

Microbial Transformations of the Fungicide Cyprodinil (CGA-219417)

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A collection of 12 microbial cultures, known to contain cytochrome P-450 monooxygenase or other degradative enzymes, was screened for their ability to degrade the Novartis Crop Protection Inc. developmental fungicide cyprodinil (CGA-219417; 4-cyclopropyl-6-methyl-*N*-phenyl-2-pyrimidinamine). Ten of the 12 cultures produced a monohydroxylated metabolite in yields ranging from 1.2 to 35.6%. The filamentous fungus, *Beauveria bassiana* ATCC 7159, produced a methoxylated glycoside of the monohydroxylated metabolite with a yield of 80%. Dihydroxylated metabolites and a molecular cleavage product, 4-cyclopropyl-6-methyl-2-pyrimidinamine, were also detected in certain cultures. The overall results of the study indicated that cyprodinil was readily metabolized by a variety of microbial species. Metabolites generated by these cultures can potentially be used as analytical reference standards to support animal, plant, and soil metabolism studies.

Keywords: Cyprodinil; microbial transformations; metabolite identification; reference standards

INTRODUCTION

The use of common microbes to synthesize potential metabolites of agrochemicals in sufficient quantities can provide an important alternative to conventional chemical synthesis or to predict environmentally relevant degradation pathways. Examples from the published literature include insecticides (Sariaslani *et al.*, 1987; Maloney *et al.*, 1988) and herbicides (Schocken *et al.*, 1989; Dietrich *et al.*, 1995; Liu *et al.*, 1996; Kulowski *et al.*, 1997). Most interestingly, these common microorganisms can metabolize xenobiotics in a manner remarkably similar to that shown by mammals, birds, fish, soil, and, to some extent, plants. This similarity in metabolite profile is largely explained by the presence of the enzyme cytochrome P-450 monooxygenase in these organisms. This enzyme is capable of causing aliphatic and aromatic hydroxylations as well as N-, S-, and O-dealkylations on a wide range of xenobiotic substrates. Because of this similarity in metabolite profiles to biological systems tested in the agrochemical industry (e.g., in rat, goat, poultry, fish, plant, and soil metabolism studies), microbial cultures can be used to synthesize metabolites in sufficient quantities (i.e., milligrams) to obtain spectra for identification purposes. Subsequently, these metabolites can be generated in larger quantities (i.e., grams) through larger scale transformations to serve as reference standards in support of agrochemical metabolism and environmental fate studies. In addition to providing an alternative to chemical synthesis, the microbial approach can also be used to *predict* metabolites in soil, mammals, birds, fish, and plants *before* the bona-fide metabolism studies are conducted.

In this study, a collection of 12 microbial cultures (9 filamentous fungi, 2 streptomycetes, and 1 bacterium) was screened for their ability to generate metabolites of the developmental fungicide cyprodinil (Heye *et al.*, 1994; Figure 1). The objective was to generate metabolite reference standards by microbial rather than chemical means as well as to predict the environmental fate of this agrochemical. Selection of cultures was, in general, based on published literature demonstrating their ability to metabolize a wide variety of xenobiotics.

MATERIALS AND METHODS

Chemicals. Cyprodinil (4-cyclopropyl-6-methyl-*N*-phenyl-2-pyrimidinamine) was synthesized by Novartis Crop Protection, Inc., and had a purity of >99.9%, based on high-performance liquid chromatography (HPLC). [¹⁴C-(pyrimidinyl)]Cyprodinil was also synthesized by Novartis Crop Protection, Inc., and had a specific activity of 51.1 μ Ci/mg. Radiochemical purity was determined to be 100% by HPLC with radiometric detection (RAM). Solvents used for HPLC and extractions were purchased from Burdick and Jackson and were of HPLC grade. PIC A Low UV, an ion-pairing reagent that minimizes background interference in the low-UV range, was purchased from Waters Corp. Water was obtained with a Sybron/Barnstead NANOpure II system and meets ASTM Type IIA requirements. Soybean grits were purchased from Archer Daniels Midland Co., Decatur, IL. All other materials were of the highest purity commercially available.

Microorganisms. The microbial cultures used for this study were obtained from the American Type Culture Collection (ATCC), located in Rockville, MD (Table 1). The pure cultures were maintained on agar slant tubes containing appropriate growth media as recommended by the ATCC.

