ELSEVIER

Carbohydrate Research 275 (1995) 167–178

CARBOHYDRATE RESEARCH

Lignin–ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins ☆

John Ralph *, John H. Grabber, Ronald D. Hatfield

U.S. Dairy Forage Research Center, USDA-Agricultural Research Service, 1925 Linden Drive West, Madison, WI 53706-1108, USA

Received 3 March 1995; accepted in revised form 22 June 1995

Abstract

Active incorporation of ferulate polysaccharide esters into ryegrass lignins has been demonstrated by NMR spectroscopy of uniformly ^{13}C-labeled ryegrass. Observation, in the HMBC spectrum, of products of ferulate at its 8-position coupling with hydroxycinnamyl alcohols at the β-position (producing 8-β'-linked structures) is proof that ferulate–lignin radical cross-coupling reactions occur in vivo. Correlations of H-α' (hydroxycinnamyl alcohol moiety) with guaiacyl and syringyl 1-, 2-, and 6-aromatic carbons in 8-β' structures indicates that ferulates couple with both coniferyl and sinapyl alcohol monomers. As notable as the presence of this and other ferulate products is the absence of coupling of ferulate at its 8-position with the 5- and O-4-positions of lignin units. Such structures were significant when ferulate was biomimetically incorporated into a synthetic lignin. Since hydroxycinnamyl alcohols couple almost exclusively at their β-position in cross-coupling reactions, the 8-5′ and 8-O-4′ structures would only be formed by coupling with higher lignin oligomers (with no side-chain conjugation). Exclusive reaction of ferulates with lignin monomers is the first real evidence that ferulate polysaccharide esters in grasses are acting as initiation or nucleation sites for lignification and are critical entities in directing cell-wall cross-linking during plant growth and development.

Keywords: Lignin–ferulate cross-links; Ferulate polysaccharide esters; Ryegrass lignins; NMR; HMBC; ^{13}C-labeling

☆ Part 3 in a series: Lignin–ferulate cross-links in grasses.

* Corresponding author; e-mail: jralph@facstaff.wisc.edu. Also affiliated with the Department of Forestry, University of Wisconsin-Madison, Madison, WI 53706, USA.

Elsevier Science Ltd.
SSDI 0008-6215(95)00237-5

1. Introduction

Ferulates are implicated in cross-linking grass cell-wall polysaccharides with lignin (see references in refs [1,2]). Ferulate–polysaccharide esters, arabinoxylans feruloylated at C-5 of an α-L-arabinofuranosyl moiety, **1** (X = xylan), 'attach' to lignins during wall development. There are two fundamentally different mechanisms possible for this attachment that we now term active (Scheme 1) and passive (or opportunistic, Scheme 2) to reflect the degree with which the ferulate is involved in the incorporation process. It has always been our contention [1,3,4] that ferulate should actively incorporate into the lignin polymer via oxidative coupling (radical) processes (Scheme 1), a pathway alluded to sporadically in the literature [5–7]. However, the mechanism most cited for attachment, Scheme 2, is the mechanism in which ferulate nucleophiles **1** (at the 4-*O*-position) 'opportunistically' react with quinone methide intermediates **13** to form benzyl aryl ethers. This is a reasonable mechanism that has been demonstrated in model systems [8], although formidable new evidence shows that lignin α-ethers, traditionally estimated at 6–9% [9], are well below 0.3% in milled wood lignins [10]. Nevertheless, to expect ferulates to exist in the wall for coupling to lignins via lignin quinone methide intermediates yet remain chemically inert to the peroxidase catalyzed single-electron processes that are producing the very quinone methides with which they must react is unreasonable [1,4,11]. Wall ferulates are involved in single-electron reactions as evidenced by their dimerization via radical processes (see below) [12]. The passive mechanism has further troubling biochemical implications that were spelled out in Part 1 of this series [1]. It has not been appreciated that adoption of a passive incorporation mechanism underestimates the abundance of ferulates in the cell wall, and underestimates their role in cross-linking the wall.

Support for the active mechanism comes from recent mechanistic studies. Oxidative coupling of low molecular mass model compounds illustrated that ferulates are amenable to single-electron oxidation processes to produce dehydrodimers [2,11]. Cross-coupling of ferulates with hydroxycinnamyl alcohols was demonstrated in a particularly revealing study where FA–Ara [1,4,13,14] **1** [X = Me, methyl 5-*O*-(*E*)-feruloyl-α-L-arabinofuranoside] was introduced into a biomimetic lignification (DHP, 'dehydrogenation polymer') system [1]. Active incorporation mechanisms produced the entire complement of ferulate/coniferyl alcohol or ferulate/coniferyl alcohol-oligomer coupling products **7–12** (Scheme 1) and incorporated the ferulate intimately into the synthetic lignin [1]. Ferulate extensively coupled at its 8-position, and it was estimated from quantitative ^{13}C NMR spectra that 40% of the ferulate was 4-*O*-linked (although this

Scheme 1. The 'active' mechanism for incorporation of ferulates into lignins. Radical cross-coupling products **7–12** formed by oxidative coupling of ferulate **1** with coniferyl alcohol and guaiacyl (from coniferyl alcohol oligomers) units **3–4** (upper half of scheme), and **7b–10b** from coupling of ferulate **1** with sinapyl alcohol and syringyl (from sinapyl alcohol oligomers) units **3b–4b** (lower half of scheme). **F**, **G**, and **S** are to indicate aromatic rings originating from ferulate, guaiacyl, and syringyl units. X = xylan, L = coniferyl alcohol side-chain or generic lignin side-chain plus the remainder of the lignin oligomer/polymer. Numbering convention is as for Fig. 1. Only compounds **9** and **9b** will release ferulate on high temperature base solvolysis. Dashed arrows indicate sites available for further polymerization.

