Pathway of *p*-Coumaric Acid Incorporation into Maize Lignin As Revealed by NMR

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Abstract: NMR methods can be used to delineate detailed structural and regiochemical information on the plant cell wall and elucidate biochemical incorporation pathways. Maize lignin isolated in high yield from rind tissue of stem internodes contained high amounts of esterified *p*-coumaric acid. Available literature indicated that acylation of the lignin by *p*-coumaric acid was at the α- or γ-position of the lignin side chain, implicating two diverse biochemical pathways. Application of ^{13}C–^{1}H correlative NMR experiments to this maize lignin, synthetic α- and γ-*p*-coumaroylated lignin model compounds, and a specifically labeled synthetic coniferyl alcohol/coniferyl *p*-coumarate dehydrogenation polymer (DHP) unambiguously revealed that *p*-coumaric acid is attached exclusively at the γ-position. The biochemical implication is that lignin acylation occurs by enzymatically controlled pre-acylation of lignin monomers which are subsequently incorporated into the lignin polymer *via* oxidative coupling. "Opportunistic" acylation at the α-position *via* quinone methide intermediates is insignificant.

Introduction

NMR spectroscopy is one of the most powerful tools available to chemists and biochemists for structural elucidation, but its full power in determining plant cell wall structure remains underexploited. Modern pulsed NMR methods are utilized to assign and authenticate low molecular mass structures and provide databases for classical interpretation of polymer spectra. With regard to lignin, solution-state two-dimensional NMR[2-5] and solid-state NMR of specifically labeled substrates[6] have largely confirmed information already well established by other methods concerning the constitution and structure of wood lignins.[7] Studies on unresolved aspects of plant growth and development have often overlooked the convenient diagnostic role NMR can play in answering regiochemical questions, which can afford unambiguous insights into biochemical pathways and provide a rational basis for selection or modification of agricultural crops for improved properties.

Three *p*-hydroxycinnamic acids **1** (biosynthetic precursors of the three *p*-hydroxycinnamyl alcohol lignin monomers **2**, Figure 1), particularly ferulic acid (**1b**) in grasses, have received considerable attention[8-11] because of their intimate association with the plant cell wall and ability to function as cross-links between wall polysaccharides[12] (notably arabinoxylans) and between polysaccharides and the phenylpropanoid lignin polymer **3**.[13] For ruminant animals, covalent attachment of lignin to wall polysaccharides limits overall polysaccharide digestibility and leads to significant amounts of undigested fiber.[9,10] Ascertaining the regiochemical relationships between wall polymers, i.e. the chemical sites at which one polymer is linked to another, can lead to a more complete understanding of the biosynthetic processes involved in wall formation and provide a sound basis for selection or biochemical modification of agricultural crops such as maize (*Zea mays* L.) to improve digestibility. The structural and regiochemical details provided by NMR have recently allowed us to demonstrate that ferulate arabinoxylan esters actively participate in oxidative coupling reactions which incorporate them into lignin/hydroxycinnamic acid/polysaccharide complexes in a variety of structures.[8,14] These structures had been previously neglected in favor of simple α-feruloyl ethers resulting from "opportunistic" incorporation[8] *via* nucleophilic addition to quinone methide lignin intermediates **4**. Resolution of these divergent incorporation pathways, revealed by NMR, allows considerable biochemical insight.

Although functional roles for ferulic acid have been adduced, the role of *p*-coumaric acid (**1a**) in wall structure and development is not well understood. Small amounts of *p*-coumaric acid are esterified to arabinoxylans early in primary wall development in much the same way as ferulic acid,[15] but, later in wall development, *p*-coumaric acid is found more extensively esterified to lignin.[8,16-23] Because of the adverse effects of *p*-hydroxycinnamic acids on

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(2) Bardet, M.; Gagnaire, D.; Nardin, R.; Robert, D.; Vincendon, M. *Holzforschung* **1986**, *40*, 17–24.

(3) Lapierre, C.; Monties, B.; Guittet, E.; Lallemand, J. Y. *Holzforschung* **1987**, *41*, 51–58.

(4) Ede, R. M.; Brunow, G. *J. Org. Chem.* **1992**, *57*, 1477–1480.

(5) Fukagawa, N.; Meshitsuka, G.; Ishizu, A. *J. Wood Chem. Technol.* **1991**, *11*, 373–396.

(6) Lewis, N. G.; Yamamoto, E.; Wooten, J. B.; Just, G.; Ohashi, H.; Towers, G. H. N. *Science* **1987**, *237*, 1344–1346.

(7) Freudenberg, K. In *Constitution and Biosynthesis of Lignin*; Freudenberg, K., Neish, A. C., Eds.; Springer-Verlag: Berlin-Heidelberg-New York, 1968; pp 47–116.

(8) Ralph, J.; Helm, R. F.; 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 201–246.

(9) Jung, H. G.; Deetz, D. A. 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 315–346.

(10) Jung, H. G.; Ralph, J. In *Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants*; Akin, D. E., Ljungdahl, L. G., Wilson, J. R., Harris, P. J., Eds.; Elsevier: New York, 1990; pp 173–182.

(11) Yamamoto, E.; Bokelman, G. H.; Lewis, N. G. In *Plant Cell Wall Polymers. Biogenesis and Biodegradation*; Lewis, N. G., Paice, M. G., Eds.; ACS Symposium Series 399; American Chemical Society: Washington, DC, 1989; pp 68–88.

(12) Ishii, T. *Carbohydr. Res.* **1991**, *219*, 15–22.

(13) Lam, T. B. T.; Iiyama, K.; Stone, B. A. *Phytochemistry* **1992**, *31*, 1179–1183.

(14) Ralph, J.; Helm, R. F.; Quideau, S.; Hatfield, R. D. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2961–2969.

(15) Mueller-Harvey, I.; Hartley, R.; Harris, P. J.; Curzon, E. H. *Carbohydr. Res.* **1986**, *148*, 71–85.

(16) Smith, D. C. C. *Nature* **1955**, *176*, 267–268.

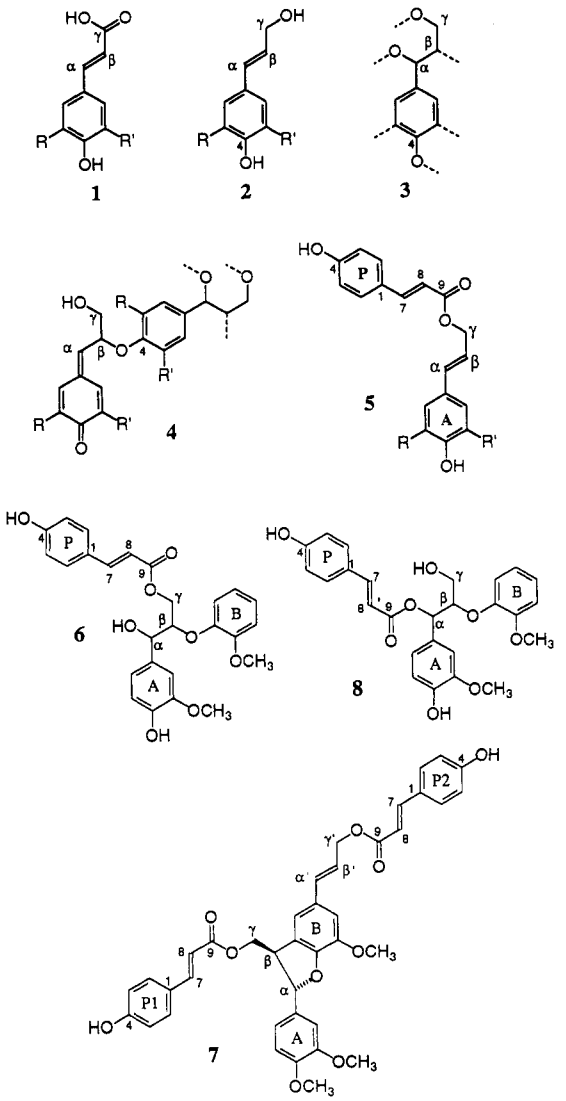

Figure 1. Plant cell wall precursors *p*-hydroxycinnamic acids **1** and monomeric *p*-hydroxycinnamyl alcohol lignin precursors **2**, a general structure of lignin **3**, quinone methide lignin intermediates **4**, *p*-hydroxycinnamyl *p*-coumarates **5**, and α- and γ-*p*-hydroxycinnamate ester model compounds **6–8** synthesized for NMR correlation work. For compounds **1**, **2**, **4**, and **5**, (**a**) R = R′ = H; (**b**) R = OCH_3, R′ = H; and (**c**) R = R′ = OCH_3.

forage digestibility, it is important to understand how *p*-coumaric acid (**1a**) is attached to lignin **3**, what biochemical process the plant invokes for its attachment, and, ultimately, how the process is controlled. If enzymatically assisted acylation of the preformed lignin polymer is discounted,[8] *p*-coumaric acid can be esterified only with hydroxyl groups on the α- or γ-side-chain carbons of lignin.[8,24] As each site implicates separate and divergent mechanisms for attachment, the problem reduces to determining the regiochemistry of *p*-coumaric acid on lignin.

