media at rapid rates. For example, pseudofirst-order removal rate constants of 0.01 to 0.17 h^{-1} were measured in systems with initial concentrations of 3–100 μM DCP and nominal plant mass of 3.5 to 4.0 gFW *L. minor* in 100 mL media. Exclusion or removal of active *L. minor* from the system halted aqueous removal of chlorophenols and eliminated suspended microbial activities as possible causative agents for observed removals.

Extracts prepared from the tissues of active plants indicated that transformation products were present in plant tissues. A chromatogram of a typical active plant extract from an experiment with DCP is shown in Figure 1. Despite numerous peaks in ultraviolet and MS traces, chlorophenol derivatives were identified from extracted ion chromatograms (EIC) for

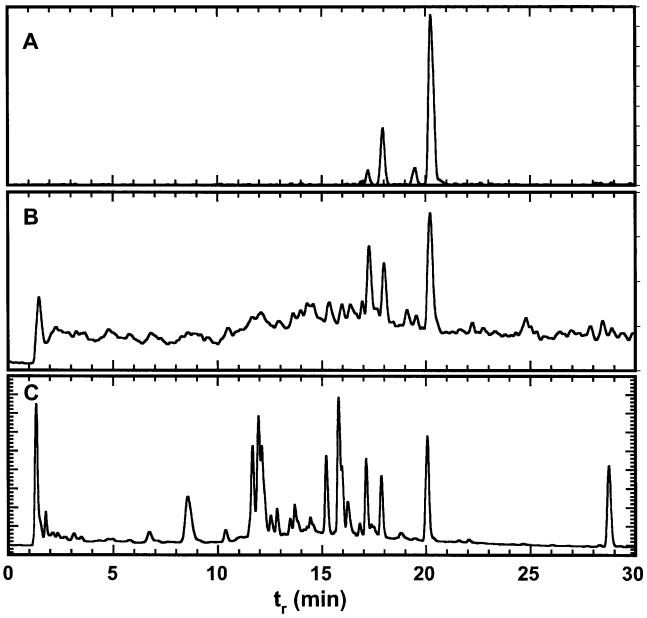

Fig. 1. Typical chromatographic trace of active plant extracts exposed to 2,4-dichlorophenol. Trace A collected by extracting 161 from trace B, the total ion chromatogram. Trace C was collected by ultraviolet absorbance at 210 nm.

mass to charge ratios (*m*/*z*) of ESI-NEG base peaks of parent phenols, i.e., *m*/*z* 161 for DCP and *m*/*z* 195 for TCP. The EIC traces were also examined for potential phase 1 metabolites: Dichlorodihydroxybenzenes (*m*/*z* 177), chlorophenols (*m*/*z* 127), chlorohydroquinones (*m*/*z* 142), and chlorodihydroxybenzenes (*m*/*z* 144). No conclusive evidence was found for any phase 1 metabolites in media or plant tissues.

Examination of plant extract ESI-NEG mass spectra of the peaks in Figure 1 showed six chromatographic peaks with recognizable dichlorophenyl signatures. The peak at 29 min was identified as DCP by comparison of retention time (t_r) to standard material. Peaks at 19.5 min and 16.8 min provided limited structural information and were not observed consistently. Remaining peaks, designated DCPA (t_r = 17.0 min), DCPB (t_r = 17.8 min), and DCPC (t_r = 21.0 min) were consistently observed. Degree of chlorination for m + 2 ion clusters in each mass spectrum was determined by comparison to hypothetical values based on relative abundance of $^{35}Cl/^{37}Cl$ ($\sim$ 3:1). It was observed that ESI-NEG spectra for DCPA, DCPB, and DCPC contained high *m*/*z* values with double the number of chlorine atoms from parent compounds. For example, the mass spectrum of DCPC had an m + 2 ion cluster *m*/*z* = 819 with four Cl atoms, and ESI-POS spectra of DCPC contained a single ion cluster at *m*/*z* 433 = 409 + 24 with two Cl atoms. Therefore, it was deduced that the compounds formed dimers in negative mode and Na^+ adducts in positive mode. With this information, nominal molecular weights of the compounds represented by these peaks were established and used to generate mass assignments in Table 1. Chromatograms of extracts from plants exposed to TCP contained analogous peaks, TCPA, TCPB, and TCPC, with nominal molecular weights consistent with addition of a single chlorine atom, relative to DCP.

