JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 7931–7937 7931
DOI:10.1021/jf902132j

Metabolism of Methoxychlor by *Cunninghamella elegans* ATCC36112

YOUNG SOO KEUM,[†] YOUN HYUNG LEE,[‡] AND JEONG-HAN KIM*,[†]

[†]Department of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea, and
[‡]Department of Horticultural Biotechnology, KyungHee University, Suwon 446-701, Korea

Methoxychlor is considered as pro-estrogen, while some of its metabolites are more potent endocrine disruptors than the parent insecticide. Major activation of methoxychlor is through cytochrome P450-catalyzed demethylation to bisphenol A-like metabolites. *Cunninghamella elegans* is a well-known fungal species with its strong resemblance of the xenobiotic metabolism of the mammalian system. In this study, the metabolism of methoxychlor was investigated with the corresponding organism. Methoxychlor was rapidly transformed to approximately 11 metabolites in phase I metabolism, including oxidation, hydroxylation, and dechlorination. Concentrations of phase I metabolites reached a maximum at 4−6 days and gradually decreased until the end of the experiments. Most metabolites from the phase I reaction were further transformed to sugar conjugates. Approximately 11 or more glucose conjugates were found in culture supernatants and gradually increased, while no glucuronides were observed throughout the experiments. Piperonyl butoxide and chlorpyrifos strongly inhibit the degradation of methoxychlor and concomitant accumulation of metabolites, indicating cytochrome P450 mediated metabolism. Little or no glycosides were detected in chlorpyrifos- and piperonyl butoxide-treated cultures. From the results, *Cunninghamella elegans* has shown strong similarities of the phase I metabolism of methoxychlor, while the conjugation reaction is different from those of animal metabolism.

KEYWORDS: Methoxychlor; endocrine disruptor; metabolism; *Cunninghamella elegans*; insecticide

INTRODUCTION

The recent development of novel pesticides ensures the safety of agricultural production. However, many classical insecticides, including organophosphorus and organochlorines, are still in use in many countries. Many organochlorine insecticides (OCs) are considered as the most notorious environmental contaminants. In addition to their recalcitrant properties, several toxicological effects are reported for OCs. For example, some OCs, including hexachlorocyclohexanes (HCHs), DDT and related analogues, and cyclodienes, are regarded as endocrine disruptors (*6*, *15*, *22*). Among several OCs, methoxychlor has interesting environmental and toxicological properties. Its mode of action is through the inhibition of neuronal signal transduction and has relatively low mammalian toxicity. It is less persistent than DDT or other OCs. Despite these promising properties, there are strong public and scientific concerns, and the use is banned in several countries (e.g., (*10*)). Major toxicological effects of methoxychlor, other than acute toxicity, are primarily related to a strong estrogenic activity of demethylated metabolites (*3*−*6*, *9*). Some of these estrogenic metabolites, including 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane and 1,1,1-trichloro-2,2-bis-(4-hydroxyphenyl)ethane (HPTE), are commonly found from mammals, birds, and fishes (e.g., (*7*, *8*, *19*, *20*, *23*)). In general, these metabolites are further transformed to other metabolites, including glucuronides or sulfates (*19*, *20*). Both biotic and abiotic factors are involved in environmental dissipation of methoxychlor (e.g., photolysis, bacteria, and white rot fungi). Dechlorination was the most dominant metabolic pathway in microorganisms (*2*, *11*, *12*, *17*, *26*, *27*).

Among the relevant soil microflora, numerous fungal species are reported as promising tools to remediate contaminated soils or other environments. For example, *Phanerochaete chrysosporium* can metabolize many structurally unrelated xenobiotics (*11*). *Cunninghamella* spp. are another important model organism in xenobiotic metabolism studies because of their metabolic resemblance with those of animals (*1*).

In this study, we investigated the metabolism of methoxychlor by *Cunninghamella elegans*. From the metabolite profile analysis, several metabolic pathways were confirmed to be common both in animals and fungi, while some metabolic features were far different from those of animals.

MATERIALS AND METHODS

Chemicals. The following reagents were obtained from Aldrich Korea (Seoul, Korea): methoxychlor, chlorpyrifos, piperonyl butoxide, bis-(trifluoromethylsilyl)acetamide-trimethylsilyl chloride (BSTFA-TMCS, sylonBFT), β-glucosidase from *Aspergillus niger*, and *n*-butylboronic acid. Potato dextrose broth (PDB) and potato dextrose agar (PDA) were from BD Korea (Seoul, Korea). Solvents, including methanol and ethyl acetate, were HPLC or higher grade. Anhydrous sodium sulfate was from Junsei (Japan). Other reagents were of the highest grade available.

*Corresponding author. Fax: 82-2-873-4415. E-mail: kjh2404@snu.ac.kr.

