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Hydroxycinnamate Conjugates as Potential Monolignol Replacements: In vitro Lignification and Cell Wall Studies with Rosmarinic Acid

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The plasticity of lignin biosynthesis should permit the inclusion of new compatible phenolic monomers, such as rosmarinic acid (RA) and analogous catechol derivatives, into cell-wall lignins that are consequently less recalcitrant to biomass processing. In vitro lignin polymerization experiments revealed that RA readily underwent peroxidase-catalyzed copolymerization with monolignols and lignin oligomers to form polymers with new benzodioxane inter-unit linkages. Incorporation of RA permitted extensive depolymerization of synthetic lignins by mild alkaline hydrolysis, presumably by cleavage of ester intra-unit linkages within RA. Copolymerization of RA with monolignols into maize cell walls by in situ peroxidases significantly enhanced alkaline lignin extractability and promoted subsequent cell wall saccharification by fungal enzymes. Incorporating RA also improved cell wall saccharification by fungal enzymes and by rumen microflora even without alkaline pretreatments, possibly by modulating lignin hydrophobicity and/or limiting cell wall cross-linking. Consequently, we anticipate that bioengineering approaches for partial monolignol substitution with RA and analogous plant hydroxycinnamates would permit more efficient utilization of plant fiber for biofuels or livestock production.

Introduction

Lignins are aromatic cell wall polymers produced by the oxidative polymerization of monolignols, principally coniferyl alcohol (CA) and sinapyl alcohol (SA) with typically minor amounts of *p*-coumaryl alcohol (PA), Figure 1a. Lignins are most abundant in vessels, tracheids, and fibrous tissues in vascular plants, where they bind, strengthen, and waterproof cell walls to provide mechanical support, enhance water transport, and help ward off plant pests. The biosynthesis and bioengineering of lignin, and its chemical and mechanical properties, have attracted significant research attention because lignin hinders agro-industrial processes such as chemical pulping of woody crops, forage digestion by livestock, and the conversion of lignocellulosic plant biomass into liquid biofuels.[1–7]

Extensive studies examining the biosynthesis and structure of lignin have revealed the substantial plasticity of lignin biosynthesis.[1,5,6,8–11] Perturbations of the monolignol pathway can lead to massive structural changes in the polymer due to incorporation of pathway intermediates and other phenolic compounds.[12–16] The malleability of lignification is further illustrated by the fact that various γ-acylated monolignols[17–20] and ferulate arabinoxylan esters are integral components of lignin in many plant species.[21,22] All these data support the well-accepted hypothesis that lignin polymerization results from a radical coupling process that is under simple chemical control.[10,13,23]

The plasticity of lignification greatly facilitates the 'design' of lignin polymers that are more amenable to processing, through the use of precursors from both the normal monolignol pathway and alternate phenolic pathways. Such monolignol replacement strategies are currently being pursued by several groups,[24–29] and we have been testing numerous plant-derived phenolics through the use of a biomimetic maize cell wall system.[24,27] These studies have, for example, demonstrated that incorporation of coniferyl ferulate with normal monolignols dramatically improves the alkaline extractability of lignin and the subsequent enzymatic hydrolysis of the fiber.[22,24] Such unique cell wall properties have been expected, as the incorporation of both ferulate and the monolignol units of the bis-phenolic conjugate results in readily cleavable ester linkages being introduced into the backbone of lignin polymer chains. In other studies, modulating the hydrophobicity of lignin by ferulates bearing hydrophilic functionali-

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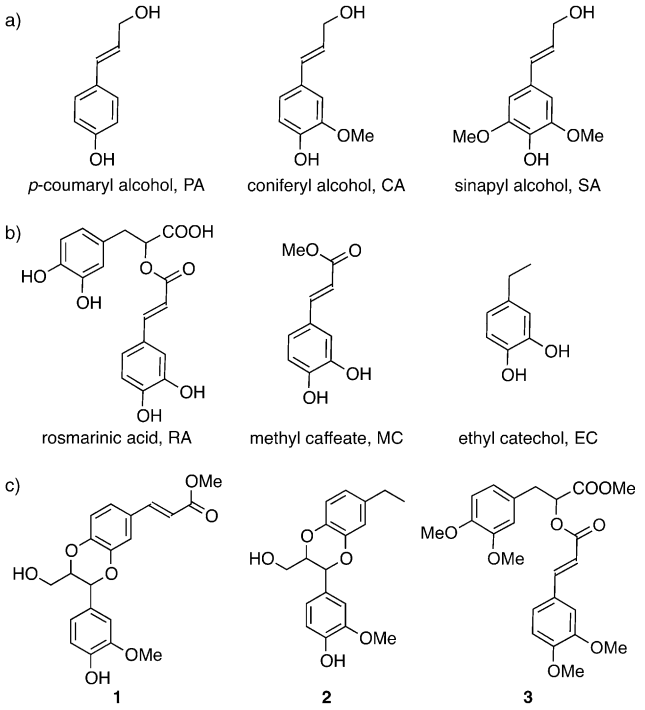

Figure 1. Structures of conventional monolignols (a), rosmarinic acid and its related catechol derivatives (b), and synthetic model compounds (c) used in this study.

ties, such as quinic acid and glycerols, facilitate the enzymatic degradation of cell walls, which can be explained by enhanced penetration and hydrolysis of the cell wall matrix.[27] Incorporation of catechols, such as caffeates and catechins, also enhance cell wall fermentability, perhaps through *o*-diphenol trapping of lignin quinone methide intermediates (QMs) to limit lignin-polysaccharide cross-linking of cell walls, as has been observed for methyltransferase-deficient angiosperms incorporating 5-hydroxyconiferyl alcohol into lignin.[9,10,30] Overall, these initial cell wall studies support the contention that some monolignol substitutes can be efficiently incorporated into cell wall lignins to dramatically affect cell wall properties. However, more fundamental studies are required to identify how these and other such unconventional phenolic monomers undergo radical coupling reactions during lignin polymerization to affect the reactivity of the lignin and the degradability of cell walls. In this paper, we focus on rosmarinic acid (RA), a bis-phenolic conjugate proposed as a lignin-engineering target several years ago (Somerville and Chapple, personal communication, 2005).

RA, an ester of caffeic acid and 3,4-dihydroxyphenyl-lactic acid (catechyl lactic acid, Figure 1 b), is well-known as a constituent in the Lamiaceae and various other plant families.[31] Based on our previous studies, RA is a promising multifunctional lignin-engineering target, possessing a readily cleavable ester linkage connecting two phenolic moieties for depolymerizing lignin,[22] free carboxylic acid functionality for modulating lignin hydrophobicity, and *o*-diphenol functionalities for limiting lignin–polysaccharide cross-linking.[9,10] In addition, recent studies have largely unveiled the genes, enzymes, and metabolites involved in the biosynthesis of RA;[31–35] such information will prove essential if researchers intend to pursue expression of RA in lignifying cell walls. Vega-Sanchez and Ronald, in their recent review,[29] highlight RA as a desirable lignin-bioengineering target for biofuel crop improvement. In the present study, we set out to validate the utility of RA as a potential monolignol replacement by testing the compatibility of RA with dehydrogenative polymerization catalyzed by horseradish peroxidase (HRP), an in vitro process that models lignin polymerization in vivo.[36–39] The chemical structures of the generated synthetic lignins (DHPs) as well as their susceptibility to biomass processing were examined by using nuclear magnetic resonance (NMR) spectroscopy and gel permeation chromatography (GPC). We then produced and characterized cell walls artificially lignified with RA, using a well-developed biomimetic maize cell wall model system,[40–42] to test whether bioengineering of plants to incorporate RA into lignin would enhance the delignification and enzymatic hydrolysis of cell walls.

