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Cell Wall Cross-Linking in Grasses by Ferulates and Diferulates

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Ferulate polysaccharide esters in grasses enter into free-radical coupling reactions in the cell wall. By radical dimerization of ferulates, polysaccharide-polysaccharide cross-linking is effected. A range of diferulate isomers are produced, only one of which had been quantitated in the past. Diferulates have been underestimated by factors of up to 20, belittling their contribution to functions in the cell wall. Both ferulates and diferulates participate in lignification reactions and become intimately bound with lignin. Under-quantitation is significant since it is not possible to release ferulate or diferulates from some of the structures generated. Overall ferulates play a significant role in cell wall development and impact upon polysaccharide utilization in grasses.

Cross-linking of plant cell wall components is expected to have a marked influence on various properties of the cell wall (1, 2). For example, in grasses, several authors have postulated the effects of isodityrosine and diferulate cross-links on cell-wall accessibility, extensibility, plasticity, digestibility and adherence (3, 4). The topic has been reviewed extensively (1, 5-10). In woody species, the cross-linking mechanism to receive most attention involves the direct attachment of polysaccharides to lignin via nucleophilic addition to intermediate lignin quinone methides during lignification. An American Chemical Society Symposium recently addressed some of these issues (11). The extent of such cross-linking and how the plant is able to exert control on this seemingly 'opportunistic' approach has yet to be fully determined.

It is well known that grasses have substantial amounts of hydroxycinnamic acids intimately associated with the cell wall, as has been detailed in a number of reviews (1, 5-10, 12). This chapter will concern itself only with ferulate, which has the major cross-linking role. It was recognized early on that ferulic acid, an ideally structured difunctional molecule, could have a role in cross-linking polysaccharides with lignin (Figures 1 and 2).

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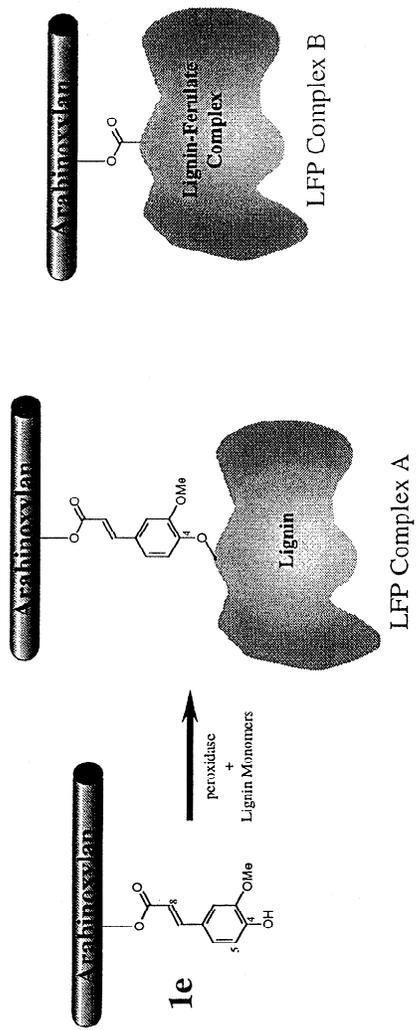


Figure 1. Ferulates, exported into the cell wall region as esters of polysaccharides, (primarily arabinoxylans in grasses), will cross-link with lignin via two mechanisms to yield a cross-linked lignin-ferulate-polysaccharide (LFP) complex from which the ferulate may or may not be releasable. The 'passive' mechanism (ferulate addition to lignin quinone methides) gives complexes (LFP complex A) from which the ferulate is fully releasable. The 'active' mechanism, where ferulate radicals cross-couple with lignin monomer or oligomer radicals, produces an LFP (Complex B) from which ferulate is only partially releasable.

Legend for all following structural Figures.
Definitions of R, L, Ara.

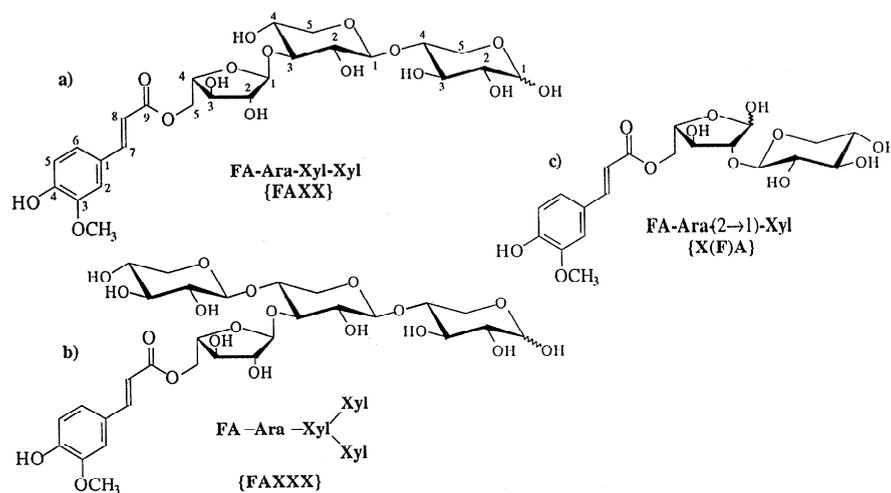
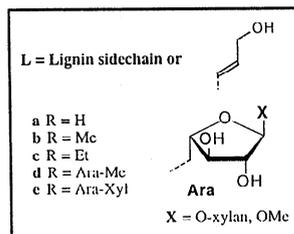