Nominal molecular weights assigned to DCPB and TCPB were identical to nominal masses of 2,4-dichlorophenyl-β-D-glucopyranoside and 2,4,5-trichlorophenyl-β-D-glucopyranoside, which were observed in axenic *L. gibba* exposed to DCP and TCP [11,12]. Likewise, nominal mass of DCPC was identical to that of 2,4-dichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside, which was observed in transgenic cotton exposed to DCP and 2,4-dichlorophenoxyacetic acid [17]. By analogy, TCPC was hypothesized to be 2,4,5-trichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside. Because no reference materials were commercially available, DCPG, DCPMG, TCPG, and TCPMG (structures shown in Fig. 2 for DCP conjugates; TCP conjugates are similar) were prepared in the laboratory. All synthesized compounds co-eluted and produced identical ESI-NEG mass spectra to extracted compounds. Positive identification was provided by comparison of the ^{1}H-NMR spectra of extracted and synthetic compounds, which were identical (Table 2). The ^{1}H signal assignments were confirmed with ^{1}H-COSY spectra (data not shown). The number of active protons expected from each structure (four for DCPG and six for DCPMG) was confirmed by direct injection of deuterated samples into ESI-interface.

No previous reports of analogs to DCPA and TCPA could be found in the literature. Dilute acid hydrolysis of DCPA and TCPA yielded DCPG and TCPG as transient intermediates, and DCP and TCP as terminal products, respectively. Therefore, it was concluded that DCPA/TCPA contained the β-glucopyranoside core structure of the other products. An isotope calculator (Mass Spec Pro Calculator, ChemSW, Fairfield, CA, USA) was used to predict possible molecular formulas based on the β-glycoside structure with CHO additions. Potential matches included tartaryl esters or pentosyl-substituted glycosides. Tartaryl esters of DCPG were prepared in the same manner as DCPMG by substituting an equimolar amount of tartaric acid for malonic acid. None of the tartaryl esters formed co-eluted with DCPA, nor shared similarity with the mass spectrum of DCPA. The ^{1}H-NMR spectra of DCPA (Table 2) contained a second anomeric proton (δ = 4.79) and an increased number of signals in the δ = 3.4 to 4.0 region consistent with an additional pentose residue. These data suggested that DCPA contained a pentosyl moiety glycosidically bound to the β-glucopyranoside core.

Acid hydrolysates of DCPA and TCPA were perbenzoylated and compared to reference chromatograms of perbenzoylated D-arabinose, L-arabinose, D-xylose, and apiose (Table 3). Based on these data, the pentosyl unit of DCPA and TCPA was determined to be apiose. The J value of the apiosyl anomeric carbon, 3.2 Hz (Table 2), was consistent with the (3-hydroxymethyl)-β-D-erythrofuranose conformation of methyl-β-D-apioside (3.7 Hz) reported by Ishii and Yanagisawa [28]. The shifts in $C6H_a'$ and $C6H_b'$ were analogous to observations with DCPMG and supported assignment of a (1→6) linkage. Furthermore, DCPA/TCPA were not reactive with Benedict's reagent, which indicated both anomeric carbons participated in glycosidic bonds.

A linkage analysis was performed by CCRC on a sample of DCPA. Apiose was the only pentose residue identified in the sample. Analysis of the mass spectrum showed that the ion distribution of the apiose residue derived from DCPA was similar to a terminal apioside residue (Table 4) reported by Brillouet et al. [29]. The chromatogram of derivatized carbohydrate residues was dominated (44% of integrated area) by 1,6-linked glucose residues further supporting designation of a glucopyranosyl-(6→1)-apiofuranoside. Therefore, DCPA and TCPA were determined to be 2,4-dichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuranoside (DCPAG) and 2,4,5-