Results and Discussion

In vitro lignin polymerization

RA (Figure 1 b) was copolymerized using a HRP/H_2O_2 oxidant with a traditional lignin monomer, CA (Figure 1 a), at different monomer feed ratios (see Table 1). To further delineate how the individual caffeate and catechyl lactic acid moieties in RA participated in radical coupling reactions, we also examined the polymerization of CA with methyl caffeate (MC) and ethyl catechol (EC), Figure 1 b. Polymer yields, average molecular weights, and dispersity data for DHPs are listed in Table 1. The HRP-catalyzed polymerization of CA alone produced a DHP (DHP-CA) in good yield with a number-averaged molecular weight of approximately 2300, which is comparable to previously reported values.[43–45] Polymerization with RA slightly increased water solubility and depressed yields, and the average molecular weights of the DHPs (DHP-CARAs). A similar tenden-

Table 1. HRP-catalyzed dehydrogenative polymerization of CA with RA, MC, and EC.

DHPs	Monomers (molar feed ratio)	Yield [%]	Molecular weight[a]			2D-HSQC signal ratio[b] [%]				
			M_n	M_w	*PD*	**A**	**B**	**C**	**D**	**E**
DHP-CA	CA (100)	86.3	2330	8130	3.49	20.9	58.9	18.1	2.1	0
DHP-CARA1	RA, CA (5:95)	78.2	1830	4520	2.47	27.1	48.5	19.4	1.6	3.3
DHP-CARA2	RA, CA (10:90)	80.0	1820	4480	2.46	25.5	41.7	18.7	3.9	10.1
DHP-CARA3	RA, CA (20:80)	81.4	1760	4150	2.36	24.2	32.5	15.0	2.5	25.8
DHP-CAMC	MC, CA (20:80)	78.6	2220	7600	3.42	17.7	50.8	15.6	2.0	13.9
DHP-CAEC	EC, CA (20:80)	62.4	1520	3520	2.32	19.3	51.0	19.9	3.2	6.5

[a] Determined by GPC with polystyrene standards, M_n: number average molecular weight, M_w: weight average molecular weight, *PD*: polydispersity index (M_w/M_n). [b] **A**, β-aryl ether; **B**, phenylcoumaran; **C**, resinol; **D**, dibenzodioxocin; **E**, benzodioxane.

cy was also observed in polymerization with EC (DHP-CAEC), whereas MC produced a DHP (DHP-CAMC) in a higher yield with a higher molecular mass. Overall, the data suggest that the polymerization ability of RA is somewhat lower than CA, which might be primarily affected by the lower reactivity of the catechyl lactic acid unit rather than the caffeate unit.

We used 2D NMR methods to obtain further detailed structural characterization of the DHPs. Incorporation of RA into DHP was first evidenced by the aromatic signals observed in the short-range ^{13}C–^{1}H correlation [heteronuclear single quantum coherence (HSQC)] spectra of DHP-CARAs; the signals from caffeate moieties (RA2, RA5, RA6, RA7, and RA8) were clearly observed, whereas signals from catechol lactic acid moieties (RA′2, RA′5, and RA′6) significantly overlapped with the predominant signals from guaiacyl (G) units derived from CA (Figure 2b). Those signals are consistent with the aromatic signals observed in spectra of DHPs prepared with MC (Figure 2c) or EC (Figure 2d), and in spectra of synthetic model dimers

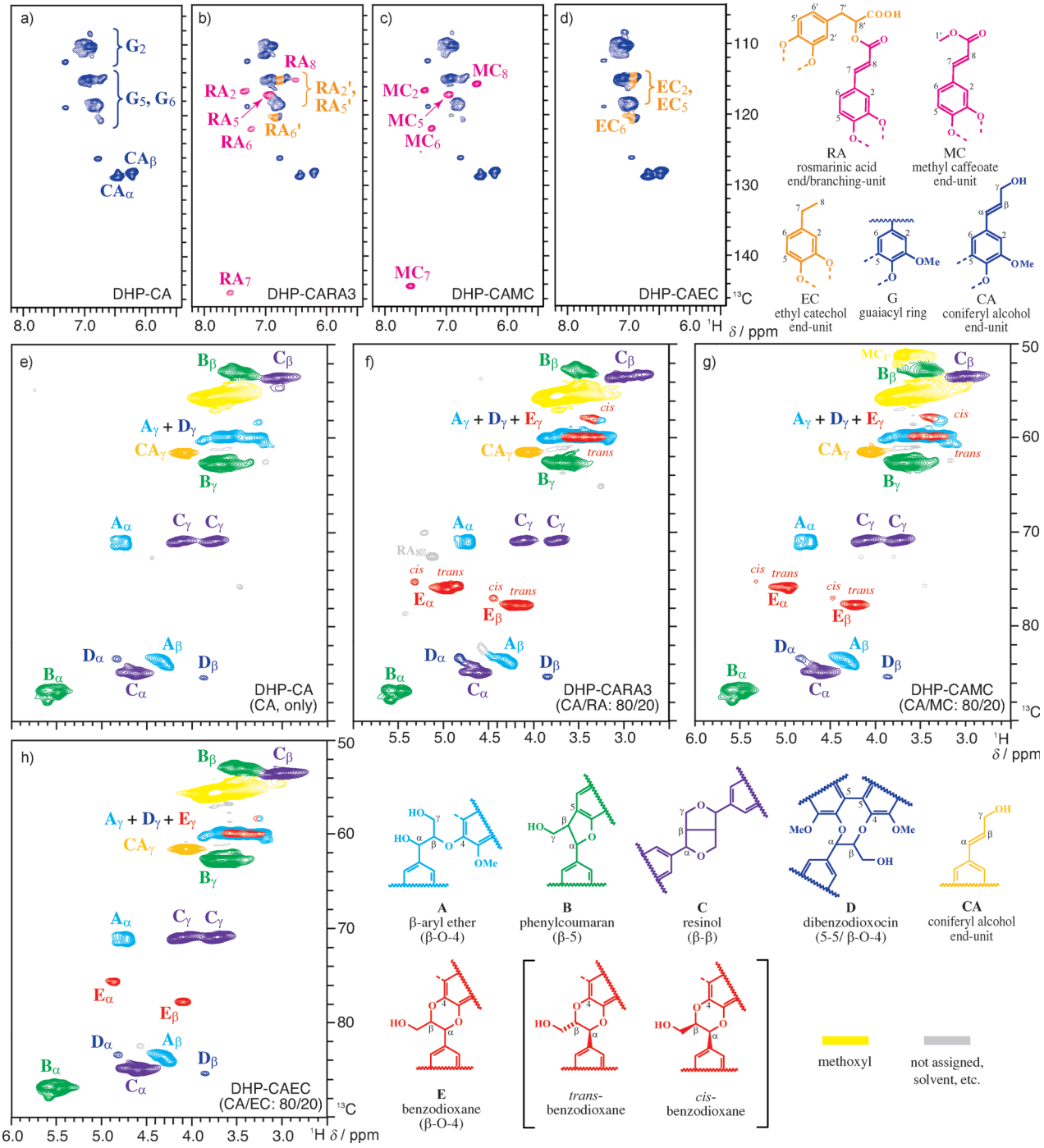

Figure 2. 2D NMR spectroscopic characterization of DHPs produced from CA with RA, MC, or EC. a–d) Aromatic regions and e–h) aliphatic regions of ^{13}C–^{1}H correlation (HSQC) spectra. DHP-CA, produced from 100 mol% CA; DHP-CARA3, produced from 80 mol% CA with 20 mol% RA; DHP-CAMC, produced from 80 mol% CA with 20 mol% MC; DHP-CAEC, produced from 80 mol% CA with 20 mol% EC.