Published on Web 08/19/2009

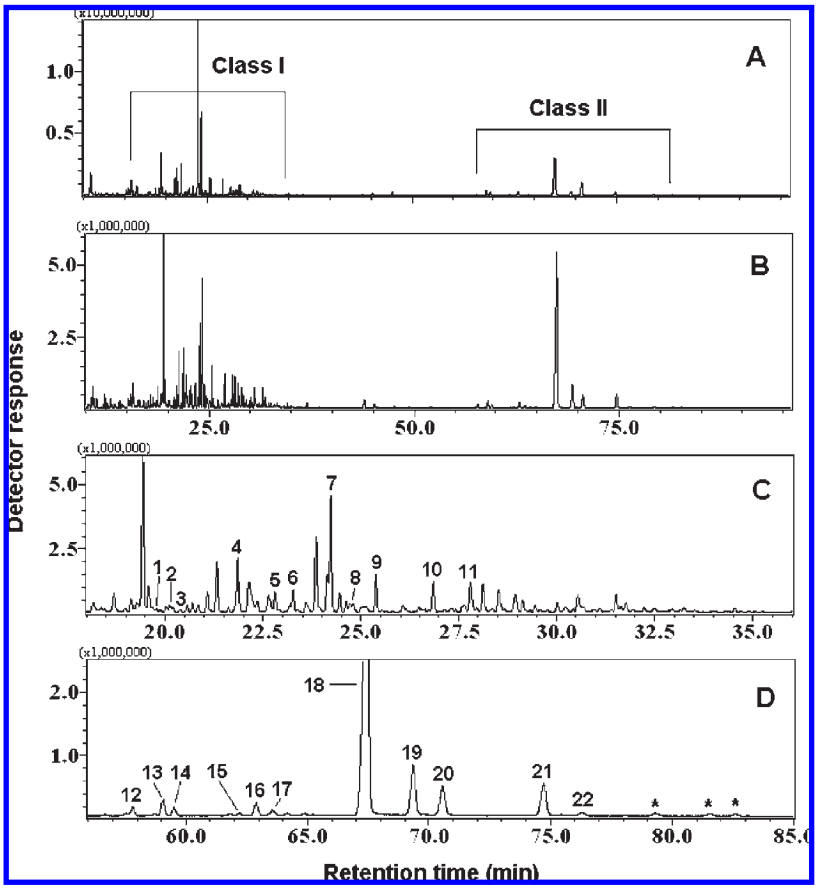

Figure 1. GC-MS total ion chromatograms of TMS-derivatized ethyl acetate extracts of culture supernatant of *Cunninghamella elegans*, treated with methoxychlor after 2 days (**A**) and 4 days (**B**) of incubation. *Y*-axis signal enhanced chromatograms of phase I (**C**) and phase II metabolite elution regions (**D**) of cultures after 4 days of incubation. Asterisks are for possible glycosides, but there is incomplete information for identification.

Culture of Fungus. *Cunninghamella elegans* ATCC 36112 was obtained from American type Culture Collection (Manassas, VA). It was typically maintained on PDA at 27 °C, while liquid cultures were on PDB at the same temperature, 180 rpm. For the degradation experiment, seed culture was prepared on PDB for 2 days. After removal of culture media, mycelia (approximately 2 g) was resuspended in fresh PDB (500 mL), supplemented with methoxychlor (2 mg in 0.5 mL of methanol), and cultured at 27 °C and 180 rpm for a predefined period. To study the effects of cytochrome P450 inhibitors, a stock solution of inhibitors (200 μL, 10 mg/L of methanol) was added and preincubated for 6 h, followed by addition of methoxychlor (2 mg/0.5 mL of methanol). Sterilized control was prepared through the same procedures but sterilized at 120 °C for 30 min. All experiments were performed in triplicate. For large-scale preparation of metabolites, three cultures (500 mL × 3, total 1.5 L) were combined and extracted after 10 days of incubation.

Extraction and Derivatization of Metabolites. Aliquots of culture with mycelium (80 mL) were diluted with saturated NaCl solution (50 mL), and pH was adjusted approximately 2−3 with 6 N hydrochloric acid. The mixture was extracted with ethyl acetate (150 mL × 2) and dried over anhydrous sodium sulfate. After removal of solvent under reduced pressure, the residue was resuspended in dry pyridine (1 mL) and derivatized with BSTFA-TMCS (200 μL) at 85 °C for 1 h. To determine the presence of catecholic metabolites, an aliquot of the organic extracts from batch culture was concentrated to dryness and redissolved in acetone (20 mL). After addition of *n*-butylboronic acid (1 mg), the mixture was gently refluxed for 3 h. Acetone was removed under reduced pressure, and the residue was dissolved in ethyl acetate (2 mL).

Hydrolysis of Conjugates. To determine the chemical structures of conjugates, aliquots of crude organic extracts from batch cultures were concentrated to dryness and redissolved in a mixture of ethyl acetate and methanol (2 mL, 1/1, v/v). Half of the solution was derivatized with BSTFA-TMCS, while another half was suspended in sodium acetate buffer (30 mL, 5 mM, pH 5.2). After addition of β-glucosidase (1 mg) from *Aspergillus niger*, the mixture was incubated at 37 °C, 100 rpm, for 6 h. The solution was then extracted with ethyl acetate (100 mL × 2). After removing organic solvent, residue was derivatized with BSFTA-TMCS.

Instrumental Analysis. Methoxychlor and metabolites were analyzed with GC-MS (Shimadzu GCMS QP-2000 and GC-2010), equipped with a DB-5MS column (60 m, 0.25 μm film thickness, 0.25 mm i.d.; Agilent Technologies, USA). Helium was a carrier gas at a flow rate of 0.85 mL/min. The column temperature were programmed as follows: 230 °C (10 min) then raised to 295 °C at a rate of 2 °C/min and held for 65 min. Temperatures of the injection port and interface were set at 285 and 280 °C, respectively. The mass spectrometer was operated at electron impact (EI) mode at 70 eV. For the analysis of *n*-butylboronic acid derivatives of metabolites, the same conditions were used except column temperature: 130 °C (10 min) and raised to 290 °C at a rate of 5 °C/min and held for 40 min.

RESULTS

Metabolism of Methoxychlor. Approximately 100 different peaks were observed in TMS-derivatized culture extracts (**Figure 1**). Peaks with characteristic chlorine isotopic mass fragments were evaluated to determine the structures and compared with those from the literature.

