Are Lignins Optically Active?

John Ralph,*[,†,‡] Junpeng Peng,[†] Fachuang Lu,[†,‡] Ronald D. Hatfield,[†] and Richard F. Helm[§]

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin 53706, Department of Forestry, University of Wisconsin—Madison, Madison, Wisconsin 53706, and Fralin Biotechnology Center, Virginia Technical Institute, Blacksburg, Virginia 24061

The accepted derivation of lignins from non(enzymatically)-controlled radical coupling reactions has been recently challenged, and it is relevant to ascertain unequivocally whether lignins are or are not (as normally assumed) optically active. Two approaches were used. First, DFRC (derivatization followed by reductive cleavage) dimers derived from β-5- and β-β-units in pine lignins, which certainly retain unaltered chiral centers (as well as β-1- and β-O-4-units where the intactness may be debated), were shown to be optically inactive by circular dichroism (CD) and chiral high-performance liquid chromatography. CD of β-5-derived dimers following enantiomeric separation readily demonstrated the sensitivity of the method. Second, no optical activity could be detected (above 250 nm to avoid carbohydrate contributions) by CD of lignin isolates from pine, kenaf, maize, or a CAD-deficient pine mutant. Representative lignins are therefore not, within limits of detection by these methods, optically active.

Keywords: *Lignin; lignin dimer; DFRC; pine, kenaf; maize; CAD-deficient pine mutant; circular dichroism; optical activity; chirality; racemic*

INTRODUCTION

Lignins are phenolic polymers produced primarily from hydroxycinnamyl alcohols (monolignols) by radical coupling reactions that are generally considered to be independent of direct enzyme control (Harkin, 1967; Freudenberg and Neish, 1968). Freudenberg and Dietrich discovered that oxidase-mediated dehydrogenation of coniferyl alcohol produced dimers that were not optically active (Freudenberg and Dietrich, 1953). Lundquist examined aspects of lignins' optical activity in some of his early acidolysis papers (Lundquist, 1970, 1973, and references cited therein). Lignin-derived β-β-dimers released from lignins by acidolytic methods are racemic. For example, pinoresinol, isolated following methanolysis of (solvent-extracted) spruce wood, was racemic (Freudenberg et al., 1965), as was syringaresinol and its derived episyringaresinol isolated following acidolysis (Lundquist, 1973). Lundquist (K. Lundquist, Chalmers University, personal communication, 1999) found other released lignin dimers (e.g., divanillyltetrahydrofuran) without optical activity. It has been convincingly demonstrated that acidolytic conditions do not scramble the stereochemistry of the important β-carbons. For example, acidolysis of (+)-pinoresinol yielded (+)-pinoresinol and (+)-epipinoresinol (Lindberg, 1950; Lundquist, 1970), (−)-2,3-divanillyl-1,4-butanediol gave (−)-3,4-divanillyltetrahydrofuran (Lundquist, 1970), acid hydrolysis of (+)-syringaresinol gave (+)-syringaresinol and (+)-episyringaresinol (Freudenberg and Sidhu, 1961), and various acid treatments of syringaresinol glycosides release optically active phenolics (Dickey, 1958). The potential for polymerized lignans (monolignol dimers that are frequently optically active) to contaminate or contribute to lignins offers a pathway to partial optical activity (Lundquist, 1973). Freudenberg and Neish clearly distinguish resin components (such as hydroxymatairesinol) from lignin precursors or intermediates (Freudenberg and Neish, 1968). Today, lignins are generally concluded to be optically inactive, but evidence appears limited to the above-mentioned observations concerning β-β-units that arise from monomer—monomer coupling and are consequently only minor components. The assertion has not been rigorously proven for the polymer or for lignins' major units.

Recently, Brunow's group showed that enantiomerically enriched dimers can result from radical coupling of chiral conjugates of lignin-related monomers (followed by removal of the conjugate), using otherwise normal peroxidase/H_2O_2 conditions (Bolzacchini et al., 1998). Such an observation opens up new possibilities for enantiomerically enriched products from radical coupling reactions in the cell wall, using chiral carbohydrate or uronic acid auxiliaries, for example (Helm et al., 1997). Lignans, dimers of monolignols, are also produced by many plants and are often in optically active forms. For example, the lignan pinoresinol and its derived matairesinol, were found only as their (−)-isomers in suspension-cultured *Pinus taeda* (Eberhardt et al., 1993).