Attachment at the α-position of the lignin side chain requires that free *p*-coumaric acid (**1a**) be present in the matrix during lignification for "opportunistic" addition[8] to quinone methide intermediates **4** formed by β-*O*-4 radical coupling between lignin monomers **2** or between a lignin monomer (β-position) and the growing lignin polymer **3** (4-position). From the chemical standpoint this represents a perfectly reasonable mechanism for attaching *p*-coumaric acid at the α-position,[23-25] but it has a number of troubling implications relating to nucleophilic competition and biochemical control.[14] Lignin monomers are generated intracellularly and move into the cell wall possibly as phenolic glycosides,[11] where glycosidases release the monomers for polymerization by oxidative phenol coupling.[7,26] *p*-Coumaric acid could presumably also be transported to the site of cell wall biosynthesis by similar methods and there combine with lignin quinone methide intermediates prior to or subsequent to deglycosylation.[11] The plant would have relatively poor control over lignin acylation since there is no enzymatic control in this strictly chemical quinone methide addition reaction. At pH values near neutral in the plant cytoplasm, *p*-coumaric acid should be able to compete effectively for the quinone methide (α-site) against water and numerous other nucleophiles present.[27] However, considerable effort has been expended to find synthetic conditions that accomplish this, but only modest (ca. 60%) yields are obtained[28] and, under physiological conditions, indications are that these yields are lower and highly dependent on pH.[27]

Attachment of *p*-coumaric acid (**1a**) at the γ-position of the lignin side chain cannot be rationalized on a purely chemical basis and must arise from a distinct biochemical pathway. Enzymatically mediated pre-esterification of *p*-coumaric acid with *p*-hydroxycinnamyl alcohol lignin monomers **2**, presumably again intracellularly, would result in *p*-hydroxycinnamyl *p*-coumarates **5** which could participate in the lignin formation by conventional oxidative coupling processes to generate a γ-*p*-coumaroylated lignin.[8,29] The cell must contain transferases for this esterification between *p*-coumaric acid and lignin monomers analogous to those it uses for feruloylation of arabinoxylans.[30-33]

While both of these mechanisms are credible routes to *p*-coumarate lignin esters, it is somewhat unlikely that two divergent mechanisms would have evolved. Previous studies, however, support the concept of parallel pathways. The most complete regiochemical studies to date[18,29,34,35] concluded, from methanolysis and UV studies and preparation of dehydrogenation polymers from coniferyl *p*-coumarate (**5b**), that bamboo lignin was esterified at both the γ- (80%) and α-positions (20%).[18,35]

Because of these uncertainties and the distinct biochemical implications of each route, we sought to resolve the regiochemical question for the C_4 plant maize. Two-dimensional ^{13}C–^1H correlative NMR experiments, with the improved sensitivity gained from proton-detected methodologies, were logical choices to provide unequivocal information, particularly when coupled with analogous studies of appropriate model compounds and synthetic lignin preparations. As a corollary, the question of

(17) Scalbert, A.; Monties, B.; Lallemand, J. Y.; Guittet, E.; Rolando, C. *Phytochemistry* **1985**, *24*, 1359–1362.
(18) Shimada, M.; Fukuzuka, T.; Higuchi, T. *Tappi* **1971**, *54*, 72–78.
(19) Monties, B.; Lapierre, C. *Physiol. Veg.* **1981**, *19*, 327–348.
(20) Atsushi, K.; Azuma, J.; Koshijima, T. *Holzforschung* **1984**, *38*, 141–149.
(21) Azuma, J.; Nomura, T.; Koshijima, T. *Agric. Biol. Chem.* **1985**, *49*, 2661–2669.
(22) Terashima, N.; Fukushima, K.; He, L.; Takabe, K. 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 247–270.
(23) Lam, T. B. T.; Iiyama, K.; Stone, B. A. *Phytochemistry* **1992**, *31*, 2655–2658.
(24) Helm, R. F.; Ralph, J. *J. Agric. Food Chem.* **1993**, *41*, 570–576.
(25) Scalbert, A.; Monties, B.; Rolando, C.; Sierra-Escudero, A. *Holzforschung* **1986**, *40*, 191–195.
(26) Harkin, J. M. In *Oxidative Coupling of Phenols*; Taylor, W. I., Battersby, A. R., Eds.; Marcel Dekker: New York, 1967; pp 243–321.
(27) Sipilä, J.; Brunow, G. *Holzforschung* **1991**, *45*, 9–14.
(28) Helm, R. F.; Ralph, J. *J. Agric. Food Chem.* **1992**, *40*, 2167–2175.
(29) Nakamura, Y.; Higuchi, T. *Cellul. Chem. Technol.* **1978**, *12*, 199–208.
(30) Fry, S. C.; Miller, J. C. In *Plant Cell Wall Polymers, Biogenesis and Biodegradation*; Lewis, N. G., Paice, M. G., Eds.; ACS Symposium Series 399; American Chemical Society: Washington, DC, 1989; pp 33–46.
(31) Iiyama, K.; Lam, T. B. T.; Meikle, P. J.; Ng, K.; Rhodes, D.; Stone, B. A. 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 621–683.
(32) Meyer, K.; Kohler, A.; Kauss, H. *FEBS Lett.* **1991**, *290*, 209–212.
(33) Mock, H. P.; Strack, D. *Phytochemistry* **1993**, *32*, 575–579.
(34) Nakamura, Y.; Higuchi, T. *Cellul. Chem. Technol.* **1978**, *12*, 209–221.
(35) Nakamura, Y.; Higuchi, T. *Holzforschung* **1976**, *30*, 187–191.

Table 1. ^{13}C NMR Chemical Shifts of *p*-Coumarate Moieties in Compounds **6–8** and in Isolated Maize Lignin (D-LPC)

	carbon								
	1	2	3	4	5	6	7	8	9
compd									
6 (*threo*)	126.89	130.94	116.66	160.63	116.66	130.94	145.57	115.22	167.15
6 (*erythro*)	126.91	130.88	116.64	160.52	116.64	130.88	145.39	115.34	167.28
7 (P1)[a]	126.97	130.94	116.68[b]	160.61[b]	116.68[b]	130.94	145.50	115.49	167.23
7 (P2)[a]	126.85	131.02	116.70[b]	160.71[b]	116.70[b]	131.02	145.91	115.08	167.23
8 (*threo*)	127.04	130.91	116.64	160.48	116.64	130.91	145.39	115.81	166.51
8 (*erythro*)	126.97	130.96	116.67	160.59	116.67	130.96	145.65	115.62	166.32
D–LPC	126.29	130.79	116.49	160.49	116.49	130.79	145.2–146.1	114.2–115.4	167.63
acetates									
6 (*threo*)	132.81	130.25	123.21	153.52	123.21	130.25	144.75	118.57	166.61
6 (*erythro*)	132.78	130.21	123.20	153.49	123.20	130.21	144.71	118.54	166.60
7 (P1)[a]	132.91	130.16	123.23	153.46[b]	123.23	130.16	144.43	119.01	166.80
7 (P2)[a]	132.77	130.21	123.23	153.53[b]	123.23	130.21	144.79	118.61	166.80
8 (*threo*)	132.82	130.22	123.21	153.52	123.21	130.22	144.91	118.76	165.90
8 (*erythro*)	132.82	130.28	123.24	153.58	123.24	130.28	145.12	118.67	165.79
D–LPC	132.64	130.09	123.13	153.32	123.13	130.09	144–146	118.3–118.9	166.4–167.1

[a] P1 and P2 refer to the two *p*-coumaroyl moieties labeled as such in structure **7**. [b] Assignments (in the same column) can be interchanged.

whether the *p*-coumarate group participates in oxidative coupling reactions can also be addressed by the same NMR approach.[36]

Results and Discussion

Isolation and Composition of Maize Rind Lignin. To facilitate the NMR experiments, field grown experimental maize inbred senescent plants were screened to identify samples with a high *p*-coumaric acid content by observation of the 4-vinylphenol peak in pyrolysis–GC–MS total ion chromatograms.[37] Crude polysaccharide hydrolase preparations were tested for the absence of esterase activity using previously synthesized model compounds.[24,38] Treatment of the ground, extracted, and ball-milled rind material with Cellulysin gave a lignin–polysaccharide complex (LPC)[39,40] containing all of the lignin and ca. 20% polysaccharides (see the Experimental Section). This was extracted with 96:4 dioxane/water[41] to give a soluble fraction (D-LPC, 42% of LPC, 81% Klason lignin) and an insoluble residue (E-LPC, 51% of LPC, 36% Klason lignin). The lyophilized D-LPC fraction was water washed (removing a surprising amount of glucose) and washed with EDTA (see the Experimental Section) to remove any trace metal contaminants. EDTA washing was found to be important in extending the NMR relaxation times sufficiently to allow useful long-range correlation spectra. The yield of "milled maize lignin" (D-LPC) is very high compared to that of similar preparations obtained from woods or other grasses.[40] Thus 65% of the total lignin was available for detailed analysis by the solution-state NMR methods described below. Analytical saponification of the D-LPC fraction (2 M sodium hydroxide, degassed, 24 h) gave a *p*-coumaric acid content of 17.4%. Similar values were obtained from quantitative ^{13}C NMR and from large-scale saponification.

