Degradation of chlorophenoxyacetic acids by the lignin-degrading fungus

*Dichomitus squalens*

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We have examined the degradation of 14C ring- and side-chain-labelled 2,4,5-trichlorophenoxyacetic acid by *Dichomitus squalens* and *Phanerochaete chrysosporium*. The effects of Mn2+ on the degradation of these radiolabelled substrates by *D. squalens* and the effect of nitrogen limitation on their degradation by *P. chrysosporium* suggested that in both fungi, side-chain cleavage was catalysed by a mechanism independent of the lignin degradation system, whereas the degradation of the aromatic ring was dependent on the lignin degradative system. Using unlabelled substrates, a pathway for the degradation of chlorophenoxyacetic acids was elucidated in *D. squalens*. Time courses for the degradation of unlabelled chlorophenoxyacetic acids by *D. squalens* demonstrated that the corresponding chlorophenol was the initial product formed. The chlorophenol intermediate was xylosylated to form the chlorophenolxyloside. In turn, the chlorophenolxyloside could be hydrolysed by an intracellular p-xylosidase to regenerate the chlorophenol. The chlorophenol product of the xylosidase reaction was oxidatively dechlorinated to form 2-chloro-p-benzoquinone which could undergo subsequent further dechlorination and ring-opening reactions, as has been reported previously for *P. chrysosporium*.

**Keywords:** chlorophenoxyacetic acids, chlorophenols, chlorophenol xyloside, white-rot fungi, p-benzoquinone.

INTRODUCTION

Chlorinated phenoxyacetic acids, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), have been used as herbicides, plant growth regulators, and defoliants (Loos, 1975). The current annual US consumption of 2,4-D is approximately 20000 tons (Hileman, 1996), and the extensive use of these compounds is recognized as an environmental and toxicological hazard. Chlorophenoxyacetic acids are teratogenic and mutagenic (Grant, 1979) and cause a variety of adverse neurological symptoms (Elo et al., 1988; Oliveira & Palermo-Neto, 1993). Furthermore, chlorophenoxyacetic acids suppress the immune system, thereby increasing the vulnerability of mammals to infectious diseases and cancers (Hileman, 1996).

Bacterial degradation of 2,4-D and 2,4,5-T in pure and mixed cultures has been reported (Kilbane et al., 1982; Sinton et al., 1986; Pieper et al., 1988; Haugland et al., 1990), as has the microbial biodegradation of 2,4,5-T in soils (McCall et al., 1981). Although bacterial biodegradation pathways for 2,4-D and 2,4,5-T have been studied (Sinton et al., 1986), much less is known about the fungal degradation of these pollutants. White-rot basidiomycetous fungi are primarily responsible for the initial depolymerization of lignin during wood decay (Kirk & Farrell, 1987; Higuchi, 1990; Gold & Alic, 1993). When cultured under ligninolytic conditions, *Phanerochaete chrysosporium*, the best-studied white-rot basidiomycete, produces two types of extracellular haem peroxidases – manganese peroxidase (MnP) and lignin peroxidase (LiP) – which, together with an H2O2-generating system, constitute the major extracellular components of its lignin-degrading system (Buswell &
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Recent studies have demonstrated that P. chrysosporium is able to degrade many chlorinated aromatic pollutants, including di, tri- and pentachlorophenols, chlorinated dibenzoxydioxins and chlorinated phenoxyacetic acids, and that Lip and MnP are involved in the initial reductase and a ring-opening dioxygenase, have also been purified and characterized (Rieble et al., 1992; Yadav & Reddy, 1992, 1993; Joshi & Gold, 1993). Several intracellular enzymes from P. chrysosporium mycelial extracts, including a quinone reductase and a ring-opening dioxygenase, have also been purified and characterized (Rieble et al., 1994; Brock et al., 1995; Brock & Gold, 1996). Both the quinone reductase and the dioxygenase presumably are also involved in lignin and pollutant degradation. In the work reported here we examined the mineralization of 2,4,5-[¹⁴C]T by D. squalens and re-examined its mineralization by P. chrysosporium. Both of these fungi degrade lignin, but they produce different complements of extracellular oxidases and peroxidases (Périé & Gold, 1991; Périé et al., 1996); therefore, it was of interest to compare their degradative activity on chlorinated compounds. In addition, we examined the initial steps in the D. squalens degradative pathways for 4-chlorophenoxyacetic acid (4-C), 2,4-D and 2,4,5-T.