Recent isolation of a "dirigent" protein from forsythia, which facilitates coniferyl alcohol radical coupling to pinoresinol in a regio- and stereoselective manner, has led the discoverers to extrapolate their lignan observations to lignins (Davin et al., 1997). In a newly proposed paradigm (Lewis and Davin, 1998), as we interpret it, lignins form from template arrays of dirigent proteins

* Address correspondence to this author at the U.S. Dairy Forage Research Center, USDA—ARS, 1925 Linden Dr. W., Madison, WI 53706-1108 [telephone (608) 264-5407; fax (608) 264-5147l e-mail jralph@facstaff.wisc.edu].

† U.S. Dairy Forage Research Center.

‡ Department of Forestry.

§ Fralin Biotechnology Center.

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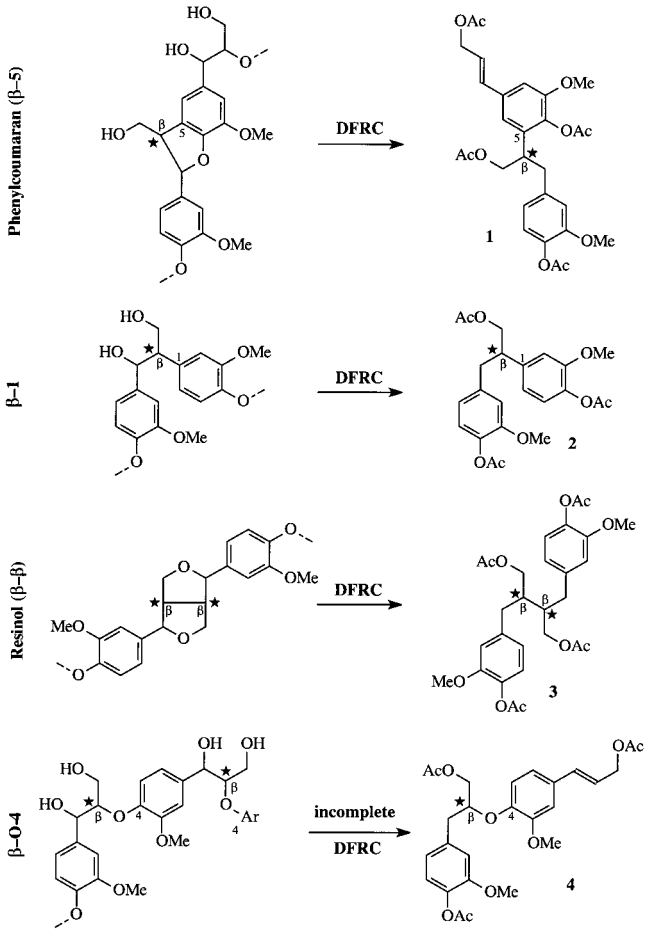

Figure 1. Dimeric DFRC products from various structural units in softwood lignins. Optical centers at β-carbons formed by radical coupling reactions during lignification are denoted with a ★. The β-O-4-dimer **4** is produced only in small amounts from incomplete ether cleavage.