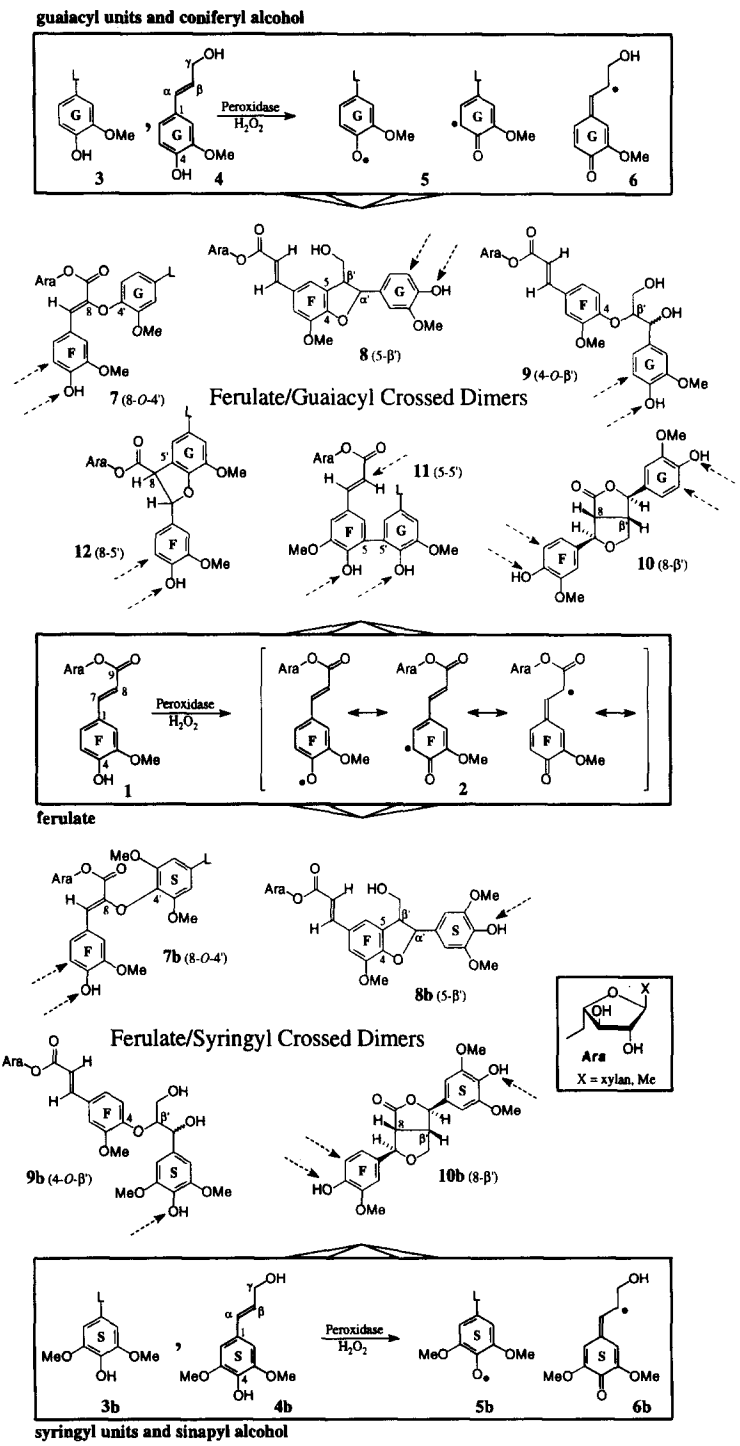
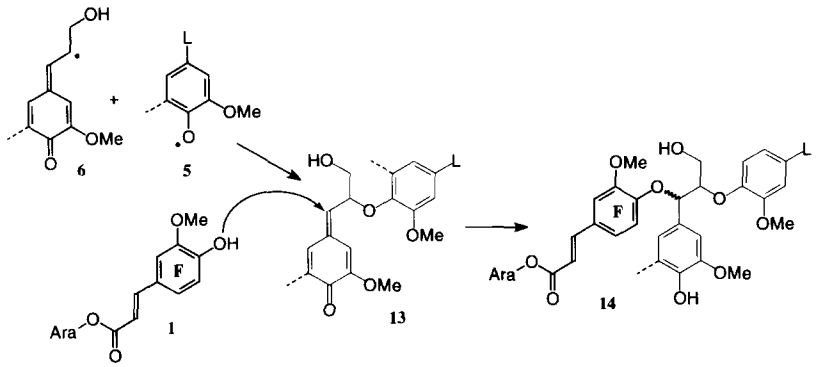

