The DFRC Method for Lignin Analysis. 4. Lignin Dimers Isolated from DFRC-Degraded Loblolly Pine Wood

Junpeng Peng, Fachuang Lu, and John Ralph*

U.S. Dairy Forage Research Center, U.S. Department of Agriculture—Agricultural Research Service, and Department of Forestry, University of Wisconsin—Madison, Madison, Wisconsin 53706

Sixteen lignin dimers were directly isolated by gel permeation and reversed-phase TLC and HPLC from loblolly pine (*Pinus taeda* L.) sapwood following large-scale application of the new derivatization followed by reductive cleavage (DFRC) procedure. Their structures were elucidated by GC/MS and NMR. These dimers included representatives from all of the common interunit linkages in softwood lignins (*β*-1, *β*-*β*, 5-5, *β*-5, and 5-O-4). The DFRC procedure efficiently cleaves *β*-aryl ethers, the major structural unit, so *β*-ethers were not found among the dimeric products. All but two of the isolated dimers were guaiacyl—guaiacyl (from coniferyl alcohol-derived units in lignin); small amounts of *p*-hydroxyphenyl/guaiacyl *β*-5 and *β*-1 dimers were characterized. Several dimers have benzaldehydes as one unit—since aldehydes are not created by the procedure, they are either present in the native lignin or are formed during the minimal preparation of the wood. The dimers isolated and identified here will eventually allow their quantitation to aid in the structural analyses of wide-ranging plant materials.

Keywords: *Lignin; lignin dimer; structural elucidation; DFRC degradation; loblolly pine wood; Pinus taeda L.; acetyl bromide; NMR*

INTRODUCTION

A method was recently introduced for cleaving α- and *β*-ethers in lignin by **d**erivatization **f**ollowed by **r**eductive **c**leavage—the DFRC method (Lu and Ralph, 1997a,b, 1998a,b). Previous papers reported on the quantification of released primary monomers (4-acetoxycinnamyl acetates) and the identification of minor monomers from degradation of isolated lignins. The data provided are analogous to those provided by analytical thioacidolysis (Rolando et al., 1992; Lapierre, 1993); both methods provide information regarding releasable ether units in lignins. Here we apply the DFRC method to loblolly pine (*Pinus taeda*) sapwood to obtain further information regarding the non-ether interunitary linkages, as is done by analyzing dimers in the extended thioacidolysis method. Determining the products in this way highlights where more mechanistic and model studies are required to understand the reactions of non-*β*-ether units. This paper presents the structural elucidation of many of the significant dimers released from a large-scale treatment of pre-extracted pine sapwood. Separation of pure components also makes them available for response factor determination for subsequent quantification of these dimers, although some may be independently synthesized in larger quantities. Analytical methodologies and structural information described here that define aspects of structure and regiochemistry are valuable aids in understanding lignin biosynthesis as well as degradation pathways.

* 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) 264-5147; e-mail jralph@facstaff.wisc.edu].

EXPERIMENTAL PROCEDURES

General. Acetonitrile (J. T. Baker, Phillipsburg, NJ) was of HPLC grade; other solvents were of AR grade. Methylene chloride and acetone were from Baker, and pyridine and acetic acid were from Mallinckrodt (Paris, KY). Reagents (AcBr, acetic anhydride, zinc) were from Aldrich (Milwaukee, WI) and were used as supplied.

Gel Permeation Chromatography. Bio-Beads S-X1 (280 g, 1% cross-linked, for 400—14 000 Da molecules, Bio-Rad Laboratories, Richmond, CA) swelled in methylene chloride (2.2 L) at room temperature for 4 h were used to pack a column. The column bed volume was ~1.8 L and measured 85 cm × 5.2 cm i.d. The beads were conditioned by eluting with 2.8 L of CH_2Cl_2. The column was connected to a UA-6 UV—vis detector with a variable path length cell set to its minimum (0.25 mm) and a Foxy 200 collector (Isco, Inc., Lincoln, NE). Gel permeation of the degraded mixture was carried out on this column: solvent, methylene chloride; detector, 280 nm; collector, 15 mL/tube (~3 min/tube).

Preparative TLC separations were made on reversed-phase (RP) DC-Fertigplatten RP-18W/UV_{254} plates with either 0.25 or 1.0 mm thickness (Macherey-Nagel Products, Germany).

Preparative HPLC chromatography was carried out on a Gilson 714 HPLC equipped with a UV detector (280 nm), using a Supelco (Bellefonte, PA) reversed-phase LC-8 column (5 *μ*m, 25.0 cm × 10.0 mm i.d.): mobile phase, acetonitrile/water; flow rate, 1.6 mL/min.

GC was run on a Hewlett-Packard (Atlanta, GA) 5980 gas chromatograph: column, 0.20 *μ*m film, 0.2 mm × 30 m SPB-5 (Supelco); He carrier gas, 1 mL/min; 30:1 split ratio; injector, 220 °C; initial column temperature 150 °C, ramped at 10 °C/min to 310 °C, hold for 10 min; total running time, 34 min. Mass spectra (EI, 70 eV) were collected on a Hewlett-Packard 5970 MS detector connected directly to a similar column in the same GC.

^1H and ^{13}C NMR, along with 2D NMR (^1H—^1H COSY and NOESY and ^{13}C—^1H HMQC and HMBC) spectra were taken on a Bruker AMX-360 instrument fitted with a 5 mm probe with normal geometry (proton coils furthest from the sample).

