JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 12379–12384 12379
DOI:10.1021/jf102980y

Metabolism of a Fungicide Mepanipyrim by Soil Fungus *Cunninghamella elegans* ATCC36112

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Mepanipyrim is a fungicide against several plant pathogens. However, no metabolic details have been established in fungi, which is the most important biomass in the natural environment. *Cunninghamella elegans* is a well-known fungal species with its strong resemblance to the mammalian xenobiotic metabolism. In this study, the detailed metabolic pathways of mepanipyrim were investigated with *C. elegans*. Approximately 87% of mepanipyrim was removed within 12 h with concomitant accumulation of nine metabolites. Structures of the metabolites were fully or tentatively identified with GC-MS and ^{1}H NMR. To determine the possible role of representative oxidative enzymes, piperonyl butoxide and methimazole were treated, and the kinetic responses of mepanipyrim and its metabolites were measured. Dose-dependent inhibition of metabolism was observed with piperonyl butoxide, while methimazole also inhibited the metabolism less effectively. The results indicate the possible involvement of cytochrome P450 and flavin-dependent monooxygenase in mepanipyrim metabolism. Comprehensive metabolic pathways can be deduced from the detailed analysis of metabolite profiles in control and inhibitor assays.

KEYWORDS: Mepanipyrim; fungal metabolism; *Cunninghamella elegans*; fungicide; inhibitor

INTRODUCTION

Environmental pollution with pesticides is now of important concern both in social communities and in industrial aspects. In general, recently developed pesticides are considered to be much safer than those with a long history (e.g., organochlorines). However, unexpected health risks have been occasionally reported. For example, fipronil is a highly selective insecticide. However, recent studies indicate its possible endocrine disruption activity in rodents (*25*). Currently, sophisticated frameworks of safety evaluation are commonly applied to ensure the safety of new pesticides, and the environmental fate studies take an important role in this task.

Mepanipyrim, *N*-(4-methyl-6-prop-1-ynylpyrimidin-2-yl)aniline, is an anilinopyrimidine fungicide, developed by Kumiai Chemical Industry Co. (*31*). It can effectively control several important plant pathogens, including *Botrytis cinerea*, *Benturia inaequaris*, and *Monilinia fructicola* (*33*). Several analogues (e.g., cyprodinil and Pyrimethanil) are also in agricultural use. Their mode of action is (a) inhibition of specific amino acid biosynthesis and/or (b) inhibition of protein sequestration (*9*, *19*, *20*). The major mode of action of mepanipyrim is proposed to be the inhibition of pathogenicity-related protein secretion (*20*). Mepanipyrim is quite a safe compound (acute oral LD_{50} > 5000 mg kg^{-1}, rat) (*31*) suggesting the least harmful effects on human or animal health, and several toxicological studies were reported (*15*−*18*, *24*).

In general, pollutants in soils are decomposed by plant, fungi, and bacteria. Biodegradation by a microorganism is one of the most important procedures of dissipation and detoxification of toxic chemicals in the natural environment (*12*). Among several macro- or microflora in the soil environment, fungal biomass is usually far higher than that of others. Consequently, their contribution in xenobiotics dissipation may also be important. *Cunninghamella elegans* (*C. elegans*), a zygomycete fungus, has frequently been used as a microbial model for mammalian xenobiotic metabolism, including drugs, pesticides, and other pollutants (*5*, *11*, *13*, *14*, *21*, *27*, *32*, *35*). *C. elegans* can produce xenobiotic-degrading enzymes, related to phase I and II metabolism (*34*).

In this study, the detailed metabolism of mepanipyrim by *C. elegans* has been investigated through metabolite profiling. The involvement of oxidative enzymes in metabolic reactions was determined with selected inhibitors.

