

Initial Steps in the Degradation of Methoxychlor by the White Rot Fungus *Phanerochaete chrysosporium*

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The white rot fungus *Phanerochaete chrysosporium* mineralized [*ring*-¹⁴C]methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] and metabolized it to a variety of products. The three most prominent of these were identified as the 1-dechloro derivative 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane, the 2-hydroxy derivative 2,2,2-trichloro-1,1-bis(4-methoxyphenyl)ethanol, and the 1-dechloro-2-hydroxy derivative 2,2-dichloro-1,1-bis(4-methoxyphenyl)ethanol by comparison of the derivatives with authentic standards in chromatographic and mass spectrometric experiments. In addition, the 1-dechloro-2-hydroxy derivative was identified from its ¹H nuclear magnetic resonance spectrum. The 1-dechloro and 2-hydroxy derivatives were both converted to the 1-dechloro-2-hydroxy derivative by the fungus; i.e., there was no requirement that dechlorination precede hydroxylation or vice versa. All three metabolites were mineralized and are therefore likely intermediates in the degradation of methoxychlor by *P. chrysosporium*.

White rot fungi such as *Phanerochaete chrysosporium* degrade a wide spectrum of organopollutants. Some of these chemicals undergo initial oxidation by the nonspecific peroxidases that are thought to function in fungal ligninolysis, but many organopollutants lack structural features that would make them susceptible to attack by ligninolytic enzymes (4, 5). The insecticide 1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane (methoxychlor) is an interesting example of this type: although it is not a peroxidase substrate, it is one of the few organopollutants to be mineralized efficiently by *P. chrysosporium* (2). To gain insight into this facet of fungal xenobiotic metabolism, we have identified several dechlorination and hydroxylation reactions that are early steps in methoxychlor degradation by *P. chrysosporium*.

Metabolites obtained from methoxychlor. *P. chrysosporium* (ATCC 24725) was grown in N-limited, rotary-shaken cultures at 39°C as described previously (6), except that Tween 80 was omitted from the growth medium. After 48 h of growth, cultures were combined pairwise, and growth medium was decanted to give 25-ml cultures that each contained approximately 30 mg (dry weight) of mycelial pellets. [*ring*-¹⁴C]methoxychlor (Sigma; 0.92 mCi mmol⁻¹, 10 μM final concentration) was then added to each of 10 replicate cultures in 50 μl of dimethyl sulfoxide. The radiochemical purity of the labeled methoxychlor was >98%, as determined by normal-phase high-performance liquid chromatography (HPLC; chromatography conditions are described below). The fungal cultures were fitted with sterile gassing manifolds to monitor the evolution of ¹⁴CO₂, which was trapped in an alkaline scintillation cocktail (9). Incubation was continued under O₂ for 7 days, at which time the cultures were combined and filtered through Miracloth to separate the mycelial pellets from the culture medium. The culture medium was adjusted to pH 7 and extracted with chloroform-acetone (1:1), after which the remaining aqueous phase was adjusted to pH 1 and reextracted with the same solvents. The mycelial pellets were extracted twice by being stirred in chloroform-acetone (1:1) for 24 h, and

then the residual mycelium was combusted to quantify nonextractable ¹⁴C-labeled material.

The neutral (pH 7) extract and the mycelial pellet extract were dried over Na₂SO₄, evaporated to dryness, redissolved in 1 ml of dichloromethane, and applied to a column of silica gel 60 (1 by 10 cm) that had been preequilibrated with dichloromethane. The column was developed with 50 ml of dichloromethane (fraction A), and then the remainder of the applied ¹⁴C was eluted quantitatively with 50 ml of dichloromethane-methanol (9:1) (fraction B). In one typical experiment, the mass balance for ¹⁴C after 7 days was as follows: mineralized, 15%; fraction A of culture medium neutral extract, 5%; fraction B of culture medium neutral extract, 8%; culture medium acidic (pH 1) extract, 13%; nonextractable water-soluble materials, 9%; fraction A of mycelial extract, 11%; fraction B of mycelial extract, 2%; mycelium-bound material, 21%; material unaccounted for, 16%.

