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Degradation of 2,4-Dichlorophenol by the Lignin-Degrading Fungus *Phanerochaete chrysosporium*

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Under secondary metabolic conditions the white rot basidiomycete *Phanerochaete chrysosporium* mineralizes 2,4-dichlorophenol (I). The pathway for the degradation of 2,4-dichlorophenol (I) was elucidated by the characterization of fungal metabolites and of oxidation products generated by purified lignin peroxidase and manganese peroxidase. The multistep pathway involves the oxidative dechlorination of 2,4-dichlorophenol (I) to yield 1,2,4,5-tetrahydroxybenzene (VIII). The intermediate 1,2,4,5-tetrahydroxybenzene (VIII) is ring cleaved to produce, after subsequent oxidation, malonic acid. In the first step of the pathway, 2,4-dichlorophenol (I) is oxidized to 2-chloro-1,4-benzoquinone (II) by either manganese peroxidase or lignin peroxidase. 2-Chloro-1,4-benzoquinone (II) is then reduced to 2-chloro-1,4-hydroquinone (III), and the latter is methylated to form the lignin peroxidase substrate 2-chloro-1,4-dimethoxybenzene (IV). 2-Chloro-1,4-dimethoxybenzene (IV) is oxidized by lignin peroxidase to generate 2,5-dimethoxy-1,4-benzoquinone (V), which is reduced to 2,5-dimethoxy-1,4-hydroquinone (VI). 2,5-Dimethoxy-1,4-hydroquinone (VI) is oxidized by either peroxidase to generate 2,5-dihydroxy-1,4-benzoquinone (VII) which is reduced to form the tetrahydroxy intermediate 1,2,4,5-tetrahydroxybenzene (VIII). In this pathway, the substrate is oxidatively dechlorinated by lignin peroxidase or manganese peroxidase in a reaction which produces a *p*-quinone. The *p*-quinone intermediate is then recycled by reduction and methylation reactions to regenerate an intermediate which is again a substrate for peroxidase-catalyzed oxidative dechlorination. This unique pathway apparently results in the removal of both chlorine atoms before ring cleavage occurs.

Chlorophenols constitute a significant category of pollutants and are major components of paper pulp bleach plant effluents (19, 31). In addition, 2,4,6-trichloro- and pentachlorophenols have been used extensively as wood preservatives and pesticides (11, 28). Finally, 2,4-dichloro- and 2,4,5-trichlorophenols are used as precursors for the synthesis of the herbicides 2,4-dichloro- and 2,4,5-trichlorophenoxyacetic acids, respectively (11, 28). The large-scale use of chlorophenols has led to the contamination of terrestrial and aquatic ecosystems, resulting in the classification of chlorophenols as priority pollutants (21, 28).

The degradation of a variety of environmentally persistent organic pollutants by the lignin-degrading basidiomycetous fungus *Phanerochaete chrysosporium* has been reported (2, 3, 16, 19). In several of these reports, the use of ^{14}C-labeled compounds demonstrated that the pollutant is mineralized. However, for the most part, the degradative pathways utilized by *P. chrysosporium* have not been examined. For example, *P. chrysosporium* has been reported to mineralize pentachlorophenol (27), whereas only the initial oxidative 4-dechlorination to the corresponding *p*-quinone by lignin peroxidase (LiP), purified from the extracellular medium of *P. chrysosporium*, has been demonstrated (17, 27). The pathway beyond this initial step has not been elucidated.

Thus, despite considerable progress in our understanding of lignin model compound degradation by *P. chrysosporium* (15, 18, 23), relatively little is known about the details of chlorophenol and other aromatic-pollutant degradation by this organism. Elucidation of these biodegradative pathways and the specific enzymes involved is necessary for the rational application of *P. chrysosporium* and other white rot fungi to development of environmental cleanup strategies.

In this report we examine the degradative pathway of 2,4-dichlorophenol by *P. chrysosporium*. We show that this pathway involves several cycles of oxidation and subsequent quinone reduction and hydroquinone methylation. The latter reactions generate a peroxidase substrate. These cycles lead to the removal of both chlorine atoms from the substrate.

MATERIALS AND METHODS

Chemicals. 2,4-Dichlorophenol (I) and 2,5-dihydroxy-1,4-benzoquinone (VII) were obtained from Aldrich. 2-Chloro-1,4-dihydroxybenzene (III) was obtained from Pfalz and Bauer.

2-Chloro-1,4-benzoquinone (II). 2-Chloro-1,4-dihydroxybenzene (III) (100 mg) was oxidized by using a molar excess of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (10) in dioxane (4 ml) at room temperature for 16 h, and the reaction mixture was filtered. The filtrate was added to H_2O (10 ml), and the mixture was extracted with chloroform. 2-Chloro-1,4-benzoquinone (II) was purified further by preparative thin-layer chromatography (TLC; Merck $60F_{254}$ silica gel) (solvent system, hexane).