MATERIALS AND METHODS

Chemicals. Mepanipyrim (purity >99%) was kindly provided by Kyungnong Ltd. in Korea. Potato dextrose agar (PDA) and potato

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Published on Web 11/03/2010

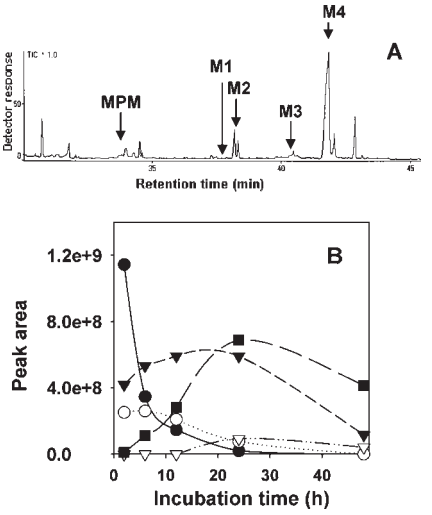

Figure 1. Representative GC-MS total ion chromatogram of culture extracts of mepanipyrim metabolism by *Cunninghamella elegans* after 48 h (**A**) and degradation and accumulation patterns of mepanipyrim and metabolites during metabolism control (**B**). Abbreviations in the insert: MPM, mepanipyrim. The structures of M1−M4 are described in **Figure 4**.

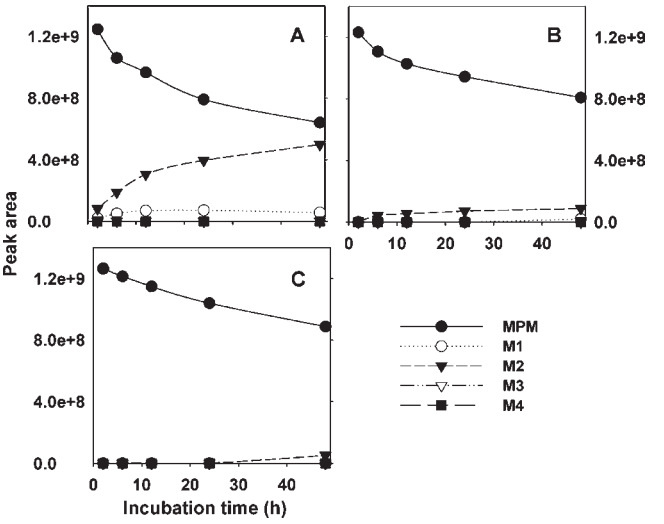

Figure 2. Degradation and accumulation patterns of mepanipyrim and metabolites by *Cunninghamella elegans* in piperonyl butoxide-treated cultures (1, 10, and 100 mg/L piperonyl butoxide for **A**, **B**, and **C**). Abbreviations in the insert: MPM, mepanipyrim.

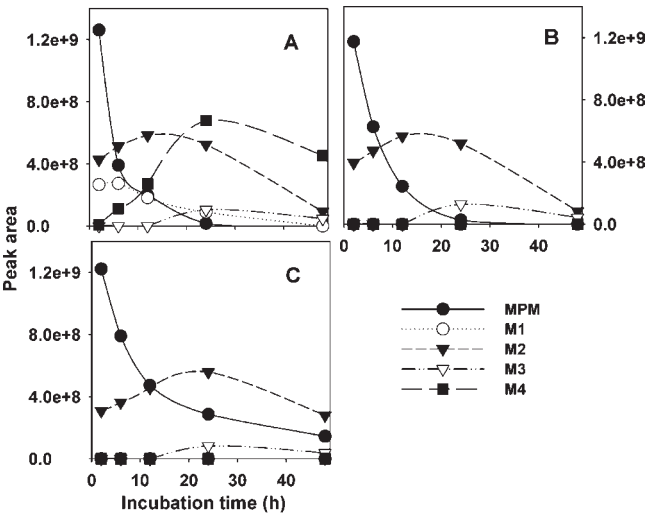

Figure 3. Degradation and accumulation patterns of mepanipyrim and metabolites by *Cunninghamella elegans* in methimazole-treated cultures (1, 10, and 100 mg/L methimazole for **A**, **B**, and **C**). Abbreviations in the insert: MPM, mepanipyrim.

dextrose broth (PDB) were purchased from BD Korea (Korea). Methimazole (MZ), piperonyl butoxide (PB), and bis(trifluoromethylsilyl) acetamide-trimethylsilyl chloride (BSTFA-TMCS, 99:1, sylonBFT) were from Aldrich Korea (Korea). Silica gel (70−230mesh) was from Merck (USA). Anhydrous sodium sulfate and sodium chloride were from Junsei (Japan). Solvents used for HPLC analysis were of the HPLC grade (Duksan, Korea). All the other reagents were of reagent grade or better.

