APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1995, p. 3407–3414 Vol. 61, No. 9
0099-2240/95/$04.00+0


# New Polymeric Model Substrates for the Study of Microbial Ligninolysis

SHINGO KAWAI,† KENNETH A. JENSEN, JR., WULI BAO, AND KENNETH E. HAMMEL*

*Institute for Microbial and Biochemical Technology, USDA Forest Products Laboratory, Madison, Wisconsin 53705*

Received 25 May 1995/Accepted 23 June 1995

**Lignin model dimers are valuable tools for the elucidation of microbial ligninolytic mechanisms, but their low molecular weight (MW) makes them susceptible to nonligninolytic intracellular metabolism. To address this problem, we prepared lignin models in which unlabeled and α-$^{14}$C-labeled β-*O*-4-linked dimers were covalently attached to 8,000-MW polyethylene glycol (PEG) or to 45,000-MW polystyrene (PS). The water-soluble PEG-linked model was mineralized extensively in liquid medium and in solid wood cultures by the white rot fungus *Phanerochaete chrysosporium*, whereas the water-insoluble PS-linked model was not. Gel permeation chromatography showed that *P. chrysosporium* degraded the PEG-linked model by cleaving its lignin dimer substructure rather than its PEG moiety. $C_\alpha$-$C_\beta$ cleavage was the major fate of the PEG-linked model after incubation with *P. chrysosporium* in vivo and also after oxidation with *P. chrysosporium* lignin peroxidase in vitro. The brown rot fungus *Gloeophyllum trabeum*, which unlike *P. chrysosporium* lacks a vigorous extracellular ligninolytic system, was unable to degrade the PEG-linked model efficiently. These results show that PEG-linked lignin models are a marked improvement over the low-MW models that have been used in the past.**

Microbial ligninolysis continues to be the subject of intensive research for a variety of reasons. One of these is its ecological importance as an essential step in the global carbon cycle—virtually all terrestrial biomass consists of lignified polysaccharides. Another is that the process is mechanistically unusual—the chemical recalcitrance, heterogeneous structure, and large size of lignin require that its biodegradation must begin with steps that are oxidative, nonspecific, and extracellular (20). Unfortunately, these complexities also make it difficult to study microbial ligninolysis. In particular, the lack of well-characterized model substrates that can be used to identify ligninolytic reactions in vivo has been a longstanding problem (3).

In past work, dimeric model compounds that represent the principal substructures of lignin have been used successfully to characterize the ligninolytic systems of white rot fungi. Models of this type provided some of the first evidence that *Phanerochaete chrysosporium* and *Trametes versicolor* cleave the lignin propyl side chain between $C_\alpha$ and $C_\beta$ (9). Dimeric models played a large role in the discovery of fungal lignin peroxidase (LiP), which cleaves lignin between $C_\alpha$ and $C_\beta$ (6, 8, 20), and they have been used recently to detect LiP activity in situ in fungus-colonized wood, where extraction and conventional assay of the enzyme is technically difficult (32).

However, dimeric lignin model compounds have the disadvantage of low molecular weight (MW). Unlike lignin, they can be taken up and metabolized intracellularly by microorganisms, which can make it difficult to determine whether the degradation products observed really reflect ligninolytic activity (3). This has been a problem, for example, in investigations aimed at finding ligninolytic activity in bacteria (14). Ideally, lignin model compounds should be macromolecular like lignin, but to facilitate product analysis, they should have simpler structures than those of the natural polymer. Here we report the synthesis of polymeric models in which the major β-*O*-4-linked substructure of lignin is covalently attached to polyethylene glycol (PEG) or polystyrene (PS). By use of the frequently studied white rot fungus *P. chrysosporium*, we show that the PEG-linked model can be used to detect ligninolytic reactions in defined medium cultures, in fungus-colonized wood, and in vitro with purified fungal LiP.

## MATERIALS AND METHODS

**Reagents.** Chemicals, all of reagent grade, were obtained from Aldrich or Sigma.

**Synthesis of dimer IV for linkage to PEG.** Compound IV was prepared by previous methods with minor modifications (Fig. 1A). First, compound II was synthesized starting with 2 to 3 g of acetovanillone (compound I) by the method of Landucci et al. (23), with hydroquinone monobenzyl ether as the B-ring precursor. When α-$^{14}$C-labeled compound IV was required, the synthesis was done with α-$^{14}$C-labeled compound I, which was prepared as described previously (23) from guaiacol and 1-$^{14}$C-acetic acid (Sigma; diluted to 0.10 mCi mmol$^{-1}$).

The aliphatic hydroxyls of compound II were then protected in a ketal exchange reaction with 2,2-dimethoxypropane (27) to give compound III (*erythro* isomer/*threo* isomer, 2:3) in an overall yield of 30 to 40% based on compound I. For one unlabeled preparation of PEG-linked dimer, the two isomers of compound III were separated by column chromatography on silica gel in hexane-ethyl acetate, 3:1, and the later-eluting *threo* isomer was selected for the subsequent steps. PEG-linked dimer made in this way was used for $^{13}$C nuclear magnetic resonance (NMR) characterization. When $^{14}$C-labeled substrate was synthesized, the isomers were not separated but rather were used together for the remainder of the synthesis.

Compound IV was prepared by hydrogenating compound III (approximately 100 mg) with 10% palladium on charcoal (50 mg) under $H_2$ (1 atm [1 atm = 101.29 kPa]) in 3 ml of $CH_3OH$-tetrahydrofuran, 2:1, for 80 min. The catalyst was removed by filtration, and compound IV was purified by preparative thin-layer chromatography (TLC) on silica gel GF (Analtech; 20 by 20 cm, 1-mm thickness) in hexane-ethyl acetate, 1:1. The yield of compound IV from compound III was about 90%.

**Preliminary test of ether linkage formation.** To model the coupling reaction,

* Corresponding author. Mailing address: USDA Forest Products Laboratory, One Gifford Pinchot Dr., Madison, WI 53705. Phone: (608) 231-9528. Fax: (608) 231-9262. Electronic mail address: kehammel@facstaff.wisc.edu.

† Present address: Department of Applied Bioorganic Chemistry, Faculty of Agriculture, Gifu University, Gifu 501-11, Japan.