Figure 2. Ferulate-oligosaccharide esters isolated from grass cell wall following dissolution by mild acid or enzyme hydrolysates: (a) *O*-[5-*O*-(*trans*-feruloyl)- α -L-arabinofuranosyl]-(1→3)-*O*- β -D-xylopyranosyl-1→4)-D-xylopyranose (FAXX), (b) *O*- β -D-xylopyranosyl-(1→4)-*O*-[5-*O*-(*trans*-feruloyl)- α -L-arabinofuranosyl-(1→3)]-*O*- β -D-xylopyranosyl-1→4)-D-xylopyranose (FAXXX), and (c) 2-*O*- β -D-xylopyranosyl-(5-*O*-feruloyl)-L-arabinose (FA-Ara-(2→1)-Xyl). Further xylose homologues of (c), X(F)AXX, and XX(F)A have also been reported.

Ferulate-esterified Polysaccharides

Ferulate **1** (Figure 3) was found linked to polysaccharides, particularly arabinoxylans, in a regiospecific manner (13-22) as has been reviewed in the literature (6-9, 23, 24). In grass arabinoxylans it is attached exclusively at C-5 of α -L-arabinofuranoside residues, which are themselves glycosidically linked to the 3-positions of a xylan backbone (17, 19, 20, 24-27). The originally reported (16) 2-attachment was corrected (19). The arabinosyl residue may be further linked at C-2 to another xylose residue (28-30) or even to another xylan chain (27). Presumably the attachment of ferulate to the arabinoxylan occurs intracellularly in conjunction with polysaccharide synthesis and the product is exported out into the cell wall (10). Isolation and characterization of FAXX, FAXXX, X(F)A (Figure 2), X(F)AXX, and XX(F)A from mild acid hydrolyzed or enzyme-degraded bamboo shoots (17, 18, 25, 26), bermudagrass (19, 30), bagasse (13, 15), barley straw (20), maize (14, 29), sugar beet (31), wheat bran (16), and fescue (28, 32) was invaluable in defining the structural characteristics. Ferulates attached to other (oligo)saccharides have been reported in other plants, particularly spinach (31, 33).

Polysaccharide Cross-linking by Ferulates: Ferulate Dimerization

The presence of ferulates attached to polysaccharide components provides a convenient mechanism for cross-linking polysaccharides. Ferulate dimerization is all that is required to effect polysaccharide-polysaccharide cross-linking. Two mechanisms have been identified. Photochemical [2+2] cycloaddition produces cyclodimers, the truxillic acids (19, 34-38). Such acids can be released from grass walls and, although most of the dimers involve *p*-coumaric acid, early speculations were that this cycloaddition was a major cross-linking mechanism (19, 34-36). As will be shown below, this was in large part due to the major portion of the other mechanism's products having been missed. It is hard to know the plant's role in effecting such cross-linking; it presumably has little control over the photo-induced dimerization other than to arrange for hydroxycinnamates to be sufficiently proximate.

Radical-mediated dimerization is the other mechanism, although it was not recognized as such earlier. A dehydrodimer of ferulic acid, the 5-5'-coupled dimer **10** (Figure 3), commonly referred to as 'diferulic acid', has been isolated and 'quantitated' (see later) from grasses by saponification for some 20 years (39-47). In 1989 Yamamoto, Bokelman and Lewis (6) remarked that radical mediation had not been proven in a manner to confer only this coupling. We too were concerned since radical dimerization by single electron metal oxidants or peroxidases/H₂O₂ produced little to none of the 5-5'-coupled dehydrodimer **10** (Figure 3) (48). The main product was always the 8-5'-coupled dimer **8**. This had been discovered and reported several times (49-51) and has been recently reconfirmed (52). The 8-8' products **12** were also formed, as well as some 8-O-4' product **9**. Our main concern was how the 5-5'-coupled dimer **10** could be formed in plants to the apparently complete exclusion of the other dimers. Such a phenomenon would appear to indicate very tight enzymatic control of coupling. As will be shown below, this is simply not the case.

Dehydrodiferulates: Dimerization of Ferulate Radicals

Figure 3 shows how ferulates would be expected to dimerize *in planta* or in *in vitro* systems. Again, a consideration of this figure confirms it to be remarkable that only the 5-5'-coupled product **10** would be formed, particularly in light of what we know about the related dimerization of coniferyl alcohol (2, 53-58). In fact, when a variety of grass wall materials were saponified and analyzed by GC/FID or GC-MS (Figure 4), it was found that the entire spectrum of expected coupling products (with the exception of the 4-O-5'-coupled dimer **17** was present (48). Furthermore, the 5-5'-coupled product **16** was not the major dimer. Other researchers were also beginning