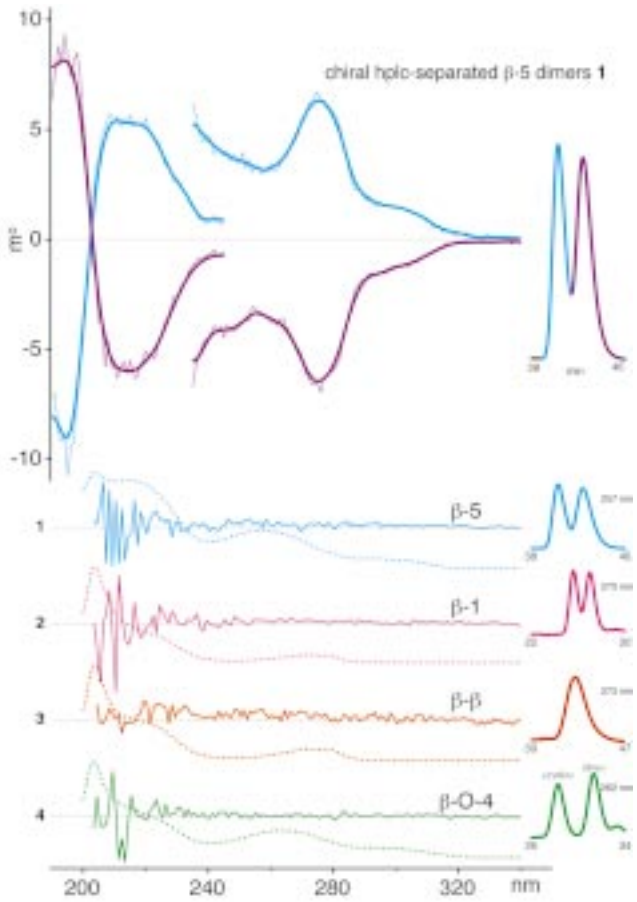

Figure 2. CD spectra of isolated DFRC dimers **1**−**4** along with the chiral-HPLC-separated β-5-dimer **1**. UV spectra are shown with dashed lines; the CD spectra are more noisy at high UV absorptions, but the CD rotations are also strongest in the region of strong UV absorption. Chiral column HPLC traces are shown to the right of the figure; the β-β-enantiomers **3** were not resolved, nor were the β-O-4-enantiomers **4**, although the *threo*- and *erythro*- (*syn*- and *anti*-) diastereomers were. The resolved β-5-enantiomers **1** showed strong CD spectra; the region from 190 to 245 nm was run using one-fourth the concentration used for the 240−350 nm region.

and are synthesized with exquisite structural control. With the possibility that the synthesized lignin chain then structurally dictates the next chain by a template-polymerization process (Sarkanen, 1998), the paradigm has the apparent necessary ingredients to bring lignin more into line with other carefully synthesized biological polymers such as cellulose and proteins. The idea is not without significant obstacles, some of which are alluded to below, and is currently without structural, biochemical, or enzymatic evidence, but it has sparked valuable debate. Although the paradigm may not require the resultant lignin to be optically active, the finding of chiral lignin would significantly strengthen the idea.

The question of lignin optical activity needs to be addressed. This paper describes a two-pronged approach to resolve the issue as unequivocally as possible for various lignins.

EXPERIMENTAL PROCEDURES

DFRC Dimers. DFRC (derivatization followed by reductive cleavage) dimers **1**−**4** (Figure 1) were isolated from pine clear sapwood as previously described (Peng et al., 1998) and repurified by HPLC (using system I, see HPLC Conditions). The compounds, ∼0.2 mg, were each dissolved in 1 mL of acetonitrile and dilutions performed until satisfactory circular dichroism (CD) spectra could be obtained without exceeding a dynode voltage of 600 V on the CD spectrometer. Concentrations for **1**−**4** used for the spectra in Figure 2 were ∼40−50 μg/mL. Chiral separation of 1.5 mg of **1**, from DFRC dimers isolated from pine (Peng et al., 1998), using system II (see below), provided the two enantiomers. Their CD spectra, Figures 2 and 3, were from 130 μg/mL for the 230−350 nm range 30 μg/mL for the 190−245 range (Figure 2).

CD. CD spectra were run on an AVIV model 62A-DS CD spectrometer (Lakewood, NJ) driven by an Apple Macintosh computer running IGOR-Pro 3 software (Wavemetrics, Lake Oswego, OR). The lamp current was set at 26 A; temperature was 25 °C; all conditions were standard. For lignin dimers, the solvent was acetonitrile (HPLC grade, Baker); for isolated lignins, the solvent was 10:3 acetonitrile/water. Sample concentrations were adjusted so that optical densities measured by the dynode were just below the maximum of 600 V at maximal absorptions in the spectral range. Spectra were typically obtained using 10 s averaging in 1 nm steps and were baseline-subtracted (using scans of the blank solvents). Smoothed and raw spectra are shown in Figures 2 and 3.