guaiacyl units and coniferyl alcohol
Peroxidase
H_2O_2
3
4
5
6
7 (8-O-4')
8 (5-β')
9 (4-O-β')
Ferulate/Guaiacyl Crossed Dimers
12 (8-5')
11 (5-5')
10 (8-β')
Peroxidase
H_2O_2
1
2
ferulate
7b (8-O-4')
8b (5-β')
Ferulate/Syringyl Crossed Dimers
9b (4-O-β')
10b (8-β')
Ara
X = xylan, Me
Peroxidase
H_2O_2
3b
4b
5b
6b
syringyl units and sinapyl alcohol

figure included the 4-*O*-α/5-β phenylcoumarans). Since only 10% of the ferulate could be released as ferulic acid by typical ester/ether cleaving reactions [15,16], the lower limit on the partitioning between active and passive mechanisms had to be 90% in that synthetic system. {Ferulates incorporated via the passive mechanism (Scheme 2), producing α-ferulate ethers **14**, would be fully releasable. In contrast, only ferulates incorporated into β-ether structures **9** are releasable from oxidative coupling products under basic ether-cleavage conditions [15,16] (4 M NaOH, 170°C).} In a biomimetic system, the quantity of ferulic acid (and diferulic acids) released by saponification was reduced by 90% when exogenously supplied hydroxycinnamyl alcohols were polymerized into non-lignified walls by wall-bound peroxidases and in situ-generated H_2O_2 [17]. Only 40% of the ferulate incorporated into lignins was recovered following hydrolysis of ether linkages, suggesting again that most of the ferulate was incorporated into the lignin via oxidative coupling mechanisms [17]. We are currently working on methods to determine the partitioning of this 40% between α- vs β-ethers from passive vs active processes, respectively, but suspect that β-ethers will predominate. Further evidence comes from the observation that the entire range of dehydrodiferulate isomers are present in grass cell walls (contrary to the prior observations of only the 5-5-coupled dehydrodimer) [12]. Ferulate radicals **2** are obviously being produced and are homo-coupling to effect polysaccharide–polysaccharide cross-linking [12]. Logically, these same radicals should cross-couple with hydroxycinnamyl radicals **5–6** to effect lignin–polysaccharide cross-linking.

More direct evidence supporting active incorporation has been difficult to obtain and, despite the emergence of the compelling evidence described above, acceptance of the idea has not been forthcoming. Application of diagnostic NMR spectroscopy has been thwarted by its insensitivity, precluding detection of polysaccharide–ferulate–lignin coupling in plants. This manuscript presents proof, from uniformly labeled ryegrass, that ferulates are indeed oxidatively coupled with lignin precursors in vivo producing ferulate-mediated polysaccharide–lignin cross-linkages.

2. Results and discussion

Ryegrass was grown in an atmosphere in which the ambient levels of CO_2 were enriched in ^{13}C to a level of $\sim$ 15%. This level provides the requisite NMR sensitivity enhancement (by a factor of 15) without severe problems caused by ^{13}C–^{13}C homonuclear coupling. A 96:4 1,4-dioxane–water-soluble lignin fraction was isolated as previously described for maize [18]. The carbon NMR spectrum (F_1 projections on the left side of Fig. 1, peak groupings labeled **A–D**) showed evidence for the same ferulate peaks as in the synthetic system [1] described above. Long-range C–H correlation via the HMBC experiment provided diagnostic correlations, Fig. 1, some of which were analogous to those evidenced in the synthetic system [1] as indicated by overlaid symbols. The most diagnostic correlation peaks, unambiguously proving that ferulates do indeed cross-couple with lignin monomers/oligomers is the 8-β'-coupling peak labeled **D**, corresponding to structures **10** and **10b** (Scheme 1). Figure 1b shows more diagnostic correlations for this structure when a long-range coupling delay of 100 ms



Scheme 2. The popular 'passive' mechanism for incorporation of ferulates into lignins. Ferulate **1** does not enter into the one-electron oxidative coupling processes; these process do, however, produce quinone methide intermediates **13** from hydroxycinnamyl alcohols and lignin monomers or oligomers. Ferulates nucleophilically add to the quinone methides, in competition with other nucleophiles in the cell wall including acids and water, to produce lignin–ferulate α-ethers (benzyl aryl ethers) **14**. Ferulate is 100% releasable from these structures by high temperature base solvolysis. L = lignin moieties as in Scheme 1.

was used; correlation intensities depend on $^{2}J_{C\text{-}H}$ and $^{3}J_{C\text{-}H}$ coupling constants. The four correlations with the carbonyl carbon are to each of the protons in the dioxabicyclo[3.3.0]octan-2-one unit that are within three bonds, namely the α-, 7-, 8-, β-protons [1,2,19]. These correlations can only arise from 8-β'-coupling reactions of ferulate radicals **2** (8-position, Scheme 1) with lignin precursor radicals **6** (β-position). Correlations with peaks **B** derive from products, undifferentiated by this experiment, of 4-*O*-coupling; namely 4-*O*-β' (cf. **9**) and 5-β'/4-*O*-α' (cf. **8**, phenylcoumarans) as well as the passive product, the 4-*O*-α' ether **14**. The strong correlations with protons at 4.2 and 4.45 ppm clearly arise from the esterified polysaccharide primary alcohol protons. The shifts are in agreement with those of the C-5-acylated protons on arabinofuranosyl moieties (triangles in Fig. 1a) [2]. Contributions from other attachment sites or other saccharides cannot be ruled out from this experiment.

