

Evidence for cleavage of lignin by a brown rot basidiomycete

Daniel J. Yelle,^{1,2} John Ralph,^{3,4} Fachuang Lu,^{3,4} and Kenneth E. Hammel^{1,5*}

¹USDA Forest Products Laboratory, Madison, WI 53726, USA.

Departments of ²Forestry, ⁴Biological Systems Engineering and ⁵Bacteriology, University of Wisconsin, Madison, WI 53706, USA.

³US Dairy Forage Research Center, USDA Agricultural Research Service, Madison, WI 53706, USA.

Summary

Biodegradation by brown-rot fungi is quantitatively one of the most important fates of lignocellulose in nature. It has long been thought that these basidiomycetes do not degrade lignin significantly, and that their activities on this abundant aromatic biopolymer are limited to minor oxidative modifications. Here we have applied a new technique for the complete solubilization of lignocellulose to show, by one-bond ¹H-¹³C correlation nuclear magnetic resonance spectroscopy, that brown rot of spruce wood by *Gloeophyllum trabeum* resulted in a marked, non-selective depletion of all intermonomer side-chain linkages in the lignin. The resulting polymer retained most of its original aromatic residues and was probably interconnected by new linkages that lack hydrogens and are consequently invisible in one-bond ¹H-¹³C correlation spectra. Additional work is needed to characterize these linkages, but it is already clear that the aromatic polymer remaining after extensive brown rot is no longer recognizable as lignin.

Introduction

Lignin comprises roughly 15% of all terrestrial biomass and must be continuously broken down to maintain the global carbon cycle, yet is one of the most recalcitrant to biodegradation of all natural polymers. Few organisms possess the extracellular mechanisms to attack its combinatorial, stereoirregular linkages, which are formed in the cell walls of vascular plants via free radical coupling reactions between various 4-hydroxycinnamyl alcohols

and the growing polymer (Boerjan *et al.*, 2003). Efficient ligninolysis is apparently confined to certain filamentous fungi that grow on the cellulose and hemicelluloses of dead plant cell walls (Kirk and Farrell, 1987). It is well documented that the porosity of intact lignocellulose is too low for cellulases and hemicellulases to penetrate, and is generally accepted that most lignocellulolytic fungi circumvent this problem by degrading some of the lignin that encrusts the polysaccharides (Blanchette *et al.*, 1997).

One important group of lignocellulolytic fungi is generally thought to contradict this model. Brown-rot basidiomycetes cause a rapidly destructive decay that is the primary cause of failure in wooden structures (Zabel and Morrell, 1992), but is also an essential source of carbon cycling and humus formation in coniferous forests (Gilbertson and Ryvarden, 1986). Extensively brown-rotted wood is markedly depleted in polysaccharides and consists largely of a partially oxidized aromatic residue derived from the lignin. As compared with sound lignin, brown-rotted lignin has a lower aromatic methoxyl content and has a higher content of phenolic hydroxyls, conjugated carbonyls and carboxyls. However, it is still polymeric, which has led most researchers to conclude that brown-rot fungi modify lignin without degrading it significantly (Kirk, 1975; Agosin *et al.*, 1989; Eriksson *et al.*, 1990; Jin *et al.*, 1990; Irbe *et al.*, 2001; Filley *et al.*, 2002).

There are two reasons why this view might be incorrect. First, there is considerable evidence that brown-rot fungi initiate lignocellulose degradation by producing extracellular hydroxyl radicals ($\cdot\text{OH}$), highly reactive, non-selective oxidants that depolymerize polysaccharides via hydrogen abstraction but also attack lignin via electrophilic addition to its aromatic rings (Goodell *et al.*, 1997; Hammel *et al.*, 2002; Suzuki *et al.*, 2006). As one consequence of the latter reaction is aryl methyl ether cleavage (Gierer *et al.*, 1992), which would explain why brown-rotted lignin is depleted in methoxyls, there is no obvious reason why the arylglycerol- β -aryl ether linkages that interconnect the majority of lignin subunits would not be analogously cleaved (Fig. 1; Tatsumi and Terashima, 1985). Second, it appears that brown-rot fungi do not depend solely on $\cdot\text{OH}$ to degrade wood polysaccharides, but rather augment this system with hydrolytic enzymes (Cohen *et al.*, 2005). If this is the case, it is unclear how the enzymes could operate without some prior ligninolysis to increase wood porosity. To address this problem, we

Received 9 January, 2008; accepted 14 February, 2008. *For correspondence. E-mail kehammel@wisc.edu; Tel. (+1) 608 2319528; Fax (+1) 608 2319262.