(Figure 1c, and Figure S1 in Supporting Information). HSQC spectra of side-chain regions resolved most of the correlations for the various inter-unit linkages in the polymer to more clearly reveal the participation of catechol monomers in lignin polymerization (Figure 2e–h). Volume integration of the contour signals allowed reasonable quantification of the different inter-unit linkages in the polymers (Table 1). In agreement with literature data,[46,47] the control DHP prepared only with CA (DHP-CA) contained mainly phenylcoumaran units **B** with moderate levels of β-aryl ether units **A** and resinol **C** units, and trace amounts of dibenzodioxocin units **D** (Figure 2e). Such typical lignin linkages were also visible in the spectra of DHP-CARAs, but the most striking difference was the appearance of new benzodioxane units **E** in DHP-CARAs (Figure 2f); the α-, β-, and γ-correlations from *trans*-benzodioxane rings, as well as lower-level contributions from *cis*-benzodioxane rings, were resolved and readily assigned by comparison with data from synthetic model dimers (Figure S1 in Supporting Information). The proportion of the benzodioxane signals increased with the portion of RA used in the polymerization (Table 1). The presence of benzodioxanes in DHP-CAMC and DHP-CAEC was also evident (Figure 2g and h), demonstrating that both types of catechols comprising RA are compatible with H-abstraction by peroxidase and subsequent radical coupling reactions with CA. Interestingly, the benzodioxane level in DHP-CAMC was significantly higher than that observed in DHP-CAEC (Table 1), implying that MC was incorporated into the lignin polymer more efficiently than EC. Polymer yield and the average molecular masses of DHP-CAMC were also higher than those observed for DHP-CAEC, further suggesting that MC is more compatible with copolymerization with CA than EC. Therefore, we deduce from these results that the reactivity of the caffeate moiety exceeds the catechyl lactic acid moiety during lignin polymerization, but both components of RA are compatible with cross-coupling reactions into the lignin polymer.

The benzodioxanes are uniquely derived from β-O-4-type radical coupling of hydroxycinnamyl alcohols (monolignols) with *o*-diphenolic substrates followed by internal trapping of QMs by the *o*-hydroxyl group (Figure 3); analogous benzodioxanes have been well authenticated as products of lignification with 5-hydroxyconiferyl alcohol in angiosperms deficient in caffeic acid *O*-methyltransferase[14,15,48–50] and, more recently, of lignification with caffeyl alcohol in *Pinus radiata* deficient in caffeoyl-CoA 3-*O*-methyltransferase.[16] Studies examining suberin and lignan biosynthesis have also identified benzodioxane-type dimers derived from in vitro radical coupling reactions of caffeic acid.[51,52] It is also possible that RA undergoes homo-coupling reactions to form several unique molecular frameworks, such as tetrahydrofuran-type 8–8-, lactone-type 8–8-, and/or unsaturated 8-O-4-coupling products, for example, analogously to the way hydroxycinnamates undergo radical-mediated dehydrodimerization.[52–54] As we could not identify conclusive NMR signals from such homo-coupling products derived from RA in DHPs, it is likely that cross-coupling reactions between CA and catechyl units in the RA monomer are the predominant pathways by which RA participates in lignin polymerization, at least when CA is a major component.

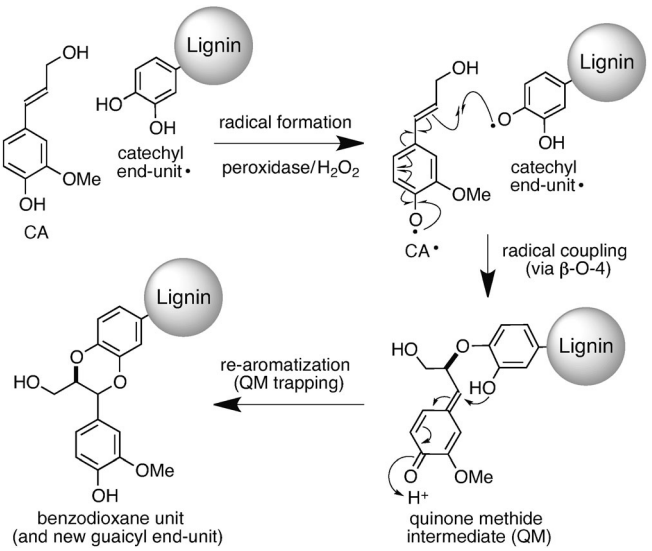

Figure 3. Generation of benzodioxane units during lignification through cross-coupling reactions between catechyl units and a monolignol (shown here using coniferyl alcohol only).

Facile depolymerization of synthetic lignins in alkali

Alkaline (pre)treatment is one of the most important methods used to remove lignins from cell walls conventionally for pulp and paper making and also for liquid fuel production in the future.[55–57] Importantly, lignification with RA introduces labile ester units into the backbone of the lignin polymer allowing it to be readily depolymerized under much milder basic or acidic conditions than is commonly suggested for biomass pretreatment.[58,59] Such lignin modification, therefore, provides an avenue for producing fibers with less lignin contamination and/or for delignification under much milder conditions with lower energy input. To test the reactivity of RA-incorporated lignins, we monitored alkaline degradation of DHP-CARAs with 0.05 M sodium hydroxide at 30 °C using NMR and GPC, with the latter run in a homogenous aqueous DMF solvent to permit characterization of products of all molecular weights (see the Experimental Section for details). As expected, GPC-derived molecular mass profiles of DHPs showed massive depolymerization of DHP-CARAs even with alkaline treatments as short as 1 min, whereas essentially no changes were observed for the control DHP-CA (Figure 4a and b). Higher levels of RA incorporation induced more striking decreases in the molecular weight of DHPs with alkaline pretreatment, clearly due to cleavage of the more frequent ester linkages introduced into the lignin polymer. NMR monitoring revealed that the subset of caffeate correlations experienced a shift upon the alkaline treatment (Figure 4c), suggesting global hydrolysis of caffeate esters (Figure 4d); similar shifts were observed for the hydrolysis of model compound **3**, the phenol etherified (and methyl esterified) analog of RA (Figure 1c, and Figure S2 in Supporting Information). Conversely, no clear changes were observed for signals from normal lignin structures (Figure S2 and S3 in Supporting Information). Earlier studies also indicated that alkaline cleavage of normal lignin linkages requires more severe condi-