2-Chloro-1,4-dimethoxybenzene (IV). 2-Chloro-1,4-dihydroxybenzene (III) (500 mg) was methylated with dimethylsulfate in refluxing acetone for 5 h as described previously (10). The acetone was evaporated, 50 ml of H_2O was added, and the product mixture was extracted with chloroform. The product was purified by preparative TLC (solvent system, CH_2Cl_2).

2,5-Dimethoxy-1,4-benzoquinone (V). 2,5-Dihydroxy-1,4-benzoquinone (VII) (500 mg) was methylated in methanol-hydrochloridic acid for 12 h at room temperature (35). The

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reaction mixture was evaporated, the products were added to water (50 ml), and the mixture was extracted with chloroform and dried over sodium sulfate. The product was purified by preparative TLC (solvent system, CH_2Cl_2). 1, 2,4,5-Tetrahydroxybenzene (VIII) and 2,5-dihydroxy-1,4-dimethoxybenzene (VI) were prepared by the quantitative reduction of 2,5-dihydroxy-1,4-benzoquinone (VII) and 2,5-dimethoxy-1,4-benzoquinone (V), respectively, by using sodium dithionite in water (10). Reaction mixtures were adjusted to pH 3.0 with dilute HCl, and products were extracted with ethyl acetate and dried over sodium sulfate. U-^{14}C-ring-labeled 2,4-dichlorophenol was obtained from Sigma.

Enzymes. LiP and manganese peroxidase (MnP) were purified from the extracellular medium of an acetate-buffered agitated culture of *P. chrysosporium* OGC101 (1) as previously described (12, 13, 32, 33). LiP concentration was determined at 408 nm by using an extinction coefficient of 133 mM^{-1} cm^{-1} (13); MnP concentration was determined at 406 nm by using an extinction coefficient of 129 mM^{-1} cm^{-1} (12).

Culture conditions. The organism was grown from a conidial inoculum at 38°C in stationary culture as described (9, 14). Unless indicated otherwise, the medium used in this study was as previously described (14, 24) with 2% glucose and either 1.2 or 12 mM ammonium tartrate as the carbon and nitrogen source, respectively. The medium was buffered with 20 mM sodium 2,2-dimethylsuccinate, pH 4.5. Cultures were incubated under air for 3 days, after which they were purged with 99.9% O_2 every 3 days.

Mineralization of 2,4-dichlorophenol (I). ^{14}C-labeled substrate (10^5 cpm, 0.01 μCi/μmol) in *N,N*-dimethylformamide was added to cultures on day 6 to avoid inhibition of growth by 2,4-dichlorophenol (I). Flasks were fitted with ports which allowed periodic purging with O_2 and trapping of evolved $^{14}CO_2$ (14, 24) in a basic scintillation fluid as previously described (24). The efficiency of $^{14}CO_2$ trapping after purging for 10 min was greater than 98%. Counting efficiency (>70%) was monitored with an automatic external standard.

Metabolism of unlabeled 2,4-dichlorophenol and metabolic intermediates. After 6 days of incubation the substrates in acetone were added to cultures to a final concentration of 250 μM. After an additional 1, 6, or 16 h of incubation as indicated, the products were isolated. Cultures were filtered on a Buchner funnel. The mycelial mat was ground in 2 g of sand, and both the mat and extracellular medium were extracted with ethyl acetate (9). The total organic fraction was washed with 10 ml of water, dried over sodium sulfate, and evaporated under reduced pressure. Products were dissolved in methanol (250 μl), and the quinone products were then reduced with sodium dithionite. The products were analyzed either directly or after derivatization. Trimethylsilylation of reduced products was carried out in bis-(*N,O*-trimethylsilyl)trifluoroacetamide–pyridine (2:1). Acetylation of reduced products was carried out in acetic anhydride-pyridine (1:1). The orthoquinone product (3-chloro-4-methoxy-1,2-benzoquinone) generated by the LiP oxidation of 2-chloro-1,4-dimethoxybenzene (IV) was also derivatized with phenylenediamine in ethanol as previously described (20).

Enzyme reactions. LiP reaction mixtures (2 ml) consisted of enzyme (10 μg), substrate (0.5 mM), and H_2O_2 (0.2 mM) in 20 mM sodium succinate, pH 3.0. MnP reaction mixtures consisted of enzyme (10 μg), substrate (0.5 mM), H_2O_2 (0.2 mM), and $MnSO_4$ (0.4 mM) in 50 mM sodium malonate, pH 4.5. Reactions were carried out at 30°C for 45 min.