Methoxychlor was extensively metabolized by *Cunninghamella elegans*, and approximately 21 or more metabolites were observed from the organic extracts (**Figures 1** and **2**, **Table 1**). These metabolites can be grouped into two different classes based on their retention times. Methoxychlor and 11 metabolites were eluted at 19−30 min (class I), while an additional 11 metabolites were observed at 59−80 min (class II). Several pairs of class I metabolites have shown common mass spectral patterns. For

example, metabolites **1**, **4**, and **7** have *m*/*z* 285 ion as base peaks, while *m*/*z* 343 ion was the most abundant ion in metabolites **6** and **9** (**Table 1**). Additional pairs of metabolites with ions of *m*/*z* 227 or 373 as base peaks were also observed throughout the experiments (metabolites **2**/**5** and **8**/**10**, respectively). Peak 5 (retention time, Rt, 22.8 min) was identified as residual methoxychlor (**5**, **Figure 1**). From the close inspection of mass spectra, **3** and **11** have also shown a characteristic chlorine-isotope pattern and are considered to be methoxychlor metabolites.

To determine the catechol metabolites, culture extracts were derivatized with *n*-butylboronic acid. One metabolite (**I**, Rt, 49.8 min) has shown characteristic mass spectra of catechols (**Figure 3**). An additional metabolite (**II**) was also suspected as a catechol metabolite. However, it was not possible to identify the structure because of strong interference.

In comparison with class I metabolites, the mass spectra of class II metabolites share a common fragmentation pattern (**Table 1**). In all peaks (**12**−**22**), the *m*/*z* 361 fragment was the most abundant ion, and several additional ions (*m*/*z* 103, 129, 147, 169, 217, 243, and 271) were found from all metabolites. Fragments, giving a characteristic chlorine isotope pattern, were minor in all class II metabolites (**Table 1**). Approximately three more peaks were observed in crude extracts (Rt's, 79−83 min, **Figure 1**). These peaks showed the above-mentioned common mass spectral patterns of class II metabolites. However, it was difficult to confirm the identities of these metabolites because of low signal intensities and interference. Crude culture extracts were incubated with β-glucosidase from *Aspergillus niger*. GC-MS analysis of the extracts before and after enzymatic treatment has shown a clear difference. Both class I and II metabolites were observed in the initial reaction mixture, but no traces of class II metabolites were detected after 6 h of incubation (**Figure 4**).

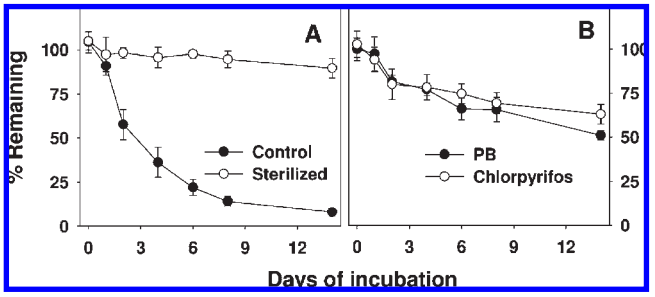

Figure 2. Degradation of methoxychlor by *Cunninghamella elegans* in no-inhibitor control and sterilized mycelia (**A**) and piperonyl butoxide and chlorpyrifos (**B**). PB, piperonyl butoxide. Results were from three replicates.

Degradation of Methoxychlor. Approximately 80% of methoxychlor was transformed into other metabolites within 8 days, where the time-dependent removal of methoxychlor was followed by typical exponential decay (**Figure 2**A). Accumulation of metabolites was accompanied by concomitant decrease of methoxychlor (**Figure 5**). However, the kinetic patterns of class I and II metabolites were quite different. The levels of class I metabolites reached maximum at 4−6 days and gradually decreased, while the concentrations of class II metabolites continuously increased until the end of experiments (**Figure 5**). For example, metabolites **7** and **9**, the most abundant class I metabolites, reached a maximum at 4 days and decreased slowly. Their relative proportions to other class I metabolites also decreased from 75 to 38% (**Figure 6**).

Effects of Cytochrome P450 Inhibitors. Two cytochome P450 inhibitors, relevant in agricultural practices, were investigated. Approximately 50−60% of methoxychlor still persisted in the culture, treated with piperonyl butoxide and chlorpyrifos even after two weeks, while only a trace amount was found in the control (**Figure 2**). The most noticeable difference between the control and inhibitor-treated sample was the formation of class II metabolites. No or only a trace amount of class II metabolites were found in inhibitor-treated samples, while the proportion of corresponding metabolites was much higher in control cultures at 14 days (**Figures 5** and **7**).

DISCUSSION

In a recent review, Asha et al. pointed out that there are strong similarities in xenobiotics metabolism between *Cunninghamella*

Table 1. Retention Times and Fragmentation Patterns of Methoxychlor and Metabolites in GC-MS