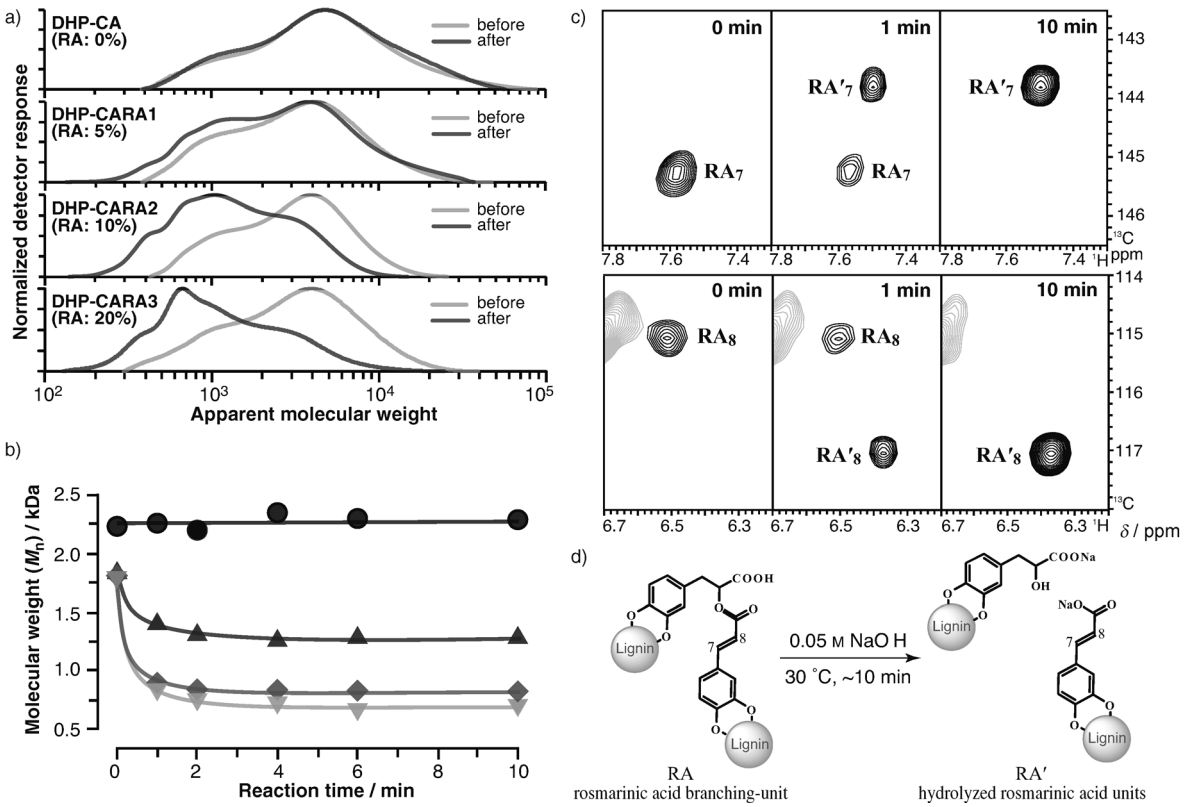

Figure 4. Alkaline degradation of DHPs produced from CA with RA. a) GPC-derived molecular mass profiles of DHPs before and after alkaline treatment (reaction time: 10 min). DHP-CA, produced from 100 mol% CA; DHP-CARA1, produced from 95 mol% CA with 5 mol% RA; DHP-CARA2, produced from 90 mol% CA with 10 mol% RA; DHP-CARA3, produced from 80 mol% CA with 20 mol% RA. b) Plots of number-averaged molecular weights (M_n) versus reaction time (● DHP-CA, 0% RA; ▲ DHP-CARA1, 5% RA; ◆ DHP-CARA2, 10% RA; ▼ DHP-CARA3, 20% RA). c) RA side-chain signals from 2D-HSQC spectra following alkaline degradation of DHP-CARA3. c) Alkaline hydrolysis of RA ester units.

tions.[55,60,61] These results support our contention that RA incorporation enables lignin polymers to be readily depolymerized under much milder basic conditions than those conventionally used for biomass processing.

Artificial lignification of maize cell walls

Before going through the laborious process of attempting to engineer the introduction of novel monomers into the plant cell wall, it is useful to be able to evaluate lignin-modified walls from a biomimetic system. Therefore, to further assess how RA incorporation affects pretreatment processing and degradability of cell walls, artificially lignified cell walls (CWDHPs) were generated using a well-developed maize cell wall model system.[40–42] Isolated maize primary walls containing bound peroxidases were artificially lignified by supplying exogenous CA and SA (Figure 1a), the normal monolignols in grass and dicot lignins, alone or in combination with RA. When added, RA comprised 35% by weight (23 mol%) of the precursor mixture, potentially yielding a shift in lignin composition comparable to that observed in some mutant or transgenic plants with altered lignin biosynthesis. At the conclusion of lignification, non-bound lignins were removed from cell walls by washing with 9:1 (*v*/*v*) acetone/water. Polymerization parameters and structural composition data for CWDHPs are listed in the Supporting Information (Table S1).

Lignification with RA somewhat reduced peroxidase activity and considerably acidified cell walls relative to controls lignified with normal monolignols. Although they received a similar mass of precursors during lignification, lignin concentrations in cell walls prepared with RA (CWDHP-CASARA) were lower than the normal lignin control (CWDHP-CASA1), but comparable to the low-lignin control (CWDHP-CASA2). UV scans of cell wall washes collected after lignification also indicated a reduced incorporation of RA-containing polymers into cell walls (Figure S4 in Supporting Information). As cell walls maintained adequate peroxidase activity throughout precursor addition, reduced lignification may be related to the somewhat lower propensity of RA to undergo oxidative coupling and the greater water solubility of its polymers, as was noted above in DHP experiments.

Formation of artificial lignins in cell walls was confirmed by performing gel-state 2D-NMR;[62] lignin signals from common units such as β-ether (β-O-4) units **A**, phenylcoumaran (β-5) units **B**, and resinol (β-β) units **C**, were visible among predominant carbohydrate signals (Figure 5). It is noticeable that β-O-4-units **A** predominated in CWDHPs, whereas β-5 **B** and β-β **C** units were more common in normal DHPs formed in the absence of a cell wall matrix (Figure 2 and Table 1). Previous stud-

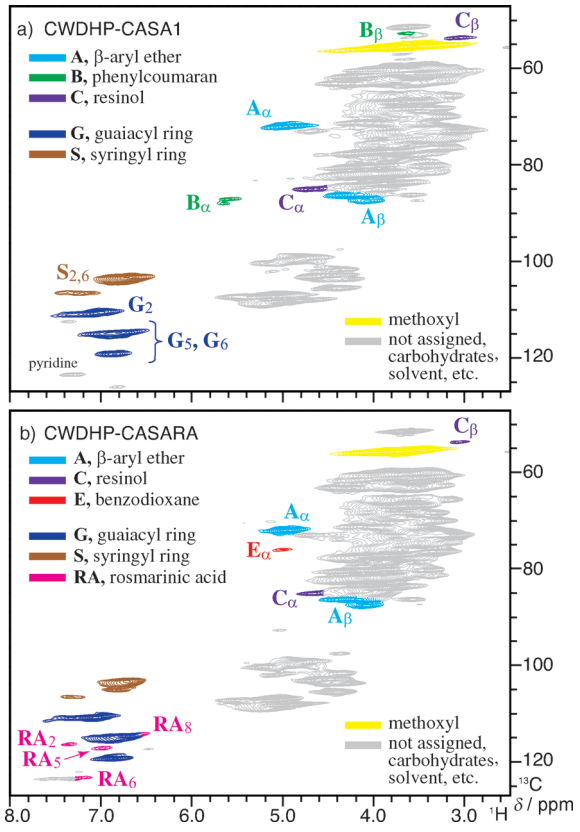

Figure 5. Gel-state 2D ^{13}C–^{1}H correlation (HSQC) NMR spectra of CWDHPs ectopically lignified with CA, SA, and RA. a) CWDHP lignified with CA (0.9 mmol monomer per ≈1.9 g nonlignified maize primary walls) and SA (0.9 mmol) alone. b) CWDHP lignified with CA (0.6 mmol) and SA (0.6 mmol) in combination with RA (0.35 mmol). For abbreviations for lignin signals, see Figure 2.

ies showed that the artificial lignins generated by this cell wall model system closely resemble natural lignins, which contain considerably larger amounts of β-ether units than normal DHPs presumably because of the enhanced endwise coupling of a monomer (radical) with the growing polymer (radical) when diffusion limits the level of monomer radicals in the lignification zone.[40] More importantly for the current study, the anticipated signals from benzodioxane units **E** and rosmarinic acid units **RA** were clearly visible in the CWDHP-CASARA spectrum (Figure 5b), but absent from lignified control cell walls (Figure 5a). The result indicates successful incorporation of RA into the cell wall lignins and the anticipated internal trapping reactions of QMs formed by β-O-4-coupling of a monomer with a caffeyl or caffeate unit. The distributions of sugar anomeric signals in the gel-state NMR spectra (Figure S5 in Supporting Information), were reflective of the sugar composition data of CWDHPs (Table S1 in Supporting Information), although crystalline cellulose is essentially invisible to these methods and is, therefore, under-represented; further studies are required for complete signal assignments in these regions. As is typical in a grass primary wall, it is most likely that cellulose, arabinoxylans, and pectic galactans comprise a major part of the polysaccharide matrix in CWDHPs.[63]