Microorganism. *Cunninghamella elegans* ATCC36112 was obtained from American Type Culture Collection (Manassas, VA). Fungal cultures were typically maintained on potato dextrose agar (PDA) at 27 °C, while the corresponding liquid cultures were performed on potato dextrose broth (PDB) at 27 °C, 170 rpm.

Metabolic Reactions of Mepanipyrim by *Cunninghamella elegans* ATCC36112. For the metabolic reaction system, the seed culture was prepared on PDB for 2 days. After removal of supernatants, mycelia (approximately 2 g) was resuspended in fresh PDB (500 mL), supplemented with mepanipyrim (5 mg), and cultured at 27 °C (170 rpm) for 48 h. For sterilized control experiment, cultures were sterilized at 120 °C for 30 min. To study the effects of metabolic inhibitors of cytochrome P450 (CYP) and flavin-dependent monoxygenase (FMO), PB and MZ were used, respectively. Stock solutions of inhibitors were added to the culture (1, 10, and 100 mg/L) and preincubated for 12 h, followed by the addition of mepanipyrim (5 mg).

Metabolic Profiling of Microbial Metabolism of Mepanipyrim. At each sampling time (2, 6, 12, 24, and 48 h after treatment), aliquots (50 mL) of the culture with the mycelium were transferred into a 500 mL separatory funnel with 15 g of NaCl and extracted with ethyl acetate (twice with 100 and 50 mL). The organic layer was combined and dried over anhydrous sodium sulfate. After removal of the solvent, the residue was resuspended in dry pyridine (0.5 mL), derivatized with BSTFA + TMCS (99:1, 200 μL) at 70 °C for 35 min, and analyzed by GC-MS.

Isolation and Identification of Metabolites by Large Scale Culture. Four of the 500 mL cultures were extracted, combined, and concentrated as described in the previous section for the identification of small metabolites (**M5**−**M9**) and isolation of major metabolites. Major metabolites **M1**, **M2**, and **M4** for ^{1}H NMR analysis were fractionated from that extract through silica gel (30 g) chromatography with 50 mL of hexane/ethyl acetate by stepwise gradient elution (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, and 30:70, v/v). Each fraction was analyzed by GC-MS after TMS derivatization. **M4** was eluted in a fraction of 30:70 (hexane/ethyl acetate), and mixture of **M1** and **M2** was collected in a fraction of 50:50 (hexane/ethyl acetate). **M1** and **M2** were further purified with HPLC (Agilent 1100 series, Santa Clara, CA) with a Luna C18 column (250 × 4.6 mm, 5 um), using a mobile phase of methanol/water (50/50, v/v) at a flow rate of 1 mL/min. Peaks were monitored by absorbance at 275 nm.

Instrumental Analysis. Mepanipyrim and metabolites were routinely analyzed with a gas chromatograph−mass spectrometer (GC-MS, QP5000, Shimadzu, Japan), equipped with a DB-5MS column (30 m, 0.25 μm film thickness, 0.25 mm i.d.; Agilent Technologies, USA). Helium was used as a carrier gas at a flow rate of 1.0 mL/min. The column temperature was programmed as follows: 80 °C (12 min), raised to 240 °C at a rate of 7 °C/min, then raised to 295 °C at a rate of 5 °C/min, and held for 40 min. Temperatures of injection port and interface were set at 290 and 270 °C, respectively. The mass spectrometer was operated at electron impact (EI) mode at 70 eV. ^{1}H NMR measurements were performed with a high resolution NMR spectrometer (Avance 600, Bruker) at 600 MHz. The samples were dissolved in $CDCl_3$ or CD_3OD.

RESULTS

Metabolism of Mepanipyrim by *C. elegans*. GC-MS analysis of the culture extract indicates that mepanipyrim was rapidly

transformed to many metabolites (**Figure 1A**). For example, the level of mepanipyrim rapidly decreased to 13% of the applied dosage in 12 h and was undetectable after 48 h (**Figure 1B**), and **M1**, **M2**, **M3**, and **M4** were observed as major metabolites. Among those, **M1** and **M2** were produced immediately (< 2 h). The concentration of **M1** reached a maximum at 6 h and then gradually decreased to trace level at 48 h. Another metabolite, **M2** gives higher GC-MS response during experiments than **M1**, and the highest accumulation of this metabolite was observed at 24 h. Only a trace quantity of **M3** was found during the initial 12 h, but it slowly increased afterward. **M4** could be observed after 4 h, and then it became the most abundant metabolite after 24 h until the end of experiments. Other metabolites (**M5**−**M9**) were hardly located on the chromatogram because of trace levels; therefore, their formation pattern could not be established. Then, the identification of those was carried out through large scale culture. In a sterilized controls experiment, no appreciable degradation was observed (data not shown).