Isolated Lignins. Pine (Ralph et al., 1997), kenaf (Ralph, 1996), maize (Ralph et al., 1994), and mutant pine (Ralph et al., 1997) lignins were isolated as previously described. Briefly, samples of *Pinus taeda* clear sapwood, *Tainung* kenaf bast fibers, mature *Zea mays* stems, and a 12-year-old CAD-deficient *P. taeda* mutant (containing knotty regions) (as well as another control pine of the same age from the same area—data not shown) were ground in a Wiley mill to pass through a 1 mm mesh screen. The ground materials were exhaustively Soxhlet-extracted with water, methanol, acetone, and chloro-

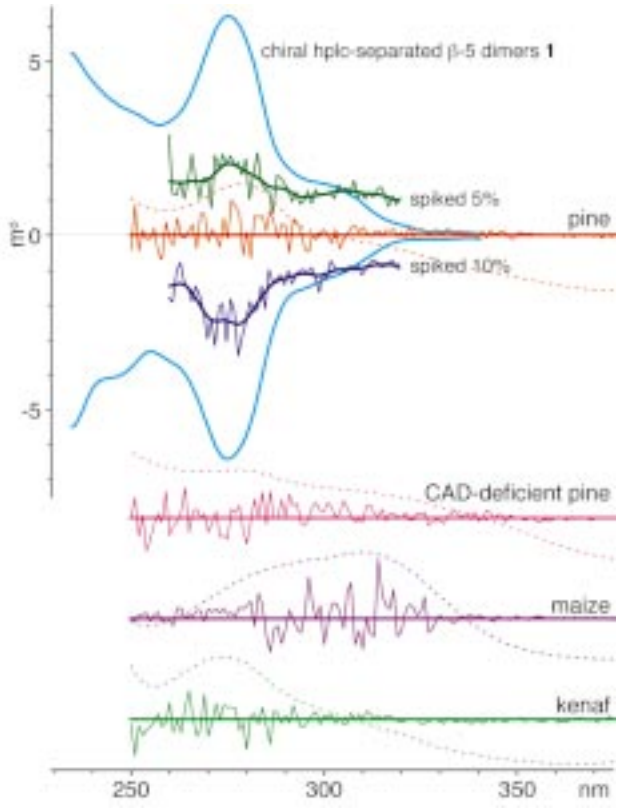

Figure 3. CD spectra of various lignin isolates, illustrating no detectable optical activity. CD spectra of the separated β-5-enantiomers **1** are shown again for comparison. To test the sensitivity of detection, a resolved enantiomer of **1** was spiked into the pine lignin sample at 5 and 10% levels; the raw data and the curve smoothed by the CD software indicate that a 5% component is reasonably easily detected (although the required level may be higher in the complex lignin sample where various units might have opposing contributions).

form or diethyl ether. The dried cell wall residues were then ball-milled for 1.5 h (three 0.5 h intervals separated by 0.5 h) using a vibratory ball mill. For the kenaf and the maize samples, excess polysaccharides were degraded using crude cellulases (Obst and Kirk, 1988; Ralph et al., 1994; Ralph, 1996). The finely divided cell wall materials were then extracted twice with 96:4 dioxane/water (Björkman, 1956). After lyophilization, the crude lignin powders were washed with distilled water and again lyophilized. Lignins were not further purified. The final yields on lignins (based on original Klason lignin) were pine, 15%; kenaf, 26%; maize, 67%; and CAD-deficient pine, 17% (and normal control pine, 12.5%). The samples contained some carbohydrates and uronics but were >90% lignin in each case. Samples, 1.6−5.5 mg, were dissolved in 0.26 mL of 10:3 acetonitrile/water and diluted again for CD. Approximate lignin concentrations used for the 250−345 nm spectra of Figure 3 were pine, 2.0 mg/mL; CAD-deficient pine mutant, 1.7 mg/mL; maize, 0.9 mg/mL; and kenaf, 2.8 mg/mL.