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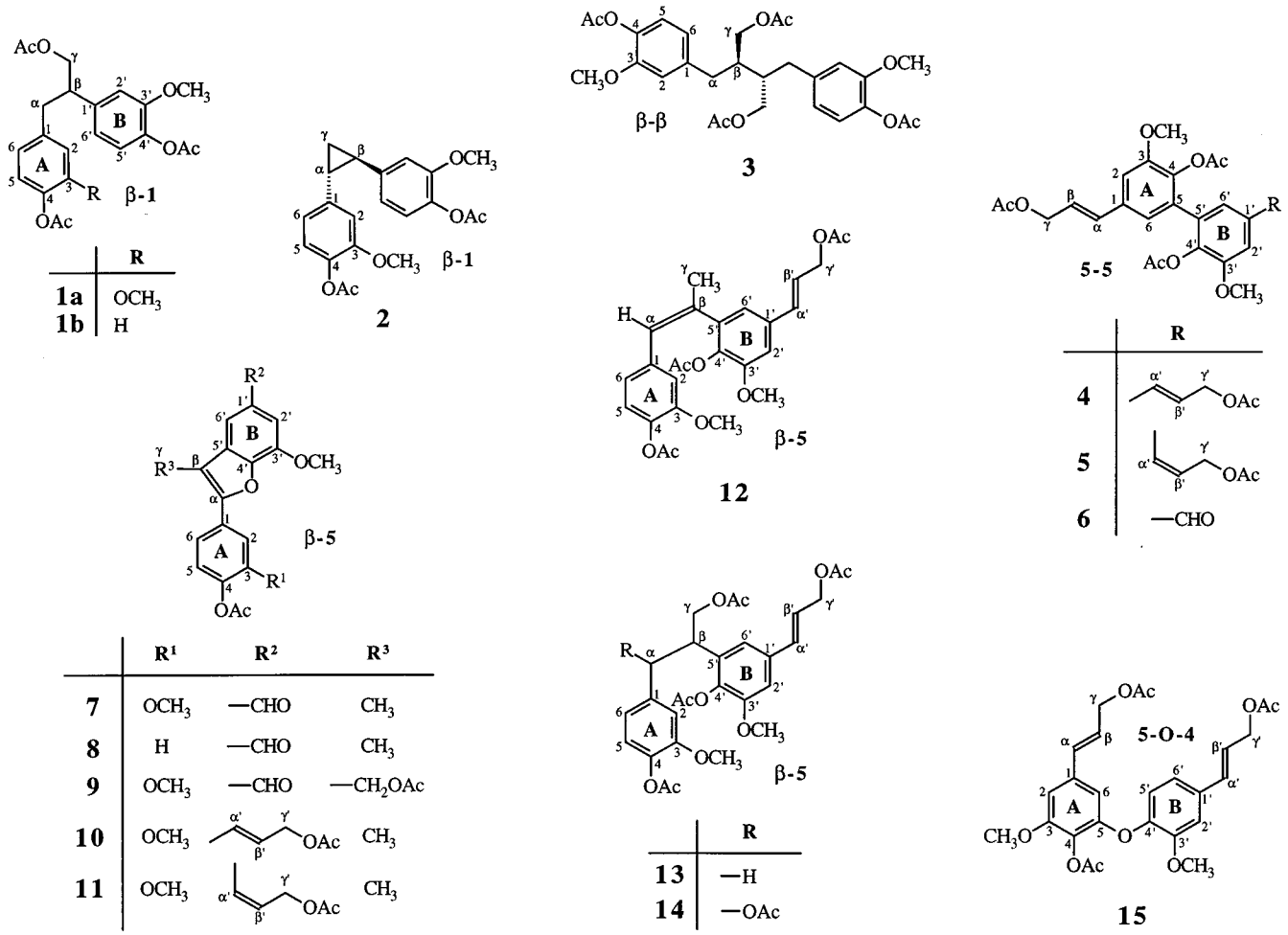


Figure 1. Structures of lignin dimeric products isolated from DFRC-degraded loblolly pine wood.

The standard conditions used for all samples were 0.1−10 mg of material in 400 μL of acetone-d_6, with the central solvent peak as internal reference (δ_H 2.04, δ_C 29.80). The carbon/proton designations are based on the standard lignin numbering system (Figure 1).

Large-Scale DFRC Treatment of Ground Pine Wood. Loblolly pine (*Pinus taeda* L.) sapwood ground to pass a 0.5 mm screen in a Wiley mill was pre-extracted with (1) acetone/water, (2) warm water, and (3) 80% EtOH and dried. The extracted milled wood (40.42 g) was treated by a scaled-up DFRC method (Lu and Ralph, 1997a) in two equal batches. Thus, ~20 g of wood was treated with 450 mL of 4:1 AcBr/HOAc for 2.5 h at 50 °C. During this time, the wood became completely solubilized. Rotary evaporation (45 °C, 20 min) removed the reagents and solvents to produce a thick syrup. This was dissolved in dioxane/HOAc/H_2O (5:4:1, 300 mL), and Zn dust (16 g) was added. After stirring for 30 min, the product was extracted with CH_2Cl_2 (200 mL × 3). Acetylation with Ac_2O/pyridine (1:1, 80 mL), followed by evaporation of the solvents under reduced pressure (aided by additions of EtOH), produced 31.56 g of degraded wood products from the two batches. Obviously, the products were largely polysaccharide-derived.

Separation and Purification of Degraded Lignin Dimers. As shown in Figure 2, the degraded wood mixture was subjected to Bio-Beads gel permeation chromatography, subsequent RP-TLC, and preparative RP-HPLC for isolation of lignin dimers.

(1) Separation of the Total Degraded Wood by Bio-Beads Gel Permeation Chromatography. Fractions (2 g) of the acetylated degraded wood mixture (31.56 g) in methylene chloride were repeatedly loaded to the Bio-Beads S-X1 column (16 times). Eluates were combined into 7 fractions based on the 7 peaks in UV chromatograms (Figure 3). Peak 7 (3.20 g) contained the major monomeric lignin acetates. Peaks 5 (3.63 g) and 6 (2.88 g) were shown to be the dimeric lignin acetates by GC/MS. Peak 5 (3.63 g) was rechromatographed on the Bio-Beads column (in two separate batches). Each tube was monitored by GC/MS and combined carefully, yielding four fractions (fractions 5.1− 5.4). Fraction 5.3 (1.12 g) and fraction 5.4 (260 mg) proved to be the major dimers (β-β, β-5, 5-5, and 5-O-4) with minor β-1 components. Peak 6 (2.88 g) was also rechromatographed on the Bio-Beads column and gave five fractions (fractions 6.1−6.5). Fraction 6.3 (1.48 g) was primarily β-1 dimers.

(2) Separation of Major Dimers by Reversed-Phase TLC. Reversed-phase TLC plates were used to try to remove the overwhelming carbohydrates and monomeric lignins. The dimer fractions (fractions 5.3, 5.4, and 6.3) were loaded onto the plates (50−120 mg/plate for 0.25 mm thickness and 150−200 mg/plate for 1.00 mm thickness). They were developed with methanol/H_2O (5:4) three times. Twenty-eight plates of 0.25 mm thickness and 11 of 1.00 mm thickness were used for the separation of the total dimer fractions. Seven major bands were visualized (254 nm UV) on the plates (from top to bottom, bands 1−7). Combination of the bands on the different plates was made according to their GC/MS profiles. The silica gel support region between bands 2 and 3 was named Si. 2 (65.6 mg) and contained a major β-1 group. The silica gel region between band 7 and the origin was named Si. 7 (21.3 mg) (Figure 2).