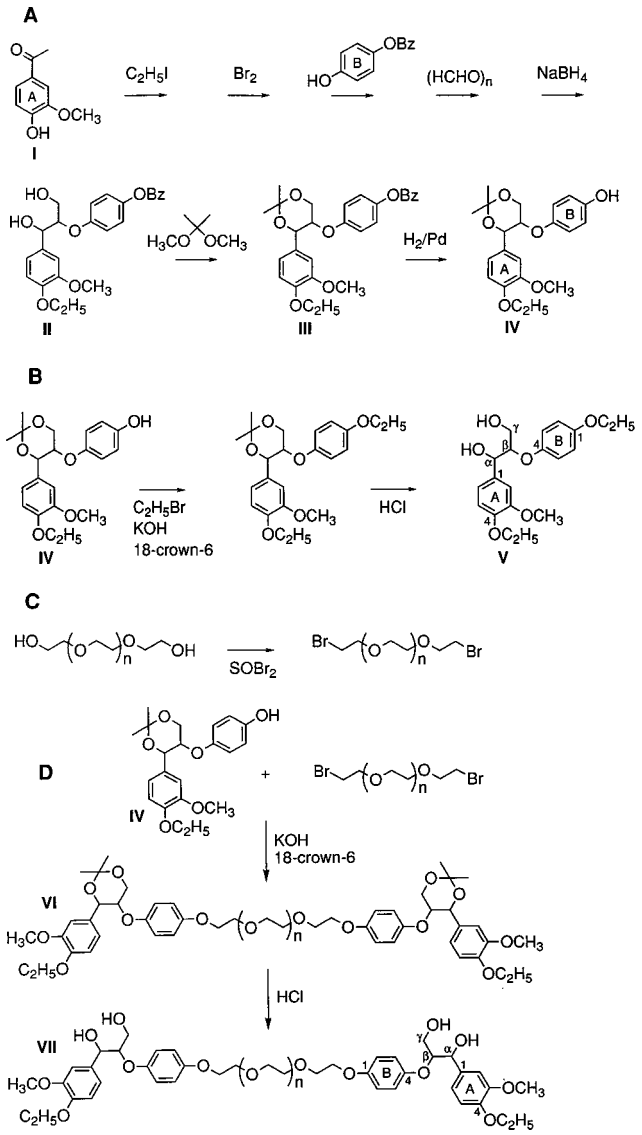


FIG. 1. Strategy for the synthesis of PEG-linked lignin model VII and its low-MW analog, compound V. (A) Preparation of compound IV by previous methods; (B) reaction of compound IV with bromoethane in the presence of base and crown ether; (C) bromination of PEG with thionyl bromide by the procedure of Bückmann et al. (2); (D) preparation of PEG-linked model VII. See Materials and Methods for details. Bz, benzyl.

compound IV was reacted with bromoethane in the presence of base and crown ether (4) (Fig. 1B). Bromoethane (10 μl, 0.13 mmol), solid KOH (6 mg, 0.11 mmol), and 18-crown-6 (15 mg, 0.057 mmol) were added to a stirred solution of compound IV (17.4 mg, 0.046 mmol) in 2.0 ml of toluene at room temperature, and stirring was continued for 25 h. The reaction mixture was then partitioned between $CH_2Cl_2$ and $H_2O$, and the $H_2O$ layer was reextracted twice with $CH_2Cl_2$. The combined $CH_2Cl_2$ layers were washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated under reduced pressure. The crude ethylated product was then treated with 2 N HCl in tetrahydrofuran for 5 h to remove the isopropylidene protecting group, after which the reaction mixture was partitioned between $CH_2Cl_2$ and $H_2O$, washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated as described for the previous step. The product was purified by preparative TLC on silica gel in $CH_2Cl_2$-$CH_3OH$, 98:2 (two cycles), to give compound V (14.5 mg, 0.040 mmol). α-$^{14}$C-labeled compound V (0.10 mCi mmol$^{-1}$) was prepared from α-$^{14}$C-labeled compound IV in the same manner as unlabeled compound V.

**Bromination of PEG.** PEG was brominated with thionyl bromide (Fig. 1C) by the procedure of Bückmann et al. (2), starting with 25 g of 8,000-MW PEG (Sigma catalog no. P 2139). The yield of bromo-PEG was 24 g. $^{13}$C NMR (δ, ppm): 30.3 (-**C**H$_2$Br), 71.2 (-O-**C**H$_2$-CH$_2$Br), 70.5 (-O-CH$_2$-**C**H$_2$-O-). Literature values: 30.2, 71.5, 70.4 (1). The $^{13}$C NMR spectrum indicated that the terminal hydroxyls of PEG were 70 to 75% brominated by this procedure.

**Linkage of compound IV to PEG.** In a typical preparation of PEG-linked model VII (Fig. 1D), brominated PEG (334 mg), solid KOH (10 mg, 0.18 mmol), and 18-crown-6 (25 mg, 0.095 mmol) were added to a stirred solution of compound IV (34 mg, 0.091 mmol) in 1.5 ml of toluene, and stirring was continued for 17 h at room temperature. The reaction mixture was then partitioned between $CH_2Cl_2$ and $H_2O$, washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated as described above to obtain the crude product, compound VI. Low-MW impurities were removed by gel permeation chromatography (GPC) on a column (1.9 by 33 cm) of Bio-beads S-X2 (Bio-Rad), with hexane-$CH_2Cl_2$, 1:1, as the eluant, to give 367 mg of compound VI. $^{13}$C NMR spectrometry showed that all of the Br present on the PEG was removed during the synthesis of compound VI. The purified polymer was then treated with 2 N HCl (approximately 1 ml) in 3 ml of tetrahydrofuran overnight at room temperature to remove the isopropylidene protecting group, after which the reaction mixture was partitioned between $CH_2Cl_2$ and $H_2O$, washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated as described above. The product was purified by GPC on the Bio-beads column to give 295 mg of PEG-linked lignin model compound VII. When α-$^{14}$C-labeled compound VII was prepared, the final specific activity of the polymeric model was $5.15 \times 10^4$ dpm mg$^{-1}$.

**Synthesis of dimers IX and XII for linkage to PS.** Two convenient methods are available for the synthesis of dimers IX and XII, that of Katayama et al. (12) (Fig. 2A), which is most suitable for large quantities, and that of Landucci et al. (23) (Fig. 2B), which is best for small-scale radiolabeled syntheses. When unlabeled PS-linked model compound was required for spectrometric analysis, compound VIII was prepared by the Katayama procedure from *p*-hydroxybenzaldehyde in a 20% yield. Compound VIII was then protected at $C_\alpha$ and $C_\gamma$ with 2,2-dimethoxypropane (27) to give compound IX in yields varying from 40 to 75%. Compound IX was separated into its *erythro* and *threo* isomers by preparative TLC on silica in hexane-ethyl acetate, 2:1 (two cycles), and the faster-running *erythro* isomer, which constituted about 80% of the total product, was selected for coupling to PS.