ID	retention time (Rt)[a]	other organism[b]	mass fragmentation pattern
1	19.692	A, F, M	402(M^+,3), 387(11), 343(18), 328(15), 285(100), 245(20)
2	20.217	A, B, M, W	310(M^+,4), 239(14), 227(100), 113(8)
3	20.425	A, M	366(M^+, 100), 351(21), 327(15), 316(43)
4	21.867	A, M	368(M^+,3), 353(1), 298(3), 285(100), 270(2)
5	22.775	-	344(M^+,3), 306(2), 309(1), 274(4), 227(100)/Methoxychlor (parent)
6	23.292	A, M	426(M^+,4), 411(2), 376(1), 343(100), 270(7)
7	24.242	A, F, H, M	402(M^+,2), 389(1), 332(2), 296(1), 285(100)
8	24.792	NR	456(M^+,7), 441(1), 373(100), 355(2), 315(15)
9	25.375	A, F, H, M	460(M^+,3), 445(2), 410(1), 390(4), 343(100), 313(1), 270(5)
10	26.867	A, H	492(M^+,6), 475(1), 456(1), 420(3), 373(100), 332(2), 285(8)
11	27.825	H, M	548(M^+, 5), 478(4), 431(100), 389(4), 343(7), 327(22), 275(8)
12[c]	57.825	NR	450(20), 398(50), 361(100), 315(46). 271(23), 243(48)
13	59.083	A, M	450(24), 429(2), 361(100), 332(75), 271(24), 243(25), 217(48)
14	59.500	A, M	450(25), 429(4), 361(100), 332(75), 271(28), 243(32), 217(50)
15	61.817	A, M	450(12), 390(75), 361(100), 217(48)
16	62.892	A	450(20), 366(60), 361(100), 332(23), 271(24), 243(26), 217(48)
17	63.567	A	456(25), 450(23), 361(100), 332(15), 271(25), 243(20), 217(40)
18	67.433	A, M	641(2), 551(1), 450(10), 368(17), 361(100), 331(9), 285(30), 271(20)
19	69.333	A, M	495(2), 450(10), 426(13), 361(100), 343(32), 271(27), 243(20)
20	70.600	NR	450(24), 361(100), 298(100), 271(20), 243(28), 217(47)
21	74.708	A, F, M	450(18), 404(13), 368(2), 361(100), 331(10), 285(48), 271(22)
22	76.050	A, F, M	462(10), 450(12), 426(3), 361(100), 343(48), 319(7), 271(25), 243(29)

[a] Retention times of TMS derivatives. [b] Metabolites, found from metabolic studies with A, bird; B, bacteria; F, fish; H, human cytochrome P450; M, mammals; W, fungi; NR, not reported (reference: Davison 1982/1984, Grifoll, Hu, Kupfer, Lee, Ohyama 2004/2005, Stuchal, Yim). [c] Metabolites (**12**−**22**) from *Cunninghamella elegans* were glycosides, while the conjugates from other organisms are glucuronides.

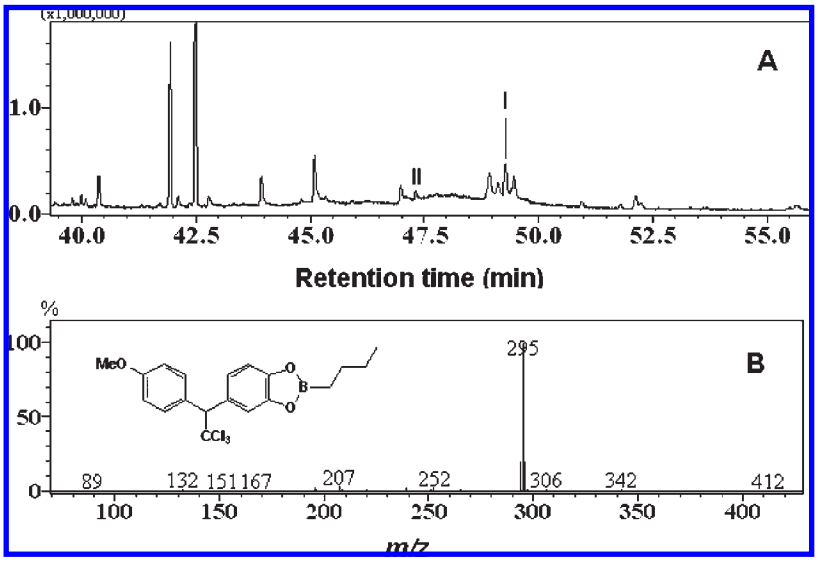

Figure 3. GC-MS total ion chromatogram of *n*-butylboronic acid derivatized organic extracts of cultures after 10 days of incubation (**A**) and mass spectrum of catecholic metabolite I (**B**).

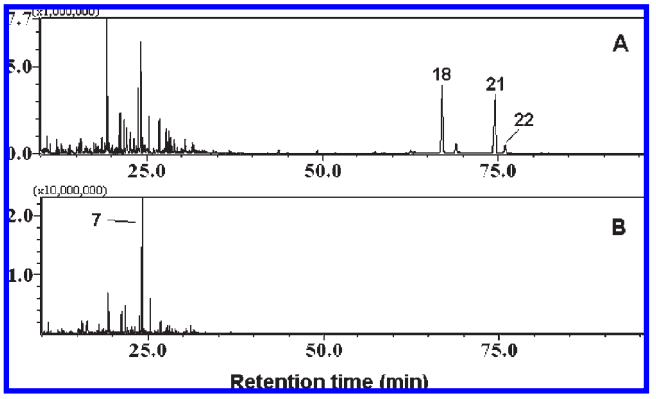

Figure 4. GC-MS chromatograms of organic extracts of cultures (14 days) before (**A**) and after treatment of β-glucosidase (**B**).

