A Highly Diastereoselective Oxidant Contributes to Ligninolysis by the White Rot Basidiomycete Ceriporiopsis subvermispora

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The white rot basidiomycete Ceriporiopsis subvermispora delignifies wood selectively and has potential biotechnological applications. Its ability to remove lignin before the substrate porosity has increased enough to admit enzymes suggests that small diffusible oxidants contribute to delignification. A key question is whether these unidentified oxidants attack lignin via single-electron transfer (SET), in which case they are expected to cleave its propyl side chains between Cα and Cβ and to oxidize the threo-diastereomer of its predominating β-O-4-linked structures more extensively than the corresponding erythro-diastereomer. We used two-dimensional solution-state nuclear magnetic resonance (NMR) techniques to look for changes in partially biodegraded lignin extracted from spruce wood after white rot caused by C. subvermispora. The results showed that (i) benzoic acid residues indicative of Cα—Cβ cleavage were the major identifiable truncated structures in lignin after decay and (ii) deplet-}

The use of mild biological agents to delignify lignocellulose with minimal loss of its valuable cellulose has been a long-standing technological goal. This process occurs in nature when certain white rot fungi delignify wood selectively, growing on its hemicel-}

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encoded proteins during growth of the fungus on ball-milled wood, the situation has not yet been evaluated for biodegradation of solid wood, and therefore, some role for LiPs cannot yet be ruled out.

To complicate the picture further, although previous work with lignin model compounds suggested that *C. subvermispora* cleaves lignin between C_α_ and C_σ_ (25, 26) consistent with the operation of a SET oxidant (23), a later analysis of wood degraded by the fungus did not reveal C_α_—C_σ_ cleavage products in the residual lignin (24). Thus, not only the nature of the ligninolytic oxidants but also the chemical route for ligninolysis remains in question for this biotechnologically promising fungus.

In principle, chemical changes in the lignin remaining after wood decay could provide clues about the poorly understood ligninolytic system of *C. subvermispora*. The major structures of lignin are well characterized (25, 26) and might differ from each other in susceptibility to particular oxidants. However, analyses of white-rotted wood have a limitation: the ability of the fungi to mineralize lignin efficiently results in very low steady-state accumulations of partially degraded structures such that almost all of the lignin present throughout decay consists of an intact polymer that has not yet been attacked (27–29). To detect degraded structures, the lignin must first be extracted with polar solvents that dissolve little of the sound polymer, after which oxidative changes can be identified in the small quantity of partially biodegraded lignin that dissolves (28). This fractionation of the lignin could lead to artifacts because some lignin structures are probably not distributed uniformly in sound wood, and it is unlikely that all parts of the wood are attacked to the same extent by the fungus or extracted with equal efficiency by the solvents. To address this issue, analyses of white-rotted lignin should ideally focus on structures that are thought to be uniformly distributed in all wood cell wall fractions.

Two structures that likely meet this criterion are the dia stereomers of the predominant arylglycerol-β-aryl ether (β-O-4) unit of lignin (structure A) (Fig. 1b). When wood is formed, structure A is biosynthesized in two steps. First, a phenoxy radical at the end of the growing lignin polymer couples with a 4-hydroxycinnamyl alcohol phenoxyl radical (shown here in its β-carbon centered resonance form) to give a new β-O-4-linked unit in which the terminal ring exists as a quinone methide and the β-carbons with their substituents occur in a 1:1 racemic mixture of enantiomers (i.e., the coupling reaction that generates a new chiral center is not stereospecific). The quinone methide then rearranges by reacting with water at its α-carbon to give an additional asymmetric center such that the newly formed structure A subunit exists as a racemic mixture of its two diastereomeric pairs of enantiomers, which can be designated RR/SS (also denoted syn or threo) and RS/SR (also denoted anti or erythro) (26).

Since local stereochemistry alone determines which face of the quinone methide reacts faster with water (Fig. 1b), the resulting threo/erythro ratio in structure A units is unlikely to vary depending on where the units are located in the wood cell wall. Rather, the threo/erythro ratio is simply characteristic of the particular 4-hydroxycinnamyl alcohols from which the lignin is biosynthesized. For softwood structure A units formed from coniferyl alcohol, this ratio is close to 1:1 and is generally thought to be invariant throughout the wood (30). Thus, although the fraction of total lignin extracted as a partially degraded polymer from a sample of white-rotted softwood is small, the threo/erythro ratio of the structure A units that this fraction had before biodegradation was likely representative of the entire sound sample. Moreover, as long as the lignin fraction analyzed is polymeric, the two diastereomers cannot be artifactually separated by solvent extraction, because they are randomly distributed and covalently linked to each other.