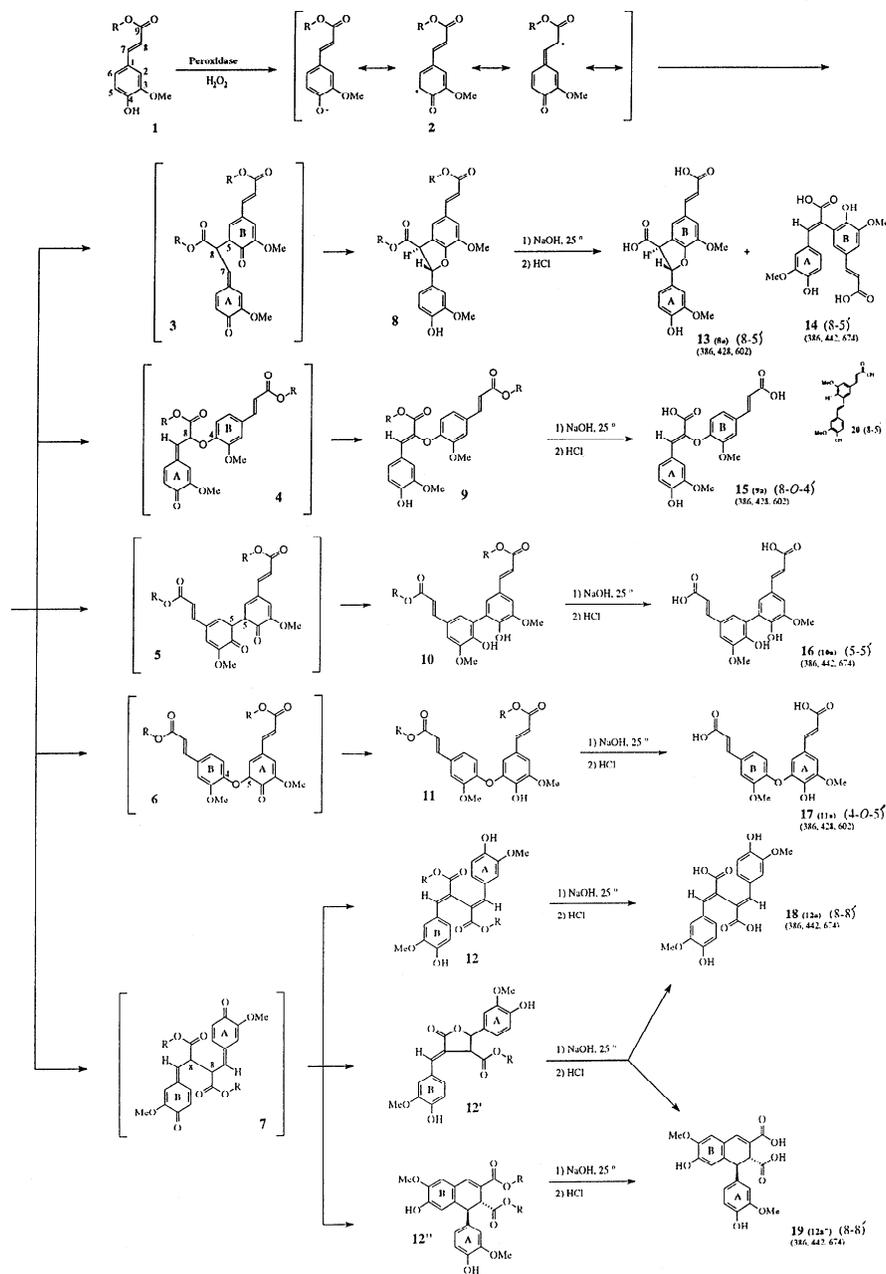


Figure 3. The general chemistry of dehydrodimer formation and saponification. Dimerization of ferulate esters via phenoxy radical 2 gives intermediates 3-7 which react in the cell wall to form dehydrodiferulates 8-12. During chemical analysis 8-12 can be saponified to dehydrodiferulic acids 13-19, also producing a small amount of compound 20. The values in parentheses below the structures represent the nominal molecular masses for the parent compound, the methylated, and the trimethylsilylated derivatives.

to suspect that there were more dehydrodimers but the two reports about the subject (59, 60) made the assumption that such dimers had all to be 5-5'-coupled. Thus the orientational isomers of Stewart *et al.* (60) certainly do not exist as discrete entities, and it is easy to show (by photochemical treatment of the 5-5'-dimer) that the claims of *cis/cis* or *cis/trans* isomers (59, 60) are not the explanation; only traces of non-*trans/trans* isomers were isolated from plant materials that we have screened (48).

Each of the radical-coupling products in Figure 3 was independently synthesized and characterized (48). GC-FID response factors were obtained, and a method was developed for quantitating all of the dimers. Figure 5 shows the results of such quantitation for four samples. More recently, an HPLC method has also been developed (61); the use of diode array detection aids in structural identification. Under-quantitation of even the 5-5'-coupled dimer **16** by possible neglect of response factors (36, 62), along with the ignorance of the other dehydrodimers **13-15, 17-20** may have led to up to a 20-fold underestimation of ferulate dehydrodimers (48). Dimers can account for as much as 70% of the total ferulate released from the wall. Clearly they play a far more significant role in wall development than previously considered. And it is not possible to quantitate all of the ferulate dehydrodimers; as we shall see below, they become inexorably covalently linked during lignification.