METHODS

Chemicals. 4-Chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP) and 2,4,5-trichlorophenol (2,4,5-TCP) were obtained from Aldrich and purified by recrystallization before use. β-Xylosidase from Aspergillus niger was obtained from Sigma. U-[¹⁴C]-Ring-labelled 2,4,5-TCP (20 mCi mmol⁻¹; 7.4 × 10⁹ Bq mmol⁻¹⁻¹) was obtained from Sigma. [¹³C]Bromoacetic acid (251 mCi mmol⁻¹; 9.3 × 10⁹ Bq mmol⁻¹⁻¹) was obtained from Amersham.

1-¹⁴C-Side-chain-labelled 2,4,5-T was prepared by the condensation of 2,4,5-TCP with [¹⁴C]bromoacetic acid in the presence of sodium hydroxide as described by Furniss et al. (1989). U-[¹⁴C]-Ring-labelled 2,4,5-T was prepared by the condensation of [¹⁴C]-ring-labelled 2,4,5-TCP with unlabelled bromoacetic acid as described by Furniss et al. (1989). The labelled substrates were purified by preparative silicic gel TLC using methanol (30%) and acetic acid (1%) in ethyl acetate (69%) as the eluent (Rₑ 0.4).

Culture conditions. Stock cultures of P. chrysosporium (OGC101) (Alic et al., 1987) and D. squalens (Karst Reid) (CBS 432.34) (Périé & Gold, 1991) were maintained on slants as described previously (Alic et al., 1987; Périé & Gold, 1991). Liquid cultures were grown from a conidial inoculum at 38°C (P. chrysosporium) or from a mycelial inoculum at 28°C (D. squalens) in stationary culture (25 ml; 250 ml flask) as described previously (Alic et al., 1987; Périé & Gold, 1991). The medium, described previously (Kirk et al., 1978; Périé & Gold, 1991), was supplemented with 2% glucose and either 1.2 mM or 12 mM ammonium tartrate, yielding high carbon–low nitrogen (HCLN) or high carbon–high nitrogen (HCHN) cultures, respectively. Cultures used for the mineralization studies were buffered with 20 mM sodium succinate (pH 4.5). Cultures used for the metabolism of unlabelled substrates were buffered with sodium acetate (pH 4.5). Cultures grown in the presence of Mn²⁺ contained a final concentration of 30 μM MnSO₄. Cultures were incubated aerobically for 5 d (3 d for P. chrysosporium) after which they were purged with 99.9% oxygen every 3 d as described previously (Kirk et al., 1978; Périé & Gold, 1991).

Mycelial mats from four stationary flasks were homogenized for 20 s in a Waring blender and used to inoculate a 2 litre flask containing 1 litre of medium supplemented with 2% glucose, 12 mM ammonium tartrate and 20 mM sodium succinate (pH 4.5). Agitated cultures were grown at 28°C on a rotary shaker (150 r.p.m.) for 96 h (Rieble et al., 1994; Brock et al., 1995).

Preparation of enzyme extracts. Mycelial pellets were harvested by vacuum filtration using Miracloth (Calbiochem), washed in ice-cold sodium phosphate buffer (pH 7.0, 20 mM) and stored at −80°C. All subsequent steps were carried out at 4°C. Routinely, 2 g wet weight of cells were broken by grinding with 5 g sand in a mortar and pestle in 20 ml 200 mM sodium phosphate buffer (pH 7.0). The homogenate was centrifuged at 10000 g for 20 min. The supernatant was recentrifuged at 150000 g for 60 min, yielding a clarified cell-free extract.