(3) Final Purification of Major Dimers by Preparative HPLC. **1a** (0.6 mg), with a trace of **1b**, was purified from a part (2.8 mg) of Si. 2 (total 65.6 mg) by HPLC; part (46 mg) of band 4 (total 152.8 mg) was separated by HPLC to give **3** (6.7 mg) and dominant β-5's **13** (13 mg) and **14** (3 mg)—the latter was a furan ring-opened α-acetoxy compound; larger amounts (24.5 mg) of this compound were also obtained by reloading band 3

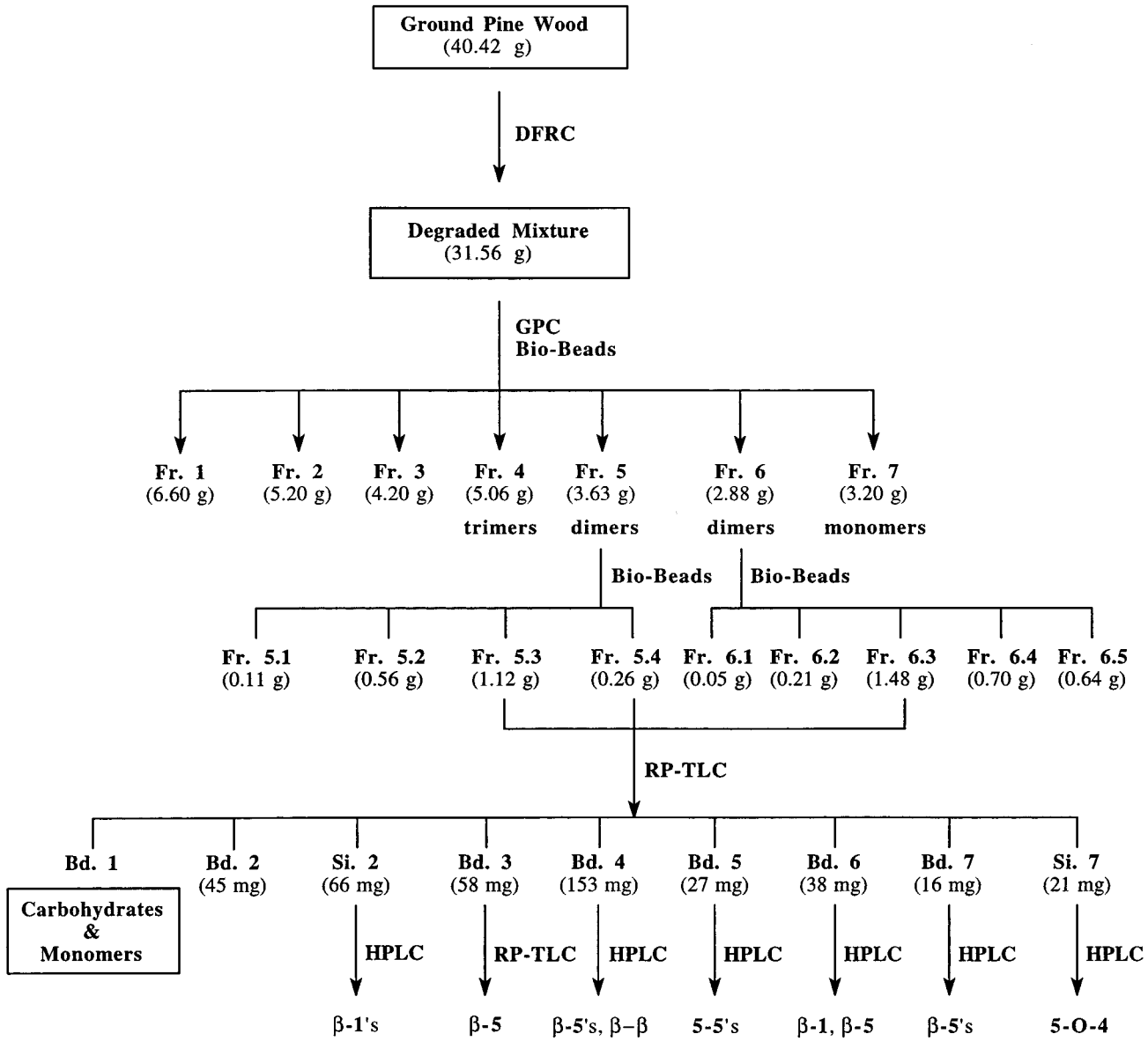


Figure 2. Separation scheme for lignin dimeric products released from DFRC-degraded loblolly pine wood.

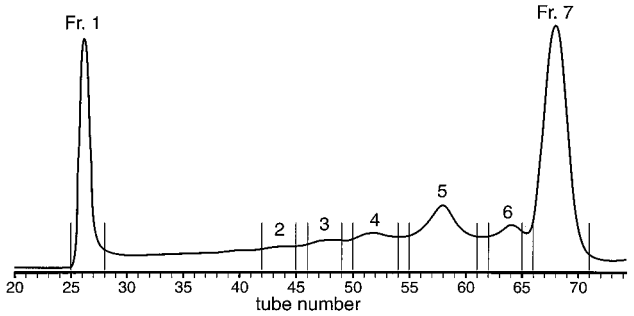


Figure 3. Gel permeation chromatogram of the crude DFRC-degraded products from loblolly pine wood, on Bio-Beads S-X1 (peak 1, lignin polymeric products that were not retained on Bio-Beads, MWs >14 000; peak 4, trimers; peak 5, dimers; peak 6, β-1 dimers; peak 7, monomers).

(58 mg) onto reversed-phase TLC (Figure 2). Band 5 (27.2 mg) gave **4** (13.2 mg), **5** (3.5 mg), and **6** (1.0 mg); 26.2 mg of band 6 (37.6 mg) yielded **2** (1.8 mg) and **12** (0.9 mg); a part (9.5 mg) of band 7 (16.4 mg) gave **7** (1.6 mg), **8** (0.1 mg), **9** (0.2 mg), **10** (0.9 mg), and **11** (0.1 mg); and Si. 7 (21.3 mg) afforded **15** (4.2 mg) by HPLC purification.

Spectra. The ^{1}H and ^{13}C NMR data of compounds **1**−**15**, assigned and authenticated via COSY, NOESY, HMQC, and HMBC spectral analyses, are given in Tables 1−3.