Two important implications regarding the cross-linking process are also revealed by this correlation experiment. Firstly, while the 8-β'-coupled product **10** is a key observation in proving that radical mechanisms are involved in ferulate incorporation into lignins, this product is the least significant in terms of cell-wall cross-linking since the previously feruloylated polysaccharide moiety is released via internal transesterification during production of structures **10** [1]. Secondly, and more importantly, the 8-β' moiety in the LPC can arise only from coupling with a lignin unit possessing both a free phenol and α,β-unsaturation; this can only be free hydroxycinnamyl alcohols **4** or **4b** or, less likely, a coniferyl alcohol linked to another coniferyl alcohol or a lignin oligomer via 5-5′ or 5-O-4′ bonds (leaving the free-phenolic conjugated 4-hydroxycinnamyl system intact). The prominence of the 8-β'-coupled products in the in vivo system, as revealed here, is an indication that ferulate may be reacting extensively with lignin monomers **4** and not, as in the DHP case, with lignin oligomers **3**. This finding may be the first evidence that ferulate functions as an initiation or nucleation site for lignification—a site

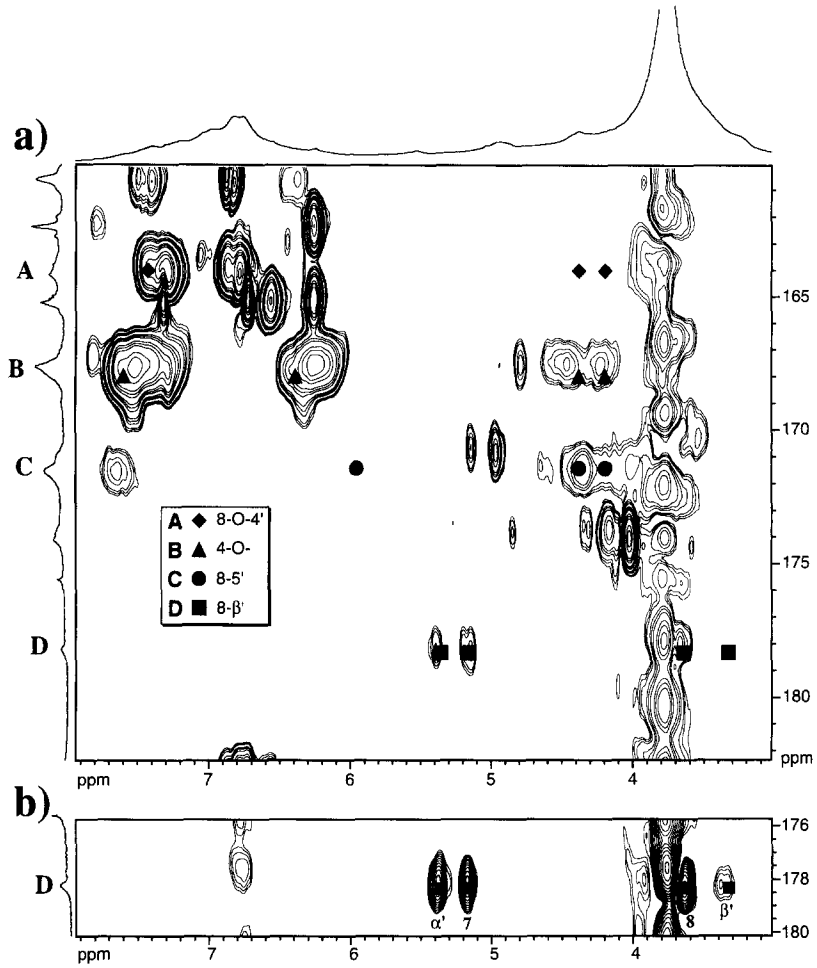


Fig. 1. (a) Small section of a long-range C–H correlation (HMBC) spectrum of uniformly ^{13}C-enriched ryegrass LPC with an 80-ms long-range correlation delay showing just the carbonyl carbon region. Processing was with gaussian apodization in t_2 as a compromise for all correlations (LB = −40, GB = 0.35). Regions are labeled corresponding to regions in a prior synthetic lignin–FA–Ara polymer [1]; **D** is the 8-β'-coupling region and **B** is the 4-*O*-β' and 4-*O*-α' region (including products arising from 5-β'/4-*O*-α', or phenylcoumaran, structures). Overlaid are the data for similar correlations in the FA–Ara DHP model system [1]. (b) The 8-β'-selection of a similar experiment run using a 110 ms long-range coupling delay which reveals the β- and 8-proton correlations more completely. Gaussian apodization in t_2 (LB = −40, GB = 0.4). Numbering convention: numbering relates to that in the original ferulate (7–9 in the side-chain) and the original hydroxycinnamyl alcohol (α', β', γ' in the side-chain). When describing a dimer by its linkage, e.g. 4-*O*-β', the first term refers to the ferulate moiety and the primed term to the lignin moiety.

at which the first coupling reactions with lignin monomers occur and from which lignification proceeds to build up the lignin–polysaccharide complex. A template theory has been advanced by ourselves [4] and others [5,6] but has been only an interesting hypothesis until this point.