Mineralization of 2,4,5-[¹⁴C]T. Ring- or side-chain-labelled substrate (3 × 10⁶ c.p.m.) in methanol was added to 25 ml stationary cultures after incubation for 5 d (D. squalens) or 3 d (P. chrysosporium). Flasks were fitted with ports which allowed periodic purging with oxygen and trapping of evolved ¹⁴CO₂ in a basic scintillation cocktail as described previously (Périé & Gold, 1991; Joshi & Gold, 1993) using a Beckman LS-6500 scintillation counter. The experiments were carried out in triplicate and the mean of the three determinations was plotted. ¹⁴CO₂ trapping efficiency after purging for 15 min was greater than 98%. Counting efficiency (> 70%) was monitored with an automatic external standard.

Metabolism of unlabelled substrates. After 8 d incubation, the substrates in dimethyl formamide (50 μl) were added to D. squalens cultures to a final concentration of 200 μM. After the indicated additional intervals, the culture medium was acidified with HCl and saturated with sodium chloride. The products were reduced with sodium dithionite. Ethyl acetate (25 ml) was added, the mixture was homogenized in a blender and the organic layer was separated. The aqueous fraction was re-extracted with ethyl acetate (3 x 50 ml). The combined organic fraction was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The products were derivatized with either acetic anhydride/pyridine (2:1) or bis(N,O-trimethylsilyl)trifluoroacetamide/pyridine (2:1) and analysed by GC or GC-MS (Joshi & Gold, 1993). Quinones were identified as described previously (Joshi & Gold, 1993). Xyloside conjugates of chlorinated phenols were also analysed directly by HPLC and by direct insertion probe MS prior to derivatization. Experiments with each substrate were carried out in triplicate with essentially identical results.

Enzymic hydrolysis of 2,4-dichlorophenol xyloside. The assay mixture (1 ml), containing 100 mM acetyl buffer (pH 6.5), β-
xylosidase (0.5 U) and 2,4-dichlorophenol xyloside (100 μM), was incubated at 27 °C for 1 h. The reaction mixture was extracted with ethyl acetate and the DCP produced was monitored by HPLC. For the analysis of xylose as a product, the assay was carried out at pH 7.2 for 24 h. The reaction mixture was filtered using an Acrodisc (Gelman Sciences) (0.2 μm) to remove insoluble protein after which excess sodium borohydride (500 μM) and NaOH (1 mM) were added and the mixture was stirred for 24 h at room temperature (Chaplin, 1986). Water was removed under reduced pressure and the xylitol produced was derivatized with acetic anhydride/pyridine (2:1). Analysis by GC-MS was performed in comparison with a standard.

**Chromatography and spectrometry.** GC-MS was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A gas chromatograph and a 30 m fused Silica column (DB-5, J & W Scientific). The oven temperature was programmed from 70 °C to 320 °C at 10 °C min⁻¹. Xylitol acetates were analysed using a 30 m Carbowax column (Altech). The oven temperature was programmed from 100 °C to 220 °C at 10 °C min⁻¹. The reaction products were also analysed by HPLC, with an HP Lichrospher 100 RP8 column, using a linear gradient of 75% acetonitrile in 0.05% phosphoric acid over 15 min with a flow rate of 1 ml min⁻¹. Products were detected at 285 nm. Yields on HPLC were obtained by using calibration curves obtained with standards. Preparative silica gel TLC was carried out using methanol (10%) and acetic acid (1%) in ethyl acetate as the eluent.