Mass spectra of acetylated dimers are quite diagnostic. Generally, aromatic acetates lose ketene (*m* 42), aliphatic side chain γ-acetates lose HOAc (*m* 60) via a McLafferty rearrangement, and α-CH_2 structures cleave to their corresponding benzylic cations, *m*/*z* 107 (for *p*-coumaryl) and 137 (for guaiacyl), while α-acetoxy guaiacyl compounds give the corresponding cation at *m*/*z* 153. The GC retention times and MS data of compounds **1**−**15** are listed in Table 4.

RESULTS AND DISCUSSION

The DFRC-degraded sapwood mixture of loblolly pine (*P. taeda* L.) was subjected to gel permeation on Bio-Beads, preparative RP-TLC, and finally preparative-scale HPLC. This led to the separation of lignin dimers **1**−**15**. These compounds represent the major structural types in softwood lignins, including β-1 (**1**, **2**), β-β (**3**), 5-5 (**4**−**6**), β-5 (**7**−**14**), and 5-O-4 (**15**). All of these dimers were isolated as acetates and were viscous oils.

Carbohydrate-derived products were the major components in the degraded wood, and the mixture con-

Table 1. ^{1}H NMR Data for DFRC Dimers 1−7

H	1a	1b	2	3	4	5	6	7
Ac Me	2.20 (s), 2.18 (s)	2.20 (s), 2.19 (s)	2.21 (s)	2.21 (s)	2.03 (s)	2.08 (s), 2.00 (s)	2.06 (s), 2.05 (s)	2.28 (s)
γ-Ac Me	1.93 (s)	1.92 (s)		2.00 (s)	2.01 (s)	2.01 (s), 2.02 (s)	2.01 (s)	
OMe	3.76 (s), 3.68 (s)	3.76 (s), 3.68 (s)	3.81 (s)	3.74 (s)	3.88 (s)	3.88 (s), 3.87 (s)	3.90 (s), 3.96 (s)	4.10 (s), 3.95 (s)
2	6.76 (d, 1.9)	6.95 (d, 8.6)	6.93 (d, 2.0)	6.87 (d, 1.9)	7.26 (d, 1.9)	7.26 (d, 2.0)	7.31 (d, 2.0)	7.52 (d, 2.0)
3		7.15 (d, 8.6)						
5	6.95 (d, 8.0)	7.15 (d, 8.6)	6.93 (d, 8.1)	6.91 (d, 8.0)				7.24 (d, 8.3)
6	6.83 (dd, 8.0, 1.9)	6.95 (d, 8.6)	6.74 (dd, 8.1, 2.0)	6.69 (dd, 8.0, 1.9)	6.89 (d, 1.9)	6.89 (d, 2.0)	6.93 (d, 2.0)	7.45 (dd, 8.3, 2.0)
α	2.94 (dd, 13.7, 11.0) 3.08 (dd, 13.7, 10.1)	2.94 (dd, 13.7, 11.0) 3.08 (dd, 13.7, 10.1)	2.22 (dd, 7.3, 6.0)	2.70 (dd, 13.9, 7.9) 2.83 (dd, 13.8, 6.7)	6.72 (dt, 15.9, 1.4)	6.70 (dt, 16.0, 1.4)	6.71 (dt, 15.9, 1.4)	
β	3.30 (m)	3.30 (m)	2.22 (dd, 7.3, 6.0)	3.82 (m)	6.39 (dt, 15.9, 6.2)	6.39 (dt, 16.0, 6.2)	6.41 (dt, 15.9, 6.2)	
γ	4.26 (d, 6.6)	4.24 (d, 6.6)	1.48 (dd, 7.3, 6.0)	4.03 (dd, 11.4, 5.5) 4.24 (dd, 11.4, 6.1)	4.70 (dd, 6.2, 1.4)	4.70 (dd, 6.2, 1.4)	4.70 (dd, 6.2, 1.4)	2.57 (s)
2′	6.96 (d, 2.0)	6.96 (d, 2.0)				7.06 (d, 2.0)	7.61 (d, 1.9)	7.45 (d, 1.4)
5′	6.87 (d, 8.0)	6.87 (d, 8.0)						
6′	6.72 (dd, 8.0, 2.0)	6.72 (dd, 8.0, 2.0)				6.73 (d, 2.0)	7.44 (d, 1.9)	7.88 (d, 1.4)
α′						6.67 (dt, 11.7, 1.5)	10.00 (s)	10.06 (s)
β′						5.82 (dt, 11.7, 6.5)		
γ′						4.84 (dd, 6.5, 1.5)		