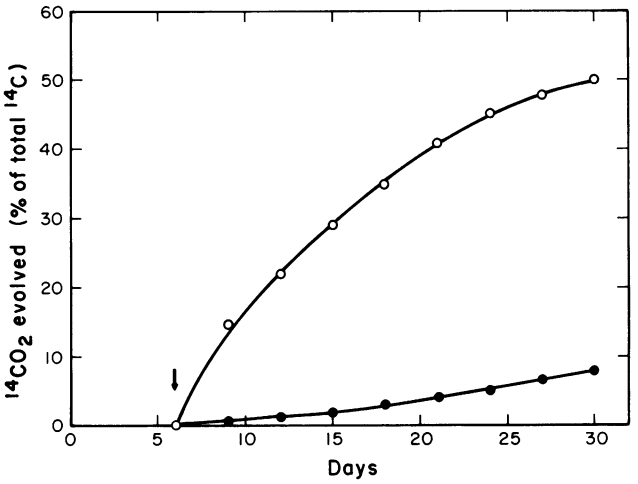

FIG. 1. Effects of nitrogen concentration on the mineralization of 2,4-[^{14}C]dichlorophenol. Stationary cultures containing 1.2 (○) and 12 (●) mM ammonium tartrate were inoculated with conidia and incubated for 6 days at 37°C after which radiolabeled substrate was added (↓). Flasks were purged with O_2, and the evolved $^{14}CO_2$ was trapped and counted as described in the text.

Chromatography and spectrometry. Gas chromatography-mass spectrometry was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A gas chromatograph and a 25-m fused silica column (DB-5; J & W Science). The gas chromatography temperature was programmed from 80 to 320°C at 10°/min. Products also were analyzed by high-performance liquid chromatography (HPLC) using a Beckman C8 Ultrasphere column and a linear gradient from 30% methanol in 3 mM acetic acid to 100% methanol.

The [^{14}C]malonic acid peak was collected and counted in a Beckman LS-3133P scintillation counter by using Beckman Ready-Solv scintillation cocktail. Silica gel TLC of radiolabeled products was performed by using benzene-methanol-acetic acid (45:8:4) as the solvent system. Autoradiography was performed with Kodak X-Omat film.

RESULTS

The mineralization of 2,4-[^{14}C]dichlorophenol (I) by cultures of *P. chrysosporium* is shown in Fig. 1. After a 24-day incubation period, approximately 50% of the substrate added to the cultures was degraded to $^{14}CO_2$ in nitrogen-limited (1.2 mM ammonium tartrate) cultures while only approximately 8% of the initial substrate was mineralized in nitrogen-sufficient (12 mM ammonium tartrate) cultures.

Metabolism of substrates. Products and their yields of the *P. chrysosporium* metabolism of various substrates and intermediates are shown in Fig. 2. Four aromatic products were identified as *P. chrysosporium* metabolites of 2,4-dichlorophenol (I): 2-chloro-1,4-benzoquinone (II), 2-chloro-1,4-hydroquinone (III), 2-chloro-1,4-dimethoxybenzene (IV), and 2,4-dichloroanisole. Several of these metabolites were prepared and added exogenously to *P. chrysosporium* cultures. 2-Chloro-1,4-hydroquinone (III), 2-chloro-1,4-dimethoxybenzene (IV), and 2,5-dimethoxy-1,4-benzoquinone (V) were identified as metabolites of 2-chloro-1,4-benzoquinone (II). 2-Chloro-1,4-hydroquinone (III) was