For the synthesis of $^{14}$C-labeled PS-linked model, α-$^{14}$C-labeled compound X was prepared by the procedure of Landucci et al. (23) (Fig. 2B), and its aldehyde group was protected with ethylene glycol so that the keto group at $C_\alpha$ could be selectively reduced to the alcohol with $NaBH_4$ (13). The aldehyde was then deprotected with HCl to give labeled compound XI in approximately a 30% yield from the starting material, α-$^{14}$C-labeled compound I. Protection of XI at $C_\alpha$ and $C_\gamma$ with 2,2-dimethoxypropane (27) afforded $^{14}$C-labeled compound XII in about a 40% yield as an 8:3 mixture of *erythro* and *threo* isomers. The mixed $^{14}$C-labeled isomers were used for coupling to PS.

**Preliminary test of the Wittig reaction.** Compound IX was reacted with benzyltriphenylphosphonium bromide to model the coupling reaction (Fig. 2C). The

TABLE 1. $^{13}$C NMR signal assignments for PEG-linked model VII and related structures

| Chemical shift of compound (δ, ppm)[a] | | | Assignment[b] |
|---|---|---|---|
| Dimer V (*threo*) | PEG-linked model VII (*threo*) | PEG | |
| 14.8, 14.9 | 14.5 | | -OCH$_2$**C**H$_3$ |
| 56.0 | 55.6 | | -O**C**H$_3$ |
| | 61.2 | 61.6 | -OCH$_2$**C**H$_2$OH of PEG |
| 61.1 | 60.6 | | -**C**$_\gamma$H$_2$OH |
| 64.0, 64.4 | 64.0 | | -O**C**H$_2$CH$_3$ |
| | 67.7 | | -OCH$_2$**C**H$_2$O-Ar of PEG |
| | 69.4 | | -O**C**H$_2$CH$_2$O-Ar of PEG |
| | 70.2 | 70.5 | Internal -O**C**H$_2$**C**H$_2$O- of PEG |
| | 72.5 | 72.5 | -O**C**H$_2$CH$_2$OH of PEG |
| 73.9 | 72.7 | | -**C**$_\alpha$HOH- |
| 84.5 | 83.7 | | -**C**$_\beta$HOAr- |
| 110.3 | 110.0 | | A2 |
| 112.6 | 112.1 | | A5 |
| 115.5 | 115.2 | | B3 and B5 |
| 118.1 | 117.6 | | B2 and B6 |
| 119.3 | 118.8 | | A6 |
| 132.3 | 132.9 | | A1 |
| 148.2 | 147.5 | | A3 or A4 |
| 149.4 | 148.9 | | A3 or A4 |
| 152.0 | 152.2 | | B1 or B4 |
| 154.1 | 153.3 | | B1 or B4 |

[a] Chemical shifts are relative to tetramethylsilane in $CDCl_3$.
[b] See Fig. 1B and D for keys to labeling of the chemical structures.

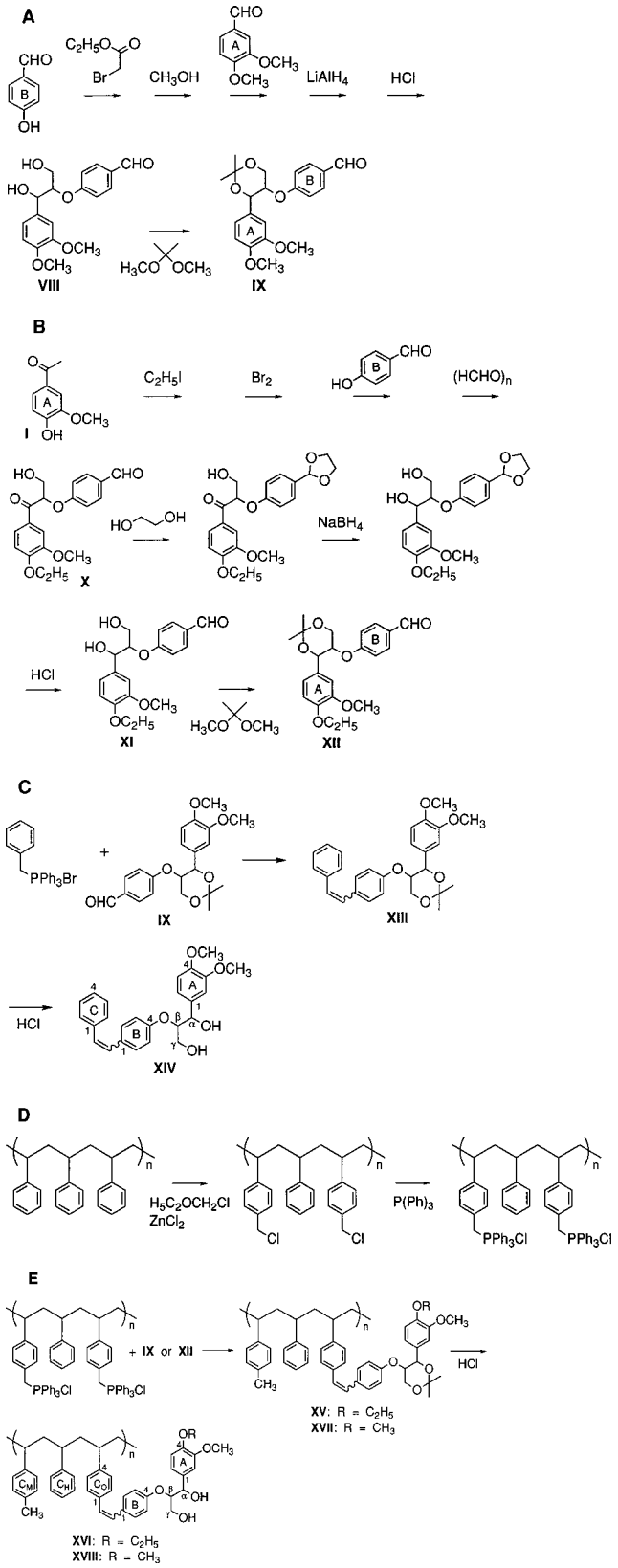


FIG. 2. Strategy for the synthesis of PS-linked lignin models XVI and XVIII and their low-MW analog compound XIV. (A) Method of Katayama et al. (12) for the synthesis of dimers IX and XII; (B) method of Landucci et al. (23) for the synthesis of dimers IX and XII; (C) reaction of compound IX with benzyltriphenylphosphonium bromide to model the coupling reaction; (D) partial chloromethylation of PS by a modification of the method of Ovchinnikov et al. (28); (E) linkage of compounds IX and XII with PS by a modification of previously published methods (11, 15). See Materials and Methods for details. Ph, phenyl. Labels on the PS rings in panel E refer to the substituent present at C-1 of each type: $C_M$, methyl substituted; $C_H$, hydrogen substituted; $C_O$, olefin substituted.