Table 1. GC/MS Retention Times and Mass Fragmentations of Mepanipyrim and the TMS-Derivatized Metabolites

compound	retention time (min)	molecular and fragment ions[a] (*m*/*z*)
Mepanipyrim	34.70	223 (M^+, 15), 222 (100),
M1	37.76	311 (M^+, 100), 296 (20), 266 (35), 221 (38)
M2	38.26	311 (M^+, 100), 296 (15), 148 (13)
M3	40.46	399 (M^+, 100), 384 (7), 368 (11), 310 (12), 179 (35)
M4	41.58	399 (M^+, 100), 384 (13), 310 (8), 170 (13)
M5	37.27	313 (M^+, 70), 299 (13), 222 (100), 199 (20), 147 (35),
M6	33.50	315 (M^+, 40), 300 (33), 256 (25), 224 (53), 199 (78), 147 (55), 117 (100)
M7	36.32	403 (M^+, 5), 388 (12), 313 (82), 300 (58), 224 (47), 199 (100),
M8	39.23	403 (M^+, 17), 388 (15), 300 (7), 287 (100)
M9	39.99	491 (M^+, 46), 401 (40), 388 (25), 312 (22), 287 (100)

[a] Values in parentheses are the relative abundance of specified fragment ions.

Effects of PB on the Degradation of Mepanipyrim. In comparison with control experiments, a large portion of mepanipyrim still persisted in the culture treated with PB. For example, residual mepanipyrim after 48 h was approximately 50−70% of the initial dose (**Figure 2A**, **B**, and **C**).

In all cultures with PB, **M3**, **M4**, and other minor metabolites were found only in trace levels or were not observed. Among others, **M2** was the major metabolite in low level PB-treated cultures (1 mg/L PB, **Figure 2A**). Its concentration gradually increased until the end of the experiment, while the similar metabolite got its maximum accumulation at 24 h and then gradually decreased in control experiments (**Figure 1B**). Concentration of **M1** in the PB-treated culture (1 mg/L) was approximately 5−10-fold less than those of the control (**Figure 2A**). Its concentration reached a maximum at 24 h and kept constant. In the case of higher level of PB-treatment (10 and 100 mg/L), **M1** was only at trace levels at 48 h or was not observed, while **M2** could be detected after 6 h (**Figure 2B** and **C**). However, the concentrations of **M2** were far less than those of the control or low PB-treatment experiments.

Effects of MZ on the Degradation of Mepanipyrim. Mepanipyrim rapidly dissipated in MZ-treated cultures (1, 10 mg/L of MZ) and was undetectable at 48 h, while significant amounts (12% of initial dose) still persisted in high levels of MZ cultures (100 mg/L) (**Figure 3**). Overall profiles of mepanipyrim and metabolites with the lowest concentration of MZ (1 mg/L) were not distinguishable from those of the control (**Figure 3A**). However, a largely different response has been observed at higher concentrations of inhibitor (10 and 100 mg/L) (**Figure 3B** and **C**). For example, **M1** and **M4** were not observed throughout the experiments for both concentrations. As for PB-treated cultures, **M2** was the major metabolite and its kinetic responses in MZ-treated cultures were almost similar to those of the control.

Identification of Mepanipyrim Metabolites. Approximately, nine metabolites of mepanipyrim were observed in culture supernatants including four major metabolites (**M1**−**M4**). Mass spectral details of those metabolites are described in **Table 1**. TMS derivatives of **M1** and **M2** have shown their molecular ion at *m*/*z* 311, which was 88 Da (-OTMS) higher than that of mepanipyrim (M^+, 223), suggesting that those metabolites may be monohydroxylated mepanipyrim. The ^{1}H NMR spectra of mepanipyrim and metabolites **M1**, **M2**, and **M4** were obtained with purified samples (**Figure 4**). The ^{1}H NMR spectrum of **M1** has shown the well conserved proton resonance of the phenyl ring (δ 7.4−7.7 ppm),