An analysis of the threo/erythro ratio for structure A units remaining in lignin after white rot is potentially useful because diastereoselectivity can serve as an indicator of an oxidant’s tendency to discriminate between chemical targets. Moreover, some relatively selective SET oxidants, including LiPs, have been shown to exhibit diastereoselectivity on structure A. Although the chemical mechanism for this phenomenon is not fully understood, it has been demonstrated repeatedly for dimeric lignin model compounds that represent structure A. When LiPs were used directly

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**FIG 1** Chemical structures and reactions discussed in the text. (a) Cleavage of lignin structure A via fungal SET oxidation, followed by autooxidation of the resulting lignin benzaldehydes. Stereochemistry is not shown for structure A. (b) Formation of structure A in the lignifying plant cell wall via free radical coupling and nucleophilic addition of water, showing routes for production of the erythro- and threo-diastereomers. OMe and MeO, methoxyl.
on these low-molecular-weight compounds, the threo-diastereomer was oxidized more rapidly (22, 31, 32). When a LiP was used in conjunction with the mediator veratryl alcohol to oxidize a small structure A model, the diastereoselectivity was preserved (31). An inorganic SET oxidant, ceric ammonium nitrate, gave a similar result, whereas oxidation of the structure A model by Fenton reagent, a highly nonselective oxidant, was not diastereoselective (31).

These results suggest that an examination of structure A threo/erythro ratios in residual lignin after attack by C. subvermispora could provide information on the chemical selectivity of the poorly understood ligninolytic oxidants that this fungus employs. However, until now, the issue of diastereoselectivity in delignification has not been addressed for any fungus on a natural lignocellulosic substrate. Here we have used two-dimensional solution-state nuclear magnetic resonance (NMR) analyses of biodegraded lignin to address three pertinent questions: (i) whether C. subvermispora cleaves lignin in wood between C₆ and C₇ consistent with a SET ligninolytic mechanism; (ii) whether the known SET oxidant LiP is diastereoselective in vitro on structure A when it cleaves polymeric lignin, as opposed to the low-molecular-weight models previously employed; and (iii) whether C. subvermispora removes structure A diastereoselectively when it cleaves lignin in wood.

**MATERIALS AND METHODS**

**Fungal cultures.** Ceriporiopsis subvermispora 90031-sp (ATCC 90467) and Phanerochaete chrysosporium ME-446 (ATCC 34541) were obtained from the Center for Forest Mycology, U.S. Forest Products Laboratory, and were maintained on malt agar. To obtain biodegraded lignins from colonized wood, the fungi were grown on wafers of white spruce sapwood (Picea glauca) (12 by 12 by 3 mm, with the large face perpendicular to the grain, ~150 mg [dry weight]). The wafers were first oven dried at 55°C for 3 days, and the dry weights for 10% of the group were recorded. The wafers were then autoclaved twice at 121°C for 50 min with an interval of 3 days between sterilizations and dried under a vacuum in a sterile desiccator jar. Sterile 20% potato dextrose broth (150 µl; 20%, wt/vol) was added to each wafer, and after the liquid was absorbed, the wafers (100 for each fungus per setup) were transferred onto 150-mm-diameter petri plates. Each plate contained 50 ml of Kirk’s B-III agar (1.5%, wt/vol) medium without glucose (33) and was overlaid with sterile nylon mesh. The wood wafers were inoculated with agar plugs containing fungal mycelium and incubated in humidified growth chambers. The cultivation temperature for P. chrysosporium was 38°C, and that for C. subvermispora was 28°C. After 6 weeks, the wafers were harvested, and excess mycelium was gently scraped from their surfaces with a razor blade. The subset of wafers for which dry weights had been recorded was oven dried as described above, and the dry weight losses for these replicates were recorded.