HPLC Conditions. HPLC was carried out on a Shimadzu 10A HPLC equipped with a diode array detector (detector range set at 190−350 nm), an autosampler, and a fraction collector. Two separation systems were used. System I used a Supelco (Bellefonte, PA) reverse-phase Supelcosil SPLC-8 column (5 μm, 250 mm × 10.0 mm i.d.): mobile phase, an isocratic elution with acetonitrile/water/THF (45:50:3); flow rate, 1.6 mL/min. System II used a Chiralcel-OD chiral column (250 mm × 4.6 mm i.d., Daicel Chemical Industries, Exton, PA): mobile phase, a gradient elution with hexane (solvent A) and 2-propanol (solvent B); flow rate, 1.0 mL/min. Initial conditions were 90% A and 10% B, which were held for 8 min followed by an increase to 20% B over 2 min, a 10 min hold, an increase to 35% B in 12 min, an 8 min hold, and then a return to initial conditions in 3 min and a 7 min hold before the next injection—total run time of 50 min per injection.

Proof That Phenylcoumarans Survive DFRC with Their β-Carbons Unscrambled. The two enantiomers of the phenylcoumaran model **5** were separated by preparative chiral HPLC (Figure 4). The fastest-eluting enantiomer [it is not known whether this was the (*R*)- or the (*S*)-isomer] of **5** (2.3 mg) in CH_2Cl_2 (5 mL) was brominated (Br_2, 50 mg; CH_2Cl_2, 0.1 mL) for 3 min to saturate the double bond and produce the 1,2-dibromo derivative **6**, which would subsequently regenerate the double bond during the reductive elimination step of the DFRC procedure (Lu and Ralph, 1997a,b). After evaporation of the solvent under reduced pressure, the brominated compound **6** was dissolved in 10% (v/v) AcBr in acetic acid solution. 2-Propanol (0.08 mL) was added to produce some HBr required for effective reaction. (HBr is required for effective reaction—fully acetylated models cannot generate any, whereas hydroxyl groups present in unacetylated models, isolated lignins, or whole cell wall samples generate sufficient HBr for reaction.) This mixture was allowed to stand for 16 h and the solvent evaporated at 45 °C under reduced pressure. The residue was dissolved in dioxane/acetic acid/water (5:4:1, 3 mL) and treated with powdered Zn (50 mg) for 30 min. Extraction with CH_2Cl_2 produced crude product (2.2 mg), which was acetylated. The desired product **1** was isolated as the major product by HPLC, 0.9 mg, ~38%. This product had GC retention time and NMR and mass spectra identical with those of the racemic dimer **1** isolated from pine wood following DFRC but gave only a single peak, the later eluting peak (Figure 4) on chiral HPLC (Chiralcel-OD, system II, see HPLC Conditions). When racemic **5** was subjected to the same bromination and DFRC reactions, chiral HPLC this time produced two peaks as expected. Because a single enantiomer of the phenylcoumaran **5** produced a single enantiomer of the product **1**, it is clear that the optical center (the β-carbon) retained in the product is unchanged from its state in the original phenylcoumaran, that is, that no scrambling had occurred.

RESULTS AND DISCUSSION

Of the various methods for determining optical activity, CD (Kagan, 1977) was chosen for its sensitivity and because the association of CD phenomena only with UV-active absorptions allows optical activity in lignins to be determined even in the presence of contaminating (optically active) polysaccharides.

Lignin DFRC Dimers. Dimeric lignin fragments were isolated from pine wood following DFRC as described previously (Peng et al., 1998) and repurified by reverse-phase HPLC (system I, see Experimental Procedures). As seen in Figure 1, the bonds originally formed in the radical coupling step remain unaltered from their native states following this degradation. Regardless of what non-stereospecific chemical reactions have occurred at other sites, the compounds retain chirality at the sites of original coupling (as is verified for phenylcoumarans, below). Determination of chirality in these compounds therefore reflects chirality in the original lignin. This is particularly clear-cut with β-5- and β-β-compounds (where the crucial bonds do not cleave) but less so for β-1's (where there are uncertainties regarding how they arise) (Ralph et al., 1998b; Setälä et al., 1999) and, perhaps, β-O-4's (where it is less clear that the β-O-4 bond has not been broken and re-formed in the production of the fragment dimers).