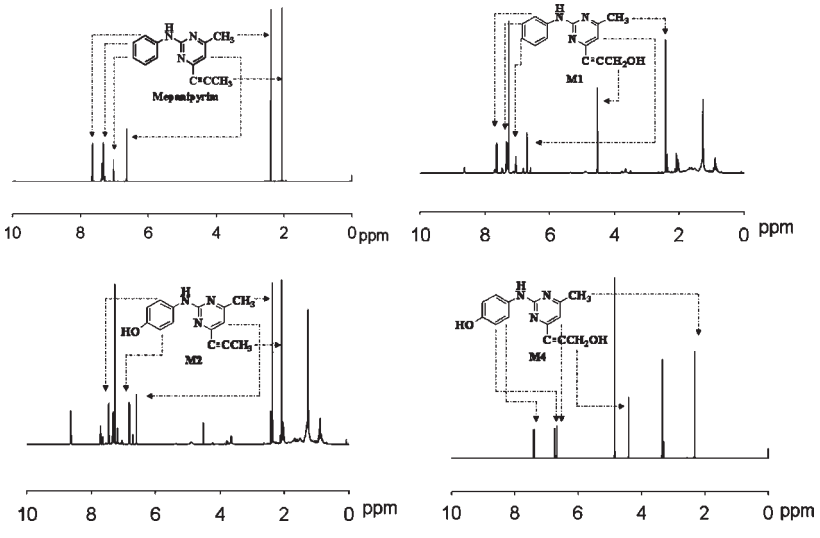

Figure 4. ^{1}H NMR spectra of mepanipyrim, **M1**, **M2**, and **M4**. Mepanipyrim, **M1**, and **M2** were dissolved in $CDCl_3$ and **M4** in CD_3OD.