**Isolation of lignins.** The biodegraded spruce wood wafers from six separate culture setups, totaling 600 wafers for each fungus, were pooled and ground to a 40-mesh size in a Wiley mill. The milled samples were solvent extracted, using a modification of a procedure reported previously (12), by stirring them successively at room temperature for 1 hour in liters of petroleum ether (1 day), chloroform (1 day), acetone (1 day), methanol (2 days), and dioxane-water (96:4) (2 days). Between extractions, the solvents were recovered by vacuum filtration through glass fiber filters, and the extracted wood was resuspended in the next solvent. Finally the dioxane-water extracts were each vacuum evaporated, dissolved in N,N-dimethylacetamide, and precipitated into 100 ml of water (pH 3, adjusted with dilute formic acid) to remove low-molecular-weight components. The precipitates were stirred for 30 min, collected by centrifugation (27,000 × g for 15 min), and lyophilized. The final extract yields based on the dry weight of degraded wood were 1.20 mg/g and 1.89 mg/g for C. subvermispora and P. chrysosporium, respectively.

To provide a nondegraded control sample, we used macromolecular milled wood lignin from white spruce, prepared by extracting ball-milled spruce wood with dioxane-water (96:4), as described previously (28). This control lignin and the two macromolecular biodegraded lignins were divided into three portions for analysis. One portion of each was assayed for noncondensed structure A units by DFRC (derivatization followed by reductive cleavage) analysis (34). The structure A yields were quantified based on the total lignin content of each sample, which was determined by the acetyl bromide method (35). The second portion was set aside without derivatization for quantitative 13C NMR analysis. The third portion, destined for two-dimensional NMR analyses, was acetylated with acetic anhydride-pyridine (1:1) overnight. The residual acetic anhydride, acetic acid, and pyridine were then removed by rotary evaporation, followed by azeotropic coevaporation with ethanol. In addition to the above-described procedure, three additional acetylated milled spruce wood lignins were added as controls for the two-dimensional NMR analyses.

**Preparation of dimeric lignin model 1.** A lignin model dimer containing a benzoic acid residue on its β-O-4-linked aromatic ring, 4-[(1,3-bis(acetoxy)-1-(3,4-dimethoxyphenyl)propan-2-yl]oxy]-3-methoxybenzoic acid (model 1), was synthesized in six steps: (i) reaction of 3,4-dimethoxyacetophenone with Br₂ in CHCl₃, at room temperature to give 2-bromo-1-(3,4-dimethoxyphenyl)ethan-1-one, (ii) coupling with methyl 4-hydroxy-3-methoxybenzoate in refluxing aceton in the presence of K₂CO₃ to give methyl 4-[2-(3,4-dimethoxyphenyl)-2-oxoethoxy]-3-methoxybenzoate, (iii) aldol coupling with formaldehyde in 1,4-dioxane in the presence of K₂CO₃ at 38°C to give methyl 4-[1-(3,4-dimethoxyphenyl)-3-hydroxy-1-oxopropan-2-yl]oxy]-3-methoxybenzoate, (iv) reduction with NaBH₄ in methanol at 0°C to give methyl 4-[(1-(3,4-dimethoxyphenyl)-1,3-dihydroxypropan-2-yl]oxy]-3-methoxybenzate, (v) hydrolysis with LiOH in tetrahydrofuran-H₂O (3:1) at room temperature to give 4-[(1-(3,4-dimethoxyphenyl)-1,3-dihydroxypropan-2-yl]oxy]-3-methoxybenzoic acid, and (vi) acetylation with acetic anhydride-pyridine (1:1) at room temperature to give a 76:24 erthro/threo mixture of model 1 in a final yield of 27%. 1H NMR (700 MHz, dimethyl sulfoxide [DMSO]-d₆) δ 7.53 to 7.48 (m, 1H, Ar-H), 7.47 to 7.45 (m, 1H, Ar-H), 7.20 to 7.15 (m, 1H, Ar-H), 7.05 to 7.01 (m, 1H, Ar-H), 6.96 to 6.95 (m, 1H, Ar-H), 6.92 to 6.89 (m, 1H, Ar-H), 5.96 to 5.89 (m, 1H, α-CH₃), 5.06 to 5.00 (m, 1H, β-CH₃), 4.22 to 3.94 (m, 2H, γ-CH₂), 3.80 to 3.78 (m, 3H, -OCH₃), 3.74 to 3.72 (m, 6H, -OCH₃), 1.97 to 1.91 (m, 6H, -COCH₃) NMR (176 MHz, DMSO-d₆) δ 171.04, 170.17, 169.38, 169.28, 167.00, 166.97, 151.61, 150.78, 149.42, 149.32, 148.95, 148.78, 148.66, 148.47, 128.56, 128.48, 124.41, 124.26, 123.83, 122.80, 119.97, 119.85, 115.44, 115.39, 112.93, 112.82, 111.47, 111.32, 110.99, 110.83, 78.71, 77.74, 74.54, 73.08, 62.58, 62.11, 59.81, 55.72, 55.67, 55.52, 55.47, 55.43, 20.79, 20.75, 70.52, 20.50.