Microbial transformations were conducted by the two-stage incubation protocol previously described by Betts *et al.* (1974). Cyprodinil (10 mg of unlabeled cyprodinil and 3 μ Ci of [¹⁴C-pyrimidinyl]cyprodinil) was dissolved in 500 μ L of ethanol and added to 24–72-h-old stage II, 50-mL cultures (200 mg/L; 0.9 mM). Cultures were harvested after 6 or 7 days. Cells were separated from broths by centrifugation (except for *Rhizopus oryzae*, for which vacuum filtration was employed). The pH of the broths was recorded and adjusted to approximately pH 6 and subsequently partitioned with 3 $\times$ 50 mL of ethyl acetate. Ethyl acetate portions were combined, concentrated by rotary evaporation at 37 $^{\circ}$ C, and dried under a stream of

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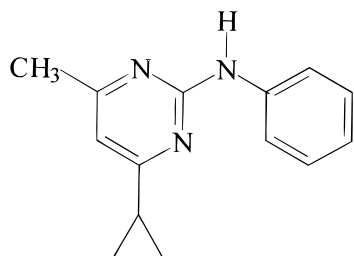


Figure 1. Chemical structure of cyprodinil (CGA-219417; MW = 225).

Table 1. Production of the Monohydroxylated Metabolite of Cyprodinil (Metabolite 1) by Microbial Cultures

microbial culture	extent of conversion (%)
<i>C. echinulata</i> var. <i>elegans</i> ATCC 36112	1.2
<i>C. echinulata</i> var. <i>elegans</i> ATCC 9245	2.0
<i>C. echinulata</i> var. <i>echinulata</i> ATCC 9244	25.6
<i>A. pseudocylindraspora</i> ATCC 24169	35.6
<i>S. griseus</i> ATCC 13273	24.8
<i>S. rimosus</i> ATCC 10970	28.0
<i>Mucor circinellus</i> f. <i>griseocyanus</i> ATCC 1207a	5.4
<i>Bacillus megaterium</i> ATCC 14581	1.5
<i>R. oryzae</i> ATCC 24563	20.5
<i>B. bassiana</i> ATCC 7159	0
<i>Phanerochaete chrysosporium</i> ATCC 24725	0
<i>Penicillium chrysogenum</i> ATCC 10002	0

nitrogen. The residue was redissolved in several milliliters of acetonitrile/water (1:1) and analyzed by HPLC-RAM and, in selected cases, by liquid chromatography/mass spectrometry (LC/MS). Cells were lysed and extracted in either acetone or methanol using a Branson Model 450 sonifier. The cell extracts were transferred to 50-mL centrifuge tubes and centrifuged at 3000 rpm for 1 h. Portions of the cell extracts were dried with a combination of rotary evaporation and a nitrogen stream, redissolved in acetonitrile/water (1:1), and analyzed by HPLC-RAM and, in selected cases, by LC/MS.

Because of substantial radioactivity remaining in the postpartitioned broths of the *Cunninghamella echinulata* var. *elegans* cultures (46–48%), postpartitioned broths were acidified with concentrated HCl to an approximate pH of 2 and repartitioned with an equal volume of ethyl acetate in an attempt to remove acidic metabolites from the aqueous phase. Afterward, a 10-mL aliquot of the postpartitioned broth from *C. echinulata* var. *elegans* (ATCC 9245) was adjusted to pH 12 with 1N NaOH and repartitioned with an equal volume of methylene chloride to remove basic metabolites from the broth. An aliquot of the acidic postpartitioned broth was concentrated under a nitrogen stream and analyzed by HPLC-RAM and LC/MS to identify and characterize metabolites. (The postpartitioned broths of *Absidia pseudocylindraspora*, *Streptomyces griseus*, and *Streptomyces rimosus* also contained radioactivity, although considerably less than the *C. echinulata* var. *elegans* cultures. Therefore, detailed characterization of the postpartitioned broths from these cultures was not pursued.)

A control consisted of cyprodinil added to the soybean grit–glucose medium in the absence of microorganisms.

Analytical Methods. Radioactivity was quantified by liquid scintillation counting (LSC) using a Beckman Model LS 5000 TD or a Beckman Model LS 1801 liquid scintillation counter calibrated with factory-prepared standards. Counting efficiencies of all experimental samples were determined using an external standard and a factory-prepared calibration curve (Beckman Instruments). All test samples were counted for a maximum of 5 min or until a 2 σ error of 5% was attained.