Diferulates are also being found in a variety of other plant materials. Waldron's group has shown very high levels in water chestnuts (63, 64), and suggests that they may be largely responsible for their crunchiness even after cooking. The light response of pine hypocotyls was tied to diferulate-mediated cross-linking of polysaccharides (65). Interestingly this group could find no 5-5'-coupled products, an observation more in line with the results of *in vitro* dimerizations. Previously, hypocotyl light responses in *Oryza* (66) and *Avena* (67) species had been noted and attributed to solely the 5-5'-dimer. Again, this may be an over-simplification, since we have recently shown that all dimers are present in their hypocotyls (68). Sugar beet has also been shown to be rich in diferulates (69, 70). Furthermore, elicitation of further cross-linking by treatment of beet pulps with peroxidase/H₂O₂ improves gelling properties (70). There is also a suggestion that, in a suspension culture system, cells compensated for reduced ferulate deposition (when grown in the presence of a phenylalanine-ammonia lyase inhibitor, 2-aminoindan-2-phosphonic acid) by increasing the extent of dehydrodimer formation (71).

Although clearly providing advantageous properties for plant growth and development, cross-linking has been thought to limit polysaccharide degradability (5-8, 23, 72-74), and this effect was essentially observed in a useful cell wall suspension cultured system (75). Higher levels of diferulates (but lower total ferulates) depress both the rate and extent of polysaccharide degradation (Figure 6) (76, 77).

Lignin-Polysaccharide Cross-linking: Ferulate Incorporation into Lignin

Verification that ferulates could be 'attached' to lignin was readily proven. Following room-temperature saponification, further ferulates were released by high temperature alkaline saponification under essentially wood pulping conditions (2-4 M NaOH, 170°C, 2 h) (73, 78-84). Ferulate release by acidolysis was also demonstrated (85-87). Lam, Iiyama and Stone provided a method of measuring ferulates that were both esterified and etherified, thus proving conclusively that ferulates were lignin-polysaccharide cross-linking agents (78, 80, 81, 88, 89). They found that all of the ferulate that was etherified was also esterified. This is expected if it is polysaccharide-ferulate esters that are exported into the cell wall. For us, it was the mechanism that was intriguing. Although various researchers had demonstrated that ferulates were 'attached' to lignin, there was little effort focused on the regiochemistry of attachment and the consequent mechanistic implications. Nearly all of the literature assumed a mechanism that we have described as 'opportunistic' or 'passive' (9, 49, 90, 91).

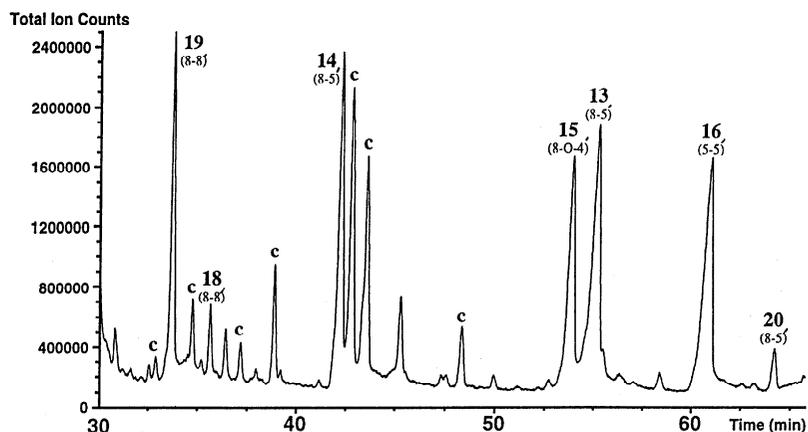


Figure 4. Total ion chromatogram from GC-MS of the dimer region of a saponified extract of primary cell walls from suspension cultured maize showing dehydrodiferulic acids **13-19** (with the exception of **17**). Peaks labeled **c** are assigned, without further authentication, to [2+2]-cyclo dimers by observation of an m/z 338 peak in their mass spectra.

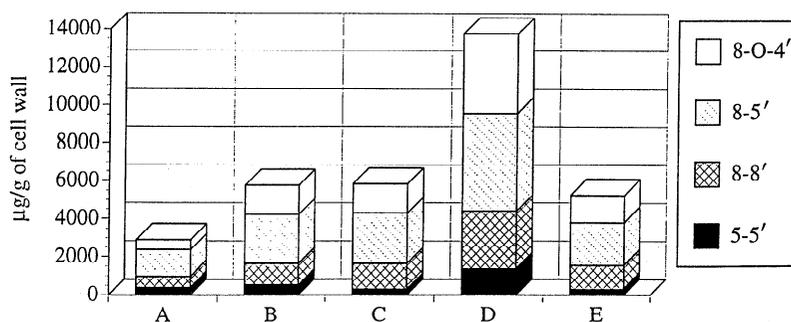


Figure 5. Composition ($\mu\text{g/g}$ of cell wall material) of dehydrodimers deriving from the individual coupling modes (5-5', 8-8', 8-5', and 8-O-4') for various plant cell wall samples: (A) suspension-cultured maize, (B) orchardgrass parenchyma, (C) orchardgrass sclerenchyma, (D) switchgrass parenchyma, (E) switchgrass sclerenchyma.