**RESULTS**

**Mineralization of 2,4,5-[14C]T by D. squalens**

A time course for 2,4,5-[14C]T mineralization by HCHN-grown cultures of *D. squalens* is shown in Fig. 1. Over the 31 d course of the experiment, approximately 65% of the side-chain-labelled substrate was converted to 14CO₂ in Mn²⁺-deficient cultures, whereas 54% of the side-chain-labelled substrate was converted in Mn²⁺-supplemented cultures. In contrast, 32% of the ring-labelled substrate was degraded to 14CO₂ in Mn²⁺-supplemented cultures, whereas only 8% of the ring-labelled substrate was converted to 14CO₂ in Mn²⁺-deficient cultures.

**Mineralization of 2,4,5-[14C]T by P. chrysosporium**

A time course for the mineralization of 2,4,5-[14C]T by *P. chrysosporium* is shown in Fig. 2. Approximately 22% and 19% of the side-chain-labelled substrate was converted to 14CO₂ after 31 d incubation in HCHN and HCLN cultures, respectively. In contrast, approximately 13% and 4% of the ring-labelled substrate was converted to 14CO₂ in HCLN and HCHN cultures, respectively. Thus, a significant nitrogen depletion effect was detected only for the mineralization of the ring-labelled substrate.

**Intermediates in the metabolism of chlorophenoxyacetic acids by D. squalens**

Two products were formed during the metabolism of 2,4-D, as detected by HPLC analysis. The major metabolite exhibited a retention time of 8.8 min, whereas the 2,4-DCP standard and the minor product exhibited a retention time of 11.4 min. GC-MS analysis identified this minor product as 2,4-DCP (Table 1). GC-MS analysis after reduction and acetylation of products also revealed the presence of the dechlorination product...
Table 1. Mass spectra of chlorophenoxyacetic acid metabolites, their derivatives and cleavage products

Products were identified from metabolism of 4-C, 2,4-D and 2,4,5-T by *D. squalens*. Cultures were incubated and extracted, and products were analysed as described in the text. The retention times and mass spectra of standard compounds were essentially identical to those of metabolites. Xylosides were analysed by direct insertion probe MS.

<table>
<thead>
<tr>
<th>Substrate or metabolite</th>
<th>Retention time (min)</th>
<th>Mass spectrum m/z (relative intensity)</th>
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<tr>
<td></td>
<td>HPLC</td>
<td>GC</td>
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<tr>
<td>4-Chloroacetoxybenzene</td>
<td>–</td>
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<tr>
<td>4-Chlorophenol xyloside</td>
<td>8.1</td>
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<tr>
<td>2,4-Dichlorophenol xyloside</td>
<td>9.8</td>
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<tr>
<td>2,4,5-Trichlorophenol xyloside</td>
<td>9.5</td>
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<tr>
<td>Xylitol penta-acetate</td>
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Fig. 3. Direct insertion mass spectrum of 2,4-dichlorophenol xyloside. The product was extracted from cultures and purified as described in the text. The spectrum of the molecular ion region is shown on an expanded scale.

2-chloro-β-benzoquinone. The major metabolite was not detected by GC-MS as its acetyl or TMS derivative. This metabolite was purified by preparative TLC (Rf 0.4) using methanol (30%) and acetic acid (1%) in 69% ethyl acetate. Direct insertion probe MS of the unknown compound indicated a molecular mass of 294 (M+) and a strong fragment ion at m/z 162 (M+-132) corresponding to 2,4-DCP. This suggests the molecular formula (C₉O₅H₁₀·H₂O) for the lost fragment. Thus, the parent compound is likely to be a monopentose conjugate of 2,4-DCP as confirmed below (Fig. 3; Table 1).