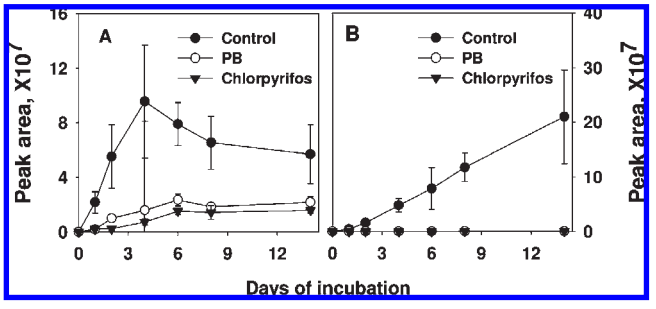

Figure 5. Accumulation of class I (**A**) and II (**B**) metabolites of methoxychlor in control and inhibitor-treated cultures. Each line indicates sum of peak area of class I (**1**−**4**, **6**−**11**) or class II (**12**−**22**) metabolites, noted in **Table 1**.

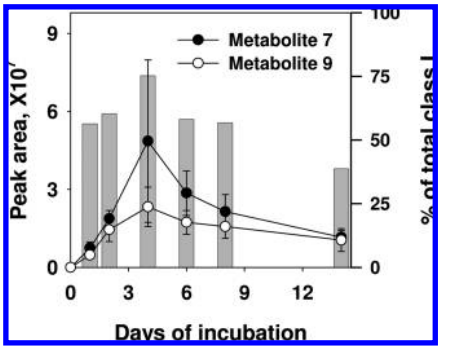

Figure 6. Time-dependent changes of the GC-MS peak area of metabolites **7** and **9** (line plot) and percent of both metabolites over total peak area of class I metabolites (bar plot).

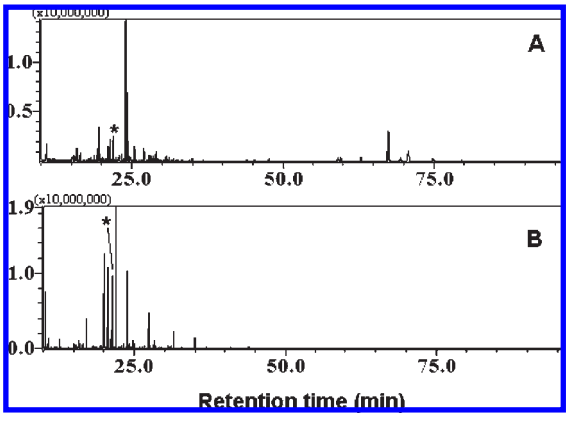

Figure 7. GC-MS total ion chromatograms of organic extracts of cultures without inhibitor (**A**) and with piperonyl butoxide (**B**) after 4 days.

spp. and animals (*1*). Although details of specific xenobiotics metabolism may be different between these biosystems, general metabolic outlines can be deduced from such a model organism, through which we can speculate toxicological studies of selected chemicals. Methoxychlor, as mentioned above, is a pro-estrogen, which can be transformed to several metabolites with endocrine-disrupting activity. In this study, a detailed metabolic profile was investigated and compared with those of animals.

Overall, 21 or more metabolites were observed during experiments. Metabolites, noted as class I (**1**−**4**, **6**−**11**) could be subgrouped by *m*/*z* of base peaks of mass spectra (*m*/*z*, 227, 285, 343, 366, 373, and 431). The fragment ion (*m*/*z* 227) corresponds to the bis(4-methoxyphenyl)methylene group. The presence of this ion indicates that metabolite **2** has the above-mentioned structures with *m*/*z* 310 as the molecular ion. These

characteristics were well-coincided with literature values of monodechlorinated methoxychlor (*7*, *8*). Mass spectra of three metabolites (**1**, **4**, and **7**) have shown *m*/*z* 285 as the base peak, which corresponds to the 4-hydroxyphenyl 4′-methoxyphenylmethylene group. Metabolite **7** with *m*/*z* 402 as the molecular ion was confirmed as monodemethylmethoxychlor, which is known as the estrogenic metabolite of methoxychlor. Isotopic mass distribution around the molecular ion of metabolite **4** (*m*/*z* 368/370/372) indicates this metabolite as monodechlorinated derivatives of **7**. The mass spectrum of **1** was very similar with **7** but was not able to confirm the structure. It may be an isomer of **7**. The fragment ion of *m*/*z* 343 was the base peak of **6** and **9**, indicating the presence of the bis(4-hydroxyphenyl)methylene group. They have shown mass spectra very similar with 1,1-dichloro-2,2-bis(4-hydroxyphenyl)ethane (**6**) and 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane (**9**) (*7*). Metabolite **3** has shown a mass spectrum very similar with that of 1,1-dichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethylene (*7*,*8*). The fragment ion of *m*/*z* 373 was the base peak of mass spectra of metabolite **8** and **10**. These two metabolites were tentatively identified as hydroxylated metabolites of monodemethoxy methoxychlor derivatives (**4** and **7**, respectively). The mass spectrum of metabolite
11 has shown *m*/*z* 431 and 548 as the base peak and molecular ion, respectively. These results indicate that **11** may have a trihydroxylated metabolite of methoxychlor. According to the previous studies, several catechol metabolites of methoxychlor were produced from animals (*8*, *13*, *16*). Three metabolites (**8**, **10**, and **11**) were considered to be catechol-type compounds. To determine whether the hydroxyl groups in polyhydroxylated metabolites are located in *ortho*-(catechol)- or nonadjacent position, the crude extracts were derivatized with *n*-butylboronic acid. Because this acid can form a stable five-membered cyclic diester only with catechols or *syn*-dihydrodiols, it is commonly used in the study of bacterial degradation of polycyclic aromatic hydrocarbons, where these diols are produced from Rieske-type dioxygenase (*21*). Among the several peaks, metabolite **8** gave characteristic mass spectral fragments of *n*-butylboronate of catechols, including the molecular ion (*m*/*z* 412), removal of the CCl_3 group (*m*/*z* 295), and sequential removal of the alkyl group (*m*/*z* 252, 238, and 227). These results indicate that metabolite **8** is 1,1,1-trichloro-2-(3,4-dihydroxyphenyl)-2-(4-methoxyphenyl)ethane. According to the above structural information, it can be stated that all class I metabolites were produced from phase I reaction of xenobiotics.