**Production and enzymatic cleavage of synthetic lignin.** An α-13C labeled synthetic lignin (dehydrogenation polymer [DHP]) was prepared from [α-13C]coniferyl alcohol (>99 atom% 13C) by using horseradish peroxidase, and a high-molecular-weight fraction of it was isolated by gel permeation chromatography as described previously (36). A colloidal dispersion of this lignin (1.5 mg) in sodium glycolate (10 mM; pH 4.51–2 methoxymethanol (7:3) was then treated with P. chrysosporium LiP (isozyme H1, 130 nmol) in the presence of H₂O₂ (0.15 mmol) and [α-13C]veratryl alcohol (>99.98 atom% 13C; 0.20 mmol), as described previously (36), in a final reaction volume of 500 ml. A control reaction was run under the same conditions without LiP. 13C-labeled lignin was required in this experiment to provide sufficient NMR signal amplitude, because ligninolysis by LiP in vitro can be performed only on dilute dispersions of lignin. Veratryl alcohol was included as a cofactor because P. chrysosporium LiP requires it to depolymerize synthetic lignins in vitro (36). The DHP lignins were recovered from the completed reactions and acetylated as described previously (36).

**NMR analysis.** Quantitative, inverse-gated, decoupled, nuclear Overhauser effect-suppressed 13C NMR spectra were acquired on a 500-MHz Bruker Avance instrument equipped with a cryogenically cooled 13C/15N [1H] probe (13C/15N coils coiled to the sample) by using Bruker’s
Measurements of structure A disappearance. Extents of structure A depletion in white-rotted lignins relative to the structure A content of sound milled wood lignin were determined by DFRC analysis, as described above. The extent of structure A depletion in DHP lignin after treatment with LiP-vernatryl alcohol relative to the structure A content in the control reaction mixture without LiP was determined by HSQC analysis. This was done by integrating the HSQC signals for structure A into the two product mixtures and then normalizing them to their total methoxyl contents determined in the same spectra. This normalization was possible because the quantities of methoxyl groups in the samples were effectively constant, since they were almost entirely attributable to the large excess (relative to lignin) of vernatryl alcohol added or to its oxidation product veratraldehyde.

Statistical analyses. For statistical analysis of the threo/erythro ratios and extents of structure A depletion, there were two major sources of variance to consider: biological variance in ligninolytic reactions and measurement variance in quantifying lignin structures.

Given the laborious procedure for sample production and lignin isolation and also the necessity to pool wood samples from many experiments to obtain enough lignin for analysis, it was not feasible to obtain biological replicates for the threo/erythro ratios or for the extents of structure A depletion. Instead, we used the variance in weight loss of the wood as a measurement of biological variance. Since weight loss in biodegrading wood involves processes in addition to ligninolysis (e.g., hemicellulose removal) that contribute additional variance, our use of weight loss variance as a proxy for variance in the threo/erythro ratio change and for variance in structure A removal likely overestimates the biological variance in our experiments. Our analysis indicated that the mean weight losses for the 600- wafer samples that we employed for lignin extraction were, with 95% confidence, within ±2% and ±3% of the mean weight losses that we determined for subsets of the wood degraded by C. subvermispora and P. chrysosporium, respectively.