One particularly striking feature of the correlation spectrum is the complete absence (within the detectable limits of this experiment) of 8-5′-coupling products **12** in region **C** (Fig. 1) and 8-O-4′ products **7** in region **A**. Such coupling is substantial in the synthetic system [1,2], and one of the major ferulate dehydrodimers (in vitro and in vivo) is the 8-5′-homocoupling product [12]. The lack of 8-5′-coupled products could be explained if it is sinapyl alcohol **4b**, not coniferyl alcohol **4**, that is first introduced into the developing cell wall (Scheme 1), although current theories are that ferulate is incorporated early in lignification while sinapyl alcohol incorporation is later [20–22]. Sinapyl alcohol is capable of coupling only at its 4- and β-positions (the 5-position is blocked by a methoxyl) and β-β or β-8 coupling of sinapyl alcohol with a number of related structures is known [11]. However, evidence that both coniferyl alcohol and sinapyl alcohol are reacting with ferulate was also obtained from the HMBC NMR experiment, as described below. Alternatively, it may be that cross-coupling of ferulate and coniferyl alcohol monomers (as opposed to oligomers) simply does not favor the 8-5′ product **12** or the 8-*O*-4′ product **7**. We have noted in a substantial database of single-electron oxidation reactions [11,23] that, even though each of the monomers favors coupling at its β-or 8-position, in non-β-β (β-8) hetero-couplings, coniferyl alcohol always dominates these encounters, coupling at its β-position and relegating the other monomer to the less-favored coupling positions (4- and 5-). Thus, in vitro and, presumably, in vivo, cross-coupling of coniferyl alcohol with other moieties rarely results in 5- or 4-*O*-linked coniferyl alcohol products—these linkages in lignins result from addition of coniferyl alcohol radicals, at their β-positions, with pre-formed dimers or oligomers in which the conjugated side-chain is no longer present (and coupling at the β-position is not possible). The absence of both 8-5′ and the 8-*O*-4′ products provides further evidence for the role of ferulates as initiation sites; those linkages are more likely to arise from coupling with lignin oligomers than with coniferyl alcohol monomers. Further studies aimed at understanding the differences between the synthetic and in vivo lignification system can shed light on temporal and mechanistic aspects of the cross-linking process and are in progress in our laboratories.

The HMBC experiment also allows us to determine if the absences of 8-5′ and 8-O-4′ products **12** and **7** are caused by reaction of sinapyl alcohol monomers **4b** rather than coniferyl alcohol **4** with ferulate. Three-bond correlations (Fig. 2) of the α- and 7-protons with carbons 2 and 6 on the aromatic ring in structures **10** vs **10b** identify the lignin monomers reacting to produce such structures. Proton-7 should correlate with ferulate F2 and F6 carbons in either **10** or **10b**. A weak two-bond correlation to carbon F1 can also be seen in Fig. 2. Correlations from the α'-proton to carbons G2′ and G6′ can be expected for coniferyl alcohol-derived structures **10** while correlations to equivalent carbons S2′/S6′ would derive from sinapyl alcohol coupling. Both sets of correlations are observed, Fig. 2. Although the contours are weak and markedly dependent on $^{3}J_{C\text{-}H}$ coupling constants, it appears that S-peaks predominate over G (i.e. structures **10b** are more prevalent than **10**). However, the clear evidence of peaks derived from coniferyl alcohol indicates that the absence of 8-5′ structures is not due to reactions solely with sinapyl alcohol. Since we know that lignin oligomers (in a DHP) will react with ferulate to form 8-5′ products **12** [1], the only explanation left is that the ferulate never sees lignin oligomers. i.e. that reaction of ferulates is (almost) entirely