Model Compounds. An important aspect of any NMR approach to structural studies of lignin is the derivation of NMR data from relevant model compounds for comparison with spectra of the polymer.[42] For this reason, an NMR database of model compounds for lignin and related plant cell wall components has been established[43] and continues to expand as relevant model compounds are synthesized and fully authenticated ^1H and ^{13}C assignment data are accumulated. In this vein, *threo*- and *erythro*-isomers of γ- and α-*p*-coumarate esters **6** and **8** of the β-aryl ether lignin model guaiacylglycerol β-guaiacyl ether, as well as the *p*-coumarate ester **7** of a phenylcoumaran model, were subjected to the correlation experiments described below for the isolated maize lignin. This provides the 2D sections, shown in Figures 3 and 5, required for assignment and authentication. The synthesis of compounds **6** and **8** has been previously described.[24] Compound **7** was synthesized by Et_3N/(dimethylamino)pyridine-mediated acylation of the 4-*O*-methyl ether of dehydrodiconiferyl

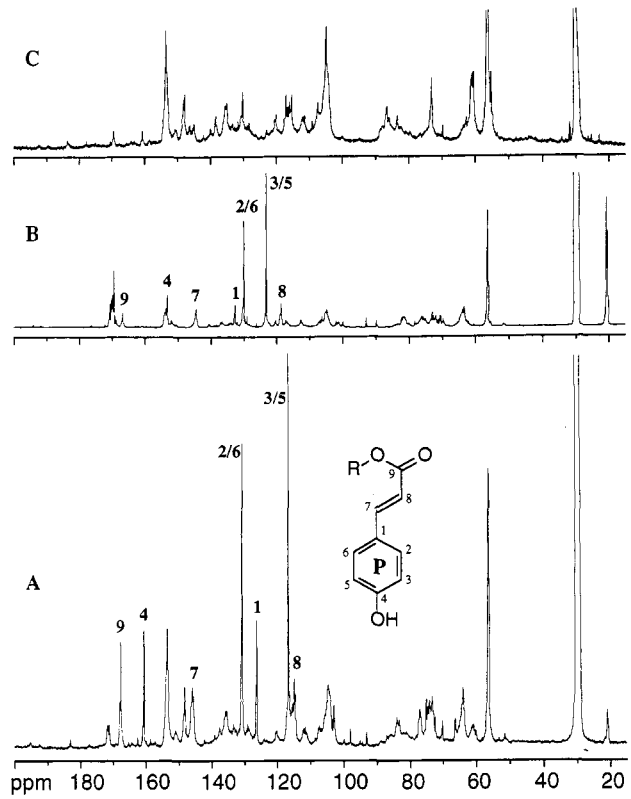

Figure 2. ^{13}C NMR spectra of D-LPC maize lignin showing the predominance of *p*-coumarate ester peaks: (A) underivatized D-LPC in 9:1 acetone-d_6/D_2O, (B) acetylated D-LPC in acetone-d_6, and (C) saponified D-LPC. Assignments are given for *p*-coumaroyl moieties; the remaining peaks are largely typical of a syringyl/guaiacyl lignin (see text).

(36) The findings documented in this paper have been presented at two meetings: Ralph, J.; Hatfield, R. D.; Quideau, S.; Helm, R. F.; Grabber, J.; Jung, H. G. *NMR of Biomaterials Symposium*, National Meeting of the American Chemical Society, Denver, CO, 1993; American Chemical Society: Washington, DC, 1993; p Cell-104. Hatfield, R. D.; Ralph, J.; Grabber, J.; Jung, H. G. *The Extracellular Matrix of Plants: Molecular, Cellular and Developmental Biology*; Keystone Symposia: Santa Fe, NM, 1993; p A-319.

(37) Ralph, J.; Hatfield, R. D. *J. Agric. Food Chem.* **1991**, *39*, 1426–1437.

(38) Hatfield, R. D.; Helm, R. F.; Ralph, J. *Anal. Biochem.* **1991**, *194*, 25–33.

(39) Minor, J. L. *J. Wood Chem. Technol.* **1986**, *6*, 185–201.

(40) Obst, J. R.; Kirk, T. K. *Methods Enzymol.* **1988**, *161*, 87–101.

(41) Björkman, A. *Sven. Papperstidn.* **1956**, *59*, 477–485.

(42) Lüdemann, H.-D.; Nimz, H. *Makromol. Chem.* **1974**, *175*, 2393–2407.

(43) Ralph, J.; Landucci, W. L.; Ralph, S. A.; Landucci, L. L. Available over Internet; send E-mail to JRALPH@FACSTAFF.WISC.EDU.

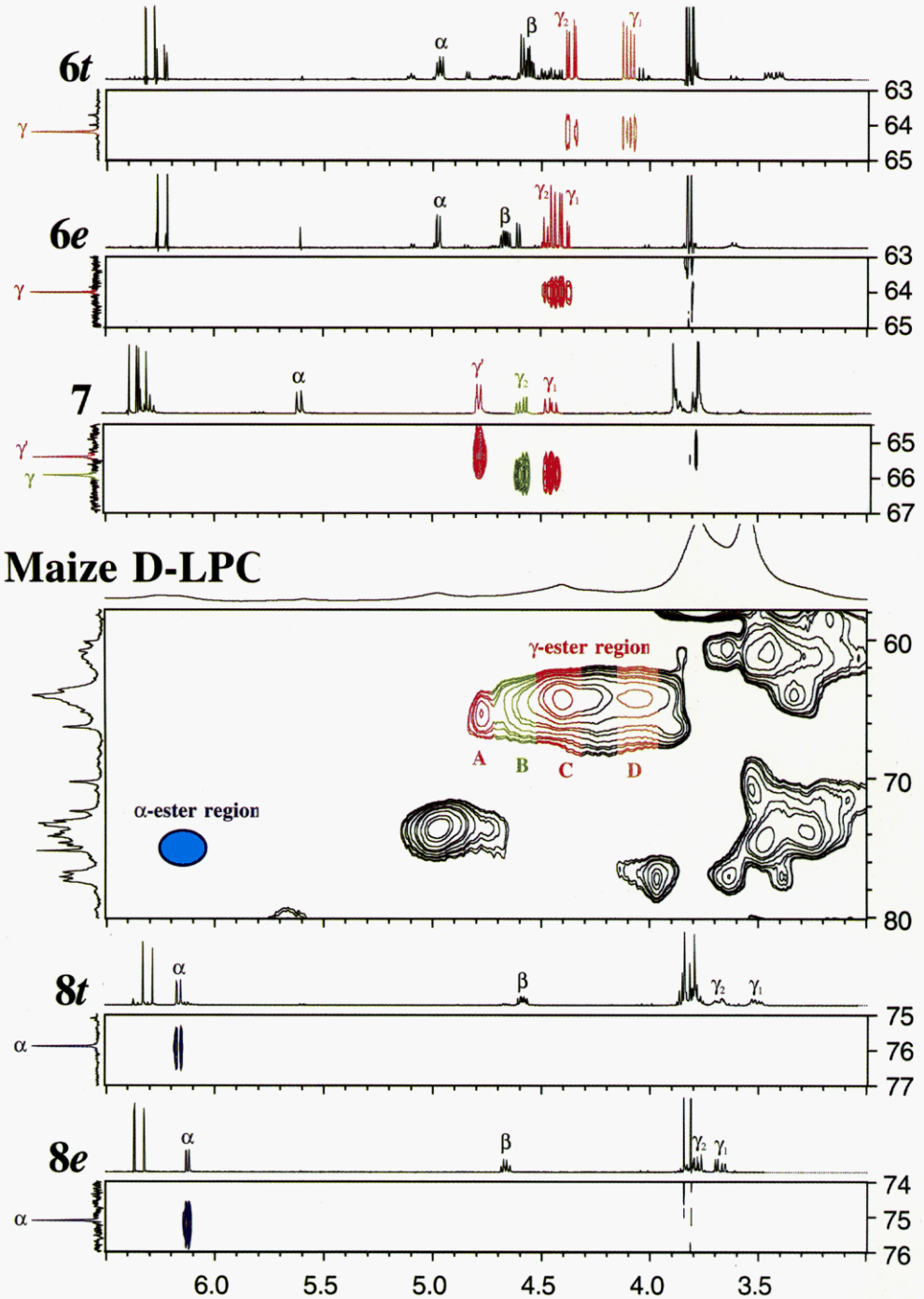

Figure 3. Partial HMQC spectrum (aliphatic regions) with ^{1}H and ^{13}C projections, of D-LPC maize lignin (central), with relevant sections of equivalent HMQC spectra of γ- (**6** and **7**) and α-ester (**8**) model compounds. Regions identified in the D-LPC correspond to the following: A, in magenta, cinnamyl *p*-coumarate end groups (γ'_C/γ'_{H}'s); B, in green, phenylcoumarans (γ_C/γ_{H2}); C, in red, β-aryl ethers (γ_C/γ_{H1} and γ_C/γ_{H2} in *erythro*-isomers, γ_C/γ_{H2} only in *threo*-isomers) and phenylcoumarans (γ_C/γ_{H1}); D, in orange, *threo*-β-aryl ethers (γ_C/γ_{H1}) [*e* = *erythro*, *t* = *threo*]. The models used here do not adequately cover the black region between regions C and D; correlations may result from syringyl analogs.

alcohol[44,45] with 4-acetoxycinnamoyl chloride, followed by deacetylation with $NaHCO_3$/methanol. [9-^{13}C]Coniferyl *p*-coumarate (**5b**), required to make the synthetic dehydrogenation lignin polymer (DHP) shown in Figure 5 was prepared by acylation of 4-(2,4-dinitrophenoxy)-3-methoxycinnamyl alcohol with 4-(2,4-dinitrophenoxy)cinnamoyl chloride.[29] The DHP was prepared using 9:1 coniferyl alcohol/[9-^{13}C]coniferyl *p*-coumarate by a method similar to that described previously.[14]

NMR Experiments. (a) Normal 1D ^{13}C NMR Spectrum. The proton-decoupled ^{13}C NMR spectra of the D-LPC lignin (Figure 2a) and its acetylated derivative (Figure 2b) are dominated by signals from *p*-coumarate esters. Table 1 lists the ^{13}C NMR chemical shifts of the *p*-coumarate ester carbons in the D-LPC lignin along with the corresponding shifts from model compounds **6–8** and their acetylated derivatives.[24,43] The chemical shifts observed in the D-LPC lignin are consistent with those of *p*-coumarate esters in which the phenolic hydroxyl is unetherified. This is confirmed by the displacement of the P-4, P-1, and P-3/5 signals observed upon acetylation. The relative sharpness of the peaks also indicates that the *p*-coumarate unit, despite its free phenolic hydroxyl group, has not been incorporated into the lignin structure and remains as a pendant, terminal group on the polymer. Other peaks in the spectra are typical of syringyl/guaiacyl lignin[46–49] with the exception of the high proportion of esterified

(44) Quideau, S.; Ralph, J. *Holzforschung* **1994**, *48*, 12–22.
(45) Quideau, S.; Ralph, J. *J. Agric. Food Chem.* **1992**, *40*, 1108–1110.