phosphonium bromide (70 mg, 0.16 mmol), cetyltrimethylammonium bromide (10 mg, 0.027 mmol), and 50% aqueous NaOH (1 ml) were added successively to a stirred solution of compound IX (*erythro* isomer, 30 mg, 0.081 mmol) in $CH_2Cl_2$ (2 ml), and the mixture was stirred vigorously under $N_2$ at room temperature for 2 h. The reaction mixture was then extracted with $CH_2Cl_2$ (20 ml, three times), and the pooled organic layers were washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated under reduced pressure. The residue was purified by preparative TLC on silica gel in hexane-ethyl acetate, 2:1, to give compound XIII (28 mg, 0.063 mmol). Compound XIII was then stirred in a mixture of 1 N HCl (1 ml) and acetone (2 ml) at room temperature for 24 h to remove the isopropylidene protecting group. The reaction mixture was extracted with $CH_2Cl_2$ as described for the previous step, and the residue was purified by preparative TLC in $CH_2Cl_2$-$CH_3OH$, 98:2, to give compound XIV (22 mg, 0.054 mmol) as a mixture of *cis* and *trans* isomers. Prior to $^{13}C$ NMR analysis, compound XIV was subjected again to preparative TLC on silica in hexane-ethyl acetate, 3:2 (three cycles), to separate the isomers. The ratio of the faster-migrating *cis* isomer to the slower *trans* isomer was 10:7.

**Chloromethylation of PS and conversion to the triphenylphosphonium chloride.** PS was partially chloromethylated by a modification of the method described by Ovchinnikov et al. (28) (Fig. 2D). Chloromethyl ethyl ether (8 ml) and anhydrous zinc chloride (700 mg) were added successively to a stirred solution of 45,000-MW PS (2 g; Aldrich catalog no. 33,165-1) in dioxane (12 ml), and the mixture was stirred for 24 h at room temperature, after which the reaction mixture was poured into 200 ml of $CH_3OH$ with vigorous stirring. (Caution: chloromethyl methyl ether should not be used for this reaction because it is highly toxic). The precipitate was collected, redissolved in toluene (approximately 10 ml), and poured into 30 ml of $CH_3OH$. The precipitated chloromethylated PS was then collected and washed with $CH_3OH$ (yield, 1.7 g). The product contained 0.22 to 0.23 chloromethyl group per aromatic ring, as shown by $^1H$ NMR spectrometry. Spectrometric data showing the chloromethyl substitution were as follows: infrared (KBr) $\nu$, 1,265 $cm^{-1}$; $^{13}C$ NMR ($CDCl_3$) $\delta$, 46.5 ppm; $^1H$ NMR ($CDCl_3$) $\delta$, 4.35 to 4.65 ppm.

The chloromethylated PS (1.5 g) was then converted to the triphenylphosphonium chloride (Fig. 2D) by treating it with triphenylphosphine (5 g) in *N*,*N*-dimethylformamide (DMF; 20 ml) for 70 h at 80°C, after which the solvent was evaporated under reduced pressure. The residue was dissolved in a minimum volume of $CH_2Cl_2$ and poured into 200 ml of diethyl ether to precipitate the product, which was collected and washed with ether (yield, 1.9 g). Conversion of the chloromethyl group to the benzyl triphenylphosphonium chloride was confirmed by infrared spectrometry, which showed virtually complete disappearance of the 1,265 $cm^{-1}$ signal and the appearance of new signals at 1,112 $cm^{-1}$ and 1,438 $cm^{-1}$.

**Linkage of compounds IX and XII with PS.** The procedure to link compounds IX and XII with PS was a modification of previously published methods (11, 15). In a typical synthesis (Fig. 2E), methyl-PS triphenylphosphonium chloride (54 mg), cetyltrimethylammonium bromide (10 mg, 0.027 mmol), and 50% aqueous NaOH (1 ml) were added successively to a stirred solution of compound XII (54 mg, 0.14 mmol) in $CH_2Cl_2$ (2 ml), and the mixture was stirred vigorously under $N_2$ at room temperature for 2 h. The reaction mixture was extracted with $CH_2Cl_2$, washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated under reduced pressure as described above for the model study. Low-MW impurities were then removed from the product by GPC on Bio-beads SX-2 as described above for the PEG-linked dimer, yielding 57 mg of compound XV. This product was treated with 2 N HCl (approximately 1 ml) in tetrahydrofuran (4 ml) for 48 h at room temperature to remove the isopropylidene protecting group. The reaction mixture was then extracted with $CH_2Cl_2$, washed with brine, dried over anhydrous $Na_2SO_4$, and evaporated under reduced pressure to give compound XVI (44 mg). The specific activity of $^{14}C$-compound XVI was $1.09 \times 10^5$ dpm $mg^{-1}$. Syntheses that started with compound IX rather than compound XII proceeded in essentially the same yield as the example given here, giving the isopropylidene protected intermediate XVII and the deprotected model XVIII.

**Oxidation of PEG-linked model VII by purified LiP.** LiP isozyme H8 from *P. chrysosporium* was purified as described previously (21). The assay (1.0 ml) contained compound VII ($7.01 \times 10^4$ dpm, 320 $\mu$M in $\beta$-*O*-4 structures), sodium glycolate (20 mM, pH 4.5), and LiP (0.3 $\mu$M). It was initiated with $H_2O_2$ (150 $\mu$M) and monitored at 308 nm until the reaction ceased after 4.1 min at 1.1 absorbance units. A sample (0.47 ml) of the reaction mixture was then injected directly onto a styrene divinylbenzene high-performance liquid chromatography (HPLC) column (Hamilton PRP-1; 5-$\mu$m particle size, 4.1 by 150 mm) to check for the formation of 4-ethoxy-3-methoxybenzaldehyde. Products were eluted at 1.0 ml $min^{-1}$ and ambient temperature, isocratically for 25 min with acetonitrile-$H_2O$-$H_3PO_4$ (30:70:0.1), followed by a 20-min linear gradient to acetonitrile-

$H_2O$-$H_3PO_4$ (60:40:0.1), and then a 5-min linear gradient to acetonitrile-$H_3PO_4$ (100:0.1). Fractions (0.5 ml) were collected and analyzed for $^{14}C$ by scintillation counting, and those eluting at the position of 4-ethoxy-3-methoxybenzaldehyde were pooled, extracted with $CHCl_3$, dried over $Na_2SO_4$, concentrated by evaporation, and analyzed by gas chromatography-electron impact mass spectrometry (GC-MS) (25).