'Passive' Attachment of Ferulates to Lignin

It has been generally assumed that the attachment of lignin to ferulates was through α -ethers **26** (Figure 7) (78), a reasonable mechanism that can readily be demonstrated by model studies (86). However, consideration of only this mechanism had the unfortunate consequence that a great deal of the diversity in cross-linking was overlooked and the quantity and importance of this cross-linking severely underestimated. This is because α -linked ferulates are fully releasable (although not necessarily quantitatively) as ferulic acid by alkaline or acidic solvolysis. As we shall see later, products of the other mechanism of lignin-ferulate attachment cannot be similarly released under these conditions.

There are additional chemical questions relating to plant control of cross-linking. The α -ferulate ethers are formed by nucleophilic attack of the ferulate phenolic hydroxyl on the α -carbon of quinone methides, intermediates formed during lignification. Recall, however, that quinone methides are produced as the product of radical coupling between a coniferyl alcohol radical and another such radical or a radical derived from a lignin dimer or higher oligomer (Figure 7). The 'passive' cross-linking mechanism denies ferulate participation in the radical-coupling process, and yet we have just seen that ferulates certainly homo-couple *via* analogous radical reactions. The ferulate must also compete for the quinone methide with other phenols that are present (including lignin monomers and oligomers), and water, a reaction which *in vitro* is capricious and low-yielding (57, 91), and extensive work by Sipilä indicates that it is a poor reaction under aqueous conditions (92-94). There are conditions that favor such ether formation in DHP's. However, it is now recognized that non-cyclic α -aryl ethers may be of very low abundance in secondary wall lignins. Prior estimates of 6-9% (95) are in fact attributing other cyclic structures to non-cyclic α -aryl ethers. Ede's elegant work (96, 97) demonstrated clearly that α -ethers are scarcely detectable in NMR spectra of milled wood lignins and that the presumed α -ethers can be attributed to dibenzodioxocins, a lignin structure recently identified by Brunow's group (98, 99). So, although ferulate may add more competitively than other phenols (this has not been tested), conditions seem to be against its competing effectively for the quinone methide. Why then would the plant choose such an uncontrolled and poor reaction for effecting what is presumably a very important process, lignin-polysaccharide cross-linking? Despite these problems, or perhaps because researchers have not sufficiently considered the alternatives, the predominant mechanism described and illustrated in the literature is this 'passive' mechanism. [We coined the term 'passive', as opposed to the 'active' mechanism described below, because ferulate must just sit around waiting for a quinone methide to which it then may chance to add. The alternative mechanism has ferulates actively participating in the lignification process.]

Active Incorporation of Ferulates in Lignins

The logical alternative (or concurrent) mechanism involves ferulate radicals **25** (Figure 8). Since feruloylated polysaccharides are present in the wall prior to lignification, peroxidases and hydrogen peroxide could couple those ferulates that are sufficiently proximate to produce a whole range of ferulate dimers, as described above. Peroxidases (or alternative oxidases) and hydrogen peroxide are required for lignification. The scant mention (6, 12, 24) that this mechanism has enjoyed recognized that β -ethers would be produced by such a mechanism, but obviously this is an over-simplification (9).

Biomimetic Lignification of a Ferulate-Polysaccharide Model

The first logical issue to address was whether ferulates would cross-couple with lignin monomers/oligomers and, if so, what types of products formed were. One way to obtain this type of information is *via* synthetic lignification, since this process will

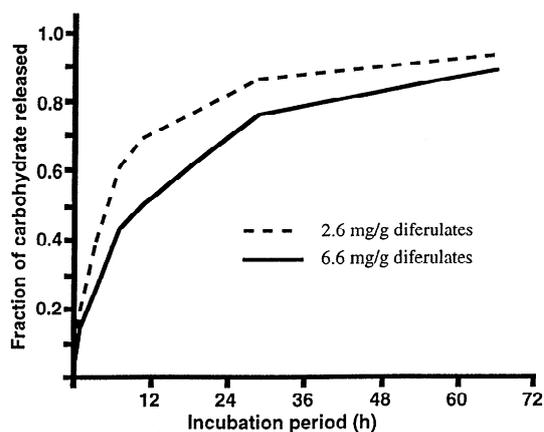


Figure 6. Total carbohydrate release from samples with differing diferulate levels.

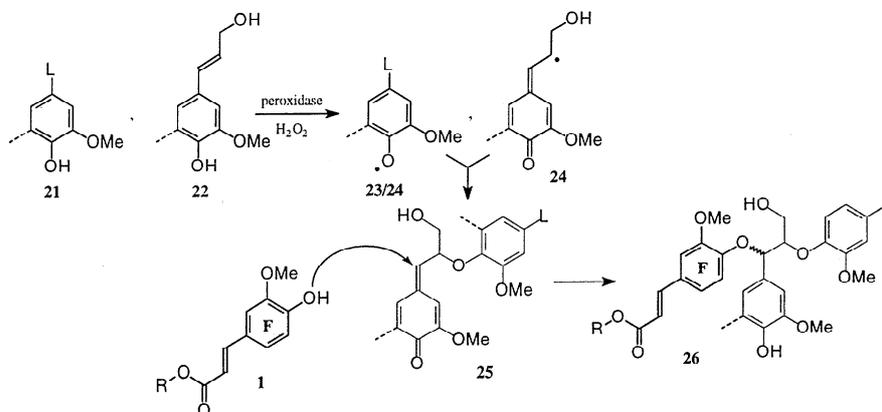


Figure 7. The popular 'passive' mechanism for incorporation of ferulates into lignins. Ferulate **1** does not enter into the one-electron oxidative coupling processes; these processes do, however, produce quinone methide intermediates **25** from coupling of a hydroxycinnamyl alcohol lignin monomer **22** with a lignin monomer or oligomer **21**. 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) **26**. Ferulate is fully releasable from these structures by high temperature basic solvolysis. L = hydroxycinnamyl alcohol sidechain or generic lignin sidechain plus the remainder of the lignin oligomer/polymer.