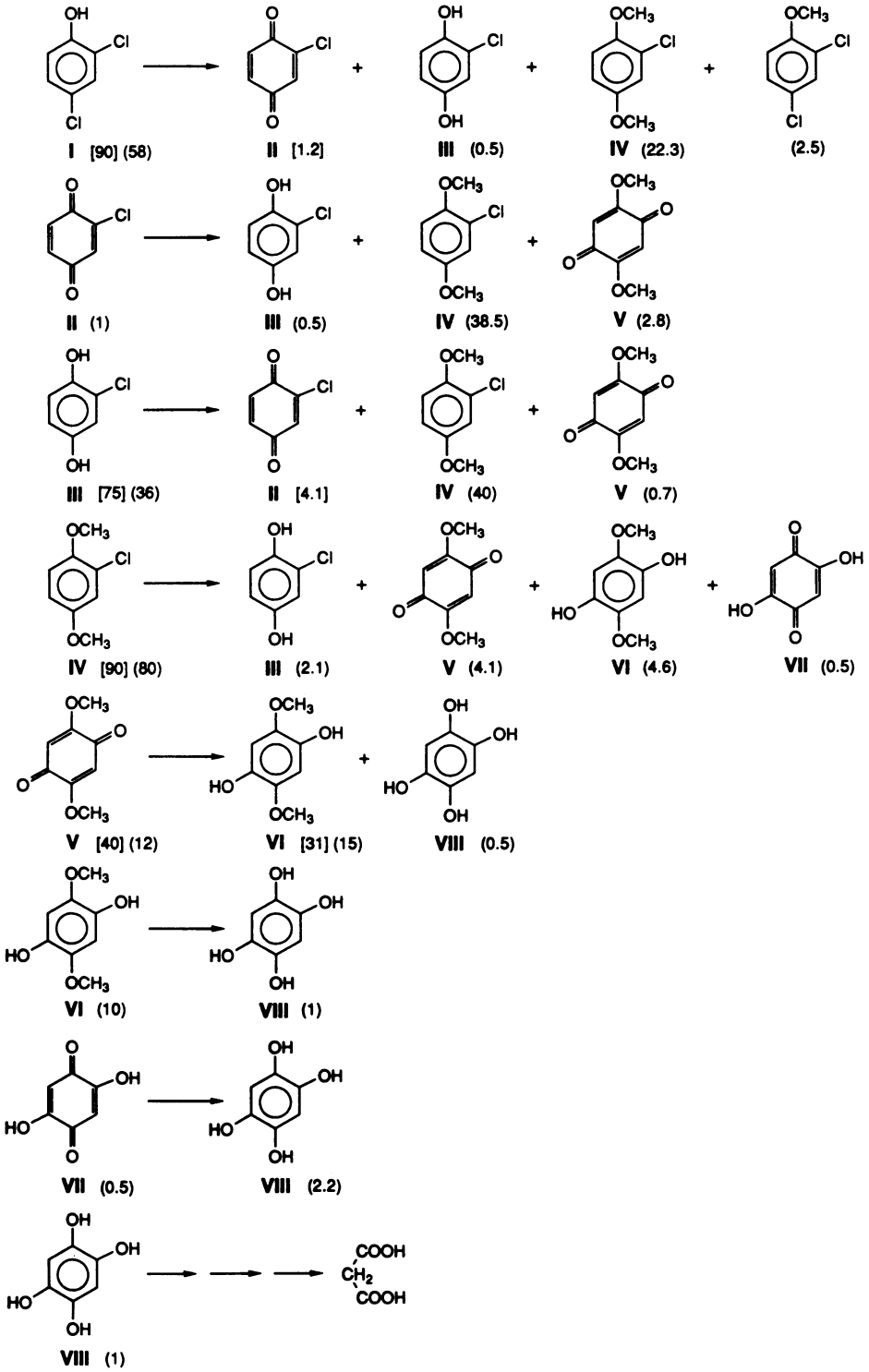

FIG. 2. Metabolites identified from the *P. chrysosporium* degradation of 2,4-dichlorophenol and pathway intermediates. Cultures were incubated and extracted, and products were analyzed as described in the text. HPLC was used to determine yields of quinones. Gas chromatography was used to determine the yields of other compounds. Percent yields of products or remaining substrate for incubations of 1 h (in brackets) and 16 h (in parentheses) are indicated.

TABLE 1. Mass spectra of metabolites and their derivatives[a]

Substrate or metabolite[b]	Mass spectrum *m/z* (relative intensity)[c]
2,4-Dichlorophenol (I)	166 (14.5), 164 (67.2), 162 (100), 126 (7.2), 98 (27.3), 73 (9.1), 63 (41.8)
2-Chloro-1,4-benzoquinone (II)	144 (23.6), 142 (100), 116 (12.7), 114 (30.9), 90 (12.7), 88 (30.9), 82 (50.9), 60 (29.1)
2-Chloro-1,4-dihydroxybenzene (III)	146 (27.2), 144 (100), 115 (12.8) 108 (12.8), 89 (7.4), 80 (98), 72 (13.8), 63 (9.1), 53 (84)
2-Chloro-1,4-diacetoxybenzene	230 (2.1), 228 (4.2), 188 (6.5), 186 (16.1), 146 (34.4), 144 (100)
2-Chloro-1,4-di(TMS)benzene	290 (17.0), 288 (35.1), 275 (6.4), 273 (11.7), 259 (6.4), 257 (11.7), 237 (11.1), 173 (13.8), 147 (29.7), 125 (21.3), 94 (11.7), 73 (100)
2-Chloro-1,4-dimethoxybenzene (IV)	174 (32.3), 172 (89.3), 159 (47.3), 157 (100), 129 (29.0), 107 (23.0), 79 (25.8), 63 (25.8)
2,5-Dimethoxy-1,4-benzoquinone (V)	168 (19.0), 153 (38.1), 139 (61.9), 125 (23.8), 111 (14.3), 95 (47.6), 69 (100), 59 (42.9)
2,5-Dihydroxy-1,4-dimethoxybenzene (VI)	170 (76.2), 155 (100), 127 (52.4), 112 (23.8), 69 (31)
2,5-Dimethoxy-1,4-diacetoxybenzene	254 (19.1), 212 (14.9), 178 (18.1), 170 (100), 155 (55.3), 118 (23.4), 95 (6.4), 85 (45.1), 69 (21.3)
2,5-Dimethoxy-1,4-di(TMS)benzene	314 (71.2), 299 (8.7), 284 (61.7), 242 (38.3), 212 (30.6), 197 (15.9), 127 (14.9), 89 (12.7), 73 (100), 59 (26.6)
1,2,4,5-Tetraacetoxybenzene	310 (10.6), 268 (20.2), 226 (69.1), 184 (84.0), 142 (100), 113 (10.6), 69 (20)
1,2,4,5-Tetra(TMS)benzene	430 (26.9), 415 (2.0), 355 (1.5), 342 (2.6), 215 (4.6), 179 (4.6), 147 (21.9), 73 (100)