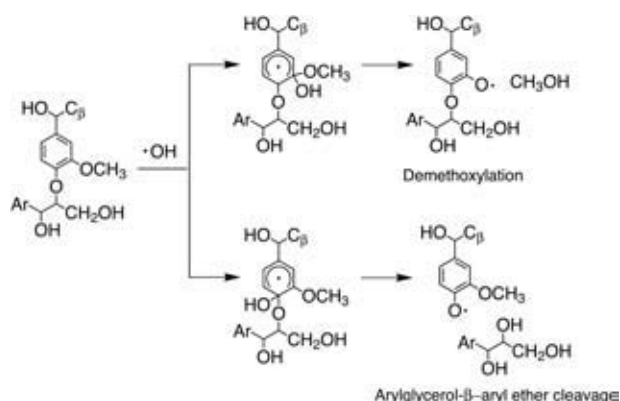


Fig. 1. Two of the many reactions expected between $\cdot\text{OH}$ and the predominant arylglycerol- β -aryl ether structure of lignin (structure A in Fig. 3). The upper pathway shows attack at the aromatic 3-position to eliminate methanol, and the lower pathway shows attack at the 4-position to eliminate a phenylglycerol. Ar, aryl.

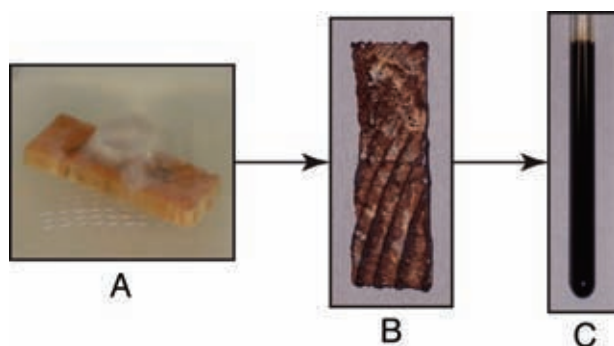


Fig. 2. Summary of the steps prior to NMR analysis, showing (A) *G. trabeum* growing on a spruce wafer 1 week after inoculation, (B) appearance of a typical spruce wafer 16 weeks after inoculation, and (C) the decayed wood sample after milling, acetylation and complete dissolution in $\text{DMSO-}d_6$.

have adopted a new technique for lignocellulose solubilization that permits, for the first time, a closer look at brown rot via two-dimensional solution-state nuclear magnetic resonance (NMR) spectroscopy.

Results and discussion

We grew the brown-rot basidiomycete *Gloeophyllum trabeum* on wafers of spruce wood for 16 weeks (Fig. 2)

Table 1. Methoxyl and uncondensed arylglycerol- β -aryl ether content in sound and decayed spruce wood.

Sample	Methoxyls g g^{-1} lignin ^a	Arylglycerol- β -aryl ethers by DFRC analysis	
		g g^{-1} lignin ^a	g g^{-1} methoxyls
Sound wood	0.168	0.111	0.661
Decayed wood	0.107	0.020	0.187

a. Klason lignin plus acid-soluble lignin.

and then selected a subset of the biodegraded wood samples that had lost $64 \pm 9\%$ ($n = 9$) of their dry weight and were 36% deficient in methoxyl groups as shown by the wet chemical analysis generally used (Table 1, see *Experimental procedures*). In these properties, our *G. trabeum*-degraded spruce samples closely resembled those analysed earlier in Kirk's seminal work, which reported an average dry weight loss of 70% and a methoxyl loss of 35% (Kirk, 1975). Our samples had lost 85% of their glucan and over 90% of their other polysaccharides, but only 16% of the residual material (Klason lignin and acid-soluble lignin) that is generally interpreted as modified but intact lignin (Table 2). These results agree well with those generally obtained after extensive decay of gymnosperm woods by *G. trabeum* (Eriksson *et al.*, 1990; Worrall *et al.*, 1997).