Table 2. ^{1}H NMR Data for DFRC Dimers 8−15

H	8	9	10	11	12	13	14	15
Ac Me	2.30 (s)	2.28 (s)	2.27 (s)	2.27 (s)	2.17 (s), 2.15 (s)	2.27 (s), 2.18 (s)	2.30 (s), 2.18 (s)	2.18 (s)
γ-Ac Me		2.09 (s)	2.08 (s)	2.08 (s)	2.01 (s)	1.90 (s), 2.02 (s)	1.89 (s), 2.03 (s)	1.99 (s), 2.03 (s)
α-Ac Me							2.07 (s)	
OMe	4.10 (s)	4.12 (s), 3.95 (s)	4.05 (s), 3.94 (s)	4.04 (s), 3.94 (s)	3.86 (s), 3.41 (s)	3.81 (s), 3.72 (s)	3.77 (s), 3.73 (s)	3.87 (s), 3.83 (s)
2	7.91 (d, 8.9)	7.61 (d, 2.0)	7.48 (d, 2.0)	7.49 (d, 1.9)	6.71 (d, 1.9)	6.86 (d, 1.9)	6.96 (d, 1.9)	6.96 (d, 1.7)
3	7.32 (d, 8.9)							
5	7.32 (d, 8.9)	7.28 (d, 8.2)	7.21 (d, 8.2)	7.21 (d, 8.3)	6.82 (d, 8.1)	6.87 (d, 8.0)	6.90 (d, 8.2)	
6	7.91 (d, 8.9)	7.49 (dd, 8.2, 2.0)	7.41 (dd, 8.2, 2.0)	7.42 (dd, 8.3, 1.9)	6.67 (dd, 8.1, 1.9)	6.72 (dd, 8.0, 1.9)	6.84 (dd, 8.2, 1.9)	6.48 (d, 1.7)
α					6.51 (dq, 1.5)	2.96 (dd, 8.0, 6.0), 3.06 (dd, 8.0, 6.8)	6.11 (d, 8.6)	6.57 (dt, 15.9, 1.4)
β						3.60 (m)	3.88 (m)	6.24 (dt, 15.9, 6.2)
γ	2.56 (s)	5.50 (s)	2.49 (s)	2.49 (s)	2.11 (d, 1.5)	4.20 (d, 6.8)	4.38 (d, 6.2)	4.63 (dd, 6.2, 1.4)
2′	7.45 (d, 1.4)	7.48 (d, 1.4)	7.11 (d, 1.4)	6.88 (d, 1.1)	7.19 (d, 1.9)	7.07 (d, 1.7)	7.03 (d, 1.9)	7.26 (d, 2.0)
5′								6.89 (d, 8.2)
6′	7.88 (d, 1.4)	8.02 (d, 1.4)	7.29 (d, 1.4)	7.11 (d, 1.1)	6.76 (d, 1.9)	7.09 (d, 1.7)	7.00 (d, 1.9)	7.02 (dd, 8.2, 2.0)
α′	10.06 (s)	10.08 (s)	6.80 (dt, 15.9, 1.4)	6.80 (dt, 11.7, 1.5)	6.60 (dt, 15.9, 1.4)	6.66 (dt, 16.0, 1.4)	6.63 (dt, 15.9, 1.4)	6.69 (dt, 15.9, 1.4)
β′			6.40 (dt, 15.9, 6.4)	5.80 (dt, 11.7, 6.6)	6.29 (dt, 15.9, 6.2)	6.36 (dt, 16.0, 6.2)	6.33 (dt, 15.9, 6.2)	6.35 (dt, 15.9, 6.3)
γ′			4.72 (dd, 6.4, 1.4)	4.90 (dd, 6.6, 1.5)	4.64 (dd, 6.2, 1.4)	4.68 (dd, 6.2, 1.4)	4.67 (dd, 6.2, 1.4)	4.69 (dd, 6.3, 1.4)

Table 3. ^{13}C NMR Data for DFRC Dimers 1−7 and 12−15

C	1a	2	3	4	5	6	7	12	13	14	15
4-Ac Me	20.5	20.5	20.5	20.3	20.3	20.3	20.5	20.4	20.5	20.5	20.2
4′-Ac Me	20.5	20.5	20.5	20.3	20.3	20.3		20.4	20.5	20.5	
γ-Ac Me	20.7		20.8	20.8	20.8	20.8			20.8	20.8	20.8
γ′-Ac Me			20.8	20.8	20.7			20.7	20.7	20.7	20.8
α-Ac Me										21.0	
3-OMe	56.2	56.2	56.1	56.5	56.5	56.5	56.6	56.6	56.0	56.2	56.6
3′-OMe	56.1	56.2	56.1	56.5	56.5	56.8	56.4	56.5	56.3	56.3	56.3
1	122.2	142.4	140.1	135.6	135.6	135.7	130.0	136.6	139.1	138.0	135.7
2	113.5	111.1	114.1	110.6	110.6	111.0	111.7	113.5	114.1	112.5	105.5
3	151.8	152.2	152.1	152.7	152.7	152.8	152.7	151.4	151.9	151.8	153.9
4	139.3	139.0	139.2	138.5	138.5	138.5	141.3	139.4	139.4	140.5	151.5
5	123.2	123.4	123.4	132.4	132.3	132.0	124.3	122.9	123.2	123.2	152.4
6	120.7	118.1	121.7	121.8	121.7	121.6	120.2	122.1	121.7	120.3	109.1
α	39.6	28.7	35.6	133.3	133.3	133.1	152.7	128.6	38.1	75.8	133.3
β	47.2	28.7	40.9	125.4	125.4	125.7	113.4	137.5	39.9	43.4	125.4
γ	67.7	18.7	64.7	65.1	65.1	65.0	9.6	26.1	67.2	64.6	64.9
1′	130.7				135.2	135.9	134.6	136.6	135.7	135.4	134.8
2′	114.4				113.3	111.5	106.2	109.7	109.2	109.6	111.9
3′	152.1				152.4	153.4	147.2	153.3	152.5	152.4	152.4
4′	141.5				138.1	143.6	146.9	138.2	139.2	139.5	145.7
5′	123.3				132.2	131.5	133.8	135.4	135.7	132.5	121.6
6′	121.8				123.6	126.3	117.8	120.5	119.1	120.1	120.6
α′					132.7	191.8	192.2	133.2	133.9	133.6	133.8
β′					127.8			125.3	124.9	125.1	124.5
γ′					61.6			65.0	65.2	65.1	65.2
4-Ac C=O	169.0	169.1	169.1	168.5	168.6	168.2	168.9	168.8	169.0	168.8	168.4
4′-Ac C=O	169.0				168.6	168.2		168.9	169.0	168.9	
α-Ac C=O										170.2	
γ-Ac C=O	170.9		171.0	170.7	170.7	170.7			171.0	170.9	170.8
γ′-Ac C=O	170.9				170.8			170.7	170.8	170.8	170.8

Table 4. GC/MS Data for DFRC Dimers 1−15

dimer	t_R, min	MS (abundance), *m/z* (%)
1a	17.56	430 (1), 388 (11), 346 (1), 328 (5), 286 (9), 209 (61), 150 (60), 137 (100)
1b	16.69	400 (2), 358 (10), 298 (31), 256 (6), 209 (100), 167 (25), 150 (24), 137 (17), 107 (91)
2	17.70	370 (9), 328 (34), 311 (2), 286 (100), 269 (13), 255 (18)
3	23.19	530 (1), 488 (14), 446 (11), 428 (1), 386 (6), 326 (3), 189 (13), 175 (5), 137 (100)
4	24.51	484 (23), 442 (6), 382 (18), 365 (3), 349 (3), 322 (100), 307 (9), 289 (18)
5	22.41	484 (19), 442 (2), 425 (2), 382 (14), 365 (3), 349 (3), 322 (100), 307 (7.5), 304 (4), 289 (14)
6	19.20	456 (2), 414 (33), 372 (19), 355 (1), 329 (2), 312 (100)
7	19.10	354 (11), 312 (100), 297 (6), 281 (3), 269 (6)
8	17.79	324 (21), 282 (100)
9	21.32	412 (20), 370 (100), 328 (4), 311 (26), 279 (74)
10	24.97	424 (31), 382 (68), 340 (22), 322 (48), 207 (100)
11	20.59	364 (31), 322 (100), 307 (8), 281 (12), 279 (19), 207 (33)
12	19.26	468 (9), 426 (26), 408 (1), 384 (8), 366 (17), 351 (1), 324 (100), 309 (14)
13	22.74	486 (24), 426 (15), 384 (5), 366 (12), 342 (2), 324 (24), 247 (20), 187 (50), 137 (100)
14	22.93	544 (7), 484 (28), 424 (12), 382 (33), 322 (13), 248 (52), 195 (50), 187 (38), 153 (100)
15	24.44	484 (11), 442 (3), 382 (14), 349 (3), 322 (100), 307 (8), 289 (20), 207 (17), 146 (12)