Each of the A fractions was subjected to normal-phase HPLC on a Lichrosorb SI-60 column (Alltech; 5-μm particle size, 4.6 by 250 mm) in hexane-tetrahydrofuran (95:5) at 1 ml min⁻¹ and ambient temperature. In some experiments, the sample was spiked with authentic standards of various possible metabolites before it was injected onto the column (see below for the provenance of the standards). The *A*₂₈₀ of the column eluate was monitored to detect the standards, and fractions were collected at 0.5-min intervals for quantitation of ¹⁴C-labeled metabolites by liquid scintillation counting. HPLC analysis of the B fractions and of the acidic extract was also attempted in various HPLC systems, but the results showed that these more polar fractions consisted of complex product mixtures that were not successfully resolved.

Figure 1 shows a typical chromatogram of fraction A from the culture medium neutral extract. Fraction A of the mycelial extract gave similar HPLC results (data not shown). The major radiocarbon peaks eluted at the same time as four of the spiked standards and therefore were identified tentatively as unchanged methoxychlor (peak II), its 1-dechloro derivative 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane (peak III), its 2-hydroxy derivative 2,2,2-trichloro-1,1-bis(4-methoxyphenyl)ethanol (peak IV), and its 1-dechloro-2-hydroxy derivative 2,2-dichloro-1,1-bis(4-methoxyphenyl)ethanol (peak VI). (See Fig. 2 for chemical structures.) Two minor radiocarbon peaks were

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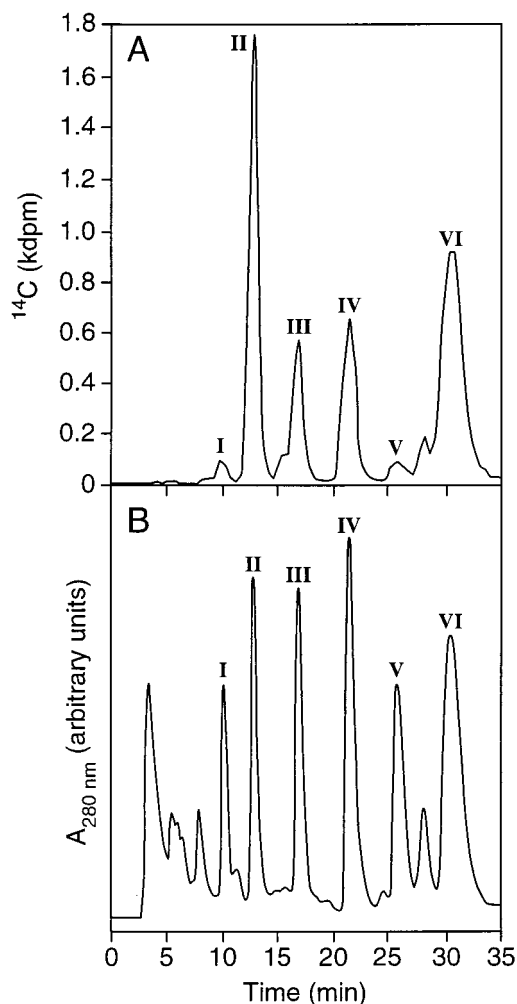


FIG. 1. HPLC analysis of nonpolar metabolites formed from [^{14}C]methoxychlor (II) by *P. chrysosporium*. (A) Radiochromatogram of fraction A from the culture medium. The total radioactivity injected onto the column was 1.6×10^4 dpm. (B) UV absorbance of chromatographic standards that were included in the same injection. Unidentified UV absorbance peaks are due to endogenous nonradioactive fungal metabolites unrelated to methoxychlor. See the text for the names of chemical structures that correspond to peaks I and III-VI.

observed to run with standards of 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane (peak I) and 4,4'-dimethoxybenzophenone (peak V), but the quantities of material recovered were too small to permit further characterization of these metabolites. The metabolite yields from the culture medium and mycelium together were as follows: peak I, 0.4%; peak II, 6.0%; peak III, 1.6%; peak IV, 1.4%; peak V, 0.2%; and peak VI, 3.4%. A control experiment with autoclaved cultures showed that no transformation of methoxychlor occurred in the absence of living fungus.

Identification of metabolites. To confirm identities of the three major metabolites, a larger number of cultures was grown and worked up as outlined above. Fraction A from the culture medium was resolved into its component peaks by preparative normal-phase HPLC in hexane-tetrahydrofuran (96:4), and the purified metabolites were compared with authentic standards by gas chromatography-mass spectrometry. Gas chromatograms were obtained on a 15-m DB-5 (nonpolar silicone polymer) fused-silica capillary column (J&W Scientific), which was operated with a temperature gradient from 140°C to 260°C at 20°C min^{-1} . The mass spectrometer (Finni-

gan MAT 4510) was operated at 70 eV in the chemical ionization mode with ammonia as the reagent gas, because electron impact spectra were not informative for the compounds under investigation.