The mass spectra of metabolites **12**−**22** (class II) showed frequent ions. The mass/charge (*m*/*z*) and intensities of these common fragments (e.g., *m*/*z* 103, 129, 147, 169, 217, 243, 271, 361, and 450) are of per-TMS derivatized hexose moieties of various conjugates. The identity of the carbohydrate group was confirmed by treating the crude extracts with *β*-glucosidase (**Figure 4**). After enzymatic hydrolysis, peaks of all class II metabolites disappeared with concomitant increase of several peaks, assigned as class I metabolites. Metabolites **12**−**22** were confirmed as glucose conjugates of class I metabolites (**1**−**4** and **6**−**11**). It is noteworthy that the mass spectra of these conjugates were very similar with those of glucuronides of methoxychlor metabolites from various animals (*7*,*8*). The mass spectra of most glucuronides contain common ions, derived from per-trimethylsilylated glucuronic acid and diagnostic ions, indicating the structural identities of aglycones. The mass spectrum of **12** has shown the dichloromethyl group (*m*/*z* 398), and this metabolite was tentatively identified as a glycoside of the hydroxylated derivative of **2**. According to the mass spectral fragments, corresponding aglycones, compound **16** was identified as the glycoside of metabolite **3**. Compounds **13** and **14** gave quite similar mass spectra. The chlorine isotopic fragment pattern indicates these metabolites as isomeric pairs of glycosides of monodechlorinated **3**, while **15** has two hydroxyl groups and a monochloroethene link. Close inspection of mass spectra of metabolites **17**−**19** indicates the presence of dichloromethyl groups with a different number of hydroxyl and methoxy groups. Metabolites **17**, **18**, and **19** were tentatively identified as glycosides of **8**, **4**, and **6**, respectively. Several ions in the mass spectra of **21** and **22** (e.g., *m*/*z* 402 and 462) suggest that the trichloromethyl group presents in these metabolites. With additional information from the literature, these metabolites were confirmed as glycosides of **7** and **9**, respectively (*7*, *8*). The mass spectrum of **20** indicates the presence of the dichloromethyl group but failed to confirm the structure. Overall, metabolites **12**−**22** were those of phase II metabolism, through which phase I metabolites are transformed into more hydrophilic conjugates of numerous primary metabolites in eukaryotic organisms.

Because of its interesting metabolic patterns and toxicological importance of metabolites, numerous studies have been published in relation to the metabolism of methoxychlor (e.g., (*4*,*5*,*7*,*8*,*13*, *10*,*14*)). Many organisms from several different taxa have shown detailed metabolic insights, indicating common pathways and some novel degradation mechanisms. Fungal metabolism of methoxychlor has been performed with several white rot fungi (*11*, *12*, *17*). However, it has to be mentioned that major degradative enzymes in white rot fungi are largely different from those of animals and other fungi. In most cases, reactive oxygen (e.g., hydroxyl radical)-generating systems are involved in the degradation of recalcitrant chemical by these fungi, while cytochrome P450 is the most common catalytic component of xenobiotic metabolism in animals and other fungal species. Most phase I metabolites (except **8**) from the present study have already been reported from animal studies (*4*, *5*, *7*, *8*, *13*, *14*, *16*, *19*, *20*). Several important mechanisms, including demethylation, hydroxylation, oxidation to alkenes, and dechlorination, are involved in the metabolisms. In many animal systems, cytochrome P450s play a major role in xenobiotic degradation, where the metabolic outcome is most of the above-mentioned metabolites. Treatment of piperonyl butoxide and chlorpyrifos strongly inhibited methoxychlor degradation and concomitant formation of metabolites. Among the trace of metabolites in inhibitor-treated cultures, a large portion of metabolites are from dehalogenation of parent pesticides (e.g., metabolite **2**−**4**), while didemethylated or hydroxylated/demethylated metabolite (**6**−**11**) levels were far less than those of control experiments (**Figure 7**). Both piperonyl butoxide and chlorpyrifos are well-known inhibitors of cytochrome P450 (*24*, *25*). Reduced formation of demethylated or hydroxylated metabolites (most of phase I metabolites) by a selected inhibitor suggests that major metabolic transformations of methoxychlor in *Cunninghamella elegans* are catalyzed by cytochrome P450. In addition, the common presence of most phase I metabolites in this fungi indicates strong resemblance with animal xenobiotics metabolism.

Among identified metabolites, formation of glycosides, rather than glucuronides, was a characteristic difference of phase II metabolism of methoxychlor between *Cunninghamella elegans* and animals. Xenobiotics−glucuronide (or sulfate) conjugations are one of the most common phase II metabolisms in animals, while glycoside formations are more common in plants and fungi. However, it has to be mentioned that *Cunninghamella* spp. can also form glucuronide or sulfates during the metabolism of selected drugs (e.g., (*18*)). Additional differences include formation of disubstituted conjugates. Ohyama et al. reported that disubstituted conjugates of methoxychlor (glucuronic acid and