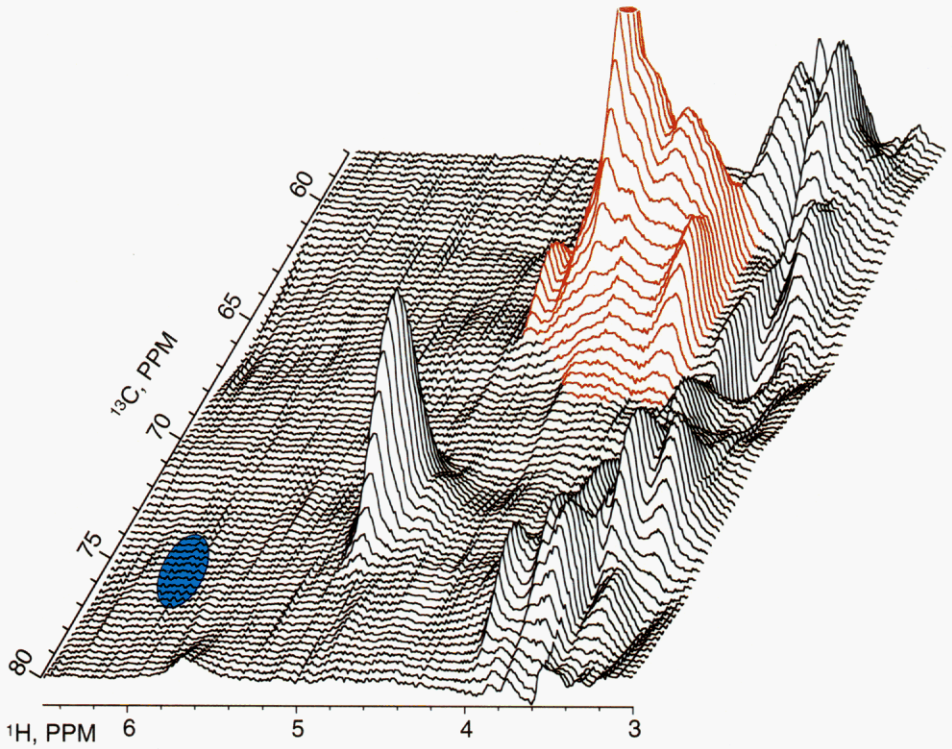

Figure 4. Stacked plot of the aliphatic region of the HMQC spectrum of D-LPC (same region as in Figure 3) showing the γ-ester correlations (red) and absence of α-ester correlations (blue region) above the noise level.

γ-positions.[46] [Syringyl and guaiacyl refer to 3,5-dimethoxy-4-hydroxyphenyl and 3-methoxy-4-hydroxyphenyl units, such as those in **2c** and **2b**, respectively]. Saponification at room temperature resulted in complete removal of *p*-coumarate ester resonances (Figure 2c). The dioxane–water insoluble E-LPC fraction was soluble in DMSO-d_6. However, the short relaxation times of the preparation, even after washing with EDTA, precluded application of 2D NMR to this sample. Nevertheless, its ^{13}C NMR spectrum showed it to be structurally similar to the soluble D-LPC fraction but with a lower *p*-coumarate ester content.

(b) One-Bond ^{13}C–^{1}H Correlation (HMQC). Application of the HMQC experiment[50] to the D-LPC sample unambiguously revealed the acylation regiochemistry. Inverse-detected experiments offer tremendously enhanced sensitivity over their normal-mode counterparts.[51] The central contour plot in Figure 3 shows a just-above-noise-level section (encompassing only the aliphatic region) of the HMQC experiment on the D-LPC sample. The huge correlation contours labeled A–D at (3.8–4.8, 62–68 ppm) are absent in lignin preparations with low *p*-hydroxycinnamate ester contents, e.g. milled wood or legume lignins, or saponified D-LPC. This contour cluster proves that the lignin acylation is at the γ-position but does not identify the nature of the ester. It may also contain minor contributions from carbohydrate primary alcohols and carbohydrates with acylated primary alcohol groups.[38,52] Chemical shifts of un-acylated lignin γ-protons are upfield in both the carbon and proton domain and are seen in the contour peak cluster to the upper right corner (3.0–3.8, 58–62 ppm) of the D-LPC spectrum in Figure 3. Authentication of these assignments is from γ-regions of the HMQC spectra (with high-resolution projections) of model esters **6** (*threo*- and *erythro*-isomers) and **7** (Figure 3, top three spectra). This region clearly indicates that γ-esters of β-aryl ethers (both isomers) and phenylcoumarans as well as cinnamyl *p*-coumarate end groups are all represented. Thus, the orange region labeled D of the contour peak in Figure 3 likely derives substantially from correlations in *threo*-β-aryl ethers (γ_C/γ_{H1}), red region C from β-aryl ethers (γ_C/γ_{H1} and γ_C/γ_{H2} in *erythro*-isomers, γ_C/γ_{H2} in *threo* isomers) and phenylcoumarans (γ_C/γ_{H1}), green region B from phenylcoumarans (γ_C/γ_{H2}), and magenta region A from cinnamyl *p*-coumarate end groups ($\gamma'_C/\gamma'_{H's}$, cf. in model **7**). Acylation therefore seems to be independent of the structural units involved. Equally important in the D-LPC HMQC spectrum is the complete absence of correlations in the esterified α-position region (ca. 6.1–6.2, 74–76 ppm), as indicated by the blue ellipse and the appropriate α-regions of HMQC spectra of α-ester models **8** (*threo*- and *erythro*-isomers, lower two spectra of Figure 3). This implies that acylation is overwhelmingly (if not exclusively) at the γ-position. No α-esters could be detected above the noise level in this region; this is particularly clear in the stacked plot of Figure 4 (blue region at high field). The HMQC experiment thus establishes that, within detectability limits, acylation is exclusively at the γ-position. The second phase is to establish that it is *p*-coumaric acid that is attached to the γ-position.

(c) Long-Range C–H Correlation (HMBC). Inverse-detected long-range C–H correlation (HMBC) correlates protons with carbons separated by two or three bonds.[53] Since these through-bond correlations span either carbon or oxygen atoms,[54] important connectivity from the ester moiety to the lignin skeleton can be established.[14,24,38,52,55,56] Figure 5 shows the section around the *p*-coumarate carbonyl carbon (P-9 in cinnamate residues) of the HMBC spectrum of the D-LPC sample along with corresponding sections from spectra of model compounds **6–8** and a synthetic dehydrogenation polymer (DHP) described below. The peaks

(46) Fernandez, N.; Mörck, R.; Johnsrud, S. C.; Kringstad, K. P. *Holzforschung* **1990**, *44*, 35–38.
(47) Scalbert, A.; Monties, B.; Guittet, E.; Lallemand, J. Y. *Holzforschung* **1986**, *40*, 119–127.
(48) Nimz, H. H.; Robert, D.; Faix, O.; Nemr, M. *Holzforschung* **1981**, *35*, 16–26.
(49) Himmelsbach, D. S.; Barton, F. E., II. *J. Agric. Food Chem.* **1980**, *28*, 1203–1208.
(50) Bax, A.; Subramanian, S. *J. Magn. Reson.* **1986**, *67*, 565–569.
(51) Sanders, J. K.; Hunter, B. K. *Modern NMR Spectroscopy. A Guide for Chemists*; 2nd ed.; Oxford University Press: Oxford, U.K., 1993; pp 314.
(52) Helm, R. F.; Ralph, J.; Hatfield, R. D. *Carbohydr. Res.* **1992**, *229*, 183–194.
(53) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.
(54) Ralph, J. *Holzforschung* **1988**, *42*, 273–275.
(55) Helm, R. F.; Ralph, J. *Carbohydr. Res.* **1993**, *240*, 23–38.
(56) Ralph, J.; Helm, R. F.; Quideau, S. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2971–2980.