[a] Products identified from the *Phanerochaete chrysosporium* metabolism of 2,4-dichlorophenol and other intermediates. Cultures were incubated and extracted, and products were analyzed as described in the text.
[b] TMS, Trimethylsilyloxy.
[c] Molecular ion is underlined.

metabolized to 2-chloro-1,4-benzoquinone (II), 2-chloro-1,4-dimethoxybenzene (IV), and 2,5-dimethoxy-1,4-benzoquinone (V). Finally, 2-chloro-1,4-hydroquinone (III), 2,5-dimethoxy-1,4-benzoquinone (V), 2,5-dimethoxy-1,4-hydroquinone (VI), and 2,5-dihydroxy-1,4-benzoquinone (VII) were identified as metabolites of 2-chloro-1,4-dimethoxybenzene (IV).

The metabolites identified in the above experiments were also added exogenously to fungal cultures, and their subsequent metabolism was examined. 1,2,4,5-Tetrahydroxybenzene (VIII) and 2,5-dimethoxy-1,4-hydroquinone (VI) were identified as metabolites of 2,5-dimethoxy-1,4-benzoquinone (V). 1,2,4,5-Tetrahydroxybenzene (VIII) was found to be a metabolite of both 2,5-dimethoxy-1,4 hydroquinone (VI) and 2,5-dihydroxy-1,4-benzoquinone (VII). Finally, malonic acid was identified as a metabolite of 1,2,4,5-tetrahydroxybenzene (VIII).

Metabolites were identified by comparing their retention

TABLE 2. Mass spectra of enzyme oxidation product derivatives

Substrate	Enzyme	Derivatized product[a]	Mass spectrum *m/z* (relative intensity)[b]
2-Chloro-1,4-dimethoxybenzene (IV)	LiP	2-Chloro-1,4-diacetoxybenzene	—[c]
		2-Chloro-1,4-di(TMS)benzene	—
		2,5-Dimethoxy-1,4-diacetoxybenzene	—
		2,5-Dimethoxy-1,4-di(TMS)benzene	—
		3-Chloro-4-methoxy-1,2-diacetoxybenzene	260 (2.0), 258 (5.9), 218 (4.5), 216 (11.9), 176 (33.3), 174 (100), 159 (21.4)
		3-Chloro-4-methoxy-1,2-di(TMS)benzene	320 (22.5), 318 (100), 305 (7.7), 303 (18.1), 290 (19.2), 258 (50), 215 (18.1), 147 (11.5), 93 (13.2), 73 (100)
		1-Chloro-2-methoxyphenazene	246 (1.2), 244 (2.1), 182 (1.2), 172 (52.1), 144 (100), 118 (37.2), 103 (7.4), 90 (24.5), 63 (16)
2,5-Dihydroxy-1,4-dimethoxybenzene (VI)	LiP or MnP	2,5-Dimethoxy-1,4-diacetoxybenzene	—
		2,5-Dimethoxy-1,4-di(TMS)benzene	—
		1,2,4,5-Tetraacetoxybenzene	—
		1,2,4,5-Tetra(TMS)benzene	—
		1-Methoxy-2,4,5-triacetoxybenzene	282 (9.6), 240 (17.0), 198 (28.7), 156 (100), 141 (11.7), 69 (7.4)
		1-Methoxy-2,4,5-tri(TMS)benzene	372 (36.2), 357 (4.3), 342 (19.2), 254 (12.8), 147 (9.6), 73 (100)
		2-Hydroxy-3-methoxyphenazene	226 (100), 211 (7.4), 197 (7.4), 163 (67.0), 172 (24.5), 155 (18.1), 144 (23.4), 132 (26.6), 118 (21.3), 102 (17.0)

[a] Products identified from the oxidation of substrates IV and VI by purified LiP and MnP. Reaction conditions and analysis were as described in the text. TMS, Trimethylsilyloxy.
[b] Molecular ion is underlined.
[c] Results are given in Table 1.