Cell wall delignification and fermentation

Enzymatic saccharification with/without alkaline pretreatment

The RA-incorporated cell wall and two control cell walls with different lignin concentrations (Table S1 in the Supporting Information) were subjected to enzymatic saccharification to comparatively assess whether RA incorporation into lignin enhances the rate and extent of structural polysaccharide hydrolysis. Prior to enzymatic hydrolysis, cell walls were pretreated in an aqueous alkaline solution to evaluate how cleaving of RA ester units affects lignin extractability and the subsequent enzymatic hydrolysis of polysaccharides. In the present study, we chose a 0.05 M NaOH treatment at 100 °C for 1 h, which is milder than that commonly used as an alkaline pretreatment of biomass for ethanol fermentation.[58,59] The alkaline pretreatment removed a fraction composed of hemicellulosic and pectic sugars, and thereby lignin levels increased considerably in the control cell walls (CWDHP-CASA1 and CWDHP-CASA2), but only slightly in RA-incorporated cell walls (CWDHP-CASARA) after the pretreatment (Figure 6a, and Table S2 in Supporting Information). Comparison of lignin loss calculated to account for the mass balance indicated that a significantly larger portion of the lignin was removed in CWDHP-CASARA than in the control cell walls (Figure 6b, and Table S2 in Supporting Information). In the gel-state NMR spectrum of alkali-pretreated CWDHP-CASARA, RA-derived benzodioxanes and caffeate aromatic signals, which were clearly present before the pretreatment (Figure 5), were no longer observable (Figure S6 in Supporting Information). This implies that RA-rich lignin fractions were solubilized into the aqueous solution by pretreatment. With the results from the alkaline degradation of RA-incorporated DHPs described above, it is likely that the al-

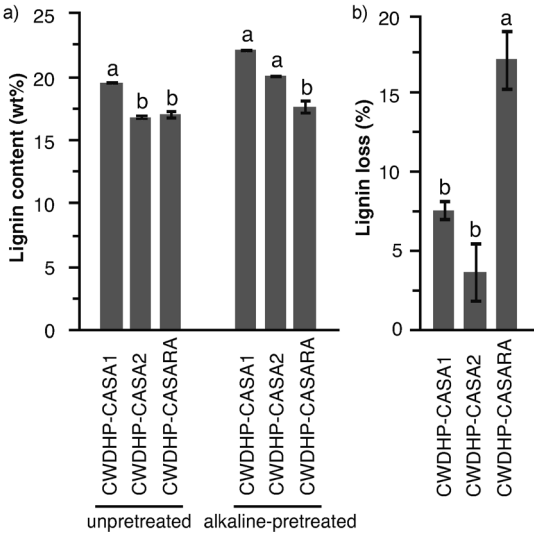

Figure 6. a) Acetyl bromide soluble lignin contents of CWDHPs lignified with CA, SA, and RA, before and after alkaline pretreatment. b) Lignin loss of CWDHPs by alkaline pretreatment. CWDHP-CASA1, produced from CA (0.9 mmol monomer per ≈1.9 g nonlignified maize primary walls) and SA (0.9 mmol); CWDHP-CASA2, produced from CA (0.7 mmol) and SA (0.7 mmol); CWDHP-CASARA, produced from CA (0.6 mmol) and SA (0.6 mmol) in combination with RA (0.35 mmol). Means within a group with unlike letters differ (LSD, $P < 0.05$).

kaline pretreatment induced lignin depolymerization through hydrolysis of RA esters to permit extraction of lignin oligomers from cell walls.

Non-pretreated and alkali-pretreated cell walls were then saccharified with appropriate loadings of fibrolytic enzymes (cellulase supplemented with hemicellulases). The relative abundances of the sugars released from the cell walls were similar for all treatments (glucose > arabinose > galactose > xylose > mannose); therefore, only total carbohydrate yields are reported and discussed below. Incorporation of RA into lignin significantly improved monosaccharide yields, especially in combination with the alkaline pretreatment (Figure 7b, and Table S2 in Supporting Information). It is reasonable that RA facilitates enzymatic hydrolysis primarily by reducing and decomposing high-molecular-mass lignin in the cell walls with the alkaline pretreatment; in addition to the lignin removal's opening up of the wall structure, the lower level of lignin phenolics that are not washed out of the wall presumably result in less enzyme adsorption and inactivation. Interestingly, even without the alkaline pretreatment, CWDHP-CASARA showed significantly higher saccharification efficiency than CWDHP-CASA2 with a similar lignin content (Figure 7a). This result suggests that factors in addition to the reduced lignin content affected sugar hydrolysis of RA-incorporated cell walls, as we will discuss below.

Table 2. Rumen bacterial fermentation characteristics of CWDHPs.

Cell walls	Gas production[a] [mL g^{-1}]			NP[b]	GRL[c]	NPAL[d]
	12 h	24 h	48 h	[mL g^{-1}]	[mL mg^{-1}]	[mg mg^{-1}]
CWDHP-CASA1	94	161	187	284	0.83	1.43
CWDHP-CASA2	111	189	224	232	0.73	1.32
CWDHP-CASARA	181	243	255	110	0.59	0.62
nonlignified cell walls	296	323	339	22	–	–
LSD[e]	53	31	26	45	0.13	0.09

[a] Total volume of fermentation gasses produced during in vitro fermentation. [b] NP at 48 h. [c] GRL, gas reduction per unit lignin calculated as [48 h $gas_{nonlignified}$ − 48 h $gas_{lignified}$]/[Klason $lignin_{lignified}$ − Klason $lignin_{nonlignified}$]. [d] NPAL, nonfermentable polysaccharide accumulation per unit lignin calculated as [$NP_{lignified}$ − $NP_{nonlignified}$]/[Klason $lignin_{lignified}$ − Klason $lignin_{nonlignified}$]. [e] LSD, least significant difference ($P = 0.05$).

Fermentation of non-pretreated cell walls by rumen bacteria

Lastly, we also assessed degradability by continuously monitoring gas production and analyzing residual non-fermented polysaccharides of CWDHPs incubated in vitro with rumen microflora. Gas production is directly linked to structural carbohydrate hydrolysis and fermentation by rumen microflora and highly correlated with biomass fermentation to ethanol.[64–66] Substituting monolignols with RA improved gas production in CWDHP-CASARA especially during the initial stages of fermentation and left less than half as much nonfermentable polysaccharides (NP) after 48 h fermentation as control cell walls lignified in the absence of RA (Table 2). Incorporation of RA also significantly reduced gas reduction per unit lignin (GRL) and nonfermentable polysaccharide accumulation per unit of lignin (NPAL), again indicating that factors in addition to lignin content contributed to enhanced cell wall hydrolysis and fermentation.

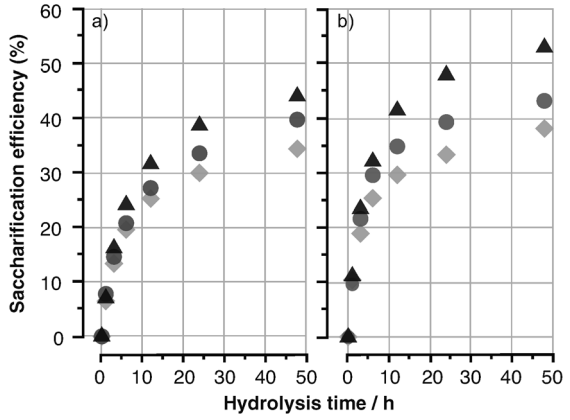

Figure 7. Time course of saccharification efficiencies (total sugar released as a percentage of total sugar in the cell wall residues) during enzymatic hydrolysis of a) non-pretreated and b) alkaline pretreated CWDHPs lignified with CA, SA, and RA. CWDHP-CASA1 (◆), produced from CA (0.9 mmol monomer per ≈1.9 g nonlignified maize primary walls) and SA (0.9 mmol); CWDHP-CASA2 (●), produced from CA (0.7 mmol) and SA (0.7 mmol); CWDHP-CASARA (▲), produced from CA (0.6 mmol) and SA (0.6 mmol) in combination with RA (0.35 mmol).