HPLC profiling was accomplished with a Waters Model 510 solvent pump equipped with a Hewlett-Packard M-1050 autosampler, an Autochrom M-112–2 gradient controller, either a Kratos 757 or a Hewlett-Packard 1049A UV detector, and a Radiomatic A-280 radioactivity detector. Metabolites were separated on a Metachem Nucleosil C₁₈ analytical column (4.6 $\times$ 250 mm) by using a 20-min linear gradient starting with 100% of solvent A [acetonitrile/water/PIC A Low UV (20:80:1)] to 100% of solvent B [acetonitrile/water/PIC A Low UV, 80:20:1] and then holding 100% solvent B for an additional 9 min. The mobile phase flow rate was 1.0 mL min⁻¹. Detection was accomplished by UV at 230 nm and by flow-through radiometric detection using a 500- μ L liquid flow cell with a cocktail flow rate (Flo-Scint II) of 3.0 mL min⁻¹.

Thin-layer chromatography (TLC) for isolating metabolites of cyprodinil was performed with Whatman 20 $\times$ 20 cm, 250- μ m thick, fluorescent TLC plates. Plates were developed to 15 cm in a solvent system comprised of methylene chloride/methanol (9:1). After evaporation of solvent from the plates was allowed for, parent compound and metabolites were located by the quenching of short-wavelength UV light. Bands corresponding to major metabolites were traced lightly with a lead pencil and scraped from the plates using a spatula. Metabolites were eluted from silica gel with 5-mL portions of ethyl acetate or a 1:1 solution of ethyl acetate/methanol.

Proton nuclear magnetic resonance spectra (¹H-NMR) were obtained on a Bruker 300 MHz at the University of Rhode Island NMR Research Laboratory under the direction of Dr. Michael A. McGregor. Samples were dissolved in deuterated methanol.

LC/MS was accomplished with a Hewlett-Packard 5989A MS engine with a particle beam interface, a Hewlett-Packard 1050 gradient pump, and a Hewlett-Packard autosampler. In most cases, chromatography included a Metachem Nucleosil (C₁₈, 10 μ m) 4.6 $\times$ 250 mm column and an isocratic mobile phase composed of acetonitrile/water (70:30) at a flow rate of 0.8 mL min⁻¹. For analysis of the postextracted broth of *C. echinulata* var. *elegans* (ATCC 9245), chromatography included a Metachem Nucleosil (C₁₈, 5 μ m) 2 $\times$ 150 mm column and a linear gradient starting at 25 mM ammonium acetate, pH 6.5 (100%), and ending at 20% 25 mM ammonium acetate, pH 6.5/80% acetonitrile. For mass spectrometry, ionization was by electron impact at 70 eV with a source temperature of 250 $^{\circ}$ C, an analyzer temperature of 120 $^{\circ}$ C, and a desolvation temperature of 55 $^{\circ}$ C. Helium pressure was 55–60 psi. In most cases, scan range was from 60 to 300 amu except in the analysis of the postextracted broth of *C. echinulata* var. *elegans* (ATCC 9245), for which the scan range was from 10 to 650 amu.

RESULTS AND DISCUSSION

The HPLC-RAM profile of the ethyl acetate extract of the broth from *S. rimosus* indicated a major metabolite (metabolite 1) eluting at 22.70 min (retention time of cyprodinil was 28.40 min). The total ion chromatogram and the electron impact mass spectrum (EI-MS) of this metabolite are provided in Figure 2, indicating a molecular ion at *m/z* 241 and a prominent fragment at *m/z* 240, suggesting a monohydroxylated metabolite of the parent compound (molecular weight of cyprodinil is 225). Subsequently, this metabolite was isolated in a greater quantity by preparative TLC (ca. 200 μ g), and a ¹H-NMR spectrum was recorded as illustrated in Figure 3. The symmetric and relatively simple proton coupling pattern in the aromatic region (signals at 6.7 and 7.4 ppm) is consistent with a para-disubstituted benzene structure (Silverstein *et al.*, 1974), indicating that the position of hydroxylation of cyprodinil appears to have occurred on the phenyl ring, para to the amino group (structure provided in the inset to Figure 3). The same metabolite was also detected in the cell extract of *S. rimosus* as well as in eight other cultures included in the microbial screen in yields ranging from 1.2 (C.