Metabolite III showed a chromatographic retention time and mass spectrum indistinguishable from those obtained for authentic 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethane. Mass spectrum: m/z (relative intensity) 332 ($M + 4 + \text{NH}_4^+$, 9), 330 ($M + 2 + \text{NH}_4^+$, 56), 328 ($M + \text{NH}_4^+$, 100), 227 ($M^+ - \text{H-CCl}_2$, 8). The standard of compound III was synthesized beforehand from anisole and dichloroacetal as described by Wiechell (16).

Metabolite IV showed a chromatographic retention time and mass spectrum indistinguishable from those obtained for authentic 2,2,2-trichloro-1,1-bis(4-methoxyphenyl)ethanol. Mass spectrum: m/z (relative intensity) 384 ($M + 6 + \text{NH}_4^+$, 0.1), 382 ($M + 4 + \text{NH}_4^+$, 0.4), 380 ($M + 2 + \text{NH}_4^+$, 4), 378 ($M + \text{NH}_4^+$, 4), 349 ($M^+ + 6 - \cdot\text{OH}$, 0.3), 347 ($M^+ + 4 - \cdot\text{OH}$, 19), 345 ($M^+ + 2 - \cdot\text{OH}$, 70), 343 ($M^+ - \cdot\text{OH}$, 77), 243 ($M^+ - \cdot\text{CCl}_3$, 100). The standard of compound IV (8) was a generous contribution from Ivar Ugi (Technische Universität München).

Metabolite VI showed a chromatographic retention time and mass spectrum indistinguishable from those obtained for authentic 2,2-dichloro-1,1-bis(4-methoxyphenyl)ethanol. Mass spectrum: m/z (relative intensity) 348 ($M + 4 + \text{NH}_4^+$, very weak), 346 ($M + 2 + \text{NH}_4^+$, very weak), 344 ($M + \text{NH}_4^+$, 0.1), 313 ($M^+ + 4 - \cdot\text{OH}$, 6), 311 ($M^+ + 2 - \cdot\text{OH}$, 66), 309 ($M^+ \cdot\text{OH}$, 100), 243 ($M^+ - \text{H-CCl}_2$, 4). Metabolite VI, alone among the products analyzed, was obtained in a quantity sufficient to obtain a ^1H nuclear magnetic resonance spectrum as well. Both the metabolite and a standard of compound VI gave the same result: δ (ppm) 3.75 (6H, s, $-\text{OCH}_3$), 5.21 (1H, s, $-\text{OH}$), 6.85 (4H, d, H_3 and H_5), 7.15 (1H, s, $-\text{CHCl}_2$), 7.55 (4H, d, H_2 and H_6). The standard of compound VI was prepared by oxidizing 3,3-bis(4-methoxyphenyl)propenoic acid with Mn(III) according to the method of Yonemura et al. (17) and was purified before analysis by preparative normal-phase HPLC as outlined above for methoxychlor metabolites. 3,3-Bis(4-methoxyphenyl)propenoic acid was prepared from 4,4'-dimethoxybenzophenone as described by Klemm and Bower (11).

Feeding experiments with isolated metabolites. To determine whether products III, IV, and VI were metabolized further by *P. chrysosporium*, they were purified by preparative normal-phase HPLC from fungal cultures that had been given [^{14}C]methoxychlor and then were supplied to new cultures (5 to 10 replicates). Each culture received 2×10^4 dpm (10 μM final concentration) of ^{14}C -labeled compound with a radiochemical purity greater than 90%. Mineralization was monitored until the rates were approximately linear and the cumulative $^{14}\text{CO}_2$ evolution was around 10%, at which point the cultures were harvested for product analysis.

HPLC analysis of fraction A from the culture extracts showed that metabolite III was converted to metabolite VI (9% in 8 days) and that metabolite IV was also converted to metabolite VI (31% in 5 days). That is, *P. chrysosporium* both dechlorinates and hydroxylates the ethyl moiety of methoxychlor without a requirement that one reaction precede the other (Fig. 2). In this respect, the initial steps of methoxychlor degradation by *P. chrysosporium* resemble the pathway proposed by Bumpus and Aust for the degradation of a related pesticide, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), by this fungus (2).