Consequently, incorporation of RA into cell wall lignins strikingly improved cell wall degradability by enzymatic hydrolysis both, with and without mild alkaline pretreatments, and improved rumen bacterial fermentation of non-pretreated cell walls. Enhancements in lignin extractability by alkaline pretreatments and the following sugar release can be reasonably attributed to lignin degradation through hydrolysis of RA ester units during the pretreatments. Although our evaluations here logically concentrated on alkaline pretreatments (because of the ready cleavage of the ester linkage under alkaline conditions), acid pretreatment also produced remarkable saccharifiability improvements (to be reported elsewhere). Therefore, it appears that incorporation of RA into lignins significantly improves saccharifiability over a range of pretreatments. Incorporating RA also improved cell wall degradations even without alkaline or acid pretreatments, indicating that RA incorporation improved inherent degradability of cell walls by affecting substrate–enzyme interactions during enzymatic hydrolysis. As previously advocated,[27,67] introducing hydrophilic functionalities, such as the carboxylic acid in RA, into lignin might enhance penetration or limit non-productive binding of hydrolytic enzymes to lignin through hydrophobic interactions. Also, the two catechol units in RA can internally trap QMs during lignification, as clearly evidenced by the presence of benzodioxane units in lignins (Figure 3); such internal re-aromatization during lignification likely limits cross-linking of lignin with polysaccharides and thereby enhances the enzymatic hydrolysis of cell walls;[9,10] nucleophilic polysaccharide additions to QMs have been considered as one of the main pathways to generate such cell wall cross-linking.[68–70] Identifying actual causative factors, however, still requires further study.

Conclusions

We have experimentally demonstrated the utility of RA as a potential partial monolignol replacement. The first part of this study utilized the in vitro HRP-catalyzed polymerization to model how RA undergoes oxidative copolymerization with conventional monolignols, and also to structurally characterize the RA-containing polymers. The efficient incorporation of RA and its related catechols into the synthetic lignins was evident; both the caffeate and catechyl lactic acid moieties act as substrates for peroxidase and are integrally cross-coupled into the polymers, generating diagnostic benzodioxane structures. Polymerization data for the two analogous mono-catechols suggests a higher reactivity of caffeate than catechyl lactic acid moieties. The impacts of RA incorporation on the alkaline degradability of DHPs is striking, with GPC and NMR data clearly showing that the caffeate esters readily undergo alkaline hydrolysis to rapidly depolymerize lignin. Copolymerization of RA with monolignols into artificially lignified maize cell walls has also been demonstrated by using gravimetric and wet chemical techniques, as well as by performing 2D-NMR spectroscopy through diagnostic benzodioxane and caffeate signals. RA incorporation considerably improves alkaline delignification and subsequent hydrolysis of cell walls by fungal enzyme preparations. It is most plausible that RA facilitates enzymatic hydrolysis chiefly by reducing high molecular mass lignin in the cell walls after mild alkaline pretreatments. Addition of RA also improves cell wall degradation by fungal enzymes and rumen microflora even without alkaline pretreatments, possibly by modulating lignin hydrophobicity and/or limiting recalcitrance ascribable to cell wall cross-linking. Although this study has utilized a maize cell wall system, comparable or greater benefits can be expected for hardwoods, softwoods, and herbaceous dicots that normally have more recalcitrance towards pretreatment processing. Overall, we anticipate that bioengineering approaches for partial monolignol substitution with RA and analogous hydroxycinnamate conjugates would permit more efficient delignification and enzymatic hydrolysis of plant cell walls, which in turn reduce energy inputs for papermaking, biofuel production, and numerous other biomass conversion processes.

Experimental Section

General

Coniferyl alcohol (CA)[71] and sinapyl alcohol (SA)[72] were synthesized according to literature methods. Horseradish peroxidase (HRP, Type VI, 250–330 U) was purchased from Sigma–Aldrich (Milwaukee, WI, USA), and enzymes used for the hydrolysis of cell walls (Novozyme Biomass Kit: cellulase, NS50013; β-glucosidase, NS50010; multi-carbohydrase complex, NS50012; xylanase, NS50030) were generously provided by Novozymes (Franklinton, NC, USA). Other chemicals were purchased from Sigma–Aldrich or Fisher Scientific (Atlanta, GA, USA) and were used as received.

Measurements

Nuclear magnetic resonance (NMR) spectra were acquired by using a Bruker Biospin (Billerica, MA, USA) AVANCE 500 (500 MHz) spectrometer fitted with a cryogenically-cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample) and spectral processing used Bruker's Topspin 3.0 (Mac) software. The central solvent peaks were used as internal reference [δ_H/δ_C: acetone, 2.04:29.8; dimethyl sulfoxide (DMSO), 2.49:39.5 ppm]. The standard Bruker implementations of one- and two-dimensional [gradient-selected COSY, heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)] NMR experiments were used for routine structural assignments of newly synthesized compounds. Adiabatic 2D-HSQC ('hsqcetgpsisp2.2') experiments for synthetic lignin (DHP) samples in a solution-state,[39] and maize cell wall (CW)DHP samples in a gel-state,[62] were performed by using the parameters described previously. Processing used typical matched Gaussian apodization in F2 (LB = −0.1, GB = 0.001) and squared cosine-bell and one level of linear prediction (32 coefficients) in F1. For an estimation of the various inter-unit linkage types in DHP, the well-resolved C_α–H_α contours ($\mathbf{A}_\alpha$, $\mathbf{B}_\alpha$, $\mathbf{C}_\alpha$, $\mathbf{D}_\alpha$, and $\mathbf{E}_\alpha$, Figure 3) were integrated; no correction factors were used.[16] Gel permeation chromatography (GPC) on DHP samples was performed by using a Shimadzu (Kyoto, Japan) LC-20 A LC system as described previously,[39] using 0.1 M lithium bromide (LiBr) in dimethylformamide (DMF) as eluent and polystyrene standards for molecular weight calibration. High-performance ion chromatography (HPIC) for sugar analysis was performed on a Dionex (Sunnyvale, CA, USA) ICS-3000 HPIC system as described previously.[73] UV spectra were collected by using a Beckman Coulter (Fullerton, CA, USA) DU7400 spectrophotometer.

Synthesis

Compound **1** (Figure 1c) was synthesized from radical coupling reactions of MC and CA through silver carbonate (Ag_2CO_3) oxidation: MC (580 mg, 3 mmol) and CA (640 mg, 4 mmol) were dissolved in acetone-toluene (90 mL, 1:2, *v/v*) and Ag_2CO_3 (1.9 g, 7 mmol) was added at room temperature. After stirring at room temperature for 13 h, additional Ag_2CO_3 (1.9 g, 7 mmol) was added, and the mixture was heated at 50 °C for 13 h. After cooling to room temperature, the solid was filtered off, and the organic solvents were evaporated under reduced pressure to give a dark solid residue, which was purified by performing silica-gel chromatography to give a colorless solid (620 mg, 1.7 mmol, 56% yield). This product was a mixture of *cis*- and *trans*-isomers of compound **1** (*cis*-**1**/*trans*-**1** = 4:96, determined by using ^{1}H NMR spectroscopy). Recrystallization from diethylether–chloroform yielded practically pure *trans*-isomer.