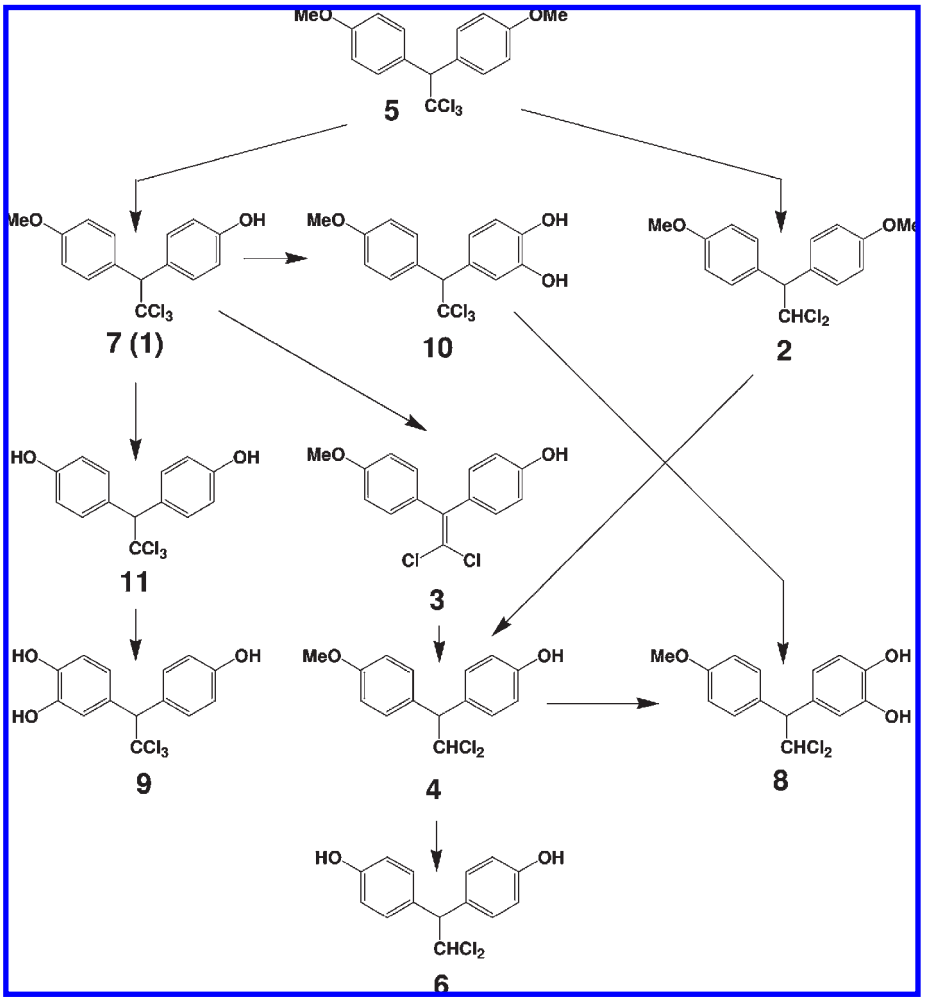

Figure 8. Proposed phase I metabolism of methoxychlor by *Cunninghamella elegans*. Several glycosides of phase I metabolites were also found (phase II metabolites).

sulfate in the same metabolite) were found in rat-liver slice experiments (*19*, *20*). However, no trace of such metabolite was observed in the present study.

Previous studies suggested that estrogenic potencies of methoxychlor metabolites (**7** and **9**) are related to the bisphenol A (4,4′-dihydroxy-2,2-diphenylpropane)-like structure (*9*). Both metabolites were the most abundant products throughout the experiment with *Cunninghamella elegans* (**Figure 1**). However, the level decreased after 4−6 days, while the concentrations of their conjugates (**21** and **22**) or other phase I metabolites with less estrogenic activity gradually increased. These results indicate that *Cunninghamella elegans* can efficiently transform methoxychlor to less toxic metabolites through phase I metabolism, which is highly similar to those of animals, followed by glycoside formation (**Figure 8**).

LITERATURE CITED

(1) Asha, S.; Vidyavathi, M. *Cunninghamella* − A microbial model for drug metabolism studies − A review. *Biotechnol. Adv.* **2009**, *27*, 16–29.

(2) Baarschers, W. H.; Bharath, A. I.; Elvish, J.; Davies, M. The biodegradation of methoxychlor by *Klebsiella pneumoniae*. *Can. J. Microbiol.* **1982**, *28*, 176–179.

(3) Blizard, D.; Sueyoshi, T.; Negishi, M.; Dehal, S. S.; Kupfer, D. Mechanism of induction of cytochrome P450 enzymes by the proestrogenic endocrine disruptor pesticide-methoxychlor: interactions of methoxychlor metabolites with the constitutive androstane receptor system. *Drug Metab. Dispos.* **2001**, *29*, 781–785.

(4) Bulger, W. H.; Muccitelli, R. M.; Kupfer, D. Studies on the in vivo and in vitro estrogenic activities of methoxychlor and its metabolites. Role of hepatic mono-oxygenases in methoxychlor activation. *Biochem. Pharmacol.* **1978**, *27*, 2417–2423.

(5) Bulger, W. H.; Feil, V. J.; Kupfer, D. Role of hepatic monooxygenases in generating estrogenic metabolites from methoxychlor and from its identified contaminants. *Mol. Pharmacol.* **1985**, *27*, 115–124.

(6) Cummings, A. M. Methoxychlor as a model for environmental estrogens. *Crit. Rev. Toxicol.* **1997**, *27*, 367–379.

(7) Davison, K. L.; Feil, V. J.; Lamoureux, C. H. Methoxychlor metabolism in goats. *J. Agric. Food. Chem.* **1982**, *30*, 130–137.