guaiacyl units and coniferyl alcohol

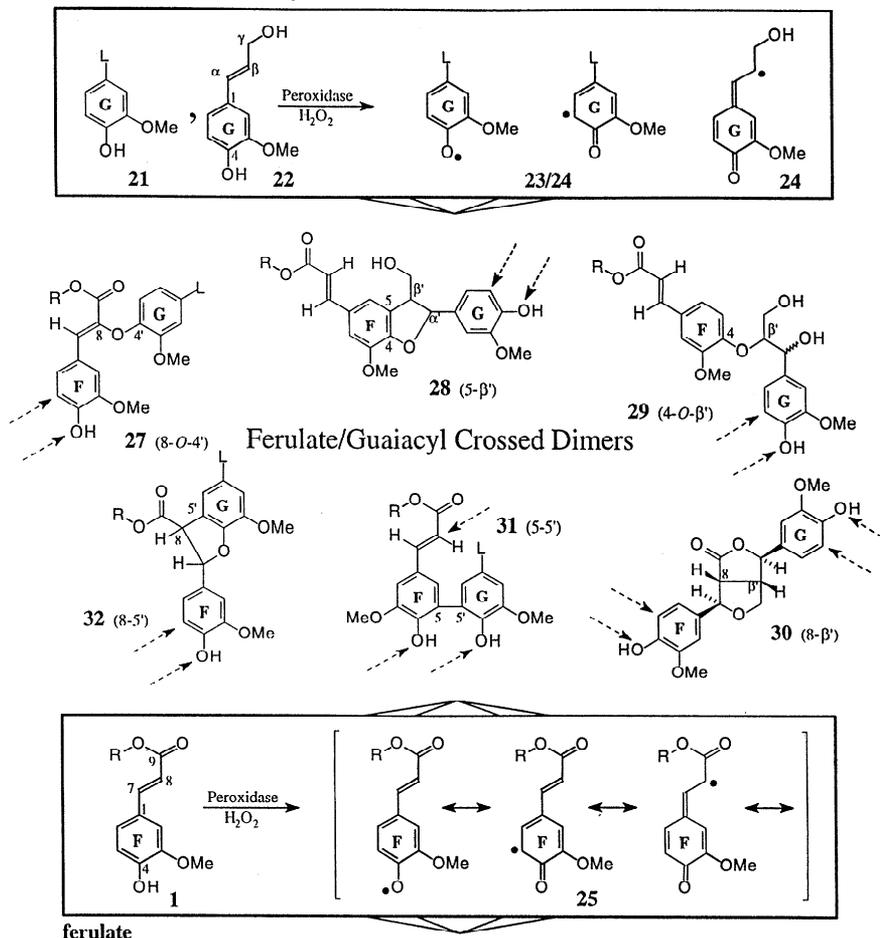
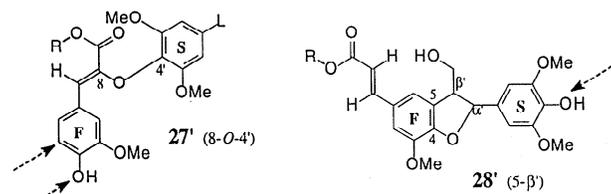
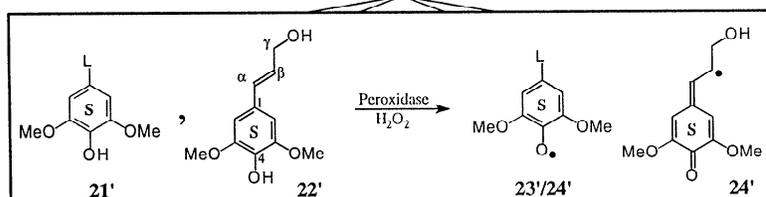
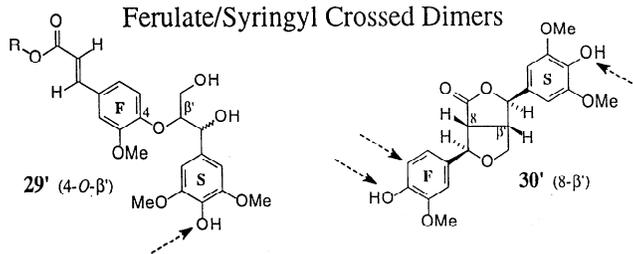


Figure 8. The 'active' mechanism for incorporation of ferulates into lignins. Radical cross-coupling products **27-32** formed by oxidative coupling of ferulate **1** with coniferyl alcohol **22** and guaiacyl (from coniferyl alcohol oligomers) units **23-24** (upper half), and **27'-30'** from coupling of ferulate **1** with sinapyl alcohol **22'** and syringyl (from sinapyl alcohol oligomers) units **23'-24'** (lower half). F, G, and S are to indicate aromatic rings originating from ferulate, guaiacyl, and syringyl units. R is defined in Figure 2, L = coniferyl alcohol sidechain or generic lignin sidechain plus the remainder of the lignin oligomer/polymer. Only compounds **29** and **29'** will release ferulate on high temperature base solvolysis. Dashed arrows indicate sites available for further polymerization.