To determine how *G. trabeum* alters the lignin in wood, we began with a wet chemical procedure that is generally called the DFRC method (for Derivatization Followed by Reductive Cleavage). DFRC analysis cleaves the predominating arylglycerol- β -aryl ether linkages of lignin in high yield to give 4-acetoxycinnamyl acetates that are then quantified by gas chromatography (Lu and Ralph, 1997a,b). The results showed that *G. trabeum* reduced the assayable arylglycerol- β -aryl ether content of the residual aromatic polymer to 18% of its original value on a Klason lignin plus acid-soluble lignin basis, and decreased it to 28% of its original value on a methoxyl basis (Table 1). It does not necessarily follow that the lignin was degraded, because non-lignolytic side-chain oxidations or free radical coupling reactions between phenolic subunits, both of which are likely in the highly oxidizing environment of brown rot, would also reduce the

Table 2. Chemical composition of sound and decayed spruce wood samples.

Sample	Klason lignin (mg)	ASL (mg)	Glucose (mg)	Mannose (mg)	Xylose (mg)	Galactose (mg)	Arabinose (mg)	Rhamnose (mg)
Sound wood								
Per gram of sound wood	273	7	451	108	60	13	11	1
Decayed wood								
Per gram of sound wood	215	20	69	8	6	3	1	nd
Per gram of decayed wood	591	54	191	22	16	8	2	nd

ASL, acid-soluble lignin; nd, not detected.

4-acetoxycinnamyl acetate yield upon subsequent DFRC analysis. However, the DFRC results make it clear that the aromatic polymer remaining after extensive brown rot by *G. trabeum* bears little resemblance to native lignin.

To identify some of the structural changes that had occurred in the brown-rotted lignin, we next applied a recently developed solubilization method that has been used successfully on native lignocellulose samples. In this procedure, the samples are ball-milled, completely dissolved in an *N*-methylimidazole/dimethylsulfoxide (DMSO) mixture, acetylated, precipitated in H₂O and finally redissolved in a suitable solvent (Lu and Ralph, 2003). We found that this approach was also applicable to extensively brown-rotted wood, which dissolved completely in DMSO after acetylation to give a nearly black solution (Fig. 2). Unlike earlier approaches that involved extracting a small proportion of the total lignin from biodegraded lignocellulose with organic solvents (Chua *et al.*, 1982), experiments with fully dissolved samples allow solution-state spectroscopic analyses to be performed without concerns that the sample may have been selectively fractionated.

We analysed the biodegraded wood and an undegraded control sample by ¹H-¹³C heteronuclear single quantum correlation (HSQC) NMR spectroscopy (Fig. 3). The advantage of an HSQC spectrum is that it correlates the chemical shift of every hydrogen atom in a molecule with the chemical shift of the carbon it is attached to, thus providing excellent peak dispersion in two dimensions and increased confidence in signal assignments. We have set the contour intensities of the aromatic signals in the two spectra to approximately equal volumes so that signals from other cell wall components can be compared visually.

As expected, the results show that polysaccharide signals were depleted relative to aromatic ones in the brown-rotted sample. However, the salient feature in the brown-rotted spectrum is the marked, non-selective loss of lignin side-chain signals relative to aromatic and methoxyl signals. For example, a quantitative comparison of the NMR signals for C_α in arylglycerol-β-aryl ether structures (structure A in Fig. 3) shows that, on a methoxyl basis, this predominant structure of lignin was depleted to 29% of its original level after brown rot (Table 3). This value agrees remarkably well with the value of 28% obtained from our wet chemical analyses (Table 1).

The decreased amplitude of lignin side-chain NMR signals after brown rot cannot have resulted simply from oxidation of the side-chains without cleavage, because most of the resulting signals would have predictable chemical shifts, yet cannot be found in the spectrum. For example, the HSQC H_β-C_β cross-peak near 5.8, 80 p.p.m. expected after benzylic oxidation of arylglycerol-β-aryl ether structures to give ketones at C_α is undetectable

(Ralph *et al.*, 2004). The result also cannot be due to cross-linking of the aromatic rings via new linkages, because these reactions would have a negligible effect on the side-chain chemical shifts (Ralph *et al.*, 2004). Therefore, it appears that lignin side-chains must be degraded during brown rot. Moreover, they are removed from the lignin even more rapidly than the methoxyls are.