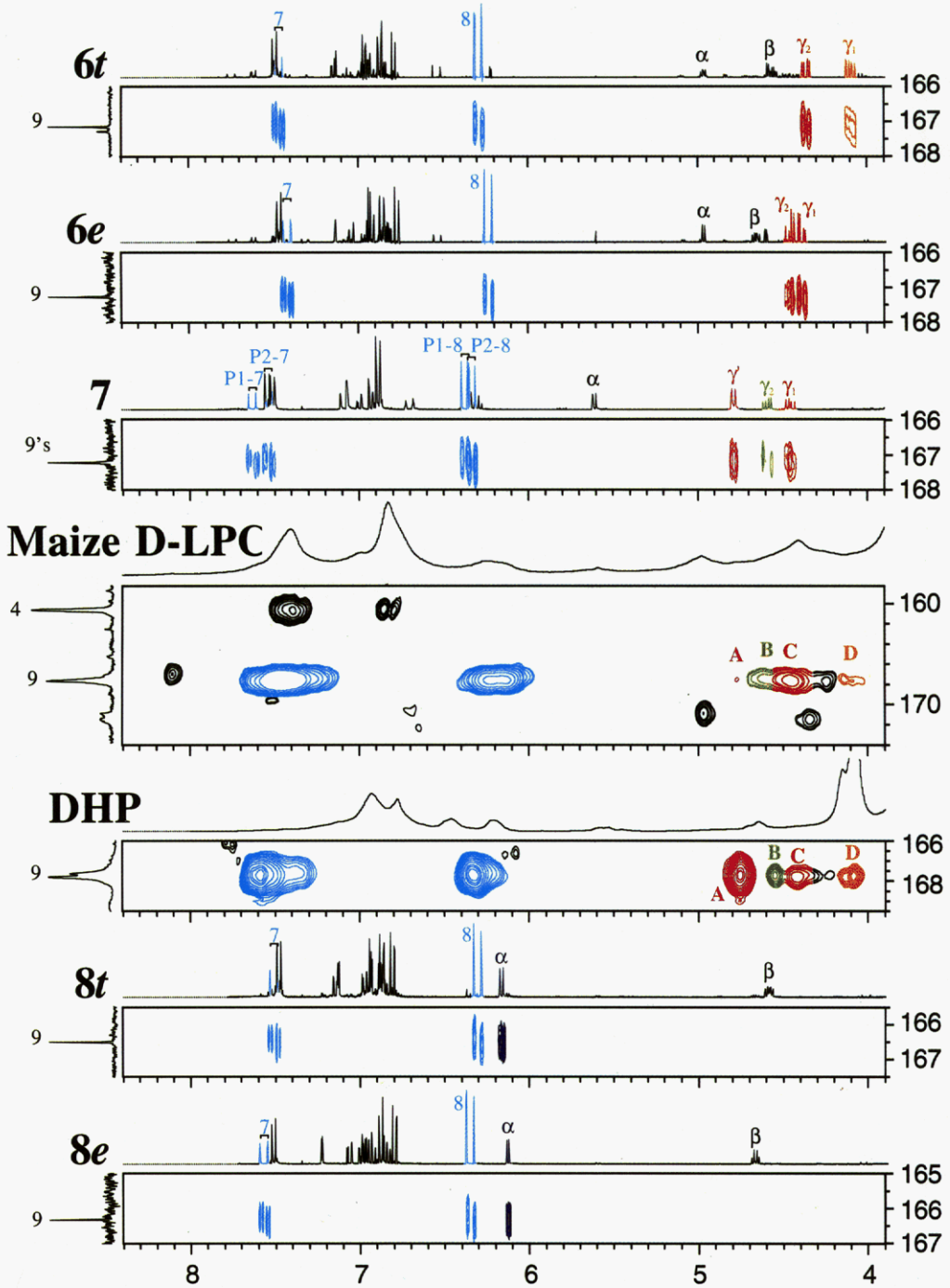

Figure 5. HMBC spectrum of D-LPC maize lignin, with appropriate sections of HMBC spectra of a coniferyl alcohol/coniferyl *p*-coumarate DHP (see text) and γ- (**6** and **7**) and α-ester (**8**) models. Regions labeled A–D correspond to correlations between the carbonyl P-9 carbon and the protons noted for the same regions in Figure 3.

correlating P-7 and P-8 protons of *p*-coumarate ester units with the ester carbonyl P-9 carbon are uninformative except to confirm that the double bond remains intact. However, correlations to protons at 4.0–4.8 ppm conclusively demonstrate that the carbonyl carbon of *p*-coumaric acid is within three bonds of the lignin γ-protons and thus that *p*-coumaric acid is esterified to the γ-position. Again, from the contour cluster labeled A–D in the D-LPC spectrum shown in the middle of Figure 5 and the model spectra shown above it, it is evident that predominant lignin units are well represented in these esterified structures including, from the small magenta correlation peak A at the left of the cluster, cinnamyl *p*-coumarate end groups (P2-9C/$\gamma'_{H's}$, cf. in model 7). It cannot be determined from the HMBC spectra alone that *p*-coumarate esters are not at the α-position since correlation peaks in this region are close to the intense peaks from P-9_C/P-8_H correlations (Figure 3, models 8). Thus, a combination of the HMQC and HMBC experiments is required to prove that *p*-coumaric acid is exclusively attached to the γ-position of the maize lignin isolate.

A comparison with the DHP spectrum reveals further important features. The DHP spectral section in Figure 5 is from a synthetic lignin polymer prepared by copolymerizing [9-^{13}C]coniferyl *p*-coumarate (**5b**) with coniferyl alcohol (**2b**). Incorporation was low due to the poor solubility of coniferyl *p*-coumarate. Although this DHP contains a large proportion of cinnamyl *p*-coumarate end groups (cf. model 7, region A), the characteristic coutours from esters of other subunits (regions B–D) indicate that this DHP has successfully modeled aspects of the *p*-coumaroylated lignin polymer from maize. As expected from related model studies,[14,44,56,57] the phenylcoumaran arising from β-5 coupling

of the coniferyl *p*-coumarate (at β) with lignin monomers or oligomers (at 5) is a predominant structure (contour peak B) and is more distinct than that in maize D-LPC. High proportions of phenylcoumarans typify oxidative dimerization of (*E*)-propenylphenols and early lignification.[44,58] More interesting is that the proportion of *threo*-β-aryl ethers (region D) is significantly higher than in maize lignin. This is a consequence of the presence of sinapyl alcohol (**2c**) during maize lignification. Guaiacylglycerol β-syringyl ethers are known[59,60] to be formed predominantly as their *erythro*-isomers which have contour peaks in region C. A further possibility is that, unlike the synthetic DHP made here from coniferyl alcohol (**2b**) and coniferyl *p*-coumarate (**5b**), sinapyl *p*-coumarate (**5c**) may also be involved in maize lignification.

Biochemical Implications. Because *p*-coumarate esters are exclusively at the γ-position of lignin side chains in maize rind lignin, and not at the α-position, the plant clearly uses an enzymatically controlled method of incorporating these esters. Free *p*-coumaric acid is apparently not present in the lignifying matrix; if present, it would likely become esterified to the α-position to some extent. No evidence for α-acylation could be detected here (Figure 4). Since *p*-coumaric acid is apparently laid down in the cell wall late in the lignification process,[22,61,62] as are syringyl units,[22,63-65] the possibility that sinapyl *p*-coumarate (**5c**) is an incorporated compound rather than, or in addition to, coniferyl *p*-coumarate (**5b**)[18] is under further investigation.

Another puzzling feature of *p*-coumaric acid attachment to lignin has escaped comment to date, *viz.* why the *p*-coumarate unit does not participate in oxidative coupling. The phenolic group on *p*-coumarate esters should be available for generation of phenoxy radicals by peroxidases for subsequent incorporation into lignin.[26] *p*-Coumaric acid and esters do in fact become incorporated into dehydrogenation polymers (DHPs). Such copolymer DHPs have been prepared using coniferyl alcohol (**2b**) and *p*-coumaric acid (**1a**),[18] coniferyl *p*-coumarate (**5b**),[34] or methyl 5-*O*-*trans*-*p*-coumaroyl α-L-arabinofuranoside (unpublished data). In each case, the *p*-coumaric acid could not be fully recovered by saponification, implying that it has been etherified or linked by carbon–carbon bonds at positions 3/5 or 8. *p*-Hydroxyphenyl units in pine compression wood lignin (a *p*-hydroxycinnamyl alcohol/coniferyl alcohol copolymer) are almost exclusively terminal units with free phenolic groups.[66] However, they are part of the lignin and are released by thioacidolysis, implying that they are incorporated into β-aryl ether structures. The fact that *p*-coumarate esters are not incorporated further into the lignin complex by phenol oxidation may be a result of the time course of lignification.[22] Some of Miksche's earlier and unpublished work[67] revealed that *p*-hydroxyphenyl units polymerize readily with guaiacyl units (derived from lignin monomer **2b**) but not with syringyl units (derived from lignin monomer **2c**). If coniferyl *p*-coumarate (**5b**) or sinapyl *p*-coumarate (**5c**) incorporation occurs when the only accessible polymerization sites are from syringyl units, the *p*-coumarate units would remain untouched. This suggests that, in maize lignin, syringyl units and *p*-coumarate esters may be laid down late in lignification, a phenomenon demonstrated in other systems.[22,61]

Conclusions

NMR reveals that maize rind lignin is a syringyl/guaiacyl copolymer with substantial amounts of *p*-coumarate esters attached exclusively at the γ-position. This strict regiochemistry implicates involvement of pre-acylated *p*-hydroxycinnamyl alcohols (*p*-hydroxycinnamyl *p*-coumarates) as substrates in the lignification process. Since this mechanism, unlike that required for production of α-esters, represents an enzymatically regulated biochemical process, there is the possibility for altering *p*-coumarate ester content by genetic manipulation either through traditional selection and plant breeding or through the application of molecular genetics. The observation that the *p*-coumaroyl moieties retain their phenolic group suggests that the *p*-coumarate ester monomers are incorporated into the lignin late in the process concurrent with, or subsequent to, incorporation of substantial amounts of syringyl lignin. The role for such high quantities of *p*-coumarate esters in maize lignins remains unexplained but is likely to involve defense mechanisms.[68]

