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New Insights into the Ligninolytic Capability of a Wood Decay Ascomycete[▽]

Semarjit Shary,[1] Sally A. Ralph,[2] and Kenneth E. Hammel[1,2]*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706,[1] and USDA Forest Products Laboratory, Madison, Wisconsin 53726[2]

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Wood-grown cultures of *Daldinia concentrica* oxidized a permethylated β-^{14}C-labeled synthetic lignin to $^{14}CO_2$ and also cleaved a permethylated α-^{13}C-labeled synthetic lignin to give C_α-C_β cleavage products that were detected by ^{13}C nuclear magnetic resonance spectrometry. Therefore, this ascomycete resembles white-rot basidiomycetes in attacking the recalcitrant nonphenolic structures that predominate in lignin.

The degradation of lignocellulose by ascomycetes, a process generally called soft rot, is an important route for carbon cycling in plant litter and soils (2). Little is known about the decay mechanisms of these fungi, in contrast to those of the better-studied lignocellulolytic basidiomycetes that degrade wood. Nevertheless, it is clear that some soft-rot fungi can degrade lignin, because they erode the secondary cell wall and decrease the content of acid-insoluble material (Klason lignin) in angiosperm wood (13, 21). Some ascomycetes have also been shown to mineralize radiolabeled synthetic lignins (known as dehydrogenative polymerizates [DHPs]), but the low extents of $^{14}CO_2$ production reported—invariably less than 10% of the lignin—indicate that the ligninolytic capabilities of ascomycetes are more limited than those of white-rot basidiomycetes (11, 17).

One possibility is that ascomycetes attack only the phenolic units in lignin, which comprise roughly 10% of the polymer and are chemically more labile than the ether-linked, nonphenolic units that make up the remainder (Fig. 1). Phenolic lignin structures are oxidized and directly cleaved by a variety of mild biological oxidants such as manganic chelates and phenol oxidases (7, 20). By contrast, the cleavage of nonphenolic lignin structures requires the action of stronger oxidants, including oxyradicals produced by the action of peroxidases or laccases on various redox mediators. The production of these more vigorous ligninolytic systems is typical of white-rot basidiomycetes (5, 12) and has not yet been convincingly shown in soft-rot ascomycetes. However, the production of phenol oxidases that could act directly on phenolic lignin is widespread among ascomycetes (11, 14, 19).

Mineralization of DHPs. To address this question, we compared the extents to which *Daldinia concentrica* mineralized phenolic and nonphenolic DHPs. *D. concentrica* is a xylariaceous ascomycete that produces phenol oxidase activity (14) and degrades angiosperm wood extensively (13). Because chemical analyses of wood degraded by this fungus have shown that it degrades syringyl lignin units faster than guaiacyl ones (Fig. 1) (13), we performed these experiments with a syringyl/guaiacyl DHP (i.e., a polymeric model of angiosperm lignin) that was ^{14}C labeled at C_β on the side chains of its syringyl units. The radiolabeled DHP, prepared as described earlier (4, 9), had a specific activity of 0.01 mCi/mmol of syringyl subunits and a syringyl/guaiacyl ratio of approximately 4:1. A portion of this phenolic syringyl/guaiacyl DHP was then permethylated with diazomethane as described earlier (8) to obtain a nonphenolic syringyl/guaiacyl DHP, i.e., a polymer in which all of the phenolic hydroxyl groups had been blocked as methyl ethers. In addition, a phenolic guaiacyl DHP (i.e., a polymeric model of gymnosperm lignin), prepared previously with ^{14}C at C_β of its side chains (0.01 mCi/mmol of guaiacyl subunits) (4), was included in the experiment for comparison.