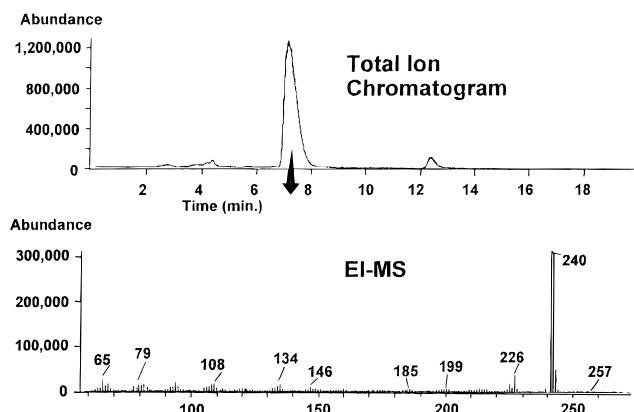


Figure 2. LC/MS of the ethyl acetate extract of the broth from *S. rimosus*.

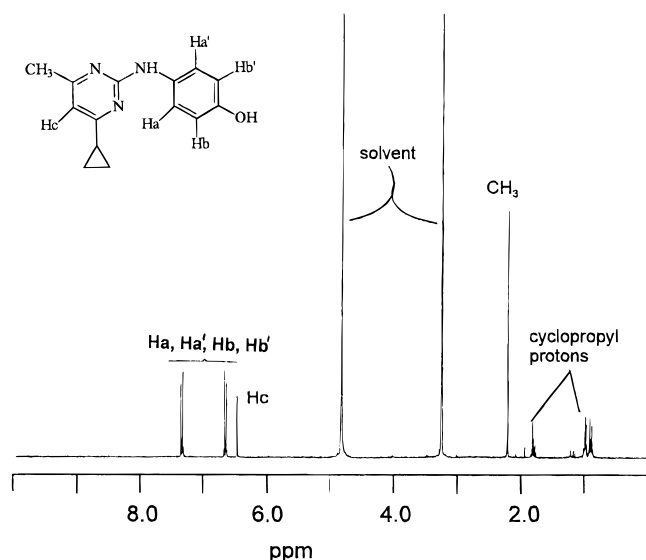


Figure 3. ^1H -NMR spectrum of the major metabolite produced by *S. rimosus*.

echinulata var. *elegans* ATCC 36112) to 35.6% (*A. pseudocylindraspora*). A summary of the production of the monohydroxylated metabolite is provided in Table 1. Additional confirmation of the identity of this metabolite (produced by *A. pseudocylindraspora*) was accomplished by cochromatography (HPLC) of the microbial metabolite with the authentic reference standard.

A predominant metabolite (metabolite 2; produced in 80% yield and present in both the broth and cell extract) from *Beauveria bassiana* had an HPLC retention time of 16.6 min. Its mass spectrum had an apparent molecular ion of m/z 241 but a shorter chromatographic retention time on LC/MS, suggesting a monohydroxylated metabolite different from the para-substituted metabolite described above. Approximately 2 mg of this metabolite was isolated by preparative TLC and a ^1H -NMR spectrum obtained, which is illustrated in Figure 4. As shown, the proton splitting pattern in the aromatic region indicated substitution para to the amino group on the phenyl ring. However, the multiplex of signals in the region of 3.0–4.0 ppm is consistent with a methoxylated carbohydrate with the anomeric proton at ca. 4.8 ppm. Thus, the ^1H -NMR spectrum indicated a monohydroxylated metabolite of cyprodinil (para-disubstituted phenyl ring moiety) which was conjugated to a sugar. The apparent molecular ion at m/z 241 (obtained by the mass spectral analysis) suggesting a

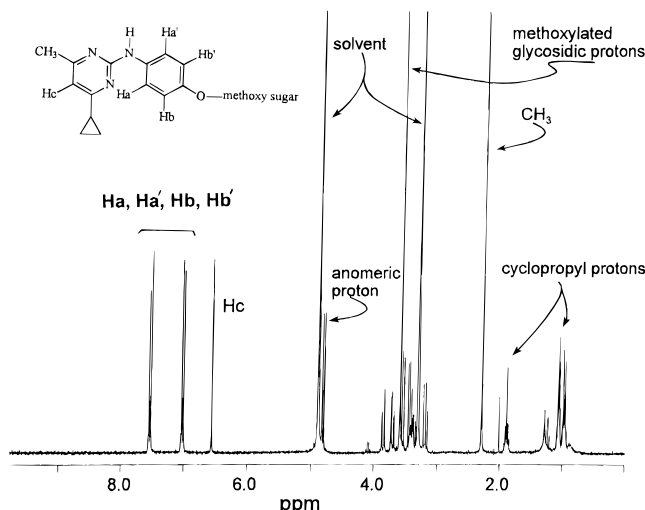


Figure 4. ^1H -NMR spectrum of the major metabolite produced by *B. bassiana*.

nonconjugated metabolite was misleading since the sugar portion apparently was lost as a result of the relatively energetic ionization method. Of note, a methoxylated sugar conjugated to a metabolite of the herbicide propham was also formed from *B. bassiana* (then called *B. sulfurescens*) and reported by Vigne *et al.* (1986).