Experimental Section

Melting points are uncorrected. Evaporations were conducted under reduced pressure at temperatures less than 42 °C unless otherwise noted. Solutions in organic solvents were dried with sodium sulfate and filtered before evaporation. Further elimination of organic solvents as well as drying of the residues was accomplished under high vacuum (10–14 N m^{-2}) at room temperature. Column chromatography was performed with silica gel 60 (230–400 mesh), and thin-layer chromatography was done on silica gel 60-F254 plates (Merck). NMR spectra of samples in acetone-d_6 or 9:1 acetone-d_6/D_2O were run at 300 or 310 K on a Bruker AMX-360 narrow-bore instrument fitted with a 10 mm dual probe ($^{13}C/^{1}H$) with normal geometry (proton coil further from the sample). The central solvent signals were used as an internal reference (^{1}H, 2.04 ppm; ^{13}C, 29.8 ppm). The maize D-LPC spectrum was run using 275 mg of EDTA-washed D-LPC lignin in 2.4 mL of 9:1 acetone-d_6/D_2O in a 10 mm tube. The acetylated spectrum (Figure 2b) used 500 mg in 2.4 mL of acetone-d_6. The HMQC spectrum of the maize D-LPC lignin (central plot of Figure 3, and Figure 4) was run using Bruker's invbtp[50] microprogram using 320 scans over 128 increments of 2K data points. Processing with cosine-bell squared apodization (Q2) in both dimensions and zero-filling to a final real matrix size of 1K × 1K resulted in digital resolutions of 3.37 and 12.2 Hz/pt in F_2 (^{1}H) and F_1 (^{13}C), respectively. Complementary spectra of model compounds requried for Figure 3 were obtained in an analogous fashion but with 8 scans per increment. The inverse long-range C–H correlation (HMBC) spectrum of maize D-LPC in Figure 5 was run using Bruker's standard inv4lplrnd sequence[53] incorporating a low-pass filter and no carbon decoupling, with 2K data points in the proton dimension and 256 increments in the carbon dimension, using 1048 scans per increment. The 90° pulse angles were 26 and 13.4 μs for ^{1}H (observe) and ^{13}C (decouple), respectively, and the long-range coupling delay was 80 ms (corresponding to optimal response for a long-range C–H coupling constant of 6.25 Hz and representing a compromise between optimal responses based on *J*'s, as low as 3.3 Hz, and the short T_1's of the sample). Optimized Gaussian apodization was applied in F_2 and unshifted squared sine-bell (Q0) apodization was applied in F_1 and the matrix zero-filled and Fourier transformed (using magnitude mode phase correction) to give a final matrix of 1K × 1K real points, resulting in digital resolutions of 3.6 and 18.1 Hz/pt. The HMBC spectrum of the DHP shown in Figure 5 was run in an analogous fashion using 100 mg of DHP (preparation described below) using 256 scans per increment. Complementary spectra of model compounds required for Figure 5 were obtained in an analogous fashion but with 64 scans per increment. Total carbohydrate was measured in samples using the phenol–sulfuric acid method[69] for neutral sugars and the 3-phenylphenol method for total

(57) Iiyama, K.; Kasuya, N.; Lam, T. B. T.; Stone, B. A. *J. Sci. Food Agric.* **1991**, *56*, 551–560.
(58) Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. *J. Chem. Soc., Perkin Trans. 1* **1994**, in press.
(59) Brunow, G.; Karlsson, O.; Lundquist, K.; Sipila, J. *Wood Sci. Technol.* **1993**, *27*, 281–286.
(60) Hauteville, M.; Lundquist, K.; Von Unge, S. *Acta Chem. Scand., Ser. B* **1986**, *B40*, 31–35.
(61) He, L.; Terashima, N. *Mokuzai Gakkaishi* **1989**, *35*, 123–129.
(62) He, L.; Terashima, N. *J. Wood Chem. Technol.* **1990**, *10*, 435–459.
(63) Terashima, N.; Fukushima, K. *Wood Sci. Technol.* **1988**, *22*, 259–270.
(64) Terashima, N.; Fukushima, K.; Tsuchiya, S.; Takabe, K. *J. Wood Chem. Technol.* **1986**, *6*, 495–504.
(65) Terashima, N.; Kukushima, K.; Takabe, K. *Holzforschung* **1986**, *40*, 101–105.
(66) Lapierre, C.; Rolando, C. *Holzforschung* **1988**, *42*, 1–4.
(67) Brunow, G.; Sipila, J.; Syrjanen, K. In *Anselme Payen Award Symposium*, National Meeting of the American Chemical Society, Denver, CO, 1993; American Chemical Society: Washington, DC, 1993; p Cell-3.
(68) Woodhead, S.; Cooper-Driver, G. *Biochem. Syst. Ecol.* **1979**, *7*, 309–310.
(69) Dubois, M.; Giles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.

uronosyl residues.[70] Sugar composition and lignin concentrations of cell walls and isolated fractions were determined using a modified Saeman hydrolysis.[71] Briefly, 40–100 mg of sample was suspended in 1.5 mL of 12 M H_2SO_4 followed by dilution to 1.6 M H_2SO_4 and hydrolysis at 100 °C for 3 h. Subsamples (100 μL) were removed prior to the 3 h heating for uronosyl assays. Inositol was added as an internal standard before cooled samples were filtered through preweighed glass fiber filters. The insoluble lignin residues were throughly washed with distilled water before drying for 48 h at 55 °C. Aliquots of the hydrolysates were neutralized with $BaCO_3$ and clarified by centrifugation and filtering, and 1–2 mL portions were dried under a stream of filter air. Neutral sugar compositions of the hydrolysates were analyzed by GLC as their alditol acetates using the procedure of Blakeney et al.[72]

Isolation of Maize Lignin Fractions. Plant materials were obtained from the Agronomy Department, University of Wisconsin—Madison, from field grown maize (*Zea mays* L.) inbreds harvested when plants had senesced and dried in the field. Several different inbreds were screened for high *p*-coumaric acid content using pyrolysis–GC–MS as previously described.[37] Samples with similar high concentrations were pooled and used for these experiments. Internode sections were isolated from maize stem samples and split longitudinally, and the pith region was scraped away from the outer rind region. These rind regions were ground through a Wiley mill (2 mm screen), then through a Udy cyclone mill (1 mm screen) before isolation of cell wall material. Cell walls were prepared using a modified Uppsala procedure.[73] Briefly, ground samples (20–25 g) were weighed into 250 mL centrifuge bottles, suspended in 80% ethanol (200 mL), and sonicated for 20 min. Bottles were centifuged (1500 *g*, 10 min), and the alcohol extract was decanted through a glass fiber filter. The ethanol extraction was repeated four times before the solvent was changed to acetone (2×), then to chloroform/methanol (2:1, 1X), and back to acetone (1×). Wall materials were allowed to air-dry for 24 h to completely remove the acetone before they were suspended in acetate buffer (20 mM acetate, pH 5.5) and heated to 90–95 °C for 2 h. The wall suspension was cooled to 55 °C, α-amylase (10 U/g CW, Sigma A3403) and amyloglucosidase (2U/g CW, Fluka 10113) were added, and the bottles were incubated at 55 °C for 3 h. At the end of 3 h, the bottles were centrifuged (1500 *g*) for 20 min and the supernatant was decanted through a glass fiber filter (2 μm). Wall residues were briefly washed with acetate buffer (2×), 80% ethanol (1×), and acetone (1×) before they were air-dried. Samples were stored in a vacuum over P_2O_5 for at least 48 h before they were ball milled using a stainless steel vibratory ball mill for 24 h.

Cellulysin was tested to be free of hydroxycinnamoyl esterases.[24,38] Milled cell walls (100 g in 250 mL centrifuge bottles, 25 g/bottle) were suspended in 20 mM acetate buffer (pH 5.0, 100 mL/bottle) and treated with Cellulysin[39,40] (Calbiochem) for 96 h with fresh enzyme and buffer being added after 24 and 48 h of incubation at 30 °C. Cellulysin solution was prepared by stirring 1 g of powder into 10 mL of acetate buffer. After being stirred for 30 min at room temperature, the mixture was centrifuged (1500 *g*, 20 min) to pellet insoluble material. Equal volumes (2.4 mL) of the clarified Cellulysin solution were added to each wall sample. Fresh enzyme was prepared each time it was added to the wall preparations. Progress of wall digestion was monitored by removing a small aliquot (500 μL) at regular time intervals, microfuging (30 s, 10 000 *g*), removing 200 μL of the clarified supernatant and resuspending the residue in fresh buffer before returning the residue to the incubation bottle. The aliquot was analyzed for total sugars[69] to determine the concentration of released sugars. This provided a convenient method for monitoring the rate and concentration of sugars released from cell wall residues and for determining when degradation was complete. The resulting lignin polysaccharide complex (LPC, 33.5 g) comprised all the lignin and ca. 20% polysaccharides. The LPC was subjected to fractionation in 96:4 dioxane/water (standard "milled wood lignin" conditions[41]), which gave a soluble fraction and an insoluble residue (E-LPC, 51% of LPC, 36% Klason lignin). The freeze-dried soluble fraction was resuspended in water, pressure-filtered through a 2-μm nylon filter, washed with warm water to remove saccharides, then with 3 mM EDTA (pH 8.0) to remove metal ion contaminants and improve relaxation times of nuclei in the sample, and with water again, and freeze-dried to yield the final material for NMR analyses (D-LPC, 42% of LPC, 81% Klason lignin).