sisted of both carbohydrates and degraded lignin products ranging from polymers to lignin monomers and monosaccharides. Even in the lignin dimer fractions eluted from the Bio-Beads column, the dimers were still overwhelmed by small sugars. All were acetylated and showed similar behavior on silica gel chromatography and were difficult to separate from each other. Bio-Beads distributed the mixture according to their molecular weight from 14 000 to 400; the dimers and similar sized carbohydrates could be separated out of the total mixture. Reversed-phase TLC adsorbed dimers more strongly than carbohydrate components so that the acetylated carbohydrates in the dimer fraction could be developed to the frontier after three elutions; meanwhile the dimers were distributed in different bands according to their structure types. This allowed isolation of the minor dimers from the overwhelming carbohydrates and monomers. With the aid of HPLC, the individual dimers were purified. The total yield of the dimers was ~1% from the total degraded wood mixture (~4% of the lignin) and individual dimers accounted for from 0.01 to 0.1% of the wood.

Structural Identification. *Compound **1a***. The MS gives a molecular ion at *m/z* 430 and a series of fragments with loss of acetyl groups, *m/z* 388 (M − 42), 346 (M − 42 − 42), 328 (M − 42 − 60), and 286 (M − 42 − 42 − 60), suggesting there are two phenolic and an aliphatic acetate in the structure, and the base peak at *m/z* 137 supports the benzyl moiety of ring A (Figure 1); the 150 mass is also diagnostic, a fragment including ring B. As shown in Table 1, ^1H NMR of **1a** gives two ABX systems in the aromatic region, one for each guaiacyl ring. At high field there is an A_2MX_2 system that is assigned to α, β, and γ protons by HMQC correlations.

*Compound **1b***. Compound **1b** was not separated from **1a** by HPLC but was clearly identified in the NMR and separated and identified by GC/MS. MS gives a molecular ion at *m/z* 400 and an analogous (to **1a**) series of acetyl group losses, suggesting that there are two phenyl and an aliphatic acetate in the structure. Compared with **1a**, **1b** had its base peak at *m/z* 107, identifying the absence of the methoxyl in ring A. As shown in Table 1, ^1H NMR of **1b** has one AB and one

ABX system in the aromatic region supporting a *p*-hydroxyphenyl ring A and a guaiacyl ring B.

*Compound **2***. NMR studies showed that **2** is a symmetrical dimer, and the high-field side chain proton shifts strongly suggested a cyclopropanoid structure. With correlations from 2D-NMR (HMQC, HMBC), the structure appears to be as shown in Figure 1. The confirmatory MS spectrum, with its molecular ion at *m/z* 370 and successive losses of 2 ketenes, suggests two phenolic and no side chain acetates.

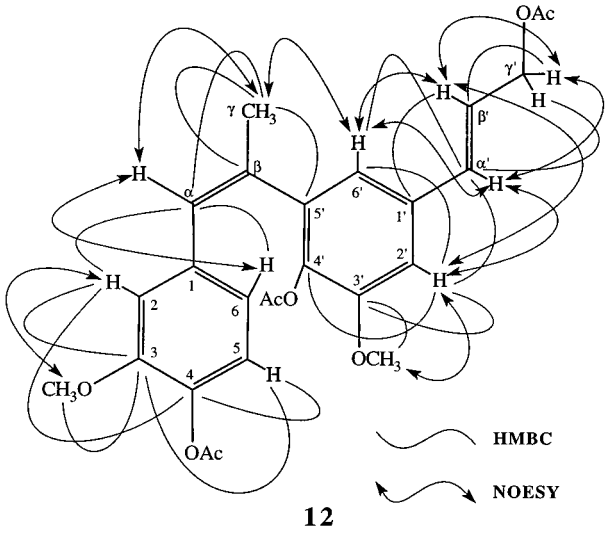
*Compound **3***. The MS gives a molecular ion at *m/z* 530 and a series of loss of acetyl groups, suggesting there are two phenolic and two aliphatic acetates in the structure. NMR studies showed **3** also has a symmetrical structure. ^{1}H NMR of **3** revealed that both rings shared the same ABX system in the aromatic region. Comparison with compound 2070 in the NMR database of lignin and cell wall model compounds (Ralph et al., 1996) confirmed **3** to be a β-β dimer (Figure 1).

*Compounds **4**−**6***. The MS of compound **4** gives a molecular ion at *m/z* 528 and a series of loss of acetyl groups, suggesting there are two phenolic and two aliphatic acetates in the structure. NMR studies showed **4** to be a symmetrical dimer. ^{1}H NMR revealed that both rings shared the same AB system in the aromatic region. Full 1D and 2D NMR confirmed **4** to be a (*trans-trans*) 5-5 dimer. Compound **5** shared the same MS molecular ion and fragmentation patterns with compound **4**. Upon comparison of ^{1}H and ^{13}C NMR spectra with those of **4**, **5** is the (*trans-cis*) isomer. Compound **6** is the corresponding monobenzaldehyde of **4** (Figure 1).

*Compounds **7**−**11***. Compounds **7**−**11** were all β-5 structures with a benzyltetrahydrofuran ring system. There is a methyl group at δ 2.57 (s) in **7** which showed correlations with three tertiary carbons at δ 152.7 (C-α), 113.4 (C-β), and 133.8 (C-5′) in an HMBC spectrum. A benzofuran skeleton was deduced on the basis of 2D NMR studies. Compound **8** is the ring A *p*-hydroxyphenyl analogue of **7**. Compound **9** is different from **7** at the γ-position with the normal acetoxymethyl group instead of a methyl group. ^{1}H NMR spectra showed protons at δ 10.06 (s, aldehyde protons) for **7** and **8** and at δ 10.08 (s, aldehyde proton) for **9**, identifying these three compounds as β-5 B-ring benzyldehydes. The aldehyde carbon of **7** correlated with protons at δ 7.45 (d, J = 1.4, H-2′) and 7.88 (d, J = 1.4, H-6′), confirming that the aldehyde group is on ring B. Both **10** and **11** have intact side chains in ring B; the former is the *trans*- and the latter is the *cis*-isomer (Figure 1).