Table 1. Negative mode electrospray mass spectral data for chlorophenol metabolites

t_r[a]	m/z[b]	Cl_x	Assignment	t_r[a]	m/z[b]	Cl_x[c]	Assignment
			DCPAG[d]				TCPAG[e]
17.0	125	1	$[M - H - 162 - HCl]^-$	20.4	195	3	$[M - H - 162]$
	161	2	$[M - H - 162]$		293	0	$[M - H - C_6H_5Cl_2]^-$
	293	0	$[M - H - C_6H_5Cl_2]^-$		489	2	$[M - H]^-$
	455	2	$[M - H]^-$		525	3	$[M + Cl]^-$
	491	3	$[M + Cl]^-$				
	(479)		$[M + Na]^+$		(513)		$[M + Na]^+$
			DCPG[f]				TCPG[g]
17.8	125	1	$[M - H - 162 - HCl]^-$	21.9	195	3	$[M - H - 162]^-$
	161	2	$[M - H - 162]^-$		357	3	$[M - H]^-$
	323	2	$[M - H]^-$		383	4	$[M + Cl]^-$
	359	3	$[M + Cl]^-$		417	3	$[M + Ac]^-$[c]
	383	2	$[M + Ac]^-$[h]		(381)		$[M + Na]^+$
	485	4	$[M + C_6H_5Cl_2]^-$				
	647	NA[i]	$[M + M - H]^-$				
	(347)		$[M + Na]^+$				
			DCPMG[j]				TCPMG[k]
20.0	161	2	$[M - H - 162]^-$	23.4	195	3	$[M - H - 162]^-$
	365	2	$[M - H - CO_2]^-$		399	3	$[M - H - CO_2]^-$
	409	2	$[M - H]^-$		443	3	$[M - H]^-$
	819	4	$[M + M - H]^-$		887	6	$[M + M - H]^-$
	(433)		$[M + Na]^+$		(467)		$[M + Na]^+$

[a] Values reported in min.
[b] Mass to charge ratios. Values in parentheses collected in positive mode.
[c] Degree of chlorination determined by comparison of percent relative abundance of m + 2 intensities to theoretical values based on the natural abundance of $^{35}Cl/^{37}Cl$.
[d] 2,4-dichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuranoside.
[e] 2,4,5-trichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuranoside.
[f] 2,4-dichlorophenyl-β-D-glucopyranoside.
[g] 2,4,5-trichlorophenyl-β-D-glucopyranoside.
[h] Ac = CH_3CO.
[i] Relative abundance values too small for comparison.
[j] 2,4-dichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside.
[k] 2,4,5-trichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside.

trichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuranoside (TCPAG), respectively (Fig. 2).

Extracted and synthetic glycosides were subjected to enzyme-catalyzed hydrolysis with β-glucosidase from almond meal. Samples containing DCPG and TCPG were rapidly hydrolyzed with complete aglycan release (up to 213 μM) in less than 1 h. However, derivatized glycosides such as DCPMG and DCPAG were resistant to hydrolysis under the same conditions. For example, a statistically insignificant fraction of DCPMG was hydrolyzed to DCP (0.7 ± 0.7% of initial integrated area). Only a small fraction of DCPAG (10.2 ± 6.3%) was released, even after extending the reaction time to 18 h. Therefore, β-glucosidase was found ineffective at accurately representing the extent and identity of glycosides formed in plant tissues.

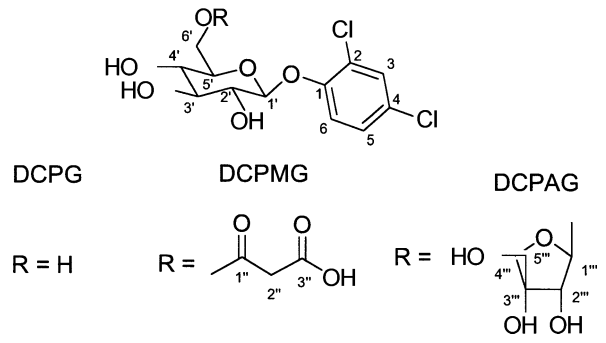

Fig. 2. Chemical structures of chlorophenol glycosides extracted from *Lemna minor*. DCPG = 2,4-dichlorophenyl-β-D-glucopyranoside; DCPMG = 2,4-dichlorophenyl-β-D-(6-O-malonyl)-glucopyranoside; DCPAG = 2,4-dichlorophenyl-β-D-glucopyranosyl-(6→1)-β-D-apiofuranoside; and 2,4,5-trichlorophenol glycosides follow a similar terminology.

Identification of metabolites in conjunction with the reaction sequence observed in Figure 3 suggested that chlorophenols were metabolized by *L. minor* via the pathway shown in Figure 4. This is consistent with the three-phase detoxification scheme previously discussed. Malonated glycosides (DCPMG and TCPMG) suggested that sequestration occurred in vacuoles of *L. minor* [13,14].