Each DHP was fractionated by gel permeation chromatography (GPC) on Sephadex LH-20 in *N*,*N*-dimethylformamide, and the excluded fractions were taken to eliminate low-molecular-mass material (≤~1 kDa) that might have been susceptible to uptake and intracellular metabolism. This step is essential in microbiological experiments with DHPs because ligninolysis is strictly an extracellular process (9). The excluded fractions were then subjected again to GPC on a 1.8- by 33-cm column of Sephacryl S-100 in *N*,*N*-dimethylformamide (15),

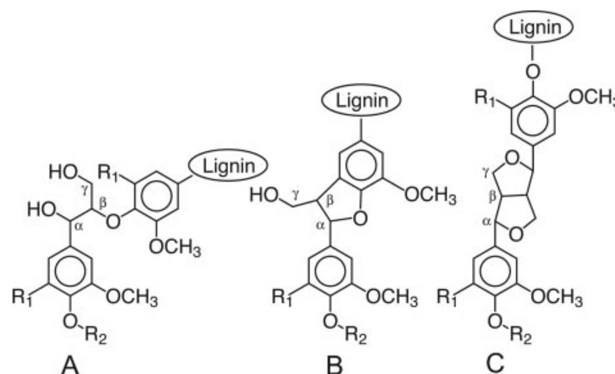

FIG. 1. Principal structures of natural lignins and DHPs. (A) Arylglycerol–β-aryl ether structures. (B) Phenylcoumaran structures. (C) Resinol structures. R_1 is H for guaiacyl structures and OCH_3 for syringyl structures. R_2 is H for phenolic structures, CH_3 for permethylated phenolic structures, and lignin for nonphenolic structures.

* Corresponding author. Mailing address: Institute for Microbial and Biochemical Sciences, USDA Forest Products Laboratory, One Gifford Pinchot Dr., Madison, WI 53726. Phone: (608) 231-9528. Fax: (608) 231-9262. E-mail: kehammel@wisc.edu.

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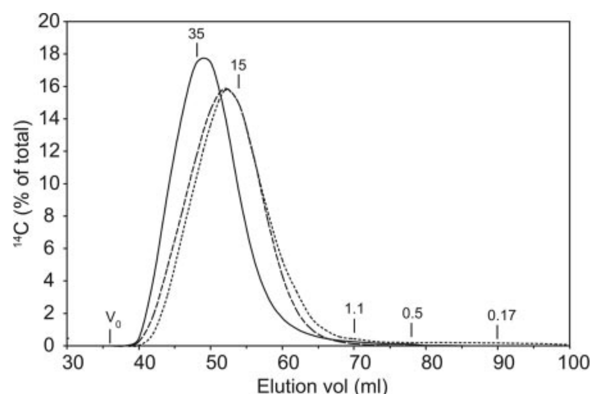

FIG. 2. Gel permeation chromatography of the radiolabeled DHPs used in mineralization experiments. The distributions shown, with their weight average molecular masses (M_w) and number average molecular masses (M_n), are as follows: phenolic syringyl/guaiacyl DHP (solid line), M_w = 33 kDa, M_n = 16 kDa; nonphenolic syringyl/guaiacyl DHP (long-dash line), M_w = 25 kDa M_n = 14 kDa; phenolic guaiacyl DHP (short-dash line), M_w = 21 kDa, M_n = 5 kDa. Elution positions and masses of standards are shown for 35-kDa, 15-kDa, and 0.5-kDa polystyrenes; a 1.1-kDa lignin model tetramer (15); and 3,4-dimethoxybenzaldehyde (0.17 kDa). V$_o$ indicates the excluded volume of the column.

and fractions eluting within the sieving range of the column were pooled so that the phenolic and nonphenolic DHPs had similar molecular mass distributions. We included this extra purification step in case the size of a DHP might influence its bioavailability. Figure 2 shows the molecular mass distributions of the three purified DHPs as determined by analytical GPC on Sephacryl S-100 in *N*,*N*-dimethylformamide–0.1 M LiCl (15).

The sized DHPs were dissolved in acetone-water (4:1), and 80,000 dpm of each was added to the end grain of replicate sterile aspen wafers (approximately 300 mg each, 3 by 10 by 30 mm, with the large face perpendicular to the grain). Additional sterile water was added to each block to bring the final volume of added water to 300 μl, and the acetone was allowed to evaporate. The wafers were then placed on spacers over potato dextrose agar in 125-ml Erlenmeyer flasks that already contained a lawn of *D. concentrica* (FP-140074-sp; Center for Forest Mycology, USDA Forest Products Laboratory). This medium was chosen because lignocellulose decay by ascomycetes is generally stimulated when auxiliary sources of carbon and nitrogen are present (21). An agar plug of the fungus was placed atop each wafer, and the flasks were stoppered with gassing manifolds. The cultures were incubated at 26°C and flushed with humidified, sterile air at 2- or 3-day intervals. The headspace gas from each flask was sparged through an alkaline scintillation cocktail to trap $^{14}CO_2$ evolved from the DHPs, and the amount of radiocarbon in each sample was then determined by scintillation counting (10).