**Fungal degradation of polymer-linked models.** Liquid cultures of *P. chrysosporium* (ATCC 24725) were grown from conidial inocula in 125-ml Erlenmeyer flasks that contained 10 ml of low-N (1.1 mM ammonium tartrate) growth medium with the basal concentration of trace elements (19). Cultures to which the PS-linked lignin model was added also contained 0.1% Tween 20. The cultures were pregrown for 72 h at 39°C in air, whereupon the previously filter-sterilized $^{14}C$-labeled polymeric model compounds were added aseptically to the surfaces of the mycelial mats. For experiments with PEG-linked model VII (six replicates), each culture received $3.90 \times 10^4$ dpm of polymer in 0.8 to 1.0 ml of $H_2O$. For experiments with PS-linked model XVI (six replicates), each culture received $2.35 \times 10^4$ dpm of polymer. The PS-linked model was dissolved beforehand in DMF, this solution was dispersed 145-fold in 0.1% aqueous Tween 20, and 1.0 ml of the dispersion was added to each mycelial mat. All cultures were fitted with gassing manifolds and incubated under $O_2$ at 39°C. The culture flask headspaces were flushed every 1 or 2 days with $O_2$, and vented $^{14}CO_2$ was trapped in an alkaline scintillation cocktail for determination of mineralized $^{14}C$ (18).

*Gloeophyllum trabeum* (ATCC 11539) was grown in 125-ml Erlenmeyer flasks that contained 2.5 g of perlite as a growth support and 15 ml of the same medium that was used for *P. chrysosporium*, except that the N source in this case consisted of 0.5 mM $NH_4NO_3$ and 0.5 mM asparagine. The flasks were inoculated with potato dextrose agar plugs of the fungus and pregrown under air at 30°C for 7 days. Mineralization experiments with compound V or VII were commenced by adding $1.3 \times 10^4$ dpm of radiolabeled compound to each of four replicate cultures in 0.5 ml of $H_2O$. The culture flasks were fitted with gassing manifolds, incubated at 30°C, and flushed with air daily to determine $^{14}CO_2$ evolution (18). For comparison, four to six replicate cultures of *P. chrysosporium* were set up with $1.4 \times 10^4$ dpm of labeled compound V or VII and then handled as described above for the other *P. chrysosporium* mineralization experiments.

Studies with solid wood cultures of *P. chrysosporium* were conducted essentially as described before (32). Autoclaved, vacuum-dried birch blocks (six replicates) were infiltrated with compound VII ($7.57 \times 10^4$ dpm) or XVI ($5.03 \times 10^4$ dpm), with 110 μl of methyl cellosolve as the solvent. The blocks were then infiltrated with a conidiospore suspension of *P. chrysosporium* in sterile, dilute (0.36% [wt/vol]) potato dextrose broth and placed on Teflon spacers over *P. chrysosporium* cultures that had been pregrown for 7 days on potato dextrose agar in 125-ml Erlenmeyer flasks. The cultures were fitted with gassing manifolds, incubated under air at 30°C, and flushed with air every 2 days for determination of $^{14}CO_2$ (18).

To detect metabolites formed by the fungus in liquid culture from PEG-linked model compound VII, five replicate cultures that originally contained $3.90 \times 10^4$ dpm of the model were harvested 8 h after it was added. The pooled medium and mycelium were stirred overnight with 2 volumes of dioxane, the mycelium was removed by centrifugation, 10 ml of DMF was added to the supernatant liquid, and the volume of the sample was evaporated to 3 ml under reduced pressure. Precipitated salts were removed from the sample by centrifugation, and the sample was subjected to GPC on a column of Sephadex LH-20 (1.9 by 33 cm) in DMF. Fractions (1.5 ml) with MWs corresponding to those of lignin monomers and dimers were then pooled and evaporated to dryness under reduced pressure. The sample was redissolved in 2.0 ml of acetonitrile-$H_2O$, 15:85, and 0.5-ml portions were subjected to reversed-phase HPLC on a Hamilton PRP-1 column at 1.0 ml $min^{-1}$ and ambient temperature. Metabolites were eluted isocratically for 15 min with acetonitrile-$H_2O$-$H_3PO_4$ (15:85:0.1), followed by a 35-min linear gradient to acetonitrile-$H_2O$-$H_3PO_4$ (65:35:0.1). Fractions (1.0 ml) were collected and analyzed for $^{14}C$ by scintillation counting. Fractions eluting at the position of 4-ethoxy-3-methoxybenzyl alcohol were pooled, extracted with $CHCl_3$, dried over anhydrous $Na_2SO_4$, concentrated by evaporation, trimethylsilylated, and analyzed by GC-MS (25).

To detect metabolites formed from compound VII by the fungus in solid wood culture, colonized wood blocks that each originally contained $7.63 \times 10^4$ dpm of the model were harvested after 5 days of incubation, pulverized in an electric coffee mill, and extracted with $CH_3OH$ in a Soxhlet apparatus (32). DMF (10 ml) was added to the extracts, which were then concentrated to 3 ml and fractionated by GPC on a column of Sephadex LH-20 (1.9 by 33 cm) in DMF. Fractions (1.5 ml) with MWs corresponding to those of lignin monomers and dimers were then pooled, worked up, and subjected to HPLC and scintillation counting as described above for the liquid culture experiments.

TABLE 2. $^{13}C$ NMR signal assignments for PS-linked model XVIII and related structures