Apiosyl residues in conjunction with xenobiotic glycosides of *L. minor* represented a novel and important discovery. Apiose is a rare carbohydrate with unusual structural properties. Apiose was first discovered as a component of a flavanoid glycoside extracted from *Petroelinum sativum* [30]. Apiose, a branched-chain pentose, is exclusively associated with plant metabolism [31] and is known to be a unique component of cell walls in *L. minor* [32–34]. Detection of this residue strongly supported that *L. minor*, and not a microorganism, was responsible for metabolites observed in this research, and close association of apiose and cell walls of *L. minor* also suggested that apiosyl glycosides of chlorophenols were indicative of a phase 3 biotransformation. This conclusion implied that other plant species would likely contain analogous glycosides with substitution of more common pentosyl units of hemicellulose, e.g., arabinose or xylose. For example, β-D-xylopyranosyl-

Table 2. Proton nuclear magnetic resonance data for chlorophenyl glycosides[a]. (The 2,4-dichlorophenyl [DCP]-glycoside abbreviations are in Fig. 2 caption)

Atom[b]	DCPG[c]	DCPMG[c]	DCPAG[c]
HC-3	7.24 (2.5) d	7.10 (2.5) d	7.28 (2.5) d
HC-5	7.02 (8.9, 2.5) dd	6.93 (9.0, 2.5) dd	7.07 (9.5, 2.5) dd
HC-6	6.92 (9.2) d	6.79 (9.2) d	6.96 (9.1) d
HC-1′	4.83 (7.4) d	4.73 (7.6)	4.86 (7.6) d
HC-2′, HC-3′[b]	3.26–3.35m	3.27–3.37 m	3.30–3.40 m
HC-4′	3.21 (9.7, 8.9) *t*[d]	3.20 (9.2, 9.4) *t*[d]	3.24 (9.1, 8.8) *t*[d]
HC-5′	3.30–3.40 m	3.46 m	3.30–3.40 m
HC-6a	3.61 (12.2, 2.29) dd	4.18 (12.4, 2.0) dd	3.72 d
HC-6b	3.47 (12.4, 5.6) dd	4.04 (12.0, 5.5) dd	3.58 d
HC-1‴			4.79 (3.2) d
HC-2‴			3.68 (3.2) d
HC-4a‴, 4b‴			3.76 m
HC-5a‴, 5b‴			3.47 m
Number of active H's[e]	4	6	6

[a] Recorded in deuterium oxide, 500 MHz. Chemical shift (δ) in ppm relative to residual water at 4.5 ppm, coupling constants (J) in Hz. d, doublet; dd, doublet-doublet; t, triplet; m, multiplet.
[b] Atom labels refer to Figure 2.
[c] Interpreted as a degenerate dd.
[d] Complex splitting makes these signals impossible to distinguish.
[e] Active protons determined by increase in mass of deuterated samples directly infused into electrospray-interface.

(6→1)-D-glucose glycosides (primeverosides) of anthraquinone were detected in cell cultures of *Morinda citrifolia* [35].

Progression of observed reactions were followed by sacrificial sampling of experimental units (Fig. 3). Uptake of DCP was characterized by a short, rapid phase followed by a slower, sustained phase consistent with a diffusion-controlled partitioning process. Both DCPG and DCPMG were detected in the first sample at approximately 1 h. The DCPAG was not detected until the second sampling point at 8 h. These results suggested that a constitutive enzyme catalyzed formation of DCPG and DCPMG, while DCPAG required induction or an undetected intermediate before the reaction sequence could continue. Both DCP and DCPG quickly reached pseudo-steady state concentrations and never accumulated to an appreciable extent. In contrast, DCPMG and DCPAG continued to accumulate over the course of the experiment. It should be noted that no metabolites were ever observed in the aqueous phase, even after volumetric concentration of 20 to 100 times. Similar results were observed in experiments with TCP (data not shown).