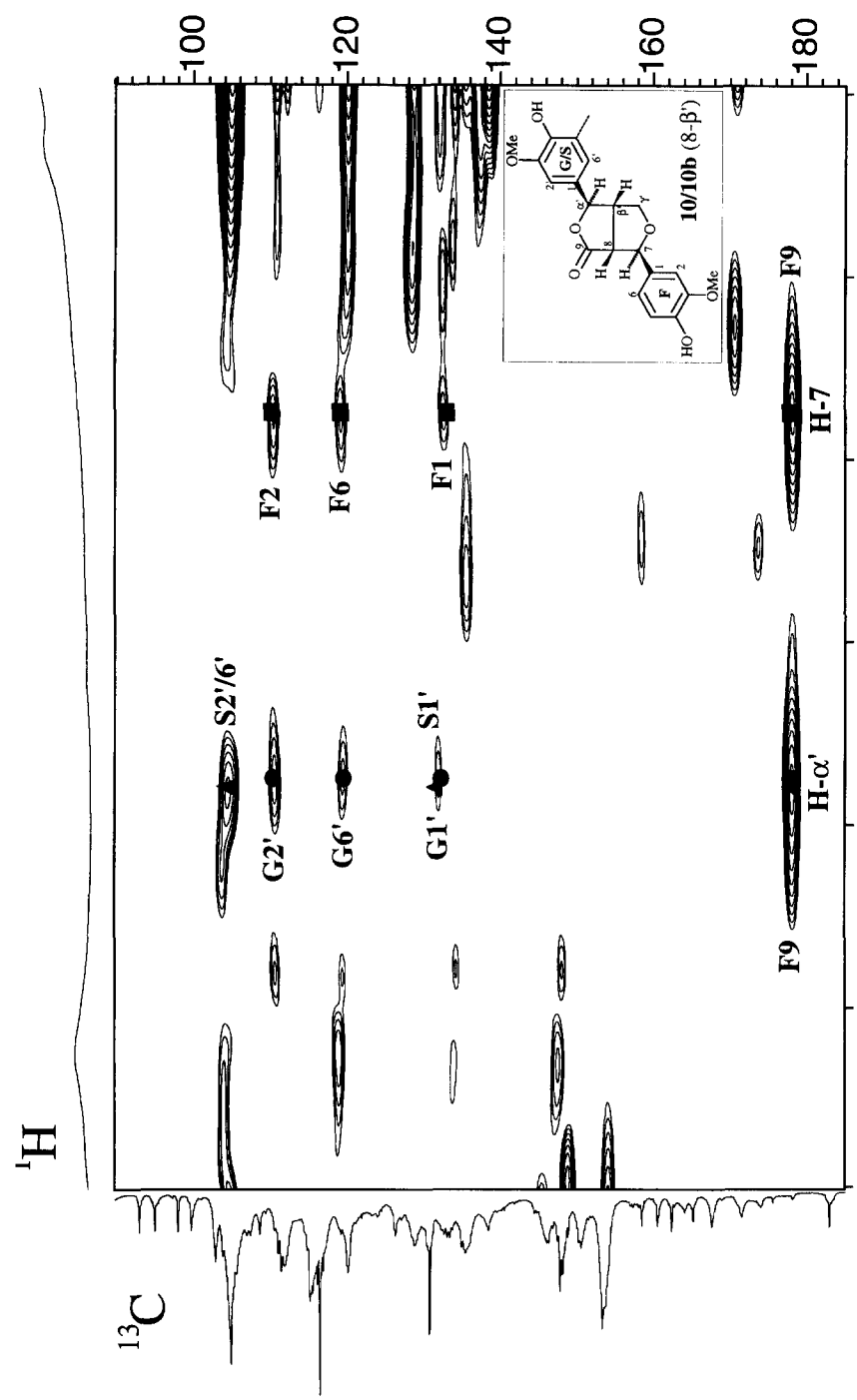


Fig. 2. Small subsection of the HMBC experiment showing correlations of protons 7 and α' with aromatic 2'- and 6'-carbons in 8-β' products **10**. Correlations with H-α indicate that ferulates couple with both coniferyl alcohol (G) and sinapyl alcohol (S). Overlaid symbols are: ■, ●, data from compound **10** [2,19]; ▲, calculated ^{13}C syringyl shifts from guaiacyl → syringyl substituent effects applied to compound **10** (C1, −0.9; C2, −5.9; C6, −15.1) taken from the recently released NMR Database for Model Compounds for Lignin and Related Cell Wall Components [25].

with lignin monomers. Exclusive reaction with coniferyl alcohol **4** or sinapyl alcohol **4b** supports the idea that lignin monomers slowly diffusing into the region of the developing wall occupied by ferulates encounter peroxidases and cross-couple with ferulates. Ferulates therefore are indeed acting as initiation sites or nucleation sites for the growth of the lignin polymer.

If it proves to be general that ferulates react solely with lignin monomers, fewer cross-linking structures would arise in vivo than in synthetic lignin systems. This is because, for coniferyl alcohol, only dimers **8** (5-β'), **9** (4-*O*-β'), and **10** (8-β') (Scheme 1) will likely be produced to the practical exclusion of dimers **7**, **11**, and **12**. Again, this results from coniferyl alcohols' propensity for coupling at its β-position. The 8-β'-products **10** will not release ferulic acid during analytical procedures, but it can be argued that this ferulic acid is less important to quantify as it, in cross-coupling with the lignin monomer, displaces the polysaccharide moiety and does not therefore represent ferulate that is involved in cross-linking polysaccharides to lignin. The ferulate in the 4-*O*-β'-structure **9** will be releasable (although not necessarily quantitatively), leaving only structures **8** that become unquantifiable cross-linking products. Assuming that sinapyl alcohol has a similar disposition for coupling at its β-position in cross-coupling encounters, structures **8b**, **9b** and **10b** are viable while **7b** is likely to be minor. The DHP studies [1,4], which revealed that the 8-*O*-4'-structures **7** (and **7b**) and the 8-5'-structures **12** form a substantial fraction of the product distribution, led us to predict even greater underestimation of ferulates by current and foreseeable analytical methods since they do not release ferulic acid. However, if ferulates are truly reacting entirely with lignin monomers, neither of these structures is likely to be significant. We continue to stress, however, that ignoring active radical mechanisms significantly underestimates ferulic acid and its role in cross-linking wall polymers. It must be cautioned that the NMR evidence described above does not derive from the entire lignin fraction. Thus partitioning of linkage types into the 1,4-dioxane–water-soluble fraction cannot be ruled out. It has been demonstrated that, in ball-milled woody species, 1,4-dioxane–water-soluble lignins, are derived predominantly from the secondary wall [24]. Our observation that sinapyl alcohol **4b**, which is incorporated later in wall development [20–22], is adding to ferulates suggests that feruloylated arabinoxylans are also laid down in the secondary wall.