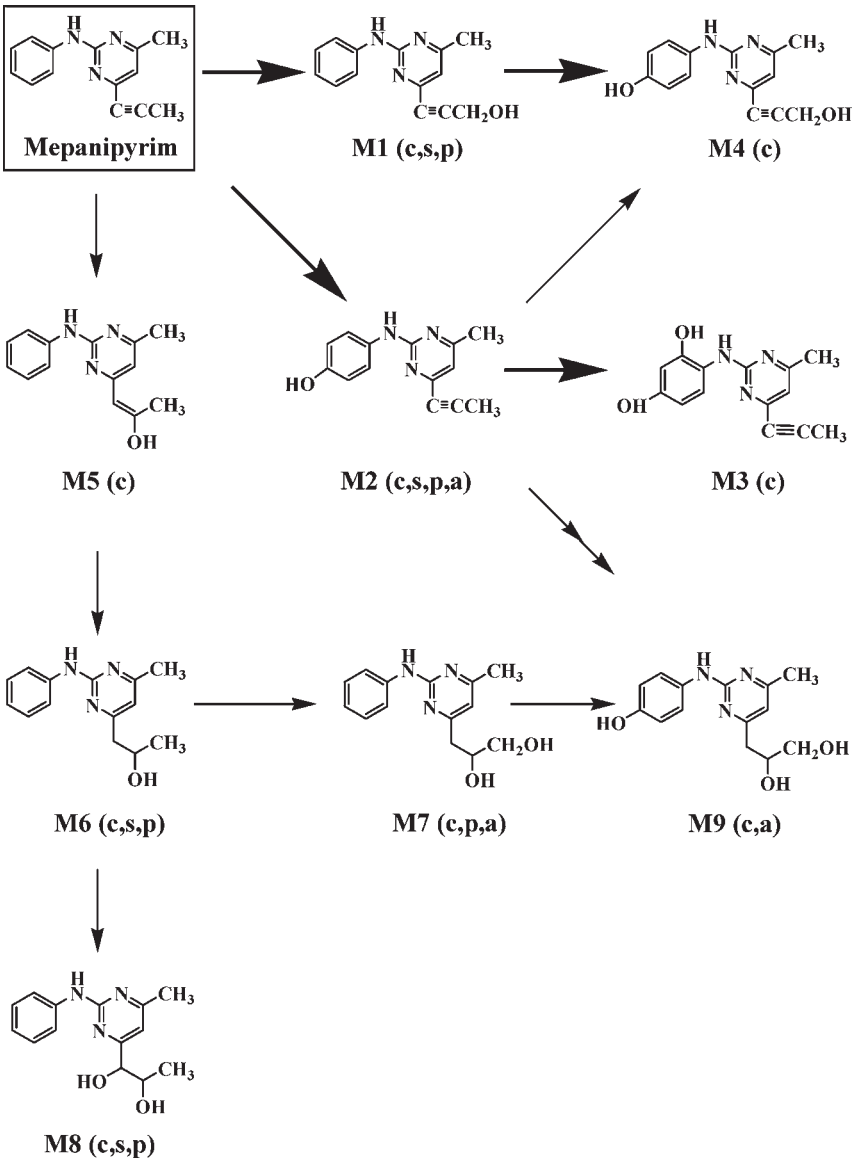

Figure 5. Proposed metabolic pathways of mepanipyrim by *Cunninghamella elegans* (c) and previously determined transformations in other environments. Major pathways are expressed as thick arrows. Abbreviations: s, soil; p, plants; and a, animals.

while the methyl groups in propynyl substituents shifted from δ 2.06 to δ 4.52, indicating the presence of a hydroxymethyl group in this moiety. On the basis of these results, **M1** was identified as 3-[6-methyl-2-(phenylamino)pyrimidin-4-yl]prop-2-yn-1-ol. In the ^{1}H NMR spectrum of **M2**, chemical shifts of substituents in pyrimidine are not changed, while there were new resonance patterns in phenyl rings, being identified as 4-[4-methyl-6-(prop-1-yn-1-yl)pyrimidin-2-yl]aminophenol. Molecular ions of **M3** and **M4** were observed at *m*/*z* 399, indicating two hydroxyl groups in corresponding compounds. **M4** shows a ^{1}H NMR feature of combined **M1** and **M2**, indicating hydroxylation at the phenyl and propynyl side chain of the pyrimidine ring, resulting in the structure of 4-[(3-hydroxy-prop-1-ynyl)-6-methyl-pyrimidin-2-yl]aminophenol. The mass spectral pattern of **M3** indicates that two hydroxyl groups may be introduced in the molecule; however, its structural identification by ^{1}H NMR failed due to the small amount of isolation.

Additional metabolites (**M5**−**M9**) were tentatively identified through mass spectral patterns and related references (*17*, *18*, *28*). They can be grouped into those with a molecular ion of *m*/*z* 313 (**M5**), 315 (**M6**), 403 (**M7**−**M8**), and 491 (**M9**). The presence of fragment ions *m*/*z* 222 and/or 224 indicates that **M5** and **M6** may have hydroxypropenyl and hydroxypropyl groups in the pyrimidine ring. Among two metabolites with *m*/*z* 403 as the molecular ion, **M7** gives a mass spectrum closely similar to that of **M6**, except for the 88 Da increase of molecular weight, again which indicates an additional hydroxyl group. The characteristic fragment ion *m*/*z* 199 corresponds to (4,6-dimethyl-pyrimidin-2-yl)-phenyl-amine. Metabolites **M8** and **M9** have shown common mass spectral features, except for their molecular ions. For example, the fragment ion *m*/*z* 287 was the base peak or was one of the most abundant ions, which may correspond to (4-methyl-6-trimethylsilyloxymethyl-pyrimidin-2-yl)phenyl-amine. These metabolites were tentatively identified as di- and trihydroxylated derivatives of **M6** (**Figure 5**).

DISCUSSION

C. elegans and other strains in the same genus can metabolize a huge number of chemicals (*3*). It is well known that cytochrome P450s (CYPs) are ubiquitous enzymes in almost all living organisms. CYPs of *C. elegans* have been implicated in the neutralization

of various polycyclic aromatic hydrocarbons (PAHs) and other environmental pollutants (*3*).

In comparison with other anilinopyrimidines (cyprodinil and Pyrimethanil), microbial metabolism of mepanipyrim is not well understood. The almost immediate onset of **M1** and **M2** accumulation indicates that these metabolites may be initial precursors of others. Comparative analysis of their structures also supports such conclusion. **M1** and **M2** were previously reported from plants, soil, or animals (*17*, *18*, *28*). It is noteworthy that cyprodinil, a cyclorpropyl analogue of mepanipyrim, has shown different metabolic patterns with the same strain (*30*). Hydroxylation of cyprodinil occurs only in the phenyl ring, while other functional groups in the pyrimidine ring remained intact during metabolism. Aromatic ring hydroxylation by a similar fungus has been observed in pyrene and benzopyrene. From the inhibitor assays, it was concluded that CYPs and epoxide hydroxylases are involved in the metabolism of these xenobiotics (*4*−*6*, *26*, *27*). Among metabolites, the onset of **M4** accumulation was observed after the maximum of **M1**, and its levels were coordinately reduced with those of the **M1** in inhibitor assay. The chemical structure of **M4** also indicates the metabolic relationship with **M1**. According to these results, it can be concluded that mepanipyrim is transformed to **M4** via **M1**.