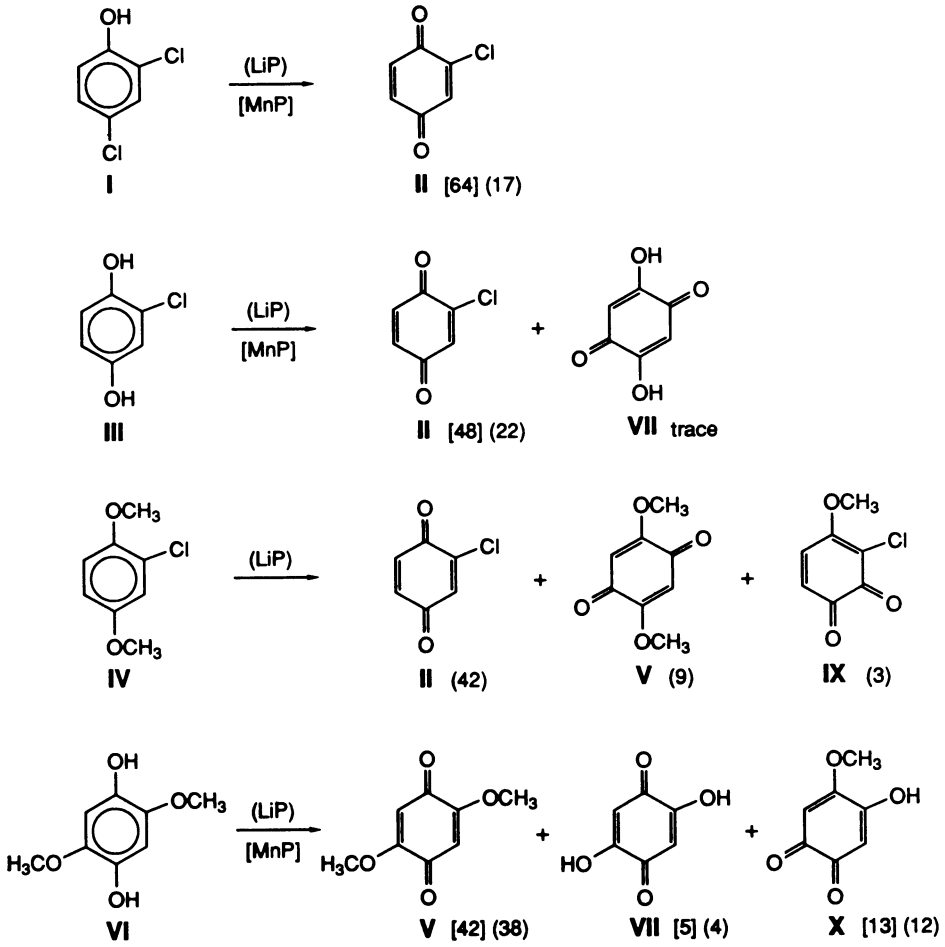

FIG. 3. Products identified from the oxidation of 2,4-dichlorophenol and several intermediates by purified LiP or MnP. Reaction conditions and identification of products were as described in the text. Percent product yields from the reactions with LiP (in parentheses) and MnP (in brackets) are indicated.

times on gas chromatography and HPLC and by comparing their mass spectra with standards. The mass spectra of these metabolites and enzyme reaction products or their derivatives are listed in Tables 1 and 2. Several cultures incubated with ^{14}C-labeled 2,4-dichlorophenol (I) on day 6 were extracted on day 9. [^{14}C]malonic acid produced by the metabolism of radiolabeled 2,4-dichlorophenol (I) was also identified by comparing its retention on HPLC and TLC. Fifteen percent of the added ^{14}C label was found in the malonic acid peak collected from the HPLC.

The LiP and MnP oxidation products of 2,4-dichlorophenol (I) and several identified metabolites are shown in Fig. 3. Both enzymes oxidized 2,4-dichlorophenol (I) to 2-chloro-1,4-benzoquinone (II). The LiP oxidation of 2,4-dichlorophenol (I) to this product has been previously reported (17). However, the results shown in Fig. 3 suggest that MnP oxidizes this substrate more efficiently than LiP. Both enzymes also oxidized 2-chloro-1,4-hydroquinone (III) to yield 2-chloro-1,4-benzoquinone (II) and a trace of 2,5-dihydroxy-1,4-benzoquinone (VII). LiP, but not MnP, oxidized 2-chloro-1,4-dimethoxybenzene (IV) to yield 2-chloro-1,4-benzoquinone (II), 2,5-dimethoxy-1,4-benzoquinone (V), and 3-chloro-4-methoxy-1,2-benzoquinone (IX). The latter was identified after derivatization with phenylene-diamine (Table 2). Finally, both enzymes oxidized 2,5-dimethoxy-1,4-hydroquinone (VI) to yield 2,5-dimethoxy-1,4-benzoquinone (V), 2,5-dihydroxy-1,4-benzoquinone (VII), and 4-hydroxy-5-methoxy-1,2-benzoquinone (X).

DISCUSSION

White rot basidiomycetous fungi are primarily responsible for the initiation of the depolymerization of lignin in wood (7, 15, 18, 23). The most well-studied white rot fungus, *P. chrysosporium*, degrades lignin during secondary metabolic (idiophasic) growth (7, 15, 23). Under ligninolytic conditions *P. chrysosporium* secretes two heme peroxidases (LiP and MnP) (15, 23) in addition to an H_2O_2-generating system (23). These two peroxidases appear primarily responsible for the oxidative depolymerization of this heterogeneous, random, nonhydrolyzable, environmentally persistent phenylpropanoid polymer. The irregular and recalcitrant nature of lignin and the fact that it contains substructures found in primary pollutants such as phenols, anisoles, biphenyls, and diarylethers led researchers to postulate that the nonspecific ligninolytic system produced by white rot fungi might be capable of oxidatively degrading persistent aromatic pollutants (2, 3, 16). Indeed, recent work has demonstrated that *P. chrysosporium* is capable of mineralizing many persistent, aromatic pollutants (2, 3, 8, 16) including chlorinated phe-