The findings described above provide a compelling endorsement of the value of prior model and DHP studies for understanding cell-wall processes but indicate the necessity for carefully examining and interpreting the results of parallel in vivo studies. In this case, the synthetic system in which peroxidase enzymes, hydrogen peroxide, ferulates, and coniferyl alcohol (and subsequently its oligomers) are all present in a homogeneous system shows the range of possible products that can be formed [1]. However, it is the plant system that suggests that ferulates react extensively with lignin hydroxycinnamyl alcohol monomers (and not pre-formed oligomers) and therefore that ferulate polysaccharide esters act as initiation sites for lignification. We are now examining suspension-cultured maize systems, expecting similarly slow diffusion of monomers into the primary walls containing ferulates and wall-bound peroxidases, to test these theories. This study is also an endorsement of not only the power but the necessity of 2D correlative NMR experiments for confirmation of structures in such complex systems

—^{13}C resonances at the right frequencies for the complete range of ferulate–lignin structures **7–12** are present (**A–D**, Fig. 1), but the correlations show, in particular, that the 8-5′-coupled product **12** is not represented. Over-interpretation of the 1D data without complementary 2D data would have led to erroneous conclusions and missed implications.

3. Conclusions

We have demonstrated that ferulate polysaccharide esters actively participate with lignin monomers in oxidative coupling pathways to generate lignin–ferulate–polysaccharide complexes during cell-wall development in ryegrass. Unlike in the passive incorporation mechanisms where ferulate is fully releasable, active incorporation of ferulates produces a range of structures, many of which will not produce releasable ferulic acid by typical solvolytic methodologies currently used for their quantitation. The observation here that active incorporation of ferulate polysaccharide esters into the lignin–polysaccharide complex is a mechanism that is occurring in vivo must alert researchers to the inescapable conclusion that ferulates in lignified grass tissues always have been, and will continue to be, underestimated to an unknown degree. Currently the only method for determining the full scope of their involvement in the LPC is via NMR, but that technique is not without significant limitations. The unanticipated finding that certain ferulate coupling products are not produced in ryegrass provides evidence for the role of feruloylated polysaccharides as initiation/nucleation sites for lignification. Feruloylated polysaccharides in grasses are therefore critical entities in directing cell-wall cross-linking during plant growth and development.

4. Experimental

Uniform ^{13}C-labeling of ryegrass.—Seeds of diploid annual ryegrass (*Lolium multiflorum* Lam. cv. Florida 80) were incubated on wet filter paper for 7 days at 2°C. Hydrated seeds were placed into fine crushed granite (20-cm diameter pots), germinated and grown in a controlled-environment chamber. Seven days after emergence, seedlings were thinned to 40 per pot and the chamber was sealed to reduce air exchange with the outside environment to $\sim 3\%\ h^{-1}$. Carbon dioxide gas (15 atom% ^{13}C, Cambridge Isotopes) was released into the chamber to maintain CO_2 at ~ 350 ppm. Plants were grown under constant irradiation with fluorescent lamps (mean photon flux density of 500 μmol $m^{-2}\ s^{-1}$ PAR at canopy height) and a temperature of 23°C and a RH of 70%. Pots were watered four to six times daily with an excess of half-strength Hoagland nutrient solution. Plants were harvested at anthesis, 44 days after seedling emergence.

Isolation of ryegrass lignin.—The dried stem and sheath material was ground through a Wiley mill (1-mm screen), the ground material was Soxhlet-extracted sequentially with water, MeOH, acetone, and $CHCl_3$ to yield 70.86 g of extractive-free material, and the lignin isolated in a similar manner to that described recently for maize [18]. From 76.5 g of ball-milled material, Cellulysin (Calbiochem, Lot 035790) treat-

ment to remove polysaccharides gave 18.64 g of enzyme lignin. Extraction with 1,4-dioxane–water (96:4) gave 5.90 g of soluble lignin. We have previously noted that the dioxane–water extract contains high amounts of monomeric glucose which needs to be washed out [18]. Consequently, the freeze-dried soluble fraction was re-suspended in water and pressure-filtered through a 10,000 MW cutoff Amicon microfilter, 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, with water again, and freeze-dried to yield the final material for NMR analyses (LPC, 4.460 g, 28% of EL, 89% lignin).

NMR spectra.—^{13}C, ^{1}H NMR, and HMBC spectra of 300 mg of the isolated lignin in 2.4 mL acetone-d_6–D_2O were recorded in on a Bruker AMX-360 using a 10-mm broadband probe of normal geometry. The central acetone peak served as internal reference (δ_H 2.04 ppm, δ_C 29.8 ppm). The inverse long-range C–H correlation (HMBC) spectrum in Fig. 1 was run using Bruker's standard inv4lplrnd sequence [26] 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 640 scans per increment. The 90° pulse angles were 27 μs and 13.4 μs for ^{1}H and ^{13}C respectively, the acquisition time was 280 ms, and the relaxation delay was 1 s. The long-range coupling delay was set at 80 ms (corresponding to a long-range C–H coupling constant of 6.25 Hz). Optimized gaussian multiplication (LB = −40, GB = 0.35) was applied in t_2 with unshifted sine-bell squared apodization in t_1 and the matrix zero-filled and Fourier transformed (using magnitude mode phase correction) to give a final matrix of 2K by 1K real points. The carbonyl region of interest was them re-processed with double the number of datapoints in each dimension (corresponding to an final total matrix of 4K by 2K real points) giving resolutions of 0.89 and 8.40 Hz per point in the ^{1}H and ^{13}C dimensions. The spectra for Figs 1b and 2 were obtained similarly using a long-range coupling delay of 110 ms (corresponding to a long-range C–H coupling constant of 4.5 Hz). Processing used LB = −40, GB = 0.4 in t_2.