Ferulate/Syringyl Crossed Dimers



syringyl units and sinapyl alcohol

Figure 8. *Continued.*

produce a range of structures that are possible from such cross-coupling reactions, but not all of those will necessarily be produced *in planta*. The most diagnostic and unambiguous method to identify such structures is NMR spectroscopy. ^{13}C -Labeling of the C-9 (ester carbonyl carbon) of the ferulate model **1d** (Figure 9) allows ready identification of any ferulate product that is linked at the 8-position (*c.f.* β -position in lignin), *i.e.* in 8- β' **30**, 8-5' **32**, and 8-O-4' **27** structures (see Figure 8). The ring-linked structures, 4-O- α' **26**, 4-O- β' **29**, and 5- β' /4-O- α' **28**, are distinguishable from the three 8-linked structures but are not resolvable with this label—a 4-label would be valuable in that case but is significantly harder to introduce.

The model ferulate chosen (49, 100) (Figure 9) was one that mimics the ferulate attached to arabinoxylans in grasses. Thus the ferulate is attached to C-5 of an α -L-arabinofuranoside; a simple methyl group replaces the xylan chain. A DHP was prepared using *ca.* 10% of this [9- ^{13}C]-labeled model **1d** and 90% coniferyl alcohol **22** (49). An inverse-detected long-range C-H correlation method, the HMBC experiment (101), was then used to correlate the labeled carbonyl carbons with protons within 3 bonds of the carbonyl. As seen in Figure 10, the (partial) HMBC spectrum is totally diagnostic, revealing that the single ferulate carbonyl resonance has produced some 4 groups of structures representing all of the expected bonding modes. Model data (49, 102) correspond exactly in both dimensions confirming the assignments. The 8- β' structure **30**, in particular, conclusively illustrates that cross-coupling between ferulate (8-position) and coniferyl alcohol (β -position) has occurred—homocoupling products are symmetrical and have only one α -proton for example. This product will be highlighted below to show that similar processes indeed occur in plants. The model and DHP data provide the necessary database to search for evidence of active ferulate participation in lignification *in vivo*.

NMR Evidence for 'Active' Mechanisms in Ryegrass

Armed with the required spectroscopic data for the array of cross-linking products that are possible (as indicated by the DHP study), it is conceivable that plant materials could be probed for such structures. Unfortunately, ferulate is present at a very low level in lignins (*ca.* 2% of the lignin) and NMR spectroscopy is a notoriously insensitive technique. Still, no other method comes close to being as structurally revealing. Solution-state NMR spectroscopy is the only consideration since it alone has the necessary resolution for these types of structural studies. We determined that uniform labeling of plant material should allow detection of these compounds. The ferulate products were sufficiently resolved from other lignin/polysaccharide peaks and the two-dimensional methods, which provide up to five pieces of concurrent data for each structure, are highly diagnostic. The alternative, labeling of say the 9-carbon of ferulate and somehow incorporating this into the plant, is fraught with problems of uptake and non-natural metabolism.

Ryegrass was grown in an environment in which the CO_2 was *ca.* 15% enriched with ^{13}C (90). Lignins were isolated and NMR spectra, particularly the HMBC approach, proved revealing. The first obvious revelation is that the 8- β' -crossed product **30** is clearly visible in the ryegrass spectrum (Figure 11). The beautiful and characteristic correlations are unmistakable and the chances of some other unrelated compound having the same five pieces of matching NMR data is highly improbable. Thus, the mechanism of active incorporation of ferulates into plant lignins was proven. A closer look at the spectrum revealed something even more intriguing. Some of the expected (from the DHP) correlation peaks, and therefore their corresponding structures, were missing. As it turns out, these are the very structures that come *only* from coupling of ferulate with preformed lignin dimers or oligomers. Only those products that arise from coupling with a coniferyl alcohol monomer radical were present. This striking observation suggests, therefore, that ferulates are acting as nucleation sites for lignification. That is, they are the sites at which building of the lignin macromolecule starts in the wall (although we cannot say that

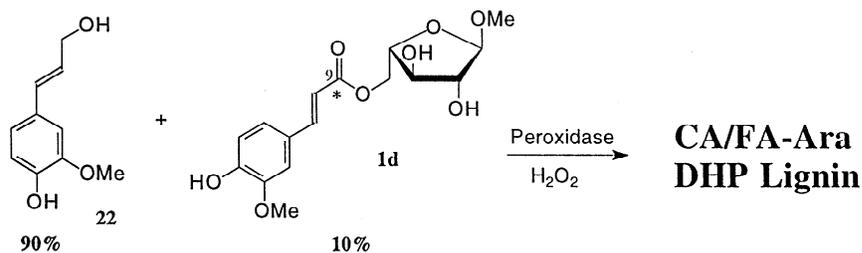


Figure 9. Preparation of FA-Ara/coniferyl alcohol copolymer DHP. The FA-Ara is strategically ^{13}C -labeled at C-9.