To estimate measurement variance in integrating the HSQC spectra to obtain threo/erythro ratios, we performed each integration five times for each sample to obtain a mean value with an error estimate. The biodegraded samples consisted of the acetylated dioxane-water lignins from 600 pooled spruce wafers for each fungus, whereas the control samples for the biodegradation study consisted of four separately prepared and acetylated milled wood lignins. The DHP lignin samples did not include replicates because the limited amount of isotope-labeled substrate available precluded this approach. However, the DHP experiment involved biochemical oxidation in vitro, not a biotreatment, so the anticipated variance between reactions is low. Our results indicated that the 95% confidence intervals for signal integration were ±2% for sound milled wood lignin, ±3% for nondegraded DHP lignin, ±7% for DHP lignin degraded by LiP, ±17% for wood lignin degraded by P. chrysosporium, and ±28% for wood lignin degraded by C. subvermispora. The relatively high variances for signal integration in biodegraded lignins reflect the marked deletion of structure A in these samples, which resulted in low signal-to-noise ratios.

To estimate measurement variance in the DFRC analyses that determined the extents of structure A depletion in biodegraded wood, we performed each analysis in duplicate. The 95% confidence intervals were found to be ±2% for wood lignin degraded by P. chrysosporium and ±5% for wood lignin degraded by C. subvermispora, in agreement with previous work that demonstrated good reproducibility for this procedure (34). To estimate measurement variance in the HSQC integrations done to determine the extents of structure A depletion in DHP lignin after treatment with LiP, we performed each integration five times. The 95% confidence interval was found to be ±10%.

As the above-described confidence intervals show, the variance in our data was chiefly attributable to errors in HSQC integration, for which replicate analyses were available. The other sources of variance, being much smaller, had a negligible effect on the composite variance estimates. We used the above-described values and Student’s t formalism (41) to calculate overall 95% confidence intervals for threo/erythro ratios and extents of structure A depletion.

RESULTS AND DISCUSSION

Lignin isolation. C. subvermispora and P. chrysosporium degraded wafers of spruce wood to similar extents in our experiments, giving mean dry weight losses of 25% ± 4% and 25% ± 7%, respectively, after 6 weeks in the subset of wafers that we analyzed. To obtain partially biodegraded lignins from the colonized wood wafers, we extracted them with a series of solvents, as described in Materials and Methods. As reported previously for P. chrysosporium (12), we found most of the extractable lignin in the methanol and dioxane-water fractions, as shown by 13C NMR analysis. These analyses also revealed relatively higher levels of low-molecular-weight material in the methanol fractions, and therefore, we restricted further work to the dioxane-water extracts, which we precipitated into water to afford macromolecular degraded lignins. To provide a nondegraded control for comparison, we employed high-molecular-weight milled wood lignin from intact spruce.

Quantitative 13C NMR spectra of the isolated lignins confirmed that all three were macromolecular, as shown by the broad signals obtained (Fig. 2). To assess the extent to which the white-rotted lignins had been degraded, we derivatized them and also the sound milled wood lignin with acetyl bromide, followed by reduc-
tive cleavage with zinc and quantification of the resulting 4-acetoxy-
cinnamyl acetates (DFRC analysis). The DFRC method quanti-
tifies the noncondensed structure A units (i.e., those present in the
unbranched linear polymer) that predominate in lignin, thus captur-
ing a high proportion of all lignin subunits (34). The results
showed that the lignins degraded by C. subvermispora and P. chrys-
osporium were 89% and 90% depleted, respectively, in noncon-
densed structure A units (Table 1).

**Major products of ligninolysis.** Quantitative 13C NMR spec-
troscopy showed that benzoic acid residues were the major iden-
tifiable degraded structures present after biodegradation by both
fungi (Fig. 2). Benzoic acids were found previously in lignin de-
graded by P. chrysosporium (12, 13) but were not reported in an
analysis of lignin degraded by C. subvermispora (24). Therefore,
we performed an additional 1H-13C HMBC experiment after acet-
ylation of the C. subvermispora and control samples. This two-
dimensional technique associates protons in a molecule with car-
bons two or three bonds distant and is thus suited for the
characterization of nonprotonated functional groups such as ben-
zoic acid carboxyls (42). The results confirmed the 13C NMR data,
showing a close correspondence between cross-peaks in the de-
graded lignin and in a threo/erythro mixture of a model compound
that represents a softwood lignin benzoic acid in a β-O-4 linkage
with the polymer (model 1) (Fig. 3).