trans-**1**: ^{1}H NMR ([D$_6$]acetone): δ = 3.50 (1H, m; Hγ), 3.71 (3H, s; 9′-OMe), 3.74 (1H, m; Hγ), 3.74 (1H, m; Hγ), 3.86 (1H, s; 3-OMe), 4.18 (1H, m; Hβ), 4.99 (1H, d, J = 8.05 Hz; Hα), 6.40 (1H, d, J = 15.90 Hz; H8′), 6.88 (1H, d, J = 8.05 Hz; H5), 6.95 (1H, d, J = 8.25 Hz; H5′), 6.96 (1H, dd, J = 8.05 and 1.95 Hz; H6), 7.12 (1H, d, J = 1.90 Hz; H2), 7.21 (1H, dd, J = 8.35 and 2.05 Hz; H6′), 7.25 (1H, d, J = 2.05 Hz; H2′), 7.58 ppm (1H, d, J = 15.95 Hz; H7′); ^{13}C NMR ([D$_6$]acetone): δ = 51.56 (9′-OMe), 56.26 (4-OMe), 61.66 (Cγ), 77.12 (Cβ), 79.83 (Cα), 111.83 (C2), 115.72 (C5), 116.56 (Cβ), 117.18 (C2′), 118.03 (C5′), 121.59 (C6), 122.97 (C6′), 128.62 (C1′), 128.84 (C1), 145.05 (Cα), 145.16 (C3′), 146.86 (C4′), 148.05 (C4), 148.47 (C3), 167.67 ppm (C9′).

cis-**1**: ^{1}H NMR ([D$_6$]acetone): δ = 4.56 (m; Hβ), 5.31 ppm (d, J = 2.80 Hz; Hα); ^{13}C NMR ([D$_6$]acetone): δ = 75.49 (Cα), 78.02 ppm (Cβ). HR–MS (ESI) calcd. for [(M−H)$^+$]: 371.1136; found: 371.1144.

Compound **2** (Figure 1c) was synthesized from radical coupling reactions between ethyl catechol (EC) and CA through Ag_2CO_3 oxidation: EC (420 mg, 3 mmol) and CA (640 mg, 4 mmol) were dis-

solved in acetone-toluene (90 mL, 1:2, *v/v*) and Ag_2CO_3 (1.9 g, 7 mmol) was added at room temperature. After stirring at room temperature for 14 h, the solid was filtered off, and the organic solvents were evaporated under reduced pressure to give a dark solid residue, which was purified by performing silica-gel chromatography to give compound **2** as a colorless solid (400 mg, 1.3 mmol, 42% yield). This product was a mixture of *cis*- and *trans*-isomers of compound **2** (*cis*-**2**/*trans*-**2** = 4:96, determined by using ^{1}H NMR). Recrystallization from diethylether–chloroform yielded practically pure *trans*-isomer.

trans-**2**: ^{1}H NMR ([D$_6$]acetone): $\delta = 1.16$ (3H, t, $J = 7.58$ Hz; H8′), 2.53 (2H, q, $J = 7.58$ Hz; H7′), 3.47 (1H, m; Hγ), 3.69 (1H, m; Hγ), 4.00 (1H, t, $J = 6.02$ Hz; γ-OH), 4.04 (1H, m; Hβ), 4.92 (1H, d, $J = 7.95$ Hz; Hα), 6.69 (1H, dd, $J = 8.15$ and 1.85 Hz; H6′), 6.73 (1H, d, $J = 1.80$ Hz; H2′), 6.79 (1H, d, $J = 8.15$ Hz; H5′), 6.86 (1H, d, $J = 8.05$ Hz; H5), 6.94 (1H, dd, $J = 8.05$ and 1.85 Hz; H6), 7.09 (1H, d, $J = 1.80$ Hz; H2), 7.78 ppm (1H, s; ph-OH); ^{13}C NMR ([D$_6$]acetone): $\delta = 16.25$ (C8), 28.67 (C7), 56.24 (OMe), 61.87 (Cγ), 77.11 (Cβ), 79.35 (Cα), 111.75 (C2), 115.65 (C5), 116.84 (C2′), 117.29 (C5′), 121.31 (C6′), 121.48 (C6), 129.40 (C1), 137.86 (C1′), 142.52 (C4′), 144.64 (C3′), 147.88 (C4), 148.41 ppm (C3).

cis-**2**: ^{1}H NMR ([D$_6$]acetone): $\delta = 4.45$ (m; Hβ), 5.24 ppm (d, $J = 2.80$ Hz; Hα); ^{13}C NMR ([D$_6$]acetone): $\delta = 76.28$ (Cα), 78.29 ppm (Cβ). HR–MS (ESI) calcd. for [(M−H)$^-$]: 315.1237; found: 315.1247.

Compound **3** (Figure 1) was synthesized by methylation of RA with dimethyl sulfate: to a solution of RA (1.4 g, 4 mmol) and dimethyl sulfate (5.7 mL, 60 mmol) in acetone (100 mL), potassium carbonate (8.3 g, 60 mmol) was added, and the mixture refluxed for 12 h. After cooling to room temperature, the solid was filtered off, and the organic solvents were evaporated under reduced pressure to give an oil, which was purified by performing silica-gel chromatography to yield compound **3** as a yellowish oil (1.4 g, 3.3 mmol, 84% yield).

^{1}H NMR ([D$_6$]acetone): $\delta = 3.06$–3.18 (2H, m; H7′), 3.69, 3.75, 3.80, 3.85, and 3.88 (15H, s; OMe), 5.25 (1H, q, $J = 8.40$ and 4.47 Hz; H8′), 6.46 (1H, d, $J = 15.90$ Hz; H8), 6.82 (1H, dd, $J = 8.15$ and 1.90 Hz; H6′), 6.86 (1H, d, $J = 8.15$ Hz; H5′), 6.94 (1H, d, $J = 1.90$ Hz; H2′), 6.99 (1H, d, $J = 8.30$ Hz; H5), 7.20 (1H, dd, $J = 8.28$ and 1.98 Hz; H2), 7.33 (1H, d, $J = 2.00$ Hz; H2), 7.64 ppm (1H, $J = 15.90$ Hz; H7); ^{13}C NMR ([D$_6$]acetone): $\delta = 37.60$ (C7′), 52.32, 55.93, 55.97, 56.02, and 56.05 (OMe), 73.77 (C8′), 110.91 (C2), 112.21 (C5), 112.54 (C5′), 114.07 (C2′), 115.37 (C8), 122.24 (C6′), 123.93 (C6), 127.92 (C1), 129.59 (C1′), 146.59 (C7), 149.33 (C4′), 150.09 (C3′), 150.56 (C3), 152.72 (C4), 166.67 (C9), 170.75 ppm (C9′). HR–MS (ESI) calcd. for [(M+Na)$^+$]: 453.1520; found: 453.1553.

HRP-catalyzed dehydrogenative polymerization

DHPs were generated through HRP-catalyzed polymerization of CA with RA, MC, and EC, using literature methods[39] with some minor modifications: monomers (total 1 mmol, feed ratio listed in Table 1) in 240 mL of acetone/sodium phosphate buffer (0.1 M, pH 6.5; 1:9, *v/v*) and a separate solution of hydrogen peroxide (1.2 mmol) in 240 mL of water were added by using peristaltic pump over a 20 h period at 25 °C to 60 mL of buffer containing HRP (5 mg). The reaction mixture was further allowed to stand at 25 °C for 4 h. After polymerization with MC and EC, the precipitate was collected by performing centrifugation (10000×*g*, 15 min), washed with ultrapure water (100 mL×3), and lyophilized to afford DHPs. After polymerization with RA, it was observed that the DHPs were partially soluble in the final reaction mixtures, probably due to the presence of hydrophilic carboxylic moieties in the polymers. Therefore, the reaction mixtures were carefully acidified (pH≈2) using 0.1 M HCl aq. at 0 °C, and the augmented precipitates were collected by centrifugation, washed with 0.01 M HCl aq. (100 mL×3), and then lyophilized to give DHPs.