Figure 2 shows CD spectra of β-5-, β-1-, β-β-, and β-O-4-dimers **1**−**4** isolated from pine lignin following DFRC, along with spectra from both enantiomers of the β-5-dimers **1** (which were separated by chiral HPLC). The spectra are somewhat noisy, particularly near 200 nm,

where the absorption is high and the total optical density of the sample limits the sample concentration that can be used for CD. However, high CD rotations are expected at high absorption wavelengths, so the sensitivity is ample. This may be seen from the 190−245 nm scans of the β-5-enantiomers *(R)*- and *(S)*-**1**, where the concentrations were reduced 4-fold and still produced pronounced CD rotations. At 280 nm, a maximum in lignin UV spectra, the noise level is sufficiently low to allow relatively sensitive determination of optical activity. Note that the CD spectra shown here are for the entire wavelength range where the sample concentration was chosen to allow the recording of spectra down to 210 nm. Subspectra from more concentrated samples were also run at higher wavelengths to improve the capability of determining activity throughout the UV spectrum (not shown). No hint of such activity was found.

Of the various linkage types, β-β-units are perhaps most likely to show optical activity. The (β-β) lignans pinoresinol and matairesinol from *P. taeda* are reported to be optically active (Eberhardt et al., 1993). There is the possibility that even carefully pre-extracted wood meal contains lignans or polymerized lignans (Sarkanen and Hergert, 1971; Hergert, 1977). One group of researchers has claimed that our observations on lignins from *P. taeda* and a CAD-deficient mutant (Ralph et al., 1997) are almost wholly due to lignan-derived components (Gang et al., 1998). The lack of any optical activity in the β-β-dimer, which was obtained from whole wood, appears to provide evidence in addition to that already at hand (Sederoff et al., 1999) that the dimers arise from true racemic lignin, rather than some poly-lignan (that would likely be optically active). Alternatively, they would have to derive from some other (nonoptically active) lignan pool. Similar conclusions arise from examining CD spectra of isolated lignins (see below).

Proof That Optical Centers Are Not Scrambled during DFRC. One of the assumptions in the above analysis of DFRC dimers is that the DFRC process itself does not scramble the optical centers that were created during the radical coupling processes of lignification. The most likely to scramble are the β-5 (phenylcoumaran) moieties, because they have the potential to reversibly eliminate HBr, producing stilbenes (Figure 4). However, stilbenes **8** produce their own diagnostic DFRC products, and significant products from HBr (re-)addition (to **7**) have not been observed in our laboratory (F. Lu, unpublished data).

A starting model compound that could be easily separated into its component enantiomers was required. The β-5-dimer **5** was a logical choice. However, it was also advantageous to produce the same product **1** as is released from lignins following DFRC; optical isomers of this compound were readily separated as described above. In lignin reactions, the side-chain double bond in **1** arises during the reductive elimination from a 2-bromo ether. This reaction pathway was not feasible to fully model; the required trimeric β-5/β-O-4 model compound has too many isomers to allow ready separation of a single enantiomer. It was recognized that the reductive elimination could be effectively simulated by simply brominating the dimeric model **5**. The phenylcoumaran moiety would then react under DFRC conditions as would such units in lignin, whereas the 1,2-dibromide would generate the requisite double bond in the Zn reductive elimination step, producing the same compound **1** as formed from lignins. Incidentally, DFRC dimer **1** has been shown to arise from the free phenolic β-5-units in lignins; etherified units produce different products (F. Lu and J. Peng, unpublished results).