Acknowledgements

We are grateful to the staff at the U.S. Dairy Forage Research Center and to the Agricultural Research Service of the U.S. Department of Agriculture for funding the AMX-360 NMR instrumentation that has made this work possible, and gratefully acknowledge support through USDA competitive grants # 92-37304-8057 and 94-02764 in the Plant Growth and Development section. We are grateful to the USDA Administrator-funded postdoc program for partial funding of JHG, to Thomas D. Sharkey and Charles A. Baum and staff at the U. Wisconsin Biotron for helping with the chamber with enriched CO_2, and Lloyd R. Nelson for providing ryegrass seeds.

Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee of the product by the USDA and does not imply its approval to the exclusion of other products that might also be suitable.

References

[1] J. Ralph, R.F. Helm, S. Quideau, and R.D. Hatfield, *J. Chem. Soc., Perkin Trans. 1*, (1992) 2961–2969.
[2] J. Ralph, R.F. Helm, and S. Quideau, *J. Chem. Soc., Perkin Trans. 1*, (1992) 2971–2980.
[3] R.F. Helm and J. Ralph, *J. Agric. Food Chem.*, 40 (1992) 2167–2175.
[4] J. Ralph and R.F. Helm, in H.G. Jung, D.R. Buxton, R.D. Hatfield, and J. Ralph (Eds), *Forage Cell-Wall Structure and Digestibility*, ASA-CSSA-SSSA, Madison, WI, 1993, pp 201–246.
[5] S.C. Fry, in B.J. Milflin (Ed.), *Oxford Surveys of Plant Molecular and Cell Biology*, Vol. 2, Oxford University Press, New York, 1985, pp 1–42.
[6] E. Yamamoto, G.H. Bokelman, and N.G. Lewis, in N.G. Lewis and M.G. Paice (Eds), *Plant Cell-Wall Polymers. Biogenesis and Biodegradation*, Vol. 399, *ACS Symp. Ser.*, American Chemical Society, Washington, DC, 1989, pp 68–88.
[7] A. Bacic, P.J. Harris, and B.A. Stone, in *The Biochemistry of Plants*, Vol. 14, Academic Press, New York, 1988, pp 297–371.
[8] A. Scalbert, B. Monties, C. Rolando, and A. Sierra-Escudero, *Holzforschung*, 40 (1986) 191–195.
[9] E. Adler, *Wood Sci. Technol.*, 11 (1977) 169–218.
[10] R.M. Ede and I. Kilpeläinen, *Res. Chem. Intermediates*, 21 (1995) 313–328.
[11] S. Quideau, *Incorporation of* p*-hydroxycinnamic acids into lignins via oxidative coupling*, Ph.D. thesis, University of Wisconsin-Madison, WI, 1994; University Microfilms International #9428350.
[12] J. Ralph, S. Quideau, J.H. Grabber, and R.D. Hatfield, *J. Chem. Soc., Perkin Trans. 1*, (1994) 3485–3498.
[13] R.D. Hatfield, R.F. Helm, and J. Ralph, *Anal. Biochem.*, 194 (1991) 25–33.
[14] R.F. Helm, J. Ralph, and R.D. Hatfield, *Carbohydr. Res.*, 229 (1992) 183–194.
[15] K. Iiyama, T.B.T. Lam, and B.A. Stone, *Phytochemistry*, 29 (1990) 733–737.
[16] T.B.T. Lam, K. Iiyama, and B.A. Stone, *Phytochemistry*, 31 (1992) 2655–2658.
[17] J.H. Grabber, R.D. Hatfield, J. Ralph, J. Zon, and N. Amrhein, *Phytochemistry*, in press.
[18] J. Ralph, R.D. Hatfield, S. Quideau, R.F. Helm, J. Grabber, and H.G. Jung, *J. Am. Chem. Soc.*, 116 (1994) 9448–9456.
[19] S. Quideau and J. Ralph, *J. Chem. Soc., Perkin Trans. 1*, (1993) 653–659.
[20] N. Terashima, K. Fukushima, L.-F. He, and K. Takabe, in H.G. Jung, D.R. Buxton, R.D. Hatfield, and J. Ralph (Eds), *Forage Cell-Wall Structure and Digestibility*, ASA-CSSA-SSSA, Madison, WI 1993, pp 247–270.
[21] N. Terashima, K. Fukushima, S. Tsuchiya, and K. Takabe, *J. Wood Chem. Technol.*, 6 (1986) 495–504.
[22] N. Terashima, K. Fukushima, and K. Takabe, *Holzforschung*, 40 (1986) 101–105.
[23] S. Quideau and J. Ralph, *Holzforschung*, 48 (1994) 12–22.
[24] P. Whiting and D.A.I. Goring, *Svensk Papperstidn.*, 84 (1981) R120–R122.
[25] J. Ralph, W.L. Landucci, S.A. Ralph, and L.L. Landucci, *NMR Database of Model Compounds for Lignin and Related Plant Cell-Wall Components*, available from the author by e-mail.
[26] A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, 108 (1986) 2093–2094.