| Chemical shift of compound (δ, ppm)[a] | | | | Assignment[b] |
|---|---|---|---|---|
| Trimer XIV (*erythro*/*cis*) | Trimer XIV (*erythro*/*trans*) | PS-linked model XVIII (*erythro*) | PS | |
| | | 21.0 | | Ar**C**$H_3$ on PS |
| | | 40.4 | 40.4 | **C**$(CH_2)_2$Ar of PS |
| | | 44.1 | 44.1 | C(**C**$H_2)_2$Ar of PS |
| 55.9 | 55.9 | 55.9 | | -O**C**$H_3$ |
| 61.5 | 61.6 | 61.6 | | -**C**$_\gamma H_2$OH |
| 74.0 | 74.0 | 74.0 | | -**C**$_\alpha$HOH- |
| 81.8 | | 81.6 | | -**C**$_\beta$HOAr- in *cis* linkages |
| | 82.1 | 82.1 | | -**C**$_\beta$HOAr- in *trans* linkages |
| 109.5 | 109.5 | 109.6 | | A2 |
| 111.1 | 111.1 | 111.1 | | A5 |
| 116.0 | | 116.0 | | B3 and B5 in *cis* linkages |
| | 116.7 | 116.8 | | B3 and B5 in *trans* linkages |
| 118.6 | 118.7 | 118.7 | | A6 |
| | | 125.7 | 125.6 | $C_H$1 in PS |
| 127.0 | 127.3 | | | C4 of compound XIV |
| 128.2, 128.8 | 126.3, 127.8 | 127.7, 128.0 | 127.6, 127.9 | C2, C3, C5, and C6 |
| 129.2, 129.4 | 127.3, 127.9 | Overlap with PS | | Olefinic |
| | 128.6 | 128.7 (approx) | | B2 and B6 in *trans* linkages |
| 130.3 | | 130.2 | | B2 and B6 in *cis* linkages |
| 130.8 | | 131.1 | | B1 in *cis* linkages |
| | 131.3 | 131.6 | | B1 in *trans* linkages |
| 132.9 | 132.9 | 132.9 | | A1 |
| | | 134.9 | | $C_O$1 and $C_M$1 in PS |
| 137.4 | 137.5 | | | C1 of compound XIV |
| | | 145.2 | 145.2 | C4 of PS |
| 148.8 | 148.8 | 148.8 | | A3 or A4 |
| 149.1 | 149.1 | 149.1 | | A3 or A4 |
| 156.7 | | 156.6 | | B4 in *cis* linkages |
| | 157.4 | 157.2 | | B4 in *trans* linkages |

[a] Chemical shifts are relative to tetramethylsilane in $CDCl_3$.
[b] See Fig. 2C and E for keys to labeling of the chemical structures.

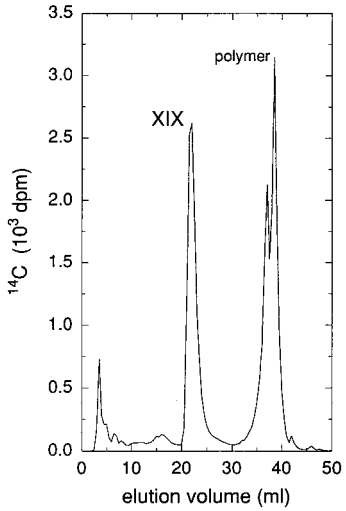


FIG. 3. Reversed-phase HPLC analysis of the products obtained after oxidation of $^{14}C$-labeled PEG-linked model VII with LiP and $H_2O_2$.

## RESULTS

**Structures of the models.** The PEG-linked model VII was analyzed by $^{13}C$ NMR spectrometry, and comparison of its spectrum with spectra of the low-MW model V and of PEG showed that all of the major signals obtained were attributable to the lignin model dimer or the PEG backbone (Table 1). Comparison of the -$OCH_2CH_2OAr$ and -$OCH_2CH_2OH$ signal amplitudes in the $^{13}C$ NMR spectrum indicated that each PEG molecule carried approximately 1 to 1.5 lignin model dimer of a possible total of 2, with the remaining end groups on the PEG consisting of hydroxyls. Compound VII was soluble in $H_2O$, $CH_3OH$, and $CH_2Cl_2$ but not in hydrocarbons.

$^{13}C$ NMR spectrometry also confirmed the structure of PS-linked models XVI (data not shown) and XVIII (Table 2). The lignin model dimer was connected to the PS via both *cis* and *trans* vinylic bonds, with the latter type predominating. Also attached to the PS were methyl groups, which resulted from decomposition of the methyl-PS triphenylphosphonium salt without linkage of the lignin model. Calculations showed that roughly 5 to 10% of the phenyl moieties in the PS had lignin model attached, about 5 to 10% carried methyl groups, and the remainder were unsubstituted. Compounds XVI and XVIII were insoluble in $H_2O$ but soluble in a variety of organic solvents.

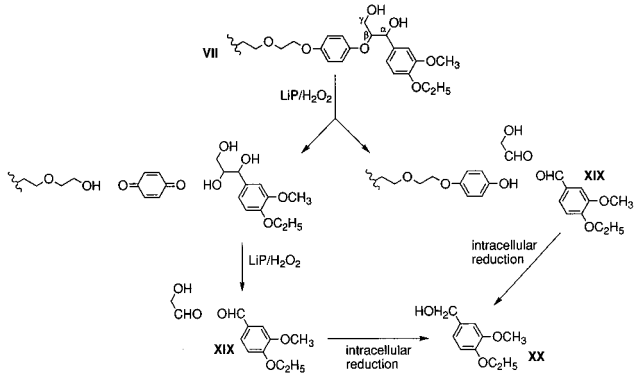


FIG. 4. Cleavage of model VII by *P. chrysosporium* LiP, followed by fungal reduction of the cleavage product compound XIX to give compound XX.

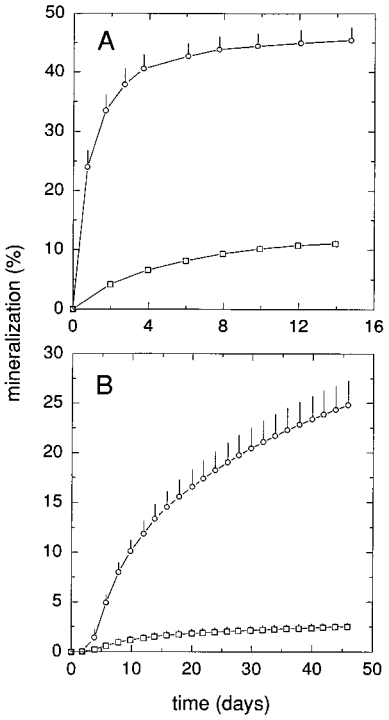


FIG. 5. Mineralization of $^{14}C$-labeled PEG-linked model VII (○) and of $^{14}C$-labeled PS-linked model XVI (□) by *P. chrysosporium* in liquid (A) and wood block (B) cultures. Error bars show 1 standard deviation of the sample.

**Cleavage of polymer-linked lignin models by LiP.** LiP oxidized the PEG-linked model VII rapidly in a reaction that gave an easily monitored $A_{308}$ increase. HPLC analysis showed that most of the oxidized substrate underwent $C_\alpha$-$C_\beta$ cleavage to give 4-ethoxy-3-methoxybenzaldehyde (compound XIX), which accounted for 72% of the $H_2O_2$ supplied (Fig. 3 and 4). GC-MS of the collected HPLC peak confirmed the identification: *m/z* (relative intensity) 180 ($M^+$, 45), 152 (-CO, 37), 151 (-HCO, 100), 137 (3), 123 (4), 119 (1), 109 (7). In addition, the reaction generated a small quantity of unidentified polar products, which probably included 4-ethoxy-3-methoxyphenylglycerol and 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropan-1-one from LiP-catalyzed cleavage of the aryl ether linkage. It is likely that some $C_\alpha$ oxidation of the lignin model to give the uncleaved ketone also occurred (22). This product, still linked to PEG, presumably eluted from the HPLC column at 34 to 41 ml with the starting material (Fig. 3).