A key question, apparently not yet discussed in the literature, is
whether the presence of benzoic acid residues in white-rotted
lignin actually signifies cleavage of the polymer. Although benzoic
acids have not been found in sound lignin, low levels of benzalde-
hydes are already present (Fig. 2). In principle, some of these could
be oxidized by the fungus to benzoic acids without ligninolysis
and then become concentrated in the sample during solvent frac-
tionation. However, the preexisting benzaldehydes in sound
lignin are terminal rather than internal units of the lignin polymer
(43). It is unlikely that many such terminal units or products de-
rived from them remain in the sample after 90% of the original
structure A units have been destroyed, which was the case for our
biodegraded lignins. Therefore, the benzoic acids observed after
extensive fungal decay likely result from C
\[\text{O}--\text{C}\]
cleavage of the
lignin.

Previous work has shown that the SET oxidant LiP operating in
conjunction with the mediator veratryl alcohol also catalyzes
C
\[\text{O}--\text{C}\] cleavage in vitro when DHP lignin is used as the substrate,
although in this case, the products are benzaldehydes (36). The
presence of the truncated residues as acids rather than aldehydes
during white rot of wood (Fig. 1a) presumably reflects the insta-
ility of benzaldehydes in oxidizing environments (44), in combi-
nation with the fact that wood biodegradation experiments re-
quire much longer times than in vitro DHP depolymerizations
(weeks versus hours). Our data thus provide evidence that SET
oxidation of lignin aromatic rings contributes to ligninolysis by C.
subvermispora as well as P. chrysosporium.

**Diastereoselectivity of attack on structure A.** To compare the
relative extents of fungal attack on threo and erythro structure A in
spruce wood, we obtained two-dimensional 1H-13C HSQC spec-
tra of the acetylated samples to associate protons in the lignin with
the carbons directly attached to them (42). The results showed
that threo structure A had been depleted more than erythro struc-
ture A in the white-rotted lignins, with this effect being much

| Wood sample and expt | Yield of 4-acetoxy-3-
methoxycinnamyl acetatea (g/g lignin) | 4-acetoxy-3-
methoxycinnamyl acetatea (μmol/g lignin) |
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Nondecayed</td>
<td>1 0.1664 630.4</td>
<td>2 0.1662 629.5</td>
</tr>
<tr>
<td>Decayed by C. subvermispora</td>
<td>1 0.0190 72.0</td>
<td>2 0.0205 77.7</td>
</tr>
<tr>
<td>Decayed by P. chrysosporium</td>
<td>1 0.0169 64.2</td>
<td>2 0.0172 65.3</td>
</tr>
</tbody>
</table>

a Duplicate determinations for each pooled sample are shown.
more pronounced in the sample degraded by *C. subvermispora* (Fig. 4). Using the same NMR method, we also found that *P. chrysosporium* LiP operating in conjunction with veratryl alcohol on /H9251-/H9251-13C-labeled DHP lignin depleted threo structure A more than it depleted erythro structure A (Fig. 5).

Table 2 shows the observed structure A threo/erythro concentration ratios and associated extents of structure A removal, with 95% confidence intervals included, for the two biodegraded lignins compared with nondegraded wood lignin and for the DHP lignin treated *in vitro* with LiP-veratryl alcohol compared with DHP lignin from a control reaction without LiP. Although the structure A threo/erythro ratios during these delignification experiments are expected to decrease as a function of structure A removal, the ratios for degraded lignins in Table 2 can be directly compared because the extents of structure A removal were not significantly different among the three oxidative treatments.

The results show that all three ligninolytic processes were diastereoselective, giving threo/erythro ratios at approximately 90% structure A depletion that were significantly lower than the initial values of 0.94 to 0.96. The ratios of 0.69 to 0.73 for lignins degraded by *P. chrysosporium* or by *P. chrysosporium* LiP in the presence of veratryl alcohol were not significantly different from each other, whereas removal of structure A by *C. subvermispora* was significantly more diastereoselective than the other two processes, with a ratio of 0.35. The similar diastereoselectivities exhibited by *P. chrysosporium* on wood and by LiP-veratryl alcohol on DHP lignin appear consistent with a ligninolytic role for this enzyme-mediator system in fungi that produce it. By contrast, the diastereoselectivity exhibited by *C. subvermispora* on wood is too high to be accounted for by LiP-veratryl alcohol.