New, oxidatively cleaved side-chain structures that might take the place of those depleted during brown rot are not evident in our spectrum. For example, the HSQC H_β-C_β cross-peak near 5.4, 72 p.p.m. expected for acetylated phenylglycerols appears to be absent (Ralph *et al.*, 2004), even though phenylglycerols are likely products from the attack of ·OH at the aromatic 4-position of arylglycerol-β-aryl ether structures (Fig. 1). The only new structures clearly apparent in the HSQC spectrum after brown rot are in the aromatic region, where shifts to higher p.p.m. along the ¹H coordinate have occurred (Fig. 3B). These signals may reflect additional acetoxyl substituents on the aromatic rings (Ralph *et al.*, 2004), which would be consistent with the earlier observation that brown-rotted lignin has an increased phenolic content (Kirk, 1975).

The disappearance of lignin side-chains during decay by *G. trabeum* must be reconciled with the facts that brown-rotted lignin retains most of its aromatic residues and is still polymeric (Kirk, 1975; Agosin *et al.*, 1989; Jin *et al.*, 1990). We surmise that the lignin was first depolymerized, but later reconnected via radical coupling of the phenolic units that become enriched during attack on the aromatic rings by ·OH. As these coupling reactions occur chiefly between unsubstituted carbons of two lignin rings or between an unsubstituted carbon of one ring and the phenoxyl oxygen of another ring, the resulting products would include biphenyls and diphenyl ethers. Our NMR experiment could not have detected these products, because their linkages contain no hydrogens and consequently are invisible in HSQC spectra. Although longer-range NMR procedures exist to show correlations between carbons and hydrogens that are two or three bonds distant, we have found that the NMR relaxation time of dissolved brown-rotted wood is too short to obtain adequate spectra by these methods (data not shown).

In addition, the lack of discernible new side-chain structures in our HSQC spectrum of decayed wood probably reflects the fact that ·OH exhibits very little regioselectivity in reactions with aromatic compounds (Walling, 1975; Gierer *et al.*, 1992). As a result, the fungus probably oxidized many of the lignin substructures at multiple sites to give a large number of products so dispersed across the spectrum that they fail to stand out above the baseline noise. During this study, we considered the possibility that an examination of less extensively decayed wood specimens would circumvent this problem, but the results