Determination of *p*-Coumarate. *p*-Coumaric acid (and ferulic acid) was released from walls and lignin fractions (10–20 mg) by saponification with NaOH (2 M, 1.25 mL) at room temperature for 20 h under N_2. Samples were acidified with excess 12 M HCl and extracted with ether. Dried extracts were silylated with BSTFA (25 μL) and pyridine (25 μL) for 30 min at 60 °C. Trimethylsilylated derivatives were quantified by GC (Perkin Elmer 8500) using a 0.25 mm × 30 m DB-1 (J&W Scientific) column and a flame ionization detector with He as a carrier gas (0.7 mL/min, 40:1 split ratio). The column temperature was held at 170 °C for 1 min and then increased to 280 °C at 10 °C min^{-1}. The injector and detector were set at 325 °C. The surprisingly high content of *p*-coumaric acid was also confirmed by a large-scale saponification. Thus, maize D-LPC (672 mg) was stirred under nitrogen with degassed NaOH (2 M, 40 mL) overnight. Complete dissolution of the lignin was almost immediate. Following acidification with excess 3 M HCl, the precipitated lignin residue was filtered off through a 2 μm nylon filter, under pressure, and washed with warm water to solubilize the *p*-coumaric acid. The solution was first extracted into methylene chloride to give a yellow oil containing all of the ferulic acid, other unidentified components, and a small amount of *p*-coumaric acid (24 mg, 3.6%). Extraction into ethyl acetate gave an almost pure (by NMR) fraction of *p*-coumaric acid which crystallized spontaneously upon solvent removal (125.5 mg, 18.7%). A crude estimate of 20% *p*-coumaric acid was also gained from quantitative ^{13}C NMR.

Model Compounds. Conifery alcohol was prepared as previously reported.[45] Model compounds **6** and **8** were prepared as described elsewhere.[24] NMR data are given in the same reference, and full data in three solvents appear in the recently released Plant Cell Wall NMR Database.[43]

Preparation of [9-^{13}C]-*p*-Coumaric Acid. Preparation from *p*-hydroxybenzaldehyde and ethyl diethylphosphono[1-^{13}C]acetate was completely analogous to that recently described for [9-^{13}C]-labeled ferulic acid.[14] The product was columned using 1:1 EtOAc/$CHCl_3$ containing 1% HOAc and crystallized from water. ^1H NMR: δ 6.32 (1H, dd, $J_{P\text{-}8/P\text{-}7}$ = 15.95 Hz and $J_{P\text{-}8/^{13}C\text{-}9}$ = 2.7 Hz, P-8), 6.88 (2H, m, P-3/5), 7.53 (2H, m, P-2/6), 7.59 (1H, dd, $J_{P\text{-}7/P\text{-}8}$ = 15.95 Hz and $J_{P\text{-}7/^{13}C\text{-}9}$ = 6.9 Hz, P-7).

Preparation of [9-^{13}C]Coniferyl *p*-Coumarate (5b). Phenol protection of both coniferyl alcohol and [9-^{13}C]-*p*-coumaric acid by treatment with 2,4-dinitrofluorobenzene and aqueous sodium bicarbonate, and generation of the chloride of the 2,4-dinitrophenyl derivative of [9-^{13}C]-*p*-coumaric acid by treatment with thionyl chloride, were accomplished as described by Nakamura and Higuchi for their synthesis of coniferyl *p*-coumarate.[29]

Coniferyl alcohol 2,4-dinitrophenyl ether [4-(2,4-dinitrophenoxy)-3-methoxycinnamyl alcohol] (1.86 mmol, 81%) was isolated as pale yellow fine crystals from acetone, mp 131.5–133.5 °C (lit[29] 149–150 °C). ^1H NMR: δ 3.82 (3H, s, A3-OMe), 3.91 (1H, t, *J* = 5.5 Hz, γ-CH_2OH), 4.26 (2H, td, *J* = 5.3, 1.6 Hz, γ's), 6.49 (1H, dt, *J* = 15.9, 5.0 Hz, β), 6.67 (1H, dt, *J* = 15.9, 1.6 Hz, α), 7.09 (1H, d, *J* = 9.3 Hz, DNP-6), 7.14 (1H, dd, *J* = 8.2, 1.9 Hz, A-6), 7.25 (1H, d, *J* = 8.2 Hz, A-5), 7.32 (1H, d, *J* = 1.9 Hz, A-2), 8.41 (1H, dd, *J* = 9.3, 2.8 Hz, DNP-5), 8.84 (1H, d, *J* = 2.8 Hz, DNP-3).

[9-^{13}C]-*p*-Coumaric acid 2,4-dinitrophenyl ether (0.62 mmol, 93% yield) was isolated as off-white fine crystals from hot acetone, mp 256.4–257.4 °C (lit.[29] 247–248 °C). ^1H NMR: δ 6.55 (1H, dd, $J_{P\text{-}8/P\text{-}7}$ = 16.0 Hz and $J_{P\text{-}8/^{13}C\text{-}9}$ = 2.6 Hz, P-8), 7.33 (2H, m, P-3/5), 7.38 (1H, d, *J* = 9.3 Hz, DNP-6), 7.71 (1H, dd, $J_{P\text{-}7/P\text{-}8}$ = 16.0 Hz and $J_{P\text{-}7/^{13}C\text{-}9}$ = 6.9 Hz, P-7), 7.86 (2H, m, P-2/6), 8.52 (1H, dd, *J* = 9.3, 2.8 Hz, DNP-5), 8.89 (1H, d, *J* = 2.8 Hz, DNP-3).

[9-^{13}C]-*p*-Coumaroyl chloride 2,4-dinitrophenyl ether (0.31 mmol, quantitative yield) was isolated as a yellow oil. ^1H NMR: δ 6.64 (1H, dd, $J_{P\text{-}8/P\text{-}7}$ = 15.6 Hz and $J_{P\text{-}8/^{13}C\text{-}9}$ = 5.2 Hz, P-8), 7.16 (1H, d, *J* = 9.2 Hz, DNP-6), 7.19 (2H, m, P-3/5), 7.69 (2H, m, P-2/6), 7.83 (1H, dd, $J_{P\text{-}7/P\text{-}8}$ = 15.6 Hz and $J_{P\text{-}7/^{13}C\text{-}9}$ = 8.8 Hz, P-7), 8.39 (1H, dd, *J* = 9.2, 2.7 Hz, DNP-5), 8.88 (1H, d, *J* = 2.7 Hz, DNP-3).

Coniferyl alcohol 2,4-dinitrophenyl ether (117.5 mg, 0.34 mmol) was dissolved in CH_2Cl_2 (2 mL, dried by passage through alumina). Triethylamine (70 μL, 0.50 mmol) and 4-(dimethylamino)pyridine (8 mg, 0.07 mmol) were successively added to give a rust-colored solution, which was then added dropwise under nitrogen to a stirred suspension of [9-^{13}C]-*p*-coumaroyl chloride 2,4-dinitrophenyl ether (107.7 mg, 0.31 mmol) in dry CH_2Cl_2 cooled in an ice–water bath. The ice–water bath was removed, and stirring was continued at room temperature for 1 h, after which time the reaction mixture was diluted with CH_2Cl_2 and

(70) Blumenkratz, N.; Asboe-Hansen, G. *Anal. Biochem.* **1973**, *54*, 484–489.
(71) Saeman, J. F.; Moore, W. E.; Millet, M. A. In *Cellulose*; Whistler, R. L., Ed.; Academic Press: New York, 1963; Vol. 3, pp 54–69.
(72) Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. *Carbohydr. Res.* **1983**, *113*, 291–299.
(73) Theander, O.; Westerlund, E. A. *J. Agric. Food Chem.* **1986**, *34*, 330–336.

successively washed with water, 0.5 M hydrochloric acid, and saturated aqueous NaCl. Standard processing gave a yellow foam, which readily crystallized from acetone to afford the bis(2,4-dinitrophenyl) ether of coniferyl *p*-coumarate as pale yellow fine crystals (106.6 mg, 52.5%). The mother liquor was concentrated, and the residue was subjected to silica gel chromatography [$CHCl_3$/EtOAc (9:1)] to give an additional 57.4 mg of the desired bis(2,4-dinitrophenyl) ether (81% total yield), mp 164.5–167.4 °C (lit.[29] 171–172 °C). ^{1}H NMR: δ 3.83 (3H, s, A3-OMe), 4.89 (2H, ddd, $J_{\beta\gamma}$ = 6.2, $J_{\gamma/^{13}C\text{-}9}$ = 3.4 Hz and $J_{\alpha\gamma}$ = 1.4 Hz, γ's), 6.55 (1H, dt, J = 15.9, 6.2 Hz, β), 6.64 (1H, dd, $J_{P\text{-}8/P\text{-}7}$ = 16.0 Hz and $J_{P\text{-}8/^{13}C\text{-}9}$ = 2.4 Hz, P-8), 6.83 (1H, dt, J = 15.9, 1.4 Hz, α), 7.10 (1H, d, J = 9.3 Hz, DNP-6/A), 7.21 (1H, dd, J = 8.2, 1.9 Hz, A-6), 7.29 (1H, d, J = 8.2 Hz, A-5), 7.34 (2H, m, P-3/5), 7.39 (1H, d, J = 9.3 Hz, DNP-6/P), 7.42 (1H, d, J = 1.9 Hz, A-2), 7.78 (1H, dd, $J_{P\text{-}7/P\text{-}8}$ = 16.0 Hz and $J_{P\text{-}7/^{13}C\text{-}9}$ = 6.9 Hz, P-7), 7.89 (2H, m, P-2/6), 8.41 (1H, dd, J = 9.3, 2.8 Hz, DNP-5/A), 8.52 (1H, dd, J = 9.2, 2.8 Hz, DNP-5/P), 8.84 (1H, d, J = 2.8 Hz, DNP-3/A), 8.89 (1H, d, J = 2.8 Hz, DNP-3/P).