A sample (0.5 mg) of this pure major metabolite was hydrolysed in 0.01 M HCl at 90 °C for 15 min. The reaction mixture was cooled and extracted with ethyl acetate. HPLC analysis demonstrated the formation of 2,4-DCP (retention time 11.4 min). After acid hydrolysis of the metabolite as described above, water was removed under reduced pressure; the crude product mixture was reduced with sodium borohydride over 24 h, derivatized with acetic anhydride/pyridine (2:1), and analysed by GC-MS on a Carbowax column. The mass spectrum of the major product was identical to that of authentic xylitol acetate (Table 1), indicating that the original metabolite was xylose-conjugated chlorophenol (DCPX). Enzymic hydrolysis of the DCPX with β-xylanosidase yielded 2,4-DCP quantitatively, as determined by HPLC. GC-MS analysis of the exhaustively reduced and acetylated enzymic hydrolysate yielded xylitol acetate as the only sugar derivative (Table 1). This indicates that the sugar is linked to the phenolic group of the aromatic moiety via a β-glycosidic bond. The DCPX structure is shown in Fig. 6. Enzymic hydrolysis of 2,4-DCPX, using the clarified crude extract from *D. squalens* mycelia, also resulted in the formation of 2,4-DCP and xylose, suggesting that a β-xylanosidase is produced by these cells. Furthermore, 24 h after the addition of DCPX to cultures of *D. squalens*, 2,4-DCP was detected in the culture medium. The structures of the 4-chlorophenol xyloside and the 2,4,5-trichlorophenol xyloside were determined in a similar manner (Table 1).
Time courses for the metabolism of chlorophenoxyacetic acids

(i) 4-Chlorophenoxyacetic acid. A time course for the conversion of 4-C by D. squalens is shown in Fig. 4. When the substrate was added to 8-d-old cultures, it was consumed within approximately 15 h. The 4-CP metabolite peak reached a maximum of approximately 30% yield in 8 h, after which it declined slowly. A second metabolite, 4-chlorophenol xyloside (4-CPX) (Table 1), was detected only after 8 h, reaching a maximum of 40% yield after 15 h, and then declining. The time course for the appearance and disappearance of these two metabolites suggests a precursor–product relationship between 4-CP and 4-CPX.

(ii) 2,4-Dichlorophenoxyacetic and 2,4,5-trichlorophenoxyacetic acids. A time course for the conversion of 2,4-D by D. squalens is shown in Fig. 5(a). When the substrate was added to 8-d-old cultures, it was consumed within approximately 36 h. 2,4-DCP was the first product observed reaching a maximum of 13% yield after 12 h. As the level of 2,4-DCP declined, 2,4-DCPX was formed, the latter reaching a maximum of 54% yield after 25 h. Similar results were obtained with 2,4,5-T (Table 1).

The possible precursor–product relationship between the chlorophenol and the chlorophenol xyloside intermediates was tested in a direct experiment. A time course for the conversion of 2,4-DCP to the DCP-xyloside by D. squalens is shown in Fig. 5(b). When 2,4-DCP was added to 8-d-old HCHN cultures, the substrate was consumed in 20 h. The major metabolite detected was 2,4-DCPX, which reached a maximum of 40% yield after 20 h, after which it slowly declined.

DISCUSSION
The main pathway reported for bacterial degradation of 2,4-D involves an initial ether cleavage followed by oxidative dechlorination of the resulting dichlorophenol and ortho cleavage of the aromatic ring (Sinton et al., 1986). Variations on this pathway, involving an initial reductive dechlorination followed by ether cleavage, also have been reported (Sinton et al., 1986). In the bacterium Alcaligenes eutrophus, the enzyme catalysing the initial ether cleavage has been identified as a 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase (Fukumori & Hausinger, 1993). Although mineralization of chlorophenoxyacetic acids has been reported in P. chrysosporium (Ryan & Bumpus, 1989; Yadav & Reddy, 1992), little has been reported on the degradation pathway. The degradation of chlorophenoxyacetic acids has not been reported previously in D. squalens.


There has been disagreement concerning the involvement of the lignin-degrading system in the degradation of chlorinated phenoxyacetic acids by P. chrysosporium (Ryan & Bumpus, 1989; Yadav & Reddy, 1992). Furthermore, the pathway for the initial steps in
degradation of these pollutants by white-rot fungi has not been reported previously.