*Compound **12***. ^{1}H NMR of **12** gives an ABX pattern for a guaiacyl ring A and an AX system for ring B (Table 2). An HMBC experiment showed a methyl group at δ 2.11 (d, 1.5 Hz) correlating with carbons at δ 128.6 (C-α), 137.5 (C-β), and 135.4 (C-5′), suggesting that **12** is also a β-5 dimer. NOESY spectra of **12** suggested that the α−β double bond was *cis*-substituted by the two aromatic rings. Diagnostic NOESY and HMBC correlations for compound **12** are shown schematically in Figure 4.

*Compound **13***. ^{1}H NMR of **13** gives an ABX for ring A aromatic protons and two doublets for ring B (Table 2). An HMBC experiment showed that the proton at δ 7.09 (d, J = 1.7 Hz, H-6′) correlates with the carbon at δ 39.9 (C-β), protons at δ 4.20 (d, J = 6.8 Hz, H-γ), 2.96 (dd, J = 8.0, 6.0 Hz, H-α), and 3.06 (dd, J = 8.0, 6.8 Hz, H-α) correlate with the carbon at δ 135.7 (C-5′), and the proton at δ 3.60 (m, H-β) correlates with the carbon at δ 139.2 (C-4′), suggesting that **13** is the β-5 dimer shown in Figure 1.



12

Figure 4. Major correlations in HMBC and NOESY spectra of dimer **12**.

*Compound **14**.* The base peak at *m/z* 153 in its mass spectrum revealed that **14** is an α-acetoxy compound. An HMBC experiment showed that the proton at δ 7.00 (d, J = 1.9 Hz, H-6′) correlates with carbon at δ 43.4 (C-β), the protons at δ 4.38 (d, J = 6.2 Hz, H-γ) correlate with carbons at δ 43.4 (C-β), 75.8 (C-α), and 132.5 (C-5′), the proton at δ 6.11(d, J = 8.6 Hz, H-α) correlates with carbons at δ 112.5 (C-2), 120.3 (C-6), 132.5 (C-5′), 138.0 (C-1), and 170.2 (α-Ac C=O), proton at δ 3.88 (m, H-β) correlates with carbons at δ 120.1 (C-6′), 132.5 (C-5′), 139.5 (C-4′), and 138.0 (C-1) suggesting that **14** is an α-acetoxy β-5 dimer.

*Compound **15**.* The mass spectrum of this compound gives a molecular ion at *m/z* 484. The base peak at *m/z* 322 arises from losses of one phenolic and two aliphatic acetates. ^{1}H NMR of **15** shows a 5-substituted system for ring A and a normal guaiacyl ring B. Each maintains a 3-carbon side chain with a trans α−β double bond. HMQC and HMBC spectra confirmed **15** is a 5-O-4 dimer.

Structural Implications. Only a trace of a β-ether dimer and no noncyclic α-ethers were detected in the degradation products, indicating that the DFRC method, even on this large scale, is efficiently cleaving the α- and β-ether bonds in this softwood lignin. Although there may be many other minor dimers than have been isolated, the major structures identified reflect all of the common linkages in softwood lignins and provide insights into details of the pine lignin structure. Intact side chains on most dimeric products obtained here provide information about the original linkages and where the ether linkages occur; the corresponding dimeric compounds produced by hydrogenolysis (Sakakibara, 1992) or thioacidolysis (Lapierre, 1993) may lose such information. In the DFRC method, an unsaturated side chain arises only if that side chain was originally a β-ether, whereas any endgroup side chain (−CH=CH−CH_2OH) produces quite different products following DFRC treatment (Lu and Ralph, 1998a). The 5-5-dimers **4** and **5** and the 5-O-4 dimer **15** can only come from two β-ether units that have coupled, i.e., from coupling of two preformed β-ether dimers or higher oligomers. Whether these 5-5 units were present in the

newly identified dibenzodioxocin (Karhunen et al., 1995a,b, 1996) structures cannot be ascertained. Model studies to understand the DFRC cleavage of non-β-ether units in lignins is now required. Products from 5 to 5 dimerization of coniferyl alcohol could not be found—it is well-known that coniferyl alcohol prefers coupling at its β-site, so such products are unlikely.

Aldehydes are well-known for the phloroglucinol lignin staining reaction (Sarkanen and Ludwig, 1971; Adler et al., 1948). Whether they are present in native lignins or are a product of the isolation procedure has been debated (Sarkanen and Ludwig, 1971). Aldehydes are not created during the DFRC procedure. Therefore, the aldehydes in dimers **6**−**9** must have been present in the original lignin or created during the minimal preparation of the wood. We have found that cinnamaldehyde endgroups react reasonably efficiently under the DFRC procedures to give diagnostic products that can be located in small amounts in DFRC product chromatograms of lignins (Lu and Ralph, 1998a). Benzaldehydes come through the procedure intact or as benzyl acetates. Aldehydes appear in dimers from which they are reasonable products of radical coupling of the preformed aldehyde, but they could also arise from postcoupling oxidations. It is well established that vanillin favors coupling at the 5-site; 5- dehydrodivanillin can be prepared almost quantitatively from vanillin with peroxidase and hydrogen peroxide (Baumgartner and Neukom, 1972). The dimers isolated here contain the vanillin unit coupled at the 5-position. However, the identification of vanillin itself and vanillyl acetate in the monomers (Lu and Ralph, 1998a) implies that it (or its cinnamaldehyde or coniferyl alcohol precursor) can also be 4-O-etherified.

Unusual benzofuran structures in dimers **7**−**11** were unanticipated. While they clearly arise from β-5 units, the exact mechanism of their formation is not clear. Analogous compounds arise from ozonation reactions (Tanahashi et al., 1975). Phenylcoumaran (β-5) units appear to react via a variety of pathways to give structures **10**−**14**, of which **13** is major.

Currently there is still debate on the source of β-1 units; they can scarcely be found in NMR spectra of milled wood lignins (Ede et al., 1990, 1996), yet can be significant products in solvolysis dimers from the same lignins (Higuchi, 1990). The DFRC procedure appears to give β-1 products **1** and **2** at similar levels to thioacidolysis, although no attempts at true quantitation are presented here.