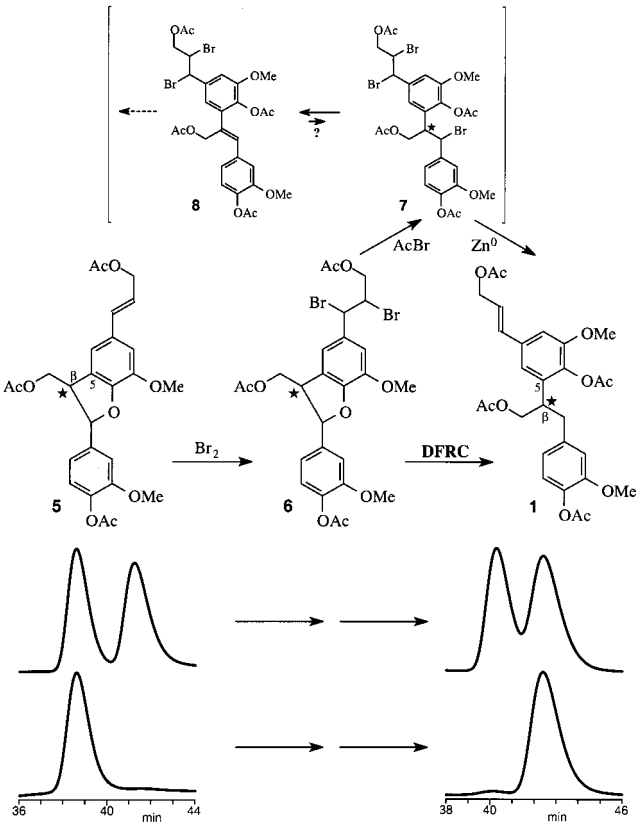

Figure 4. Proof that the DFRC method does not scramble the crucial β-carbon stereochemistry in β-5-units. Dimeric model **5** was first brominated so that the unsaturated side chain would be generated, giving the same β-5-product **1** as isolated from DFRC of lignin. The potential for scrambling the center by HBr elimination from the bromo intermediate **7** is shown but, as noted in the text, is not anticipated. Chiral HPLC (bottom traces) indicates that a single enantiomer of **5** produces a single enantiomer of **1** following DFRC, proving that the optical center is not scrambled.

When racemic **5** was taken through the bromination and DFRC steps (Lu and Ralph, 1997a,b), product **1** was indeed the major product. Chiral column HPLC showed that the two enantiomers of **5** produced two enantiomers of **1** in equal amounts (Figure 4) as expected. When the separated faster-eluting enantiomer of **5** was treated the same way, only the slower-eluting enantiomer of **1** resulted. Clearly, the optical center had been retained throughout the process; insignificant scrambling had occurred. Therefore, the assumption made above that the bond formed during the radical coupling step of lignification remained intact is valid. This is logically also true for the chiral centers in β-β-units but, as noted above, less clear for β-1-units, because their origin in lignins is still unclear, and perhaps β-O-4-units. Because β-O-4-units are almost completely cleaved during DFRC and because the optical isomers of products **4** were not resolved by our HPLC conditions, their optical activity retention through DFRC was not examined. Nevertheless, it is clear from at least the β-β- and β-5-dimers that no optical activity is present in these lignin units in the *P. taeda* examined.

Isolated Lignins. A valuable and exploitable feature of CD spectra is that CD is associated only with UV bands (Kagan, 1977). Unlike with other optical measurements (direct optical rotation or optical rotatory dispersion), CD spectra can therefore be run on samples containing chiral impurities, providing the UV spectrum of the component of interest has diagnostic absorptions well separated from those of the chiral contaminants. Isolated lignins always contain contaminating carbohydrates, which are optically active. However, the UV spectra of carbohydrates do not extend much above 200 nm. A major lignin peak of interest is at 280 nm, with shoulders out at around 300+ nm. Acetylated lignins were dismissed from consideration as acetylated carbohydrate absorptions can reach up to 250 nm. The range 250−375 nm was sufficiently diagnostic for underivatized lignins while devoid of carbohydrate interference.

Isolated lignins were dissolved in 10:3 acetonitrile/water, a particularly good solvent for UV studies. CD spectra of maize, kenaf, pine, and CAD-deficient mutant pine lignins all showed no detectable optical activity (Figure 3). Improved signal-to-noise ratio was obtained by longer averaging in noisy subregions (not shown).