The HPLC results also showed that *P. chrysosporium* did not metabolize compound III, IV, or VI to 4,4'-dimethoxybenzophenone (compound V), even though compound V appears to have been produced at a low level from the parent compound, methoxychlor (Fig. 1). Bumpus and Aust proposed that *P.*

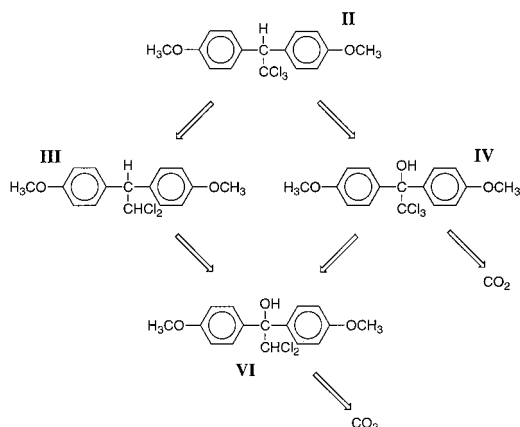


FIG. 2. Routes demonstrated for the dechlorination, hydroxylation, and mineralization of methoxychlor (II) by *P. chrysosporium*. See the text for the names of structures III, IV, and VI.

chrysosporium mineralizes dechlorinated and hydroxylated DDT metabolites via a benzophenone intermediate (2), but our results do not indicate the operation of such a pathway in methoxychlor degradation.

The ¹⁴CO₂ evolution data (Fig. 3) show that metabolites III, IV, and VI are likely intermediates in the mineralization of methoxychlor. Compound IV was mineralized as rapidly as methoxychlor was, whereas the downstream metabolite VI was mineralized more slowly. These results suggest that some of metabolite IV was mineralized without passing through the pathway that includes metabolite VI (Fig. 2). The high variability in mineralization results among cultures that received compound III makes firm conclusions impossible for this metabolite, but the data suggest that it was mineralized at approximately the same rate that metabolite VI was. Of course, our results do not rule out the possibility that there exist other routes for methoxychlor mineralization which do not proceed via any of the metabolites we were able to detect. The highly

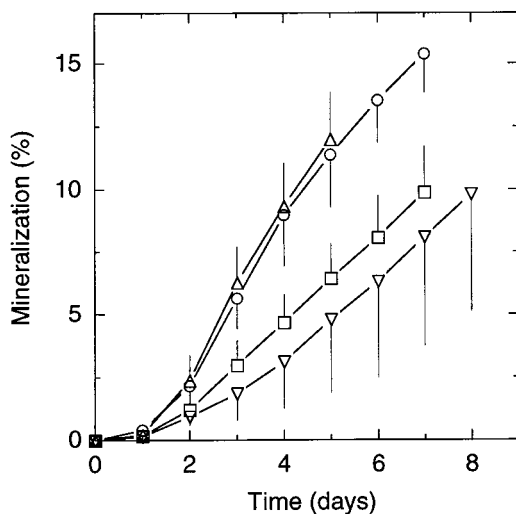


FIG. 3. Mineralization of [¹⁴C]methoxychlor (○) and of ¹⁴C-labeled metabolites III (▽), IV (△), and VI (□) by *P. chrysosporium*. Error bars represent 1 standard deviation of the sample.

branched, complex nature of xenobiotic metabolism in *P. chrysosporium* has been noted by other researchers (7, 15).

Conclusions. Compound III was previously shown to occur as a product of methoxychlor metabolism by a bacterium, *Klebsiella pneumoniae* (1), and in uncharacterized mixed microbial cultures as well (13). Demethylation of the aromatic rings in methoxychlor, a reaction known to be catalyzed by cytochrome P-450 monooxygenases (10, 12), has also been noted in mixed microbial cultures (13). However, to our knowledge, the benzylic hydroxylation of methoxychlor and compound III to give metabolites IV and VI has not previously been observed in a biological system.

It is possible that the fungal 1-dechlorination and 2-hydroxylation of methoxychlor play a role in the removal of this pesticide from contaminated soils. Although *P. chrysosporium* is not a soil fungus, related *Phanerochaete* species that produce rhizomorphs grow aggressively in soil (3, 14). We do not yet know the mechanisms for these reactions, but it is probably safe to predict that they do not involve direct action by fungal ligninolytic peroxidases, which our preliminary results show have no effect on methoxychlor (data not shown).

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