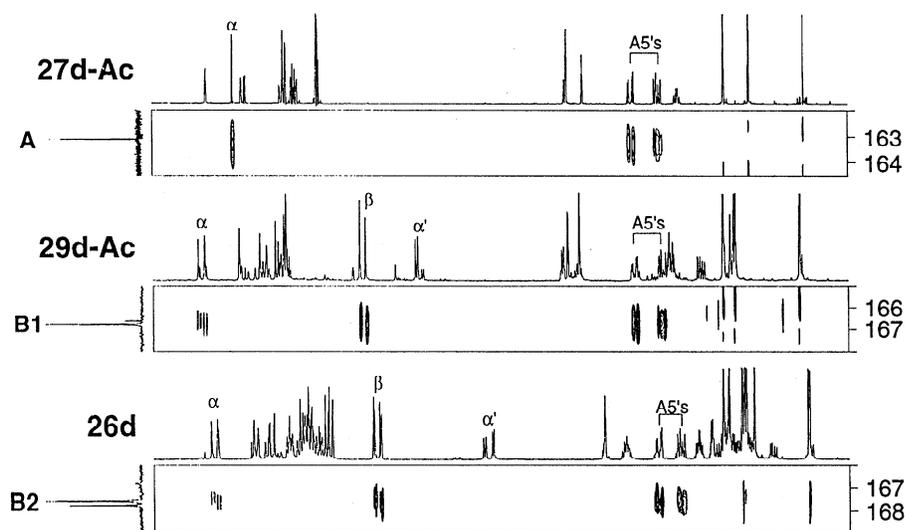


Figure 10. Portion of inverse-detected long-range 2D ^{13}C - ^1H correlation spectrum of FA-Ara/coniferyl alcohol DHP showing just the carbonyl carbon region. The 1D carbon and proton spectra along the axes are from quantitative 1D experiments. Peak groupings are assigned to structures in Figure 8. Authentication of assignments is shown *via* similar regions of the HMBC spectra of model compounds. Regrettably, compounds **27d-Ac** and **29d-Ac** are fully acetylated and consequently do not model the parent compounds precisely, particularly with regard to the arabinosyl proton shifts. Compound **30** was not obtained in pure form; the spectrum slice shown is from a mixture of the required compound **30** along with pinoresinol and the dilactone.

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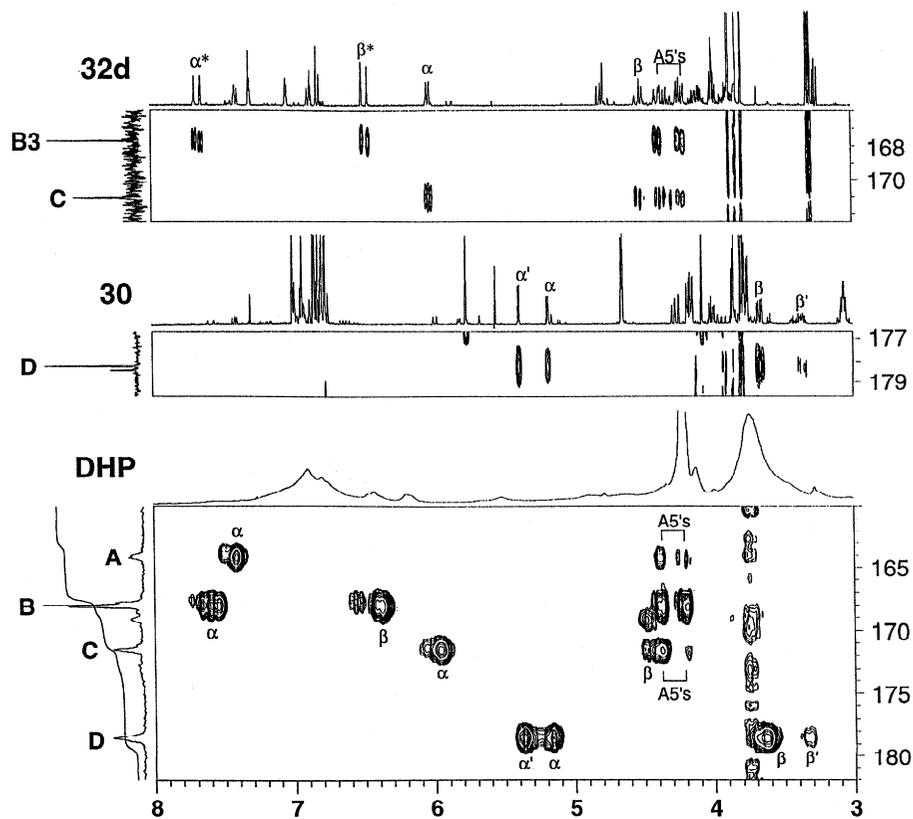


Figure 10. *Continued.*