An indication of the level of a chiral component required to observe optical activity can be gained from spiking studies. Figure 3 shows CD spectra from pine lignin with ~5 and ~10% levels of an isolated enantiomer of the β-5 dimer **1** added. The optical activity can be readily seen at the 5% level. Admittedly, with several potential chiral centers in lignin, and the possibility of non-reinforcing optical CD spectra, the ability to determine chirality at levels as low as 5% may still be difficult under our experimental conditions. With unlimited access to long-term-averaging, improved signal-to-noise ratios should allow an improvement of these determinations.

The maize lignin has high UV absorption centered at 310 nm, resulting in considerable noise in the CD spectrum in that region. The aldehyde-rich CAD-deficient pine lignin also has absorptions extending out to ~350 nm. The (normal) pine and kenaf lignins have the more normal distinct 280 maximum. The CAD-deficient pine mutant (Ralph et al., 1997) was of particular interest because it has been suggested that this isolated lignin is merely a polymerized lignan artifact (Gang et al., 1998), an issue that we address more fully elsewhere (Sederoff et al., 1999). If this were so, the mutant's lignans must be (unexpectedly?) optically inactive because the isolated lignin/poly-lignan contains no hint of optical activity.

Implications for Lignification. As suspected by Freudenberg and others many years ago, lignin appears to be racemic, fully in accord with its generation from radical coupling reactions that are without direct enzymatic control. We have seen no experimental data that require a precisely controlled synthesis of structurally defined lignin, that is, data that cannot be supported by simply recognizing that the plant does exquisitely, temporally and spatially, control the supply of monolignols, oxidizing enzymes, and oxidative species (H_2O_2 for example). The matrix environment of the polymerization affects the interunit linkage composition, as has been well demonstrated in synthetic lignification experiments (Brunow et al., 1993; Terashima et al., 1996). The regulated differences in lignification in various cells and various regions of the cell, in wounding or in stress, demand nothing more than changes in monomer supply (Sederoff et al., 1999).

Producing a variety of structures/stereochemistries is an asset in plant defense responses (Denton, 1998). Unrestricted free radical coupling maximizes the structural diversity while possibly optimizing the functionality of the lignin macromolecule. In light of the above, the recently advocated bold new paradigm (Lewis and Davin, 1998) for absolute structural and stereochemical control of lignification may be an overextrapolation of the researchers' lignan findings (Davin et al., 1997). The proposal cites two convoluted explanations for the "perceived lack of optical activity of lignins" (Lewis and Davin, 1998). One explanation, that two types of complementary proteins encode opposite optical isomers, requires the plant to adopt an energetically excessive mode of creating an optically active lignin polymer only to carefully negate that structural feature via a complementary set of proteins for which additional complementary biochemical pathways must also be supported. This effort is to produce two complementary lignin polymers when each has identical physical properties, identical to those of the racemic mixture. The template argument, by which replication of the lignin chain forms complementary mirror images, has less serious detractions, although no evidence of any structural replication ability has yet appeared. Because lignins are found intimately associated with hemicelluloses, it is not clear how discontinuities in the alignment of one lignin chain might contribute to excess optical activity (of the incompletely replicated section). It appears to us that nature has already solved the problem more elegantly by using "uncontrolled" radical coupling reactions. Until experimental evidence is provided, the new (anti-Ockham's razor) paradigm for lignification is an interesting discourse currently without compositional, structural, or enzymatic evidence.

Conclusions. Our inability to detect optical activity in lignins by two approaches, as presented here, seems to strengthen the view that lignin is indeed synthesized by the plant without direct (regio- or) stereocontrol over the exact course of radical coupling events. A plant's ability to produce a cell wall polymer with appropriate properties for water transport, defense, and other tasks, with a compositional (and structural) flexibility that is determined by monomer supply, is presumably well suited to surviving environmental, gravitropic, and biological stresses. Indeed, the recently noted abilities of plants to remain viable by circumventing biogenetic obstacles placed upon their lignin biosynthetic pathways, in a single generation, without the benefit of evolution, appears to be an endorsement of their flexible strategy (Ralph et al., 1997, 1998a; Boudet, 1998; Sederoff et al., 1999).

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