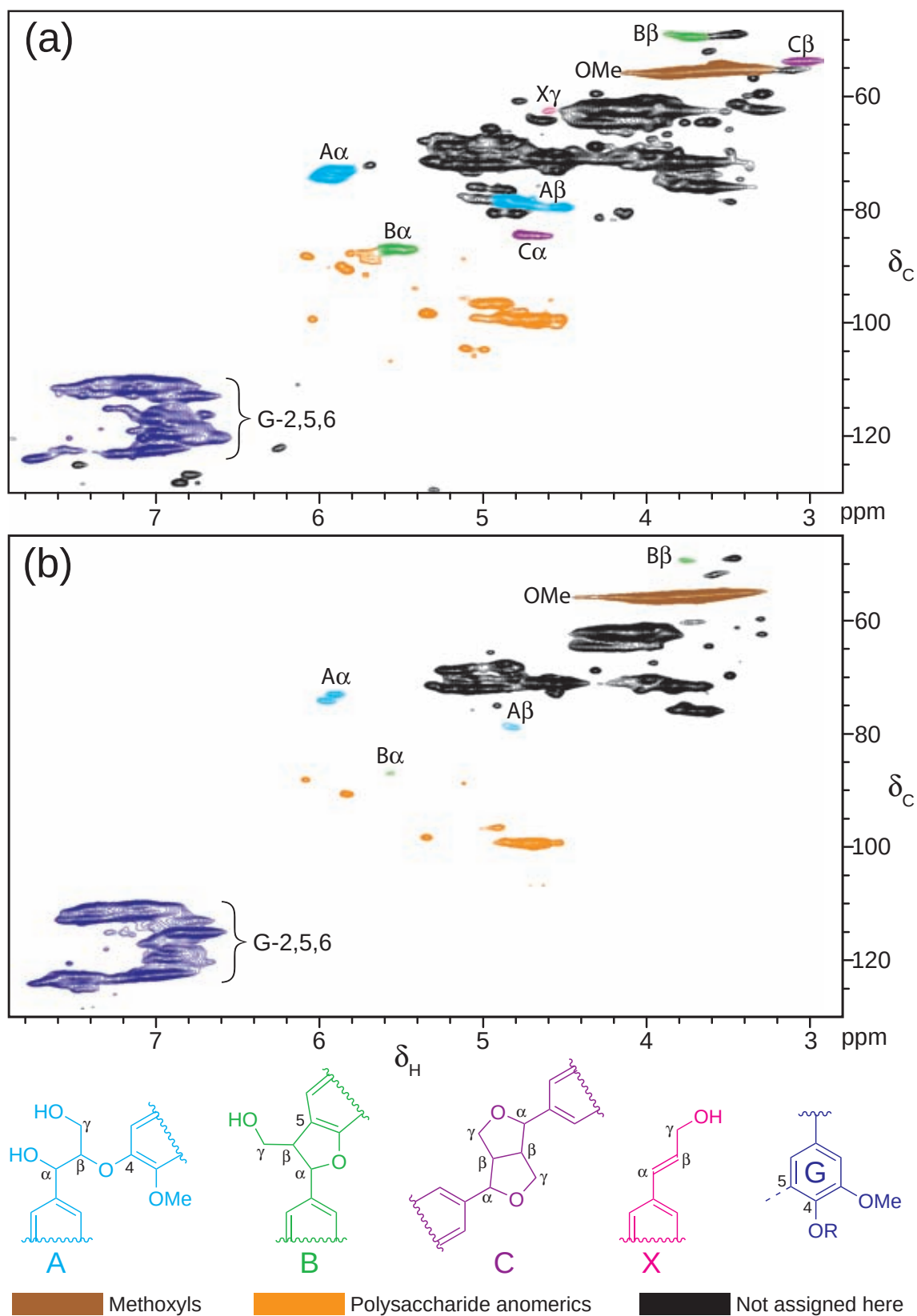


Fig. 3. HSQC NMR spectra of acetylated sound spruce wood (A) and acetylated, brown-rotted spruce wood (B) in DMSO- d_6 . Most of the unassigned structures are attributable to non-anomeric carbon–hydrogen bonds in polysaccharides. OMe: methoxyl. R: lignin polymer or H.

Table 3. Relative integrals of contours in HSQC spectra of acetylated, solubilized spruce wood.

Lignin structure	Sound wood integral	Decayed wood integral
OMe	1.00	1.00
A α	0.079 (69)	0.023 (70)
B α	0.024 (21)	0.007 (21)
C α	0.011 (10)	0.003 (9)

See Fig. 3 for abbreviations used for structures. Numbers in parentheses show what per cent of the sum of the integrals for structures A α , B α and C α is accounted for by the integral for each individual structure.

showed that new side-chain structures were also undetectable after 2–3 weeks and 7–10% weight loss (data not shown). One solution to this problem in future work might be to supplement the wood beforehand with a synthetic lignin enriched at specific positions with ^{13}C , which would amplify potentially interesting signals in the NMR spectra (Shary *et al.*, 2007).

Experimental procedures

Reagents and fungal cultures

Chemicals, all reagent grade or better, were obtained from Aldrich (Milwaukee, WI). *Gloeophyllum trabeum* (ATCC 11539) was grown as described earlier on wafers of spruce wood (*Picea glauca*, 3 × 10 × 30 mm, each approximately 300 mg dry weight) that had been cut with the large face perpendicular to the grain (Suzuki *et al.*, 2006).

Sample preparation and wet chemical analyses

After 16 weeks of decay, those wafers that had lost 50–70% of their dry weight were pooled, dried overnight at 50°C and ground in the presence of dry ice using a coffee grinder. Samples were then taken for analyses of total sugars (Davis, 1998), Klason lignin (ASTM, 1996), acid-soluble lignin (TAPPI, 1991), methoxyl content (Chen, 1992; Zakis, 1994) and uncondensed arylglycerol- β -aryl ether content (Lu and Ralph, 1997a,b), which were performed as described in the references cited.

A 300 mg sample of the remaining milled wood was then added to a Retsch PM100 planetary ball-mill equipped with a 50 ml ZrO₂ cup, eight 10-mm-diameter ZrO₂ balls and three 20-mm-diameter ZrO₂ balls. The wood was milled three times at 300 r.p.m. for 20 min, with a 10 min pause for cooling between each run. A sample of undegraded spruce wood (500 mg) was treated similarly, except it required milling nine times at 600 r.p.m. for 20 min, with a 10 min pause between each run.