We grew these cultures on wood wafers because weight loss in the substrate provides an independent test of whether the fungus has expressed its entire lignocellulolytic system. The extensive loss that occurred over the 12-week experiment—55% on average—leaves no doubt that this was the case. The mineralization data (Table 1) show that the phenolic syringyl/guaiacyl DHP was degraded more extensively than the phenolic guaiacyl DHP, in agreement with previous analyses of the lignin from wood degraded by *D. concentrica* (13). Nevertheless, the results establish that this fungus can degrade guaiacyl lignin structures extracellularly, because the proportion of ^{14}C with a molecular mass less than 1 kDa in our guaiacyl DHP was only about 2% (Fig. 2). Most interestingly, the data also show that blockage of the phenolic groups in the syringyl/guaiacyl DHP had no significant effect on its biodegradation. Therefore, contrary to our initial hypothesis, attack on nonphenolic lignin structures is a significant route for ligninolysis by *D. concentrica*.

Identification of cleavage products in a nonphenolic DHP. To address the question of how *D. concentrica* cleaves nonphenolic lignin structures, we supplemented aspen wafers with a nonphenolic DHP enriched with ^{13}C at C_α of its propyl side chain, extracted this DHP from the wood after decay, and then identified chemical changes at C_α by ^{13}C nuclear magnetic resonance (NMR) spectrometry. The [α-^{13}C]DHP was a guaiacyl polymer prepared earlier (4) with a ^{13}C content greater than 99% at C_α. It was first permethylated to block its phenolic structures and then fractionated on Sephadex LH-20 as described above to provide a high-molecular-mass fraction. The ^{13}C NMR spectrum of this fraction was obtained, after which two sterile aspen wafers (dry weights: 334 mg and 350 mg) were each infused with 9 mg of it in acetone-water and then inoculated with *D. concentrica* as described above. At the same time, five replicate cultures were set up on aspen wafers (average dry weight: 385 ± 22 mg) without [α-^{13}C]DHP so that the contribution of endogenous lignin to the NMR spectra could be assessed. The cultures were covered with aluminum foil and incubated at 26°C and 70% relative humidity for 7 weeks.

At the conclusion of the experiment, the two supplemented wafers had a combined dry weight of 464 mg and showed weight losses of 31% and 33%, compared with a mean value of 46% ± 3% for the five control wafers without [α-^{13}C]DHP. Apparently, the presence of DHP inhibited wood decay somewhat, but it is evident that a complete biodegradative system was nevertheless expressed in supplemented wafers. Two wafers, weighing a total of 461 mg, were chosen from the decayed set lacking [α-^{13}C]DHP, and each pair was then ball milled. The milled wood samples were treated with a crude cellulase

TABLE 1. Mineralization of β-^{14}C-labeled synthetic lignins by *D. concentrica* on wood

Polymer added	Amt mineralized ± SD (%) at:			Wt loss in wood ± SD (%) at 12 wk
	4 wk	8 wk	12 wk	
Nonphenolic syringyl/guaiacyl DHP (n = 6)	6.2 ± 2.7	10.4 ± 4.1	11.3 ± 4.4	56.1 ± 10.1
Phenolic syringyl/guaiacyl DHP (n = 5)	7.5 ± 2.4	12.3 ± 3.9	13.3 ± 4.1	51.4 ± 10.2
Phenolic guaiacyl DHP (n = 5)	2.1 ± 0.6	4.0 ± 1.1	4.6 ± 1.2	58.4 ± 6.4