Monitoring alkaline degradation of DHPs

GPC monitoring was conducted as follows: 0.2 M sodium hydroxide (NaOH, 300 μL) aqueous solution was added to a solution (900 μL) containing DHP (6.0 mg) in DMF at 30 °C. After initiating the reaction, aliquots (150 μL) of the reaction mixtures were periodically sampled, mixed with DMF (1350 μL) containing 0.1 M LiBr and 0.1 M acetic acid to terminate the reaction, cooled at 4 °C, and directly subjected to GPC analyses. For NMR monitoring, DHP (20 mg) was dissolved in DMF (4.5 mL) and 0.2 M NaOH aqueous solution (1.5 mL) was added at 30 °C. After the desired reaction time, the mixture was poured into 0.1 M aq. HCl (200 mL) at 0 °C. Resultant precipitates were collected by filtration through a nylon membrane (pore size, 0.45 μm), washed with 0.01 M aqueous HCl (200 mL), and lyophilized to yield typically 16–20 mg powder. The obtained powder was dissolved in [D$_6$]DMSO and subjected to NMR analyses.

Preparation of artificially lignified maize cell walls

Freshly prepared fully hydrated nonlignified primary cell walls (≈1.9 g dry weight) from maize cell suspensions[40] were stirred in water (300 mL) containing 3 mM $CaCl_2$. Prior to lignification, cell wall ferulates were dimerized through wall-bound peroxidases by adding dilute H_2O_2 (0.3 mmol in 10 mL of water, ≈2 eq per mol of cell wall ferulate) by using a peristaltic pump over a 30 min period followed by stirring for an additional 30 min. Cell wall suspensions were then artificially lignified by adding two levels of a two-component mixture of CA and SA (each at 0.7 or 0.9 mmol) or by adding a three-component mixture of CA and SA (each at 0.6 mmol) with RA (0.35 mmol). Lignin precursors (prepared in 10 mL dioxane and 90 mL of water) and dilute H_2O_2 (1.1 eq per mol of monolignol or catechin unit prepared in 110 mL of water) were added separately by using a peristaltic pump, initially at 20 mL h^{-1} for 1 h, followed by 10 mL h^{-1} for 3 h, and then completed at 5 mL h^{-1} to mimic the proposed progression of lignin formation in plants. Treatments were replicated twice in independent experiments, and nonlignified controls were stirred in a solvent mixture similar to the final makeup of the lignification reaction media. Following monolignol addition, the cell wall peroxidase activity was visually assessed through guaiacol–H_2O_2 staining,[42] and the acidity of the cell wall suspension was measured with a pH meter. Following additions, the cell walls were stirred for an additional 12 h, stored several days at 4 °C, and then filtered and resuspended four times with acetone/water [400 mL of 9:1 (*v/v*)] in fritted-glass Büchner funnels (≈5 μm retention) to remove DHPs not bound to cell walls. Cell walls were then dehydrated by five washes with acetone (400 mL), briefly subjected to vacuum to remove excess acetone, and transferred to sample jars. After setting overnight in a hood to evaporate the acetone, cell walls were dried at 55 °C and then weighed to estimate, by difference, the mass of lignin precursors polymerized into cell walls. Subsamples from acetone/water filtrates were dried in vacuo, redissolved in 1:1 (*v/v*) dioxane-water, and subjected to UV spectroscopy to estimate dehydrogenation products washed out of cell walls.

Cell wall analysis

For carbohydrate analysis, dried cell walls (10 mg) were treated with H_2SO_4 (0.5 mL, 72%) at room temperature for 2 h, and then the mixture was diluted to 3% acid concentration followed by autoclaving (≈0.1 Pa, 121 °C) for 1 h. After cooling to room temperature, the hydrolysate was analyzed for sugars by performing HPIC,[73] and for uronic acid by using an *m*-hydroxydiphenyl colorimetric assay.[74] Lignin content was determined as acetyl bromide soluble lignin (ABSL),[75,76] and/or as acid-insoluble lignin by using the Klason method.[77] All analyses were performed at least three times, and the data reported here are mean ± standard errors. For gel-state NMR analyses, dried cell walls (100 mg) were ball milled (4×5 min, 5 min cooling cycle, total milling time of 35 min) by using a Retsch PM100 ball mill vibrating at 600 rpm with ZrO_2 vessels (50 mL) containing ZrO_2 ball bearings (10 mm×10). The recovered ball-milled cell walls were then transferred into NMR tubes, swollen in [D_6]DMSO/[D_5]pyridine (4:1, *v*/*v*), and subjected to 2D-HSQC experiments.[62]

Alkaline pretreatment and enzymatic hydrolysis

Cell walls were soaked in 0.05 M sodium hydroxide (10% loading on cell wall, *w*/*w*) for 2 h at 25 °C, which was followed by heating (boiling water bath) at 100 °C for 1 h. The ratio of liquid-to-cell wall used was 50. After pretreatment, cell walls obtained were thoroughly washed with water until the pH of the mixture was neutral. Cell walls were centrifuged intermittently during dilution (1500×*g*; 15 min) to prevent the loss of cell walls. Finally, the solids were freeze-dried. The lignin loss was calculated according to Equation (1):

$$\text{loss}(\%) = \left(1 - \frac{\text{lignin}_{\text{PCW}} \times \text{substrate}_{\text{recovered}}}{\text{lignin}_{\text{UCW}}}\right) \times 100 \quad (1)$$

in which $\text{lignin}_{\text{PCW}}$ corresponds to the lignin content of pretreated cell walls and $\text{lignin}_{\text{UCW}}$ to the lignin content of un-pretreated cell walls. Enzymatic hydrolysis was conducted at a substrate consistency of 2.0% (*w*/*v*) in sodium acetate buffer (pH 4.8). Enzyme loadings were cellulase 15 FPU (FPU = filter paper unit) per g glucan, β-glucosidase 30 CBU (CBU = cellobiase unit) per g glucan, multi-carbohydrase complex 15 PGU (PGU = polygalacturonase unit) per g cell wall, and xylanase 15 FXU (FXU = Farvet xylan unit) per g cell wall, respectively. Tetracycline (0.16%, *w*/*v*), was added to prevent microbial contamination. Substrates (untreated or pretreated cell walls) were pre-incubated at 50 °C in buffer for 24 h prior to the addition of enzymes. The hydrolysis was conducted at 50 °C in a shaker (300 rpm) for 48 h. Samples were collected intermittently and analyzed for sugar concentration by using HPIC.[73] Each experiment was repeated at least two times. The released sugars at each time interval were used to calculate the saccharification efficiency of the substrate. The saccharification efficiency was calculated by using Equation (2):

$$\text{efficiency}(\%) = \frac{\text{sugar}_{\text{released}}}{\text{sugar}_{\text{substrate}}} \times 100 \quad (2)$$

where $\text{sugar}_{\text{released}}$ represents the amount of sugar released after each time interval and $\text{sugar}_{\text{substrate}}$ the amount of potential sugar in the substrate.

Fermentation by rumen microflora

Cell walls were incubated at 39 °C in 60 mL sealed bottles with phosphate-bicarbonate buffer (5.7 mL), reducing agent (0.3 mL), and diluted rumen inoculum (4 mL).[78] Gas production during fermentation was monitored continuously by using pressure transducers. Blank-corrected gas production data from three to four independent fermentation runs were fitted with a dual-pool exponential model to estimate the kinetics of microbial hydrolysis and fermentation of cell walls.[78] Kinetic parameters were then used to estimate the volume of gas produced at 8, 24, and 48 h. Freeze-dried residues remaining after cell-wall degradation by rumen microflora were dissolved in 12 M H_2SO_4 at 25 °C for 2 h and analyzed for nonfermentable polysaccharides by using the phenol-sulfuric acid assay,[79] with corrections for inoculum contamination and sugar recovery. The recovery of sugars from nonfermentable polysaccharides was estimated by running unfermented nonlignified cell walls through the 12 M H_2SO_4 dissolution/phenol–sulfuric acid assay procedure.

Statistical analysis

Data for CWDHPs were subjected to an analysis of variance according to a randomized complete block design by running PROC GLM (Version 9.2, Statistical Analysis System, SAS Institute Inc., Cary, NC, USA). If *F*-tests were significant ($P \leq 0.05$), then treatment means were compared by using the least significant difference (LSD) option at $P = 0.05$.

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