Only two dimers derived from the minor *p*-coumaryl units in lignin were isolated in low amounts. The β-1 product **1b** must result from coupling of a *p*-coumaryl alcohol monomer (at β) with a preformed guaiacyl β-ether dimer or higher oligomer. Similarly, the β-5 product **8** must result from coupling of a *p*-coumaryl alcohol monomer (again at β) with another guaiacyl unit. Determining whether *p*-coumaryl alcohol units actually have a higher propensity to couple with guaiacyl units (rather than hydroxycinnamyl alcohol monomers) or whether the products of such reactions are simply more easily released from lignins awaits further data.

Conclusions. The DFRC method can play a role in identifying traditional and novel or new lignin constituents. It has recently been invaluable in screening CAD-deficient pine mutants for increased levels of dihydroconiferyl alcohol-derived units (Ralph et al., 1997). It produces extraordinarily clean products for β-aryl ether units in lignin, cleaving them efficiently, yielding low molecular weight lignin fragments. Other interunit structures in lignin are also altered by the DFRC conditions and, regrettably, each linkage type does not give a single product. However, the major 5-5, β-β, β-1, β-5, and 5-O-4 structures are readily identified in the dimeric fraction, as are several minor β-5 structures with unusual benzofuran skeletons. As with the extended thioacidolysis method, quantification of these released dimers will provide useful insights into lignin structure and structural changes occurring under various chemical and biological treatments. Additionally, the minor products (e.g. aldehydes) observed here and in the monomer fraction (Lu and Ralph, 1998a) are interesting indicators of minor structural elements in lignins.

Now that dimers have been identified and have been shown to be reasonable products from the chemistry involved, we will optimize conditions for quantitation of major dimers from small samples. A combination of normal and reversed solid-phase extraction followed by GC/FID or GC/MS detection should allow development of a convenient method for dimers in small samples (~30−50 mg of wood sample), once response factors are determined. Preliminary trials suggest that the DFRC-degraded products of loblolly pine wood, with the originally overwhelming carbohydrates and monomers removed by solid-phase extraction, give a clean profile with most dimers separated on the GC chromatogram. It is hoped this emerging method can be utilized for identification, quantification, and comparison of dimers in wide-ranging plant materials.

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LITERATURE CITED

Adler, E.; Björkquist, K. J.; Häggroth, S. Über die Ursache der Farbreaktionen des Holzes. *Acta Chem. Scand.* **1948**, *2*, 93−94.

Baumgartner, J.; Neukom, H. Enzymatische Oxydation von Vanillin. *Chimia* **1972**, *26*, 366−368.

Ede, R. M.; Brunow, G.; Simola, L. K.; Lemmetyinen, J. Two-dimensional proton−proton chemical shift correlation and J-resolved NMR studies on isolated and synthetic lignins. *Holzforschung* **1990**, *44*, 95−101.

Ede, R. M.; Ralph, J.; Torr, K. M.; Dawson, B. S. W. A 2D NMR investigation of the heterogeneity of distribution of diarylpropane structures in extracted *Pinus radiata* lignins. *Holzforschung* **1996**, *50*, 161−164.

Higuchi, T. Lignin biochemistry: biosynthesis and biodegradation. *Wood Sci. Technol.* **1990**, *24*, 23−63.

Karhunen, P.; Rummakko, P.; Sipilä, J.; Brunow, G.; Kilpeläinen, I. Dibenzodioxocins; a novel type of linkage in softwood lignins. *Tetrahedron Lett.* **1995a**, *36*, 169−170.

Karhunen, P.; Rummakko, P.; Sipilä, J.; Brunow, G.; Kilpeläinen, I. The formation of dibenzodioxocin structures by oxidative coupling. A model reaction for lignin biosynthesis. *Tetrahedron Lett.* **1995b**, *36*, 4501−4504.

Karhunen, P.; Rummakko, P.; Pajunen, A.; Brunow, G. Synthesis and crystal structure determination of model compounds for the dibenzodioxocine structure occurring in wood lignins. *J. Chem. Soc., Perkin Trans. 1* **1996**, 2303−2308.

Lapierre, C. Application of new methods for the investigation of lignin structure. In *Forage Cell Wall Structure and Digestibility*; Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J., Eds.; ASA-CSSA-SSSA: Madison, WI, 1993; pp 133−166.

Lu, F.; Ralph, J. Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers. *J. Agric. Food Chem.* **1997a**, *45*, 2590−2592.

Lu, F.; Ralph, J. The DFRC method for lignin analysis. Part 1. A new method for β-aryl ether cleavage: lignin model studies. *J. Agric. Food Chem.* **1997b**, *45*, 4655−4660.

Lu, F.; Ralph, J. The DFRC method for lignin analysis. Part 2. Monomers from isolated lignins. *J. Agric. Food Chem.* **1998a**, *46*, 547−552.

Lu, F.; Ralph, J. The DFRC method for lignin analysis. Part 3. NMR studies. *J. Wood Chem. Technol.* **1998b**, *18*, in press.

Ralph, S. A.; Ralph, J.; Landucci, W. L.; Landucci, L. L. NMR Database of Lignin and Cell Wall Model Compounds. Available over the Internet at http://www.dfrc.wisc.edu/software.html or send e-mail to jralph@facstaff.wisc.edu, 1996.

Ralph, J.; MacKay, J. J.; Hatfield, R. D.; O'Malley, D. M.; Whetten, R. W.; Sederoff, R. R. Abnormal lignin in a loblolly pine mutant. *Science* **1997**, *277*, 235−239.

Rolando, C.; Monties, B.; Lapierre, C. Thioacidolysis. In *Methods in Lignin Chemistry*; Dence, C. W., Lin, S. Y., Eds.; Springer-Verlag: Berlin, 1992; pp 334−349.

Sakakibara, A. Hydrogenolysis. In *Methods in Lignin Chemistry*; Dence, C. W., Lin, S. Y., Eds.; Springer-Verlag: Berlin, 1992; pp 350−368.

Sarkanen, K. V.; Ludwig, C. H. *Lignins, Occurrence, Formation, Structure and Reactions*; Wiley-Interscience: New York, 1971.

Tanahashi, M.; Nakatsubo, F.; Higuchi, T. Structural elucidation of bamboo lignin by acidolysis and ozonolysis. *Wood Res.* **1975**, *58*, 1−11.

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