**Depletion of structures B and C in decayed lignin fractions.** The HSQC analyses also showed that structures B (phenylcoumarans) and C (pinoresinols) were markedly depleted relative to structure A in the white-rotted lignins (Fig. 4). However, this result is of uncertain significance because the relative levels of structures A, B, and C are probably not constant throughout the wood cell wall, in contrast to the uniform relative levels expected for threo and erythro structure A. The current view of lignin biosynthesis is that the secondary wood cell wall lignifies chiefly via end-wise polymerization, whereas the middle lamella between wood cells lignifies partly via bulk polymerization (26, 45). Condensed units such as structures B and C are relatively more abundant in lignins formed via bulk polymerization (46) and are consequently likely to be concentrated in the middle lamella. We cannot rule out the possibility that middle lamella lignin was underrepresented in our extracted biodegraded lignins relative to the milled wood lignin control.

**Conclusion.** Our results show that the ligninolytic oxidants of *C. subvermispora* are collectively more diastereoselective on structure A than are currently known fungal agents. This finding is consistent with a role in selective white rot, as it shows that the *C. subvermispora* oxidants exhibit specificity for their chemical tar-

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**FIG 3** 1H-13C HMBC NMR spectra obtained on acetylated lignins from control and degraded spruce wood and from a threo/erythro mixture of lignin model I, showing correlations between aromatic H2/H6 and carbons two to three bonds away. Assigned cross-peaks are shown in black and are labeled to associate them with particular carbons and protons in lignin benzoic acids and in model I, which are depicted below their respective spectra. Each assignment states whether the contour is attributed to the threo (t)- or erythro (e)-diastereomer, followed by the numbered positions of the carbons and protons responsible. Unassigned contours are shown in gray. OMe, methoxyl.
gets, unlike nonselective fungal oxidants such as Fenton reagent. Identification of these selective agents will be a significant challenge. One possibility is that a secreted \textit{C. subvermispora} enzyme, perhaps a peroxidase or laccase, oxidizes an unknown mediator to a diffusible oxidant that can infiltrate the sound wood cell wall. If so, the responsible ligninolytic radical must be more diastereoselective on structure A than the veratryl alcohol cation radical is.

TABLE 2 Diastereoselectivity of structure A removal

<table>
<thead>
<tr>
<th>Sample</th>
<th>Structure A depletion (% $\pm$ 95% confidence interval)</th>
<th>Structure A threo/erythro ratio $\pm$ 95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondecayed spruce lignin</td>
<td>0 $\pm$ 0</td>
<td>0.94 $\pm$ 0.02</td>
</tr>
<tr>
<td>Lignin from spruce decayed by \textit{C. subvermispora}</td>
<td>89 $\pm$ 5</td>
<td>0.35 $\pm$ 0.10</td>
</tr>
<tr>
<td>Lignin from spruce decayed by \textit{P. chrysosporium}</td>
<td>90 $\pm$ 3</td>
<td>0.73 $\pm$ 0.13</td>
</tr>
<tr>
<td>Nondegraded DHP lignin</td>
<td>0 $\pm$ 0</td>
<td>0.96 $\pm$ 0.03</td>
</tr>
<tr>
<td>DHP lignin degraded by LiP-veratryl alcohol</td>
<td>85 $\pm$ 9</td>
<td>0.69 $\pm$ 0.07</td>
</tr>
</tbody>
</table>
because our results show that the diastereoselectivity of structure A removal by *C. subvermispora* on wood was significantly higher than the diastereoselectivity on synthetic lignin exhibited in *vitro* by the LiP-veratryl alcohol system.

Recent work suggests three possible enzyme-mediator systems that could contribute to selective ligninolysis by *C. subvermispora*. (i) A LiP acts in concert with an oxidized fungal metabolite. The low transcript levels found for the encoding genes do not favor this hypothesis (5), but it cannot yet be excluded. (ii) A laccase or manganese peroxidase oxidizes wood-derived phenols to produce phenoxyl radical oxidants that attack lignin. However, currently known phenolic mediator systems oxidize lignin model compounds in very low yields (47), so their diastereoselectivity cannot be tested. (iii) A manganese peroxidase generates ligninolytic oxidants from unsaturated lipids (21, 48–51). Although this system cleaves both synthetic lignin and lignin model compounds, the proximal oxidants that are responsible have not yet been identified (52), and whether they show diastereoselectivity on lignin remains unclear.

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