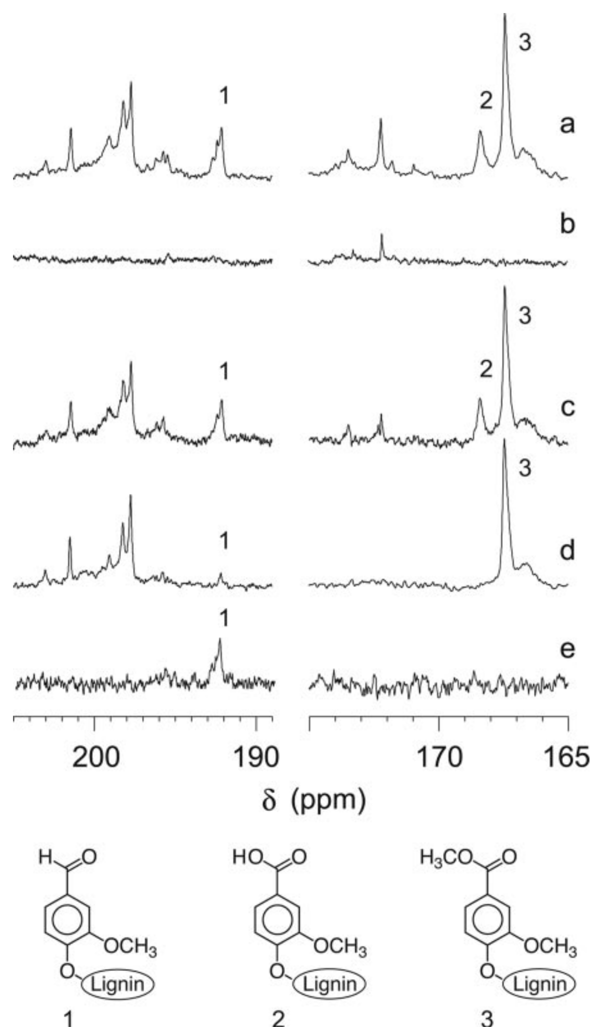

FIG. 3. ^{13}C NMR results showing (a) a spectrum of the extract from wood decayed by *D. concentrica* in the presence of permethylated guaiacyl [α-^{13}C]DHP, (b) a spectrum of the extract from decayed wood that received no DHP, (c) the difference between spectra a and b, (d) a spectrum of undegraded, permethylated, guaiacyl [α-^{13}C]DHP, and (e) a DEPT spectrum of the extract from wood decayed in the presence of permethylated guaiacyl [α-^{13}C]DHP. Spectra a and b were normalized with respect to the amount of wood lignin present by equalizing the signal from C-2/C-6 in syringyl aromatic carbons (guaiacyl DHP does not contribute in this region). Spectra a and d were normalized with respect to the amount of DHP present by equalizing the signal from C_α in resinol structures (this carbon was present at a very low abundance in the wood lignin). The samples were dissolved in 400 μl of acetone-d_6–D_2O (4:1), and the spectra were obtained with a Bruker DPX 250-MHz spectrometer (62.9 MHz for ^{13}C). The qualitative experiments shown in spectra a to d were acquired with a standard power-gated sequence, a 0.5-s delay, and 150,000 scans. Quantitative experiments (not shown) were performed with an inverse-gated sequence, a 15-s delay, a 90° pulse, and 25,000 scans. For spectrum e, the standard Bruker DEPT135 sequence was run for 30,000 scans. In all cases, the acetone signal at 29.8 ppm was used as the internal reference. Structures corresponding to signal assignments 1 to 3 are shown below the spectra. The assignments were confirmed by measuring carbonyl chemical shifts (δ) for 3,4-dimethoxybenzaldehyde, 3,4-dimethoxybenzoic acid, and 3,4-dimethoxybenzoate methyl ester. The new signals at 172 to 174 ppm in spectrum c may represent aliphatic carboxyl groups resulting from C_α-aryl cleavage of the DHP (1).

mixture (Cellulysin; Calbiochem, San Diego, CA) for 48 h thrice as described elsewhere (16), after which the lignin-enriched fraction was extracted for 24 h thrice with dioxane-water (9:1) to solubilize as much lignin as possible. The two wafers originally supplemented with [α-^{13}C]DHP yielded 97 mg of solubles, whereas the two unsupplemented wafers yielded 82 mg.