In the fungus *A. pseudocylindraspora*, two major metabolites of cyprodinil were produced. The major metabolite, accounting for a 35.6% yield, was identified as metabolite 1. Its identity was based on ^1H -NMR and chromatographic and mass spectral comparisons to the authentic reference standard. The other metabolite (metabolite 3), produced in a 12% yield and somewhat more polar, had a ^1H -NMR spectrum indicating a pattern of a para-disubstituted phenyl ring with loss of the proton (ca. 6.5 ppm) signal on the pyrimidine ring. The EI-MS of this metabolite indicated a molecular ion of m/z 257, suggesting a dihydroxylated metabolite. Both metabolites of *A. pseudocylindraspora* are presented in Figure 5, which also depicts pathways of cyprodinil metabolism in the various microbial cultures.

The ethyl acetate extract of the broth of both isolates of the fungus *C. echinulata* var. *elegans* (ATCC 36112 and ATCC 9245) had comparable radiocarbon contents (13.3 and 14.5% of the dose) and showed similar HPLC-RAM profiles. LC/MS analysis from *C. echinulata* var. *elegans* ATCC 36112 indicated polar metabolites with molecular ions at m/z 241 and 257, suggesting mono- and dihydroxylated metabolites of cyprodinil. On the basis of the EI-MS fragmentation pattern (i.e., prominent fragment ions at m/z 148 and 149, representing an unsubstituted methylcyclopropylamino pyrimidine moiety), the position of dihydroxylation is thought to occur on the phenyl ring rather than on the pyrimidine or cyclopropyl rings (Figure 5; metabolite 4). LC/MS analysis of ATCC isolate 36112 also indicated a metabolite with an apparent molecular ion of m/z 149 (Figure 5; metabolite 5). This would be consistent with a molecular cleavage product of cyprodinil. The HPLC retention time of the authentic reference standard (14.88 min) was similar to that of the microbial metabolite (14.80 min), providing additional confirmation.

Both fungal isolates showed significant amounts of radiocarbon (46–48%) remaining in the broth after ethyl acetate partitioning. Therefore, extracted broths were adjusted to an approximate pH of 2 and repartitioned with ethyl acetate. A small portion of radiocarbon was