Dissolution of the milled spruce samples closely followed the procedure introduced by Lu and Ralph (2003). From each sample, 100 mg was taken and added to 2 ml of DMSO and 1 ml of *N*-methylimidazole. The samples were dissolved with stirring, acetylated with 600 μ l of acetic anhydride for 1.5 h and precipitated quantitatively in 400 ml of water with stirring

for 30 min. The settled precipitate was collected by vacuum filtration through a 0.2 μ m pore size nylon membrane, washed twice by filtration with 200 ml of distilled water and dried in a vacuum oven at 50°C.

We noted some differences between sound and decayed spruce in the dissolution and derivatization steps. First, the decayed wood required overnight stirring in DMSO/*N*-methylimidazole for complete dissolution, whereas the undecayed sample dissolved within 3 h. Second, the decayed sample gave a nearly black solution in DMSO/*N*-methylimidazole, as compared with the amber colour of dissolved, undecayed spruce. Third, filtration of the decayed sample after acetylation and precipitation was considerably slower than that of the undecayed sample, which suggests a finer particle size in the decayed sample. Finally, although both acetylated samples were soluble in DMSO, only the undecayed sample was soluble in chloroform.

Solution-state NMR spectroscopy

One-bond ^1H - ^{13}C correlation (HSQC) NMR spectra were acquired at 300 K on a Bruker BioSpin (Rheinstetten, Germany) DMX-750 instrument equipped with a cryogenically cooled 5 mm TXI $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ gradient probe with inverse geometry (proton closest to the sample). Samples (approximately 60 mg each) of ball-milled, acetylated wood were added to 5-mm-diameter NMR tubes and dissolved in 500 μ l of DMSO-*d*₆. The central DMSO solvent peak was used as an internal reference ($\delta_{\text{C}} = 39.5$ p.p.m., $\delta_{\text{H}} = 2.49$ p.p.m.). HSQC experiments had the following parameters: sweep width, 8.6–2.4 p.p.m. in F₂ (^1H) using 1864 data points (acquisition time, 200 ms) and 160–40 p.p.m. in F₁ (^{13}C) using 512 increments (F₁ 'acquisition time', 11.3 ms). The number of scans was 32 with a 1 s interscan delay, the d24 delay was set to 1.72 ms, and the total acquisition time was 5 h 34 min. Processing used typical matched Gaussian apodization in F₂ and a squared cosine-bell in F₁. Integration calculations of the two-dimensional contours in HSQC spectra were accomplished with Bruker TopSpin v. 2.0 software (Ralph *et al.*, 2006).

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References

- Agosin, E., Jarpa, S., Rojas, E., and Espejo, E. (1989) Solid-state fermentation of pine sawdust by selected brown-rot fungi. *Enzyme Microb Technol* **11**: 511–517.
- ASTM (1996) Standard test method for acid-insoluble lignin in wood, D 1106. In *Annual Book of ASTM Standards*. West Conshohocken, PA, USA: ASTM (American Society for Testing and Materials) Press.
- Blanchette, R.A., Krueger, E.W., Haight, J.E., Akhtar, M., and