To investigate the responsible enzyme in mepanipyrim metabolism, PB and MZ were treated in the culture because PB is a well-known CYP inhibitor (*11*), while MZ is also a well-known FMO inhibitor (*23*). As expected, PB strongly inhibited the dissipation of mepanipyrim and the formation of four major metabolites, while treatment of MZ gives significant inhibition only at the highest dose. The result indicates that FMO may have a minor role in mepanipyrim metabolism. Interestingly, the concentrations of **M2** and **M3** were not significantly changed by MZ, while the formation of **M1** and **M4** was strongly inhibited (**Figure 3**). The result suggests that mepanipyrim is transformed to **M3** via **M2** and that FMOs are involved only in certain parts of mepanipyrim metabolism, while CYPs have a wide array of catalytic activities. Similar trends of metabolic transformation of **M1** and **M2** were reported in soils, plants, and animals (*17*, *18*, *25*). These results has supported that **M7** and **M8** were downstream metabolites of **M6** and that **M7** eventually transformed to **M9**. **M2** was relatively stable to further degradation. However, the continuous formation of **M9** suggests a slow but evident metabolism of **M2**. As mentioned above, CYPs have major role of **M2** formation. Further oxidation on the propynyl group by CYP and/or FMO may produce **M9**.

Acetylenic compounds are generally considered to be highly reactive in natural environments. For example, many polyacetylenes from Asteraceae plants have strong phytotoxicity (*2*). However, the triple bond in mepanipyrim was relatively stable than the other functional groups. All major metabolites (**M1**−**M4**) still reserve the acetylenic bond as it is. In addition, themethyl group in pyrimidine remained intact over most metabolites. It is well known that *C. elegans* preferentially oxidizes the alkyl side chain over the aromatic ring in alkyl PAHs (*4*). Collectively, the metabolism of mepanipyrim in *C. elegans* was quite different from those of soils and plants. For example, major metabolic pathways in tomato seedlings and soil are through **M5** to **M8** (*17*, *18*). However, a similar pathway takes a minor role in *C. elegans* (**Figure 5**).

In addition to phase I metabolism, *Cunninghamella* spp. can catalyze almost all phase II reactions, including sulfation, glucosylation, and glucuronide formation (*1*, *11*, *13*, *27*). Among several fungi, *Beauveria bassiana* could produce a glycoside conjugate of cyprodinil, while most strains of *C. elegans* could not catalyze a similar reaction (*30*). In this study, no trace of either glucosides or glucuronide of mepanipyrim were observed even in prolonged cultures. The results indicate that hydroxylated metabolites of mepanipyrim may not be subjected to phase II reactions. However, it cannot be ruled out that there may be other types of reactions (e.g., sulfate conjugation). A recent study has shown that cyprodinil sulfate was the major phase II metabolite from rats (*22*). Further investigation with other extraction methods and LC-MS will be addressed to solve the problems.

In addition to conjugate formation, it is well known that large portions of environmental contaminants are transformed to nonextractable residues. For example, cyprodinil in plants and soils become rapidly nonextractable residues (*7*, *8*, *29*). Kang et al. reported that cyprodinil was rapidly incorporated into insoluble polymers by laccase from *Trametes villosa* (*10*). Although, it is not clear whether *Cunninghamella* spp. can produce such enzymes, similar reactions are highly probable in the natural environment, probably by other fungi or plants.

In summary, this is the first report of microbial metabolism of mepanipyrim, and a detailed analysis indicates that *C. elegans* can effectively metabolize the fungicide by phase I oxidative enzymes (CYP and FMO) through several different pathways, including those common in plants, soils, or animals.

Supporting Information Available: Mass spectra of mepanipyrim and TMS derivatized metabolites (**M1**−**M9**) were presented in supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review July 30, 2010. Revised manuscript received October 5, 2010. Accepted October 6, 2010.