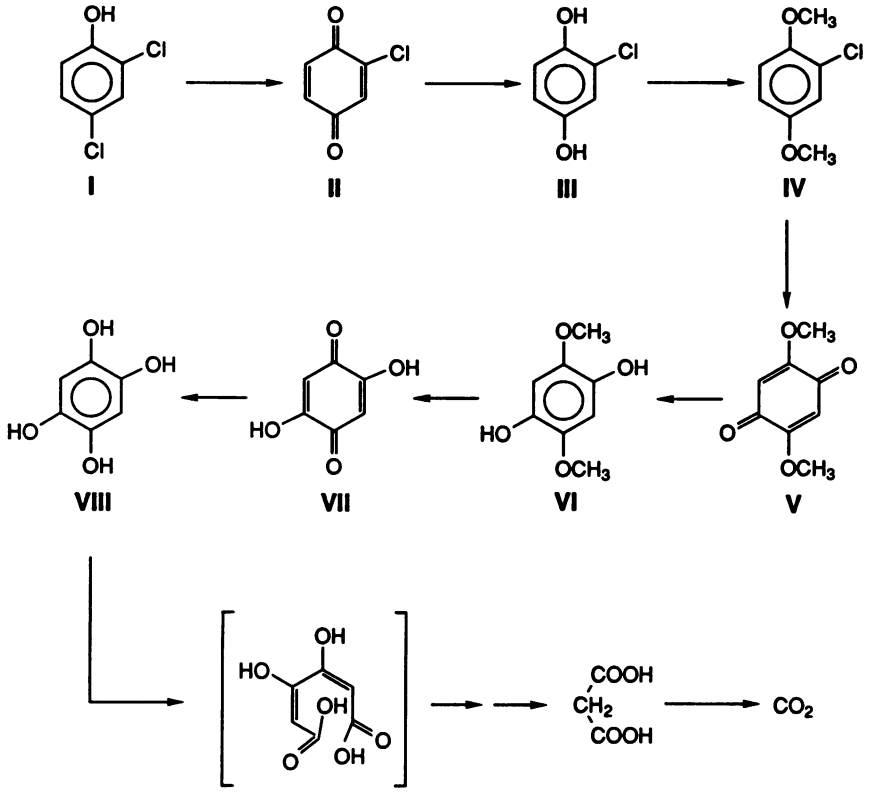

FIG. 4. Proposed pathway for the degradation of 2,4-dichlorophenol by *P. chrysosporium*.

nols (19, 27). In addition, previous work (17, 27) demonstrated that LiP could catalyze the first oxidative dechlorination step in the degradation of several chlorinated phenols. However, except for this initial step, fungal chlorophenol degradative pathways have not been previously elucidated.

Our results demonstrate that *P. chrysosporium* extensively mineralizes 2,4-dichlorophenol only under nutrient nitrogen-limiting conditions (Fig. 1), suggesting that the lignin-degradative system is, at least in part, responsible for the degradation of this pollutant. Similar results have been reported for the degradation of pentachlorophenol (27). Sequential identification of the primary metabolites produced during 2,4-dichlorophenol degradation and subsequent identification of the secondary metabolites produced after addition to cultures of synthesized primary metabolites have enabled us to propose a pathway for the oxidation of 2,4-dichlorophenol by *P. chrysosporium*. The first step in the proposed pathway is the oxidation of 2,4-dichlorophenol (I) to 2-chloro-1,4-benzoquinone (II) (see Fig. 4). This reaction has been previously shown to be catalyzed by LiP (17). However, our results (Fig. 3) suggest that MnP oxidizes this substrate more efficiently than LiP, and presumably, both enzymes are involved in the degradative pathway. 2-Chloro-1,4-benzoquinone (II) is not a substrate for either LiP or MnP. Both 2-chloro-1,4-hydroquinone (III) and 2-chloro-1,4-dimethoxybenzene (IV) were identified as metabolites of 2-chloro-1,4-benzoquinone (II). Since both LiP and MnP are extracellular enzymes, we propose that 2-chloro-1,4-benzoquinone (II) is taken up by the cells and reduced to 2-chloro-1,4-hydroquinone (III). 2-Chloro-1,4-hydroquinone (III) is then methylated to produce 2-chloro-1,4-dimethoxybenzene (IV). Both the 2-chloro-1,4-hydroquinone (III) and 2-chloro-1,4-dimethoxybenzene (IV) are metabolized by *P. chrysosporium*, yielding the oxidized 2,5-dimethoxy-1,4-benzoquinone (V), suggesting that these compounds are substrates for either LiP or MnP. Both MnP and LiP oxidize 2-chloro-1,4-hydroquinone (III) (Fig. 3). While the corresponding 2-chloro-1,4-benzoquinone (II) is the major product, a significant amount of the nonchlorinated product 2,5-dihydroxy-1,4-benzoquinone (VII) is also detectable. LiP oxidizes 2-chloro-1,4-dimethoxybenzene (IV) to the nonchlorinated product, 2,5-dimethoxy-1,4-benzoquinone (V), demonstrating that 2-chlorodimethoxybenzenes, as well as dimethoxybenzenes (22), are substrates for LiP (Fig. 3). The fungus readily converts 2-chloro-1,4-hydroquinone (III) to 2-chloro-1,4-dimethoxybenzene (IV) and dechlorinates 2-chloro-1,4-dimethoxybenzene (IV) to yield the unchlorinated products 2,5-dimethoxy-1,4-benzoquinone (V), 2,5-dihydroxy-1,4-dimethyoxybenzene (VI), and 1,2,4,5-tetrahydroxybenzene (VIII). These important reactions apparently compete effectively with the futile oxidation of 2-chloro-1,4-hydroquinone (III) and 2-chloro-1,4-dimethoxybenzene (IV) back to 2-chlorobenzoquinone (II). These results also indicate that this organism can remove both chlorine atoms from the aromatic ring before ring opening takes place. The essential steps in the proposed pathway are, first, the oxidative 4-dechlorination catalyzed by either LiP or MnP and then a sequence of proposed steps, including the uptake of the *p*-benzoquinone 2-chloro-1,4-benzoquinone (II), reduction by a quinone reductase, and methylation of the resultant hydroquinone to regenerate a peroxidase substrate. The apparent relocalization of the activated product to the extracellular medium allows a subsequent dechlorination step catalyzed by LiP.