- Akin, D.E. (1997) Cell wall alterations in loblolly pine wood decayed by the white-rot fungus *Ceriporiopsis subvermispora*. *J Biotechnol* **53**: 203–213.
- Boerjan, W., Ralph, J., and Baucher, M. (2003) Lignin biosynthesis. *Annu Rev Plant Biol* **54**: 519–546.
- Chen, C.-L. (1992) Determination of methoxyl groups. In *Methods in Lignin Chemistry*. Lin, S.Y., and Dence, C.W. (eds). Heidelberg, Germany: Springer, pp. 465–472.
- Chua, M.G.S., Chen, C.-L., Chang, H.M., and Kirk, T.K. (1982) ^{13}C NMR spectroscopic study of spruce lignin degraded by *Phanerochaete chrysosporium*. 1. New structures. *Holzforschung* **36**: 165–172.
- Cohen, R., Suzuki, M.R., and Hammel, K.E. (2005) Processive endoglucanase active in crystalline cellulose hydrolysis by the brown rot basidiomycete *Gloeophyllum trabeum*. *Appl Environ Microbiol* **71**: 2412–2417.
- Davis, M.W. (1998) A rapid modified method for compositional carbohydrate analysis of lignocellulosics by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD). *J Wood Chem Technol* **18**: 235–252.
- Eriksson, K.-E., Blanchette, R.A., and Ander, P. (1990) *Microbial and Enzymatic Degradation of Wood and Wood Components*. Berlin, Germany: Springer.
- Filley, T.R., Cody, G.D., Goodell, B., Jellison, J., Noser, C., and Ostrofsky, A. (2002) Lignin demethylation and polysaccharide decomposition in spruce sapwood degraded by brown rot fungi. *Org Geochem* **33**: 111–124.
- Gierer, J., Yang, E., and Reitberger, T. (1992) The reactions of hydroxyl radicals with aromatic rings in lignins, studied with creosol and 4-methylveratrol. *Holzforschung* **46**: 495–504.
- Gilbertson, R.L., and Ryvarden, L. (1986) *North American Polypores*. Oslo, Norway: Fungiflora.
- Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., et al. (1997) Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *J Biotechnol* **53**: 133–162.
- Hammel, K.E., Kapich, A.N., Jensen, K.A., and Ryan, Z.C. (2002) Reactive oxygen species as agents of wood decay by fungi. *Enzyme Microb Technol* **30**: 445–453.
- Irbe, I., Andersone, I., Andersons, B., and Chirkova, J. (2001) Use of ^{13}C NMR, sorption and chemical analyses for characteristics of brown-rotted Scots pine. *Int Biodeter Biodegr* **47**: 37–45.
- Jin, L., Schultz, T.P., and Nicholas, D.D. (1990) Structural characterization of brown-rotted lignin. *Holzforschung* **44**: 133–138.
- Kirk, T.K. (1975) Effects of a brown-rot fungus, *Lenzites trabea*, on lignin in spruce wood. *Holzforschung* **29**: 99–107.
- Kirk, T.K., and Farrell, R.L. (1987) Enzymatic combustion – the microbial degradation of lignin. *Annu Rev Microbiol* **41**: 465–505.
- Lu, F.C., and Ralph, J. (1997a) DFRC method for lignin analysis. 1. New method for β -aryl ether cleavage: lignin model studies. *J Agric Food Chem* **45**: 4655–4660.
- Lu, F.C., and Ralph, J. (1997b) Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers. *J Agric Food Chem* **45**: 2590–2592.
- Lu, F.C., and Ralph, J. (2003) Non-degradative dissolution and acetylation of ball-milled plant cell walls: high-resolution solution-state NMR. *Plant J* **35**: 535–544.
- Ralph, J., Akiyama, T., Kim, H., Lu, F.C., Schatz, P.F., Marita, J.M., et al. (2006) Effects of coumarate 3-hydroxylase down-regulation on lignin structure. *J Biol Chem* **281**: 8843–8853.
- Ralph, S.A., Ralph, J., and Landucci, L.L. (2004) *NMR Database of Lignin and Cell Wall Model Compounds*. [WWW document]. URL <http://ars.usda.gov/Services/docs.htm?docid=10491>.
- Shary, S., Ralph, S.A., and Hammel, K.E. (2007) New insights into the ligninolytic capability of a wood decay ascomycete. *Appl Environ Microbiol* **73**: 6691–6694.
- Suzuki, M.R., Hunt, C.G., Houtman, C.J., Dalebroux, Z.D., and Hammel, K.E. (2006) Fungal hydroquinones contribute to brown rot of wood. *Environ Microbiol* **8**: 2214–2223.
- TAPPI (1991) Acid-soluble lignin in wood and pulp. Useful method 250. In *TAPPI Useful Methods*. Atlanta, GA, USA: TAPPI (Technical Association of the Pulp and Paper Industry) Press.
- Tatsumi, K., and Terashima, N. (1985) Oxidative degradation of lignin. 7. Cleavage of the β -O-4 linkage of guaiacylglycerol- β -guaiacyl ether by hydroxyl radicals. *Mokuzai Gakkaishi* **31**: 316–317.
- Walling, C. (1975) Fenton's reagent revisited. *Acc Chem Res* **8**: 125–131.
- Worrall, J.J., Anagnost, S.E., and Zabel, R.A. (1997) Comparison of wood decay among diverse lignicolous fungi. *Mycologia* **89**: 199–219.
- Zabel, R.A., and Morrell, J.J. (1992) *Wood Microbiology: Decay and Its Prevention*. San Diego, CA, USA: Academic Press.
- Zakis, G.F. (1994) *Functional Analysis of Lignins and Their Derivatives*. Atlanta, GA, USA: TAPPI (Technical Association of the Pulp and Paper Industry) Press.