Removal of the 2,4-dinitrophenyl protecting groups (0.23 mmol) was then accomplished by treatment with piperazine in refluxing benzene as previously described.[29] Purification by silica gel chromatography [$CHCl_3$/EtOAc (9:1)] gave [9-^{13}C]coniferyl *p*-coumarate (**5b**) (60.3 mg, 80%) as a syrup, which was crystallized from $CHCl_3$/light petroleum, mp 102–104 °C (lit.[29] 69.5–70.5 °C). ^{1}H NMR: δ 3.86 (3H, s, A3-OMe), 4.79 (2H, ddd, $J_{\beta\gamma}$ = 6.5, $J_{\gamma/^{13}C\text{-}9}$ = 3.3 Hz and $J_{\alpha\gamma}$ = 1.3 Hz, γ's), 6.25 (1H, dt, J = 15.8, 6.5 Hz, β), 6.37 (1H, dd, $J_{P\text{-}8/P\text{-}7}$ = 16.0 Hz and $J_{P\text{-}8/^{13}C\text{-}9}$ = 2.5 Hz, P-8), 6.65 (1H, dt, J = 15.8, 1.3 Hz, α), 6.80 (1H, d, J = 8.1 Hz, A-5), 6.87–6.93 (3H, m, P-3/5 and A-6), 7.10 (1H, d, J = 1.9 Hz, A-2), 7.53 (2H, m, P-2/6), 7.64 (1H, dd, $J_{P\text{-}7/P\text{-}8}$ = 16.0 Hz and $J_{P\text{-}7/^{13}C\text{-}9}$ = 6.8 Hz, P-7). ^{13}C NMR: δ 56.30 (A3-OMe), 65.48 ($J_{\gamma/P\text{-}9}$ = 2.3 Hz, γ), 110.33 (A-2), 115.58 ($J_{P\text{-}8/P\text{-}9}$ = 76.6 Hz, P-8), 115.83 (A-5), 116.70 (P-3/5), 121.20 (A-6), 121.82 ($J_{\beta/P\text{-}9}$ = 1.8 Hz, β), 127.04 ($J_{P\text{-}1/P\text{-}9}$ = 7.3 Hz, P-1), 129.49 (A-1), 130.88 (P-2/6), 134.90 (α), 145.45 ($J_{P\text{-}7/P\text{-}9}$ = 1.7 Hz, P-7), 147.76 (A-4), 148.50 (A-3), 160.53 (P-4), 167.27 (m, P-9).

Coniferyl Alcohol/[9-^{13}C]Coniferyl *p*-Coumarate DHP. Coniferyl alcohol (**2b**) (457 mg, 2.54 mmol) and [9-^{13}C[coniferyl *p*-coumarate (**5b**) (51 mg, 0.15 mmol) were dissolved in 1,4-dioxane (20 mL) and added with stirring to 200 mL of phosphate buffer (0.01 M, pH 6.6, degassed), containing 351 units of horseradish peroxidase (EC 1.11.1.7, Type II, Sigma). A second solution, containing commercial hydrogen peroxide (250 μL of 30% solution), was prepared in 220 mL of phosphate buffer. The two solutions were simultaneously added with stirring, at room temperature, to 100 mL of phosphate buffer. The additions were accomplished using a double-channel Masterflex peristaltic pump, at the rate of 3 mL/h. The reaction mixture was kept in the dark. Some [9-^{13}C]-coniferyl *p*-coumarate (**5b**), which had precipitated out, was redissolved in 1,4-dioxane (5 mL) and added to the reaction mixture at the same rate as previously. After additions were complete, 351 units of horseradish peroxidase were again added to the reaction mixture, which was left stirring for ca. 70 h. The resulting brownish suspension was then filtered through nylon 6,6 membrane (2 μm) and thoroughly washed with distilled water. The insoluble DHP polymer was taken up in distilled water and freeze-dried to give an amorphous beige powder (345 mg, 68%).

Preparation of Compound 7. The γ-ester model compound **7** was synthesized from the phenylcoumaran β-5-coupled dehydrodiconiferyl alcohol, which was prepared as previously described.[44] Phenol methylation of this dehydrodiconiferyl alcohol was performed with diazomethane. Acylation of the methylated dehydrodiconiferyl alcohol (16.4 mg, 0.0044 mmol) was then accomplished with 4-acetoxycinnamoyl chloride (25 mg, 0.111 mmol, freshly prepared as previously described)[52] using the procedure described for the preparation of **5b**. Processing and purification by PLC [$CHCl_3$/EtOAc (1:1)] gave the acetylated derivative of γ-ester **7** (20.9 mg, 64%, not opitmized). ^{1}H NMR: δ 2.26 (6H, s, 2 × $OCOCH_3$), 3.76 (6H, s, A3-OMe and A4-OMe), 3.88 (3H, s, B3-OMe), 3.88 (1H, m, β), 4.48 (1H, dd, J = 11.1, 7.5 Hz, γ_1), 4.61 (1H, dd, J = 11.1, 5.4, Hz, γ_2), 4.81 (2H, dd, J = 6.5, 1.3 Hz, γ's), 5.61 (1H, d, J = 7.2 Hz, α), 6.32 (1H, dt, J = 15.8, 6.5 Hz, β'), 6.50 (1H, d, J = 16.0 Hz, P2-8), 6.54 (1H, d, J = 16.0 Hz, P1-8), 6.71 (1H, dt, J = 15.8, 1.3 Hz, α'), 6.93 (1H, d, J = 8.2 Hz, A-5), 7.00 (1H, dd, J = 8.2, 2.0 Hz, A-6), 7.08 (2H, br d, J = 2.0 Hz, A-2 and B-2), 7.11 (1H, br s, B-6), 7.16–7.21 (4H, m, P1-3/5 and P2-3/5), 7.58 (1H, d, J = 16.0 Hz, P2-7), 7.68 (2H, m, P2-2/6), 7.70 (1H, d, J = 16.0 Hz, P1-7), 7.72 (2H, m, P1-2/6). ^{13}C NMR: δ 20.94 (2 × $OCOCH_3$), 51.21 (β), 56.08 (A3-OMe), 56.14 (A4-OMe), 56.41 (B3-OMe), 65.71 (γ'), 66.15 (γ), 89.17 (α), 110.97 (A-2), 112.28 (B-2), 112.63 (A-5), 116.35 (B-6), 119.53 (A-6), 122.17 (β'), 129.17 (B-5), 131.47 (B-1), 134.41 (A-1), 134.99 (α'), 145.42 (B-3), 149.44 (B-4), 150.45 (A-3), 150.50 (A-4), 169.45 (2 × $OCOCH_3$). Shifts of *p*-coumaroyl moieties are reported in Table 1.

Deacetylation was performed with aqueous saturated sodium bicarbonate in methanol/water (1:1) (2 mL). A few drops of acetone were added for complete dissolution, and the solution was stirred overnight at room temperature, then diluted with EtOAc, washed with aqueous NH_4Cl, and processed to give **7** as an oil in quantitative yield. ^{1}H NMR: δ 3.77 (6H, s, A3-OMe and A4-OMe), 3.88 (3H, s, B3-OMe), 3.85 (1H, m, β), 4.45 (1H, dd, J = 11.1, 7.6 Hz, γ_1), 4.58 (1H, dd, J= 11.1, 5.4 Hz, γ_2), 4.78 (2H, dd, J = 6.5, 1.2 Hz, γ's), 5.61 (1H, d, J = 7.1 Hz, α), 6.31 (1H, dt, J = 15.8, 6.5 Hz, β'), 6.33 (1H, d, J = 15.9 Hz, P2-8), 6.37 (1H, d, J = 15.9 Hz, P1-8), 6.70 (1H, dt, J = 15.8, 1.2 Hz, α'), 6.88 (4H, m, P1-3/5 and P2-3/5), 6.93 (1H, d, J = 8.3 Hz, A-5), 7.00 (1H, dd, J = 8.3, 2.0 Hz, A-6), 7.07 (2H, br d, J = 2.0 Hz, A-2 and B-2), 7.11 (1H, br s, B-6), 7.51 (2H, m, P2-2/6), 7.53 (1H, d, J = 15.9 Hz, P2-7), 7.54 (2H, m, P1-2/6), 7.63 (1H, d, J = 15.9 Hz, P1-7). ^{13}C NMR: δ 51.33 (β), 56.09 (A3-OMe), 56.13 (A4-OMe), 56.41 (B3-OMe), 65.42 (γ'), 65.92 (γ), 89.15 (α), 110.93 (A-2), 112.25 (B-2), 112.65 (A-5), 116.36 (B-6), 119.44 (A-6), 122.40 (β'), 129.21 (B-5), 131.51 (B-1), 134.52 (A-1), 134.78 (α'), 145.42 (B-3), 149.42 (B-4), 150.43 (A-3), 150.51 (A-4). Shifts of *p*-coumaroyl moieties are reported in Table 1.

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