Our results demonstrate that *D. squalens* extensively mineralizes 2,4,5-T under nitrogen-sufficient conditions. Mineralization also occurs under low nitrogen conditions, although to a lesser extent (data not shown). We have examined the degradation of both side-chain- and ring-labelled 2,4,5-T by *D. squalens*. In cultures grown in the absence of Mn\(^{2+}\), approximately 65% of the side-chain-labelled 2,4,5-T is converted to \(^{14}\)CO\(_2\) during the 31-day incubation period. In cultures grown in the presence of 30 μM Mn\(^{2+}\), approximately 54% of the 2,4,5-T is mineralized (Fig. 1). In light of our previous results on lignin degradation by *D. squalens* in the presence and absence of Mn\(^{2+}\) (Perié & Gold, 1991), these experiments suggest that the ligninolytic system is not involved in the initial cleavage of this chlorophenoxyacetic acid to its chlorophenol, nor is it involved in the subsequent metabolism of the side-chain intermediate. In contrast to the results obtained with side-chain-labelled 2,4,5-T, approximately 32% of the ring-labelled substrate is degraded to \(^{14}\)CO\(_2\) when cultures are grown in the presence of Mn\(^{2+}\), but only 8% is degraded to \(^{14}\)CO\(_2\) in cultures grown in the absence of Mn\(^{2+}\) (Fig. 1). These results suggest the involvement of MnP in the degradation of the chlorinated aromatic ring, as previously reported for *P. chrysosporium* (Valli & Gold, 1991; Joshi & Gold, 1993).

Both side-chain- and ring-labelled 2,4,5-T are degraded less efficiently by *P. chrysosporium* than by *D. squalens* (Fig. 2). Only 22% of the side-chain-labelled 2,4,5-T is degraded by *P. chrysosporium* during the course of the experiment, compared to 65% by *D. squalens*. Likewise, with the ring-labelled substrate, only 13% is mineralized by *P. chrysosporium*, compared to 32% by *D. squalens*. It has been well established that the lignin-degrading system of *P. chrysosporium* is repressed in cultures grown under high nitrogen conditions (Kirk et al., 1978; Kirk & Farrell, 1987; Gold & Alic, 1993). With side-chain-labelled 2,4,5-T, the extent of mineralization by *P. chrysosporium* is slightly greater in cultures grown in high nitrogen medium, suggesting that, as with *D. squalens*, the presumed initial cleavage of 2,4,5-T and subsequent degradation of the side-chain intermediate are not catalysed by the lignin-degrading system. In contrast, with ring-labelled substrate, the degradation rate is considerably greater in cultures grown under nitrogen-limiting conditions, suggesting that the degradation of the ring-labelled chlorophenol intermediates occurs under conditions of nitrogen depletion. We have shown previously that 2,4-DCP and 2,4,5-TCP are degraded by *P. chrysosporium* under nitrogen-limiting conditions (Valli & Gold, 1991; Joshi & Gold, 1993). These results suggest the involvement of ligninolytic enzymes in the degradation of the chlorinated phenolic intermediates, in agreement with results reported by Ryan & Bumpus (1989). Our results are also consistent with the results of Yadav & Reddy (1992) on the degradation of side-chain-labelled 2,4,5-T. However, Yadav & Reddy (1993) also reported that ring-labelled 2,4-D degradation by *P. chrysosporium* occurred more rapidly in cultures grown in high nitrogen. The latter results are inconsistent with those of Ryan & Bumpus (1989) and with our current and previous results (Valli & Gold, 1991; Joshi & Gold, 1993). Although this inconsistency could be due to differences among *P. chrysosporium* strains, our results with side-chain- and ring-labelled 2,4,5-T clearly suggest that ligninolytic enzymes are involved in the degradation of the chlorinated phenolic intermediates, but not in the initial cleavage reaction nor in the subsequent metabolism of the side-chain of the chlorophenoxyacetic acid substrate.