Biosynthesis of glycosidic bonds usually occurs by sequential reaction of an aglycan with a nucleotide diphosphate. Because nucleotide diphosphate formation requires at least one equivalent of ATP [36], a minimal energetic cost may be assigned to the formation of β-glucosides, DCPG or TCPG, of approximately −30.5 kJ/mol product formed. This value is the approximate free energy released by hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) under standard biological conditions: 25°C, pH 7.0 and unit activity [37]. Similarly, an additional ATP equivalent must be used to form malonyl or apiosyl derivatives. Thus, metabolites described in this research represent an energetic sink to plants storing chlorophenols in these forms. Therefore, it may be concluded that conjugation of chlorophenols reduced free energy available for primary metabolic processes associated with growth and maintenance. In addition, free-energy losses are also likely to be associated with dissipation of electrochemical potentials by free chlorophenols in plant tissues. However, these losses are mitigated by glycosylation, which effectively

Table 3. Monosaccharide indentification by high-performance liquid chromatography.[a] (Chlorophenyl-glycosides abbreviations are presented in Fig. 2 caption)

Compound	Peak 1	Peak 2	Peak 3
DL-arabinose[b]	1.41 (0.96)	1.53 (0.34)	2 (1.00)
D-xylose[c]	1.71 (1.00)[c]		
D-apiose	1.66 (0.26)	1.77 (1.00)	
D-glucose	2.70 (1.00)		
D-galactose	2.68 (1.00)	3.19 (0.36)	
DCPG[d] (glucose)	2.72 (1.00)		
DCPAG[d] (apiose)	1.69 (0.26)	1.79 (1.00)	
(glucose)	2.72 (1.00)		
TCPAG[d] (apiose)	1.67 (0.25)	1.79 (1.00)	
(glucose)	2.72 (1.00)		

[a] Retention reported relative to benzoic acid, $(t_r - t_{\text{benzoic Acid}})/t_{\text{benzoic acid}}$. Reported values are the average of two determinations. Values in parentheses are fraction of largest peak in set.
[b] D-arabinose and L-arabinose were indistinguishable by this method.
[c] The peak for D-xylose contained two unresolved compounds.
[d] Abbreviations defined in Table 1.

Table 4. Comparison of ion distribution of 1-linked apiose from Brillouet et al. [29] and pentosyl residue reported by the Complex Carbohydrate Research Center, University of Georgia (USA); dimethyl tetrachloroterephthalate (DCPA)

Brillouet et al. [29]		Sample derived from DCPA	
Mass to charge ratio	Relative abundance (%)	Mass to charge ratio	Relative abundance (%)
87	26.6	87	21.7
101	100.0	101	100.0
117	88.3	118	76.7
131	30.9	132	30.0
146	<5.0	146	5.0
161	36.2	161	40.8
173	8.5	174	14.2
201	<5.0	202	3.3
205	<5.0	205	<5.0
233	<5.0	234	<5.0

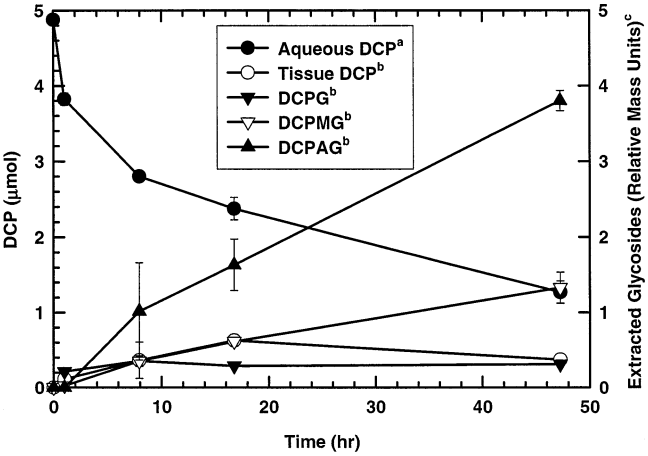

Fig. 3. Concentration profile of batch plant system inoculated with 2,4-dichlorophenol (see Fig. 2 caption for glycoside descriptions). [a] Total mass in 100-mL aqueous phase. [b] Total mass extracted from plant tissues. [c] Relative mass units were derived by normalizing peak area to the g fresh weight of plant in the reactor. 2,4-dichlorophenol (DCP); refer to Fig. 2 legend for acronym definitions.

blocks phenols from scavenging electrons from energy production.