Qualitative ^{13}C NMR analyses of these samples were obtained, and the normalized spectra (Fig. 3) were inspected for signals indicative of ligninolysis. No significant changes were discernible in the alcohol and ether range (70 to 90 ppm), corresponding to C_α in major structures (Fig. 1) of the undegraded DHP, but new structures were apparent in the carbonyl (190 to 205 ppm and 165 to 175 ppm) regions. Figure 3a shows the spectrum in these regions for the [α-^{13}C]DHP that was extracted from degraded wood, and Fig. 3b shows the spectrum in the same regions for the extract from degraded wood that had received no [α-^{13}C]DHP. Figure 3c shows the difference between these two spectra and is thus a spectrum of degraded [α-^{13}C]DHP that has been corrected for the small contribution made by material originating from the wood. To see the changes that *D. concentrica* caused in the nonphenolic [α-^{13}C]DHP, the difference spectrum in Fig. 3c should be compared with Fig. 3d, which shows the spectrum of the original, undegraded, permethylated polymer.

In [α-^{13}C]DHP recovered from degraded wood, a signal in the region characteristic of benzaldehyde carbonyls was apparent (Fig. 3a and 3c, signal 1) and was confirmed to be from a protonated (i.e., aldehyde as opposed to ketone) carbonyl in a distortionless enhancement with polarization transfer (DEPT) experiment (18) (Fig. 3e). This benzaldehyde signal was absent from the lignin extracted from degraded wood that had received no [α-^{13}C]DHP (Fig. 3b) and therefore must have originated from the DHP. Benzaldehyde residues were also present in the original, undegraded [α-^{13}C]DHP (Fig. 3d), as also found in earlier research on another DHP (3), but they occurred at a much higher level in the degraded sample.

Similarly, the [α-^{13}C]DHP from degraded wood exhibited benzoic acid carboxyl signals (Fig. 3a and c, signal 2) that were detectable neither in the lignin from wood without [α-^{13}C]DHP (Fig. 3b) nor in the original undegraded [α-^{13}C]DHP (Fig. 3d). The undegraded [α-^{13}C]DHP did originally contain benzoic acid residues, in agreement with earlier work (3), but they all appear in our spectra as methyl benzoates (Fig. 3d, signal 3) because the [α-^{13}C]DHP had been permethylated before the experiment. Since these preexisting methyl benzoates were unaffected by fungal decay (Fig. 3a and c) they cannot have been the source of the new benzoic acid residues.

There are two possible sources for new benzaldehydes and benzoic acids in a biodegraded lignin. First, if the polymer originally contained primary benzyl alcohols, these might simply become oxidized at C_α. Such reactions would not be ligninolytic, but we can rule them out as an explanation for our data because a two-dimensional ^{1}H-^{13}C heteronuclear spin quantum correlation (HSQC) NMR spectrum of the original permethylated [α-^{13}C]DHP showed that it lacked the $^1H_\alpha$-$^{13}C_\alpha$ cross-peak at a δ of 4.6, 65 ppm, that is expected for primary benzyl alcohols (data not shown; for typical NMR chemical shifts of lignin-related structures see the U.S. Dairy Forage Research Center NMR Database of Lignin and Cell Wall Model Compounds at http://ars.usda

.gov/Services/docs.htm?docid=10491). This result leaves the second explanation, which is that the new benzaldehydes and benzoic acids arose via biodegradative C_α-C_β cleavage of propyl side chains in the [α-^{13}C]DHP. A quantitative ^{13}C NMR spectrum (data not shown) showed that new benzoic acid residues were roughly 10-fold more abundant than new benzaldehyde residues in the degraded [α-^{13}C]DHP and that approximately 1% of the residual polymer had been cleaved between C_α and C_β.

Conclusion. Our results show that *D. concentrica* degrades the recalcitrant nonphenolic structures in lignin and that C_α-C_β cleavage of the lignin propyl side chain is one of the processes responsible. The ligninolytic agents that *D. concentrica* produces remain unidentified because no method has yet been found to elicit their production on any medium except wood. However, it is reasonable to predict that they resemble some of the extracellular one-electron oxidants produced by white-rot basidiomycetes, because the latter fungi and their isolated ligninolytic systems also cleave nonphenolic lignin structures between C_α and C_β (1, 4). The relatively low ability of ascomycetes to depolymerize guaiacyl lignin structures has led researchers to suggest that they produce weaker ligninolytic oxidants (13), but this inference is contradicted by the observation that guaiacyl lignin structures are actually slightly easier to oxidize than syringyl structures (6). We consider it more likely that the ascomycetes produce a relatively small quantity of ligninolytic oxidants and consequently fail to overcome the tendency that guaiacyl lignin fragments have to repolymerize during one-electron oxidation (4).

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