The resulting dechlorinated intermediate 2,5-dimethoxy-

1,4-benzoquinone (V) cannot be oxidized further by LiP or MnP. 2,5-Dimethoxy-1,4-benzoquinone (V) is, in turn, taken up by the cells and reduced to the corresponding hydroquinone 2,5-dihydroxy-1,4-dimethoxybenzene (VI) (Fig. 2). This intermediate is a substrate for both LiP and MnP (Fig. 3). The intermediate 2,5-dihydroxy-1,4-dibenzoquinone (VII) probably is also taken up and reduced to 1,2,4,5-tetrahydroxybenzene (VIII). 1,2,4,5-Tetrahydroxybenzene (VIII) undergoes ring cleavage to produce malonic acid after subsequent oxidation (Fig. 2 and 4). The first step in the cleavage of the tetrahydroxybenzene could be catalyzed by a dioxygenase. This reaction might yield the intermediate 3,4-dihydroxy-2,4-hexadiene-1,6-dicarboxylic acid which would be oxidized further to malonic acid (Fig. 4). The presumed dioxygenase responsible for the intradiol ring cleavage of 1,2,4,5-tetrahydroxybenzene may be the same enzyme which was reported to cleave 1,2,4-trihydroxybenzene (5). The biodegradation of the two *o*-quinones generated in the enzyme reactions (Fig. 3) was not determined. Presumably, they also would be subject to reduction, methylation, and further oxidative degradation reactions.

Our results strongly suggest that *P. chrysosporium* elaborates a general pathway for the oxidative degradation of chlorinated aromatic compounds which involves initial oxidative 4-dechlorination by either LiP or MnP, presumably followed by uptake of the *p*-quinone product. Subsequent reduction and methylation steps result in the regeneration of a peroxidase substrate. This process can then be repeated and is probably responsible for the dechlorination and mineralization of pentachlorophenol (17, 27). Recently we have identified 2,3,5,6-tetrachloro-1,4-dimethoxybenzene as a *P. chrysosporium* metabolite of pentachlorophenol, suggesting that a similar pathway is involved in the degradation of that pollutant. Presumably this proposed pathway evolved for the degradation of lignin metabolites. For example, the oxidative decarboxylation of the lignin metabolite vanillic acid yields 2-methoxy-1,4-hydroquinone (4, 34) and 2-methoxy-1,4-benzoquinone (12, 25). 2-Methoxy-1,4-benzoquinone is also a product of the LiP-catalyzed oxidation of the *P. chrysosporium* secondary metabolite veratryl alcohol (30). Reduction of 2-methoxy-1,4-benzoquinone to its hydroquinone has been measured in cell-free extracts (6, 30). Oxidation of 2-methoxy-1,4-hydroquinone by LiP or MnP would probably generate 2,5-dihydroxy-1,4-benzoquinone (VII). This intermediate would enter the pathway described here.

In the proposed pathway both chlorine atoms are removed by oxidative reactions before ring cleavage takes place. Indeed, peroxidase-catalyzed oxidative dechlorination followed by reduction of the quinone product results in the introduction of phenolic groups which should facilitate ring opening. This pathway contrasts with several prevalent pathways in bacteria in which phenolic groups are introduced by ring hydroxylation. Ring cleavage of the resultant chlorocatechol could generate toxic acyl halide intermediates (26, 29). We are attempting to isolate and characterize the quinone reductase(s), methyl transferase(s), and dioxygenase which are implicated by the results of this study.

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