CONCLUSIONS

The results reported here were consistent with prior reports of glycosidation as the initial step in xenobiotic assimilation by plants, e.g., [11,12,38]. Although these previous reports were based on studies of axenic plants or cell cultures, this work demonstrated that the same processes were prominent in natural plant systems. Over the time-scales investigated here, the rapid assimilation of chlorophenols did not allow selection for chlorophenol-degrading microorganisms as no microbial transformation products were detected in the media. Attributing metabolism to *L. minor* is also consistent with observed differences in nature of plant and microbial detoxification. In particular, glycosidation of xenobiotics is attributed to plants because this type of detoxification would represent a large expenditure of potential energy, reducing power and carbon sources to heterotrophic microbial populations operating under natural nutrient-limiting conditions [39]. These losses are mitigated in plant species due to the high levels of carbohydrates formed from photosynthetic activity. Location of all observed metabolites within plant tissues also supports the conclusion that all observed transformations were mediated by plant biochemistry. The strongest evidence of plant-mediated transformation was the detection of apiosides, which are exclusive to the plant kingdom [31].

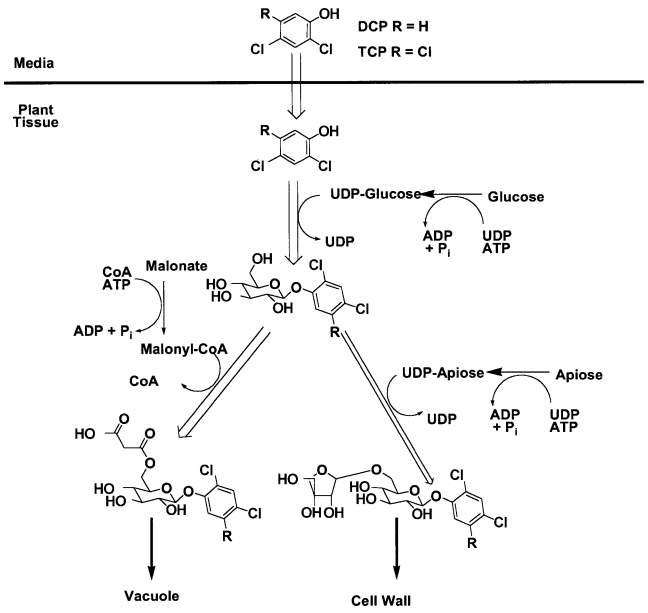

Fig. 4. Assimilation of 2,4-dichlorophenol (DCP) and 2,4,5-trichlorophenol (TCP) observed in *Lemna minor*; adenosine triphosphate (ATP)/ adenosine diphosphate (ADP); uridine diphosphoglucuronic acid (UDP).

The nature of the two glycoside derivatives, e.g., DCPMG and DCPAG, indicated the fate of DCP in tissues of *L. minor*. As stated previously, malonated glycosides have been associated with sequestration in plant-cell vacuoles [13,14]. Observed malonated glycosides, DCPMG and TCPMG, in plant tissues were consistent with these reports, and localization within plant tissues was counterindicative of microbial metabolism in media or on plant surfaces.

With *L. minor*, DCP and TCP transport into tissues was directly observed with media and tissue measurements. Subsequent formation of β-glycosides required accumulation of parent chlorophenols in tissues. In turn, β-glycosides of DCP or TCP served as precursors for glycosyl derivatives, i.e., malonyl and apiosyl glycosides of the parent compound. The ultimate fate of DCP and TCP was sequestration into cell walls as apiosyl glycosides and vacuoles as malonyl glycosides. In the sequestered form, the chlorophenols were less toxic to plants. No dechlorination was observed for either substrate. In natural systems, this work implied that considerable attention should be directed toward assessing contaminant uptake and sequestration in exposed flora. In addition to metabolites found in this research, it is quite likely that a large number of similar glycosides of contaminants probably await discovery in other species. Also, evidence was presented that reliance on enzyme catalyzed hydrolysis provided misleading information on the extent of glycoside conjugation in plant tissues.

In retrospect, strong structural similarities of xenobiotic conjugates reported here and natural products suggested an improved strategy for finding phytotransformation products of xenobiotics. Natural products chemically related to xenobiotics can provide new insight into the variety and extent of products. For example, chlorophenolic glycosides reported herein, have natural product analogs such as flavanoids, flavanols, and triterpenoids, which have all been found to form glycosides in multiple species, e.g., *Dirca palustris* [40], *Bidensandicola andicola* [41], and *Proteaceae* species [42]. Therefore, if natural product analogs can be identified, it is reasonable to assume that xenobiotic glycosides can be formed in similar systems. Furthermore, the breadth of these types of reactions across species further suggested that plant roles in xenobiotic fate may be sorely underestimated.

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