(8) Davison, K. L.; Lamoureux, C. H.; Feil, V. J. Methoxychlor metabolism in chickens. *J. Agric. Food Chem.* **1984**, *32*, 900–908.

(9) Gaido, K. W.; Maness, S. C.; McDonnell, D. P.; Dehal, S. S.; Kupfer, D.; Safe, S. Interaction of methoxychlor and related compounds with estrogen receptor alpha and beta and androgen receptor: structure-activity studies. *Mol. Pharmacol.* **2000**, *58*, 852–858.

(10) Gray, L. E., Jr.; Ostby, J. S.; Ferrell, J. M.; Sigmon, E. R.; Goldman, J. M. Methoxychlor induces estrogen-like alterations of behavior and the reproductive tract in the female rat and hamster: effects on sex behavior, running wheel activity and uterine morphology. *Toxicol. Appl. Pharmacol.* **1988**, *96*, 525–540.

(11) Grifoll, M.; Hammel, K. E. Initial steps in the degradation of methoxychlor by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **1997**, *63*, 1175–1177.

(12) Hirai, H.; Nakanishi, S.; Nishida, T. Oxidative dechlorination of methoxychlor by lignolytic enzymes from white rot fungi. *Chemosphere* **2004**, *55*, 641–645.

(13) Hu, Y.; Kupfer, D. Metabolism of the endocrine disruptor pesticide-methoxychlor by human P450s: pathways involving a novel catechol metabolite. *Drug Metab. Dispos.* **2002**, *30*, 1035–1042.

(14) Kapoor, I. P.; Metcalf, R. L.; Nystrom, R. F.; Sangha, G. K. Comparative metabolism of methoxychlor, methiochlor, and DDT in mouse, insects, and in a model ecosystem. *J. Agric. Food Chem.* **1970**, *18*, 1145–1152.

(15) Kojima, H.; Katsura, E.; Takeuchi, S.; Niiyama, K.; Kobayashi, K. Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. *Environ. Health Perspect.* **2004**, *112*, 524–531.

(16) Kupfer, D.; Bulger, W. H.; Theoharides, A. D. Metabolism of methoxychlor by hepatic P450 monooxygenases in rat and human. 1. Characterization of a novel catechol metabolite. *Chem. Res. Toxicol.* **1990**, *3*, 8–16.

(17) Lee, S.-M.; Lee, J.-W.; Park, K.-R.; Hong, E.-J.; Jeung, E.-B.; Kim, M.-K.; Kang, H.-Y.; Choi, I.-G. Biodegradation of methoxychlor and its metabolites by the white rot fungus *Stereum hirsutum* related to the inactivation of estrogenic activity. *J. Environ. Sci. Health B* **2006**, *41*, 385–397.

(18) Lin, L.; Huang, H.; Zhang, P.; Qi, X.; Zhong, D. Microbial transformation of dextromethorphan by *Cunninghamella blakesleeana* AS 3.153. *Chem. Pharm. Bull.* **2007**, *55*, 658–661.

(19) Ohyama, K.; Maki, S.; Sato, K.; Kato, Y. In vitro metabolism of [^{14}C]methoxychlor in rat, mouse, Japanese quail and rainbow trout in precision-cut liver slices. *Xenobiotica* **2004**, *34*, 741–754.

(20) Ohyama, K.; Maki, S.; Sato, K.; Kato, Y. Comparative in vitro metabolism of methoxychlor in male and female rats: metabolism of demethylated methoxychlor metabolites by precision-cut rat liver slices. *Xenobiotica* **2005**, *35*, 683–695.

(21) Seeger, M.; Camara, B.; Hofer, B. Dehalogenation, denitration, dehydroxylation, and angular attack on substituted biphenyls and related compounds by a biphenyl dioxygenase. *J. Bacteriol.* **2001**, *183*, 3548–3555.

(22) Soto, A. M.; Sonnenschein, C.; Chung, K. L.; Fernandez, M. F.; Olea, N.; Olea-Serrano, F. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* **1995**, *103*, 113–122.

(23) Stuchal, L. D.; Kleinow, K. M.; Stegeman, J. J.; James, M. O. Demethylation of the pesticide methoxychlor in liver and intestine from untreated, methoxychlor-treated, and 3-methylchloranthrene-treated channel catfish (*Ictalurus punctatus*): evidence for roles of CYP1 and CYP3A family isozymes. *Drug Metab. Dispos.* **2006**, *34*, 932–938.

(24) Tang, J.; Cao, Y.; Rose, R. L.; Hodgson, E. In vitro metabolism of carbaryl by human cytochrome P450 and its inhibition by chlorpyrifos. *Chem.-Biol. Interact.* **2002**, *141*, 229–241.

(25) Usmani, K. A.; Rose, R. L.; Hodgson, E. Inhibition and activation of the human liver microsomal and human cytochrome P450 3A4 metabolism of testosterone by deployment-related chemicals. *Drug Metab. Dispos.* **2003**, *31*, 384–391.

(26) Yim, Y.-J.; Seo, J.; Kang, S.-I.; Ahn, J.-H.; Hur, H.-G. Reductive dechlorination of methoxychlor and DDT by human intestinal bacterium *Eubacterium limosum* under anaerobic conditions. *Arch. Environ. Contam. Toxicol.* **2008**, *54*, 406–411.

(27) Zepp, R. G.; Wolfe, N. L.; Gordon, J. A.; Fincher, R. C. Light-induced transformations of methoxychlor in aquatic systems. *J. Agric. Food Chem.* **1976**, *24*, 727–733.

Received June 22, 2009. Revised manuscript received August 6, 2009. Accepted August 11, 2009.