LiP also oxidized the PS-linked model XVI in vitro, but the reaction was slow and could not be monitored spectrophotometrically because compound XVI was water insoluble even in the presence of surfactants. HPLC analysis of overnight reactions that included 0.1% Tween 80 showed that LiP cleaved compound XVI to give a mixture of low-MW products, including compound XIX, in a yield of several percent (data not shown).

**Fungal cleavage of polymer-linked lignin models.** *P. chrysosporium* mineralized the PEG-linked model VII in liquid medium and in solid wood cultures (Fig. 5). The rates and extents of mineralization were similar to those generally observed when the fungus mineralizes synthetic lignins under these conditions (32). Mineralization of the PS-linked model XVI was markedly slower than mineralization of compound VII in liq-

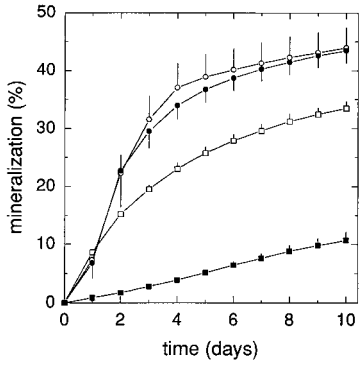


FIG. 6. Mineralization of $^{14}$C-labeled PEG-linked model VII by *P. chrysosporium* (●) and *G. trabeum* (■) compared with mineralization of low-MW $^{14}$C-labeled compound V by *P. chrysosporium* (○) and *G. trabeum* (□). Error bars show 1 standard deviation of the sample.

uid medium (Fig. 5A) and was negligible in wood cultures (Fig. 5B).

*P. chrysosporium* mineralized the PEG-linked model VII at the same initial rate that it mineralized compound V, a low-MW analog of compound VII. By contrast, the brown rot fungus *G. trabeum*, which shows only low ligninolytic activity in culture (18), mineralized compound VII at a much lower initial rate than it did compound V (Fig. 6).

In liquid culture and in wood, *P. chrysosporium* cleaved low-MW products from compound VII. GPC analysis showed that these products corresponded in size to monomeric and dimeric lignin model compounds (Fig. 7). There was little production of cleavage products intermediate in size between the starting PEG-linked model and these small products, from which we conclude that the PEG backbone was relatively stable in culture compared with the β-*O*-4-linked moiety of the model. That is, the fungus did not degrade the PEG to oligomers before it cleaved the β-*O*-4 structure of the model. No cleavage of compound VII occurred in the absence of the fungus.

Similar GPC results were obtained with the PS-linked model XVI in liquid culture, except that the recovery of soluble $^{14}$C-labeled material was low and a relatively greater quantity of apparently dimeric products accumulated (data not shown).

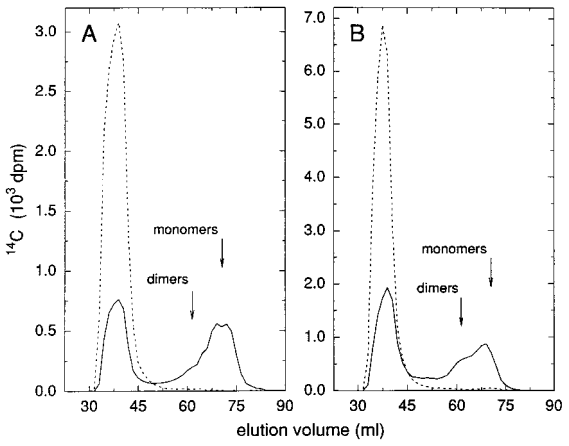


FIG. 7. GPC analysis on Sephadex LH-20 of the metabolites produced by *P. chrysosporium* from $^{14}$C-labeled PEG-linked model VII after 8 h in liquid cultures (A) and 5 days in wood block cultures (B). Solid curves show results for inoculated cultures, and dashed curves show results for uninoculated cultures.

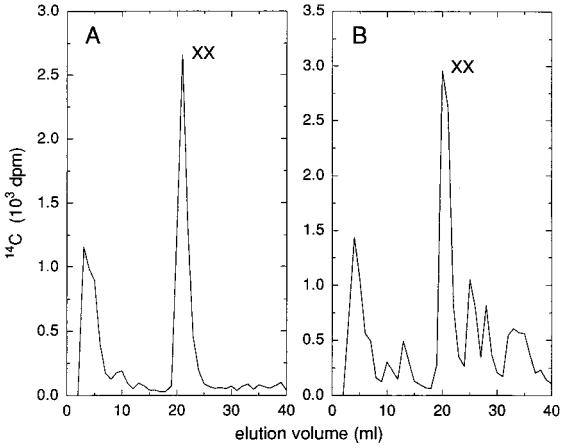


FIG. 8. Reversed-phase HPLC analysis of the low-MW metabolites produced by *P. chrysosporium* from $^{14}$C-labeled PEG-linked model VII after 8 h in liquid cultures (A) and 5 days in wood block cultures (B).

GPC analysis of compound XVI metabolites from the solid wood cultures was not attempted because the low extent of mineralization indicated that little metabolism of the model had occurred in this case.

When the low-MW material from liquid cultures that contained the PEG-linked model VII was isolated by GPC and analyzed by reversed-phase HPLC, the $C_\alpha$-$C_\beta$ cleavage product 4-ethoxy-3-methoxybenzyl alcohol (compound XX) was obtained in significant yield (Fig. 4 and 8A). GC-MS of the HPLC peak after trimethylsilylation confirmed the identification: *m/z* (relative intensity) 254 ($M^+$, 87), 239 (-$CH_3$, 7), 225 (10), 223 (8), 211 (14), 209 (20), 179 (8), 165 (100), 151 (3). After 8 h of incubation in the cultures, compound XX accounted for 13% of the $^{14}$C initially added to the experiment, for 19% of the recovered soluble $^{14}$C, and for 51% of the low-MW products recovered from the GPC column (mass balance shown in Table 3). Most of the other low-MW material obtained by GPC consisted of polar products that were not retained on the HPLC column.