With *D. squalens*, we have identified 4-CP, 2,4-DCP and 2,4,5-TCP as degradation intermediates from 4-C, 2,4-D and 2,4,5-T, respectively, by comparing their mass spectra and HPLC retention times with those of standards (Table 1). Presumably these intermediates are produced by cleavage of the ether linkage. In addition, the oxidative dechlorination of 2,4-DCP to form 2-chloro-β-benzoquinone was observed. Interestingly, the xyloside conjugate of each phenol was also identified. The structure of the xyloside was established by direct insertion probe MS of the intact conjugate and by chemical and enzymatic hydrolysis followed by HPLC and GC-MS identification of the resultant chlorophenol, which was produced in quantitative yield. In addition, the monosaccharide released by hydrolysis with β-xylosidase was identified as its xylitol penta-acetate after exhaustive reduction and acetylation, followed by GC-MS analysis (Table 1). Xylose is probably linked to the phenol by a β-glycosidic bond, because the conjugate is hydrolysed quantitatively by β-xylosidase. The xylosylation of vanillyl alcohol and methyl guaiacol by the white-rot fungus *Trametes versicolor* also has been reported (Kondo et al., 1993). However, this is the first report of the formation of a xyloside of a chlorophenol in a wood-rotting basidiomycete. In *P. chrysosporium* chlorinated phenols undergo methylation to form their respective anisoles under secondary metabolic conditions (Lamar & Dietrich, 1990; Valli & Gold, 1991; Joshi & Gold, 1993). These chlorinated anisoles would be expected to be metabolically more stable than the corresponding xylosides reported herein, and this may at least partially account for the comparatively slower rate of chlorophenoxyacetic acid metabolism exhibited by *P. chrysosporium* (Fig. 2). Under primary metabolic conditions, *P. chrysosporium* has been shown to glycosylate 9-phenanthrol (Sutherland et al., 1991). Xylosylation probably reduces the toxicity of the phenols to the fungus, allowing the conjugates to accumulate without deleterious effects. Various phenolic conjugation systems have been reported in higher eukaryotes (Mulder, 1982), and conjugation is considered to be a detoxification reaction.

The results shown in Figs 4 and 5 suggest a precursor-product relationship between the chlorophenol and the chlorophenol xyloside conjugates in *D. squalens* cultures degrading chlorophenoxyacetic acids. The time course for the degradation of 4-C (Fig. 4) suggests that 4-CP is the initial intermediate, which is
squalens sporium is versatile. All of these results suggest the pathway shown primarily during secondary metabolism to produce reaction. In xylosylated and the resulting conjugate is secreted. The degradation of the chlorinated aromatic ring of the pathway. In conclusion, our results indicate that in liquid cultures hydrolysed to regenerate chlorophenols. The latter undergo ether cleavage to form corresponding chlorophenols. The chlorophenols are xylosylated in undergo oxidative dechlorination, catalysed by manganese peroxidase, and subsequent ring cleavage (Valli & Gold, 1991; Joshi & Gold, 1993). We are attempting to identify the enzymes catalysing the ether cleavage, xylosylation and xyloside hydrolysis steps in this pathway.

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In conclusion, our results indicate that in liquid cultures D. squalens mineralizes chlorinated phenoxyacetic acids more efficiently than does P. chrysosporium. The results also suggest the involvement of ligninolytic enzymes in the degradation of the chlorinated aromatic ring of the substrate but not in the initial side-chain cleavage reaction. In D. squalens, the chlorophenoxyacetic acids undergo ether cleavage to form corresponding chlorophenols. The chlorophenols are xylosylated in D. squalens to form a xylose conjugate of the phenol, whereas in P. chrysosporium, the chlorophenols are methylated to form their corresponding anisoles (Lamar & Dietrich, 1990; Valli & Gold, 1991; Joshi & Gold, 1993). We have also shown that the xylosides are hydrolysed to regenerate chlorophenols. The latter undergo oxidative dechlorination, catalysed by manganese peroxidase, and subsequent ring cleavage (Valli & Gold, 1991; Joshi & Gold, 1993). We are attempting to


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