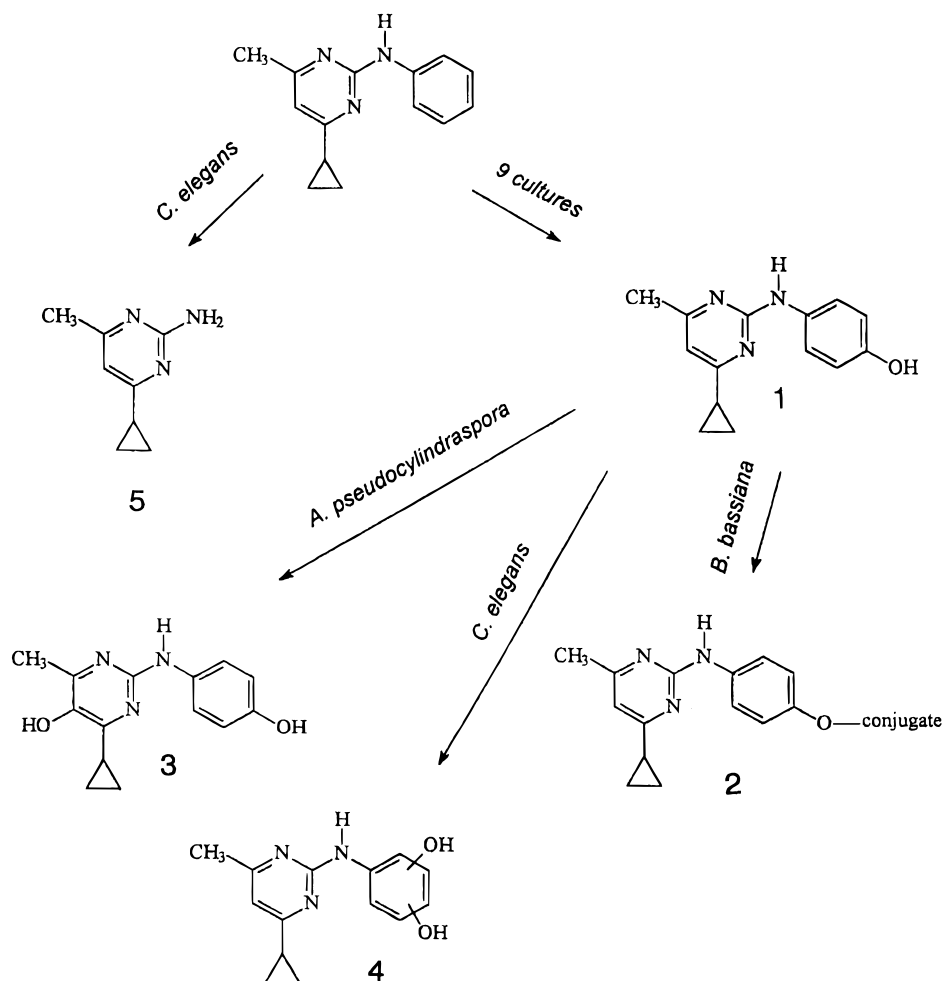


Figure 5. Pathways of cyprodinil metabolism in microbial cultures.

removed (5%), suggesting the presence of acidic metabolites. Since substantial radiocarbon remained, the extracted broth of *C. echinulata* var. *elegans* (ATCC 9245) was brought to a pH of 12 with 1 N sodium hydroxide and partitioned with an equal volume of methylene chloride. Only a small amount of radiocarbon was found in the methylene chloride (2%), suggesting that the metabolites in the extracted broth of the *C. echinulata* var. *elegans* cultures were quite polar in nature. The partitioned pH 2 broth was subsequently concentrated 10-fold under a nitrogen stream and analyzed by HPLC-RAM and LC/MS. The HPLC-RAM profile revealed the presence of polar metabolites. The LC/MS total ion chromatogram was quite complex. Mass spectra were obtained on eluting compounds, which were base-line resolved. A number of metabolites had apparent molecular ions at m/z 241 and 257, suggesting mono- and dihydroxylated metabolites of parent compound. However, since these kinds of compounds should have been partitioned into ethyl acetate, it is more likely that these metabolites represent conjugates, either hydroxylated at a variety of positions on the cyprodinil molecule or hydroxylated in similar positions but conjugated to a variety of sugars or other natural products. Since molecular ions at higher mass corresponding to an intact conjugate were not detected, it is likely that the electron impact mode of ionization was not "soft" enough (compared to alternative modes of ionization such as electrospray or thermospray) to preserve the molecular ion of the conjugates. Further efforts to define the nature of the conjugates were not pursued.

The presence of conjugates in the *B. bassiana* and *C. echinulata* var. *elegans* cultures is indicative of phase II reactions in mammalian metabolism. *C. echinulata* var. *elegans* has previously been reported to form conjugates with a variety of polycyclic aromatic hydrocarbon metabolites including anthracene (sulfate conjugate; Cerniglia, 1982), phenanthrene (sulfate and glucoside conjugates; Casillas *et al.*, 1996), fluoranthene (glucoside conjugates; Pothuluri *et al.*, 1992), pyrene (glucoside conjugates; Cerniglia *et al.*, 1986), benzo[*a*]pyrene (sulfate and glucuronide conjugates; Cerniglia and Gibson, 1979), and benzo[*e*]pyrene (sulfate and glucoside conjugates; Pothuluri *et al.*, 1996).

The recovery of radioactivity in the broth and cell extracts of the cultures used in the screen ranged from 71 to 96%. Since the majority of radioactive residues were extractable, no further efforts were made to release cell-bound materials.

The control showed minimal degradation of cyprodinil (<2%) in the soybean grit-glucose medium, indicating that observed metabolism in the screen was microbially mediated.

Thus, the results of this study indicate that the fungicide cyprodinil is readily degraded by a number of microbial species known to contain the enzyme cytochrome P-450 monooxygenase. The identification of the resulting metabolites can provide important information relevant to the metabolism of this compound in animals, soils, and higher plants.

Although microbial cultures have been used extensively for generating as well as predicting potential mammalian metabolites of pharmaceuticals (Smith and

Rosazza, 1975, 1983; Griffiths *et al.*, 1991), the approach has been used only sparingly for agrochemicals. Nevertheless, the results of this study as well as other published reports suggest the important utility of the technique.

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