Similar HPLC results were obtained in solid wood cultures after 5 days, except that other unidentified degradation products made up a larger proportion of the recoverable metabolites (Fig. 8B). Compound XX accounted for 6% of the $^{14}$C initially added to this experiment, for 10% of the recovered soluble $^{14}$C, and for 34% of the low-MW products recovered from the GPC column (mass balance shown in Table 3). The identification of compound XX in solid wood cultures could not be confirmed by GC-MS because of the large excess of low-MW wood extractives in the sample. However, it was con-

TABLE 3. Mass balance for metabolism of $^{14}$C-labeled compound VII by *P. chrysosporium*

| Fraction | $^{14}$C recovered (%) | |
|---|---|---|
| | In liquid cultures, 8 h | In wood block cultures, 5 days |
| Soluble in $CH_3OH$ | 72 | 54 |
| Mineralized | 5 | 7 |
| Insoluble | 16 | 31 |
| Glassware rinse | 2 | 1 |
| Total | 95 | 93 |

firmed by oxidizing the collected $^{14}C$-labeled HPLC peak with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (5), which gave a product that ran identically with the aldehyde XIX upon subsequent HPLC analysis (data not shown).

HPLC analysis of the low-MW metabolites produced in small quantity by liquid cultures of *P. chrysosporium* from the PS-linked model XVI was not conclusive, but compound XX was tentatively identified by its HPLC retention time as one of several products obtained (data not shown).

## DISCUSSION

Our inspiration for this project came from the work of Brunow and coworkers, who recently attached β-*O*-4 lignin models to PS beads via benzyl ether and vinylic linkages, by use of the Wittig reaction in the latter case, and who showed that polymers of this type are oxidized by LiP (15, 16, 24). One problem with these insoluble models was that low-MW aromatic compounds adhered noncovalently to the polymer beads, presumably because the hydrophobicity of PS caused it to act as a reversed-phase resin (24). Indeed, a structurally related styrene divinylbenzene polymer is the basis for the reversed-phase HPLC column that we used here to separate low-MW lignin model fragments. We anticipated that this property of PS beads would interfere with the release of lignin model metabolites, and we therefore sought to design lignin models linked to smaller soluble polymers of PEG and PS that would not adsorb low-MW cleavage metabolites.

A useful polymeric lignin model should be easy to prepare. The procedures we used for synthesis of the necessary β-*O*-4-linked dimers are well characterized and widely used, and the coupling reactions for attachment to the polymers are also technically straightforward. The syntheses are no more complicated than that of synthetic lignin, and the yield of usable high-MW polymer is probably higher because synthetic lignin preparations always contain a substantial quantity of oligomeric material that is too small for use in studies of microbial ligninolysis (17).

Lignin models for microbiological work must also be bioavailable in culture. This was the case for the water-soluble PEG-linked model VII but not for the water-insoluble PS-linked model XVI. Compound VII gave much greater rates and extents of mineralization and, unlike compound XVI, it was mineralized by *P. chrysosporium* in wood. The action of the fungus on compound VII was clearly a consequence of ligninolytic metabolism, in this case $C_\alpha$-$C_\beta$ cleavage of the lignin dimer. The first step in this reaction is ionization of the A ring or B ring of the aromatic substrate by LiP to give an aryl cation radical, which then fragments to give 4-ethoxy-3-methoxybenzaldehyde (compound XIX) or 4-ethoxy-3-methoxyphenylglycerol. The latter product is further oxidized by LiP, yielding additional compound XIX (20, 22). Compound XIX is then reduced intracellularly by the fungus to give compound XX (10), the product observed here in both liquid cultures and wood cultures (Fig. 4).

Ideally, a lignin model should be limited to extracellular attack by the organism under investigation. Previous work has shown that PEG with a MW greater than about 1,000 is unable to penetrate the cell wall of the bacterium *Bacillus megaterium* (30), the yeast *Saccharomyces cerevisiae* (31), or the oomycete *Achlya bisexualis* (26). Smaller PEGs with MW values of a few hundred can penetrate the cell walls of these organisms but are still excluded by their cell membranes. The 8,000-MW PEG that we used as a lignin model carrier was far larger than these PEGs. It undoubtedly contained some lower-MW polymer because of sample polydispersity, but we are confident that none of the PEG present had a MW less than 1,000 because it was excluded from Sephadex LH-20 during GPC (29). It is therefore unlikely that PEG-linked model compounds the size of compound VII are incorporated into the fungal cytoplasm.

The contrasting mineralization results we obtained with the brown rotter *G. trabeum* and the white rotter *P. chrysosporium* are consistent with this assessment. *G. trabeum* mineralized the polymeric model VII at a much lower initial rate than it did the low-MW model V, even though the two substrates differed only in the length of the alkoxy substituent at C-1 of their B rings. This result agrees with previous findings that *G. trabeum* mineralizes low-MW lignin models rapidly, presumably via intracellular processes unrelated to ligninolysis (7), but that it has a very limited (although detectable) ability to mineralize macromolecular synthetic lignin (7, 18). By contrast, *P. chrysosporium* mineralized compounds VII and V at the same rate, irrespective of their size, as would be expected if the initial cleavage of these compounds is extracellular.

We must note that our results do not rule out the possibility that compound VII can enter the periplasmic space of ligninolytic fungi. To our knowledge, no work has been done to assess the porosity of basidiomycete cell walls, and it is not presently clear that they will exclude PEGs with MWs of several thousand. It therefore remains possible that PEG-linked lignin models the size of compound VII might be degraded by ligninolytic systems that are trapped within the fungal cell wall. Such mechanisms, if they exist, would presumably be limited to the degradation of oligomeric lignin in fungus-colonized wood and would not have access to lignin that was still cross-linked into the secondary wood cell wall. This is a limitation of both PEG-linked models and synthetic lignins: because of their diffusibility, they may detect degradative reactions that are secondary to the first steps of fungal delignification, which must occur outside the hyphal cell wall.

Finally, a good lignin model should yield mechanistic information about how lignin is cleaved. Compound VII and related models should be useful in this regard because they have simpler structures than synthetic lignins and therefore cannot release the same plethora of low-MW products that cleaved lignins do. Of course, the elucidation of ligninolytic mechanisms through analysis of low-MW cleavage products is feasible only if the products persist in culture and are not subsequently modified beyond recognition by intracellular metabolism. If complications of this type occur, one must resort instead to functional group analysis of the leftover extracellular polymer, a much more difficult undertaking.

## ACKNOWLEDGMENTS

We are grateful to Kolby Hirth for the $^{13}C$ NMR analyses and to Ewald Srebotnik for critical comments.

This work was supported by grant DE-FG02-94ER20140 from the U.S. Department of Energy and by a grant from the Consortium for Plant Biotechnology Research, Inc., in conjunction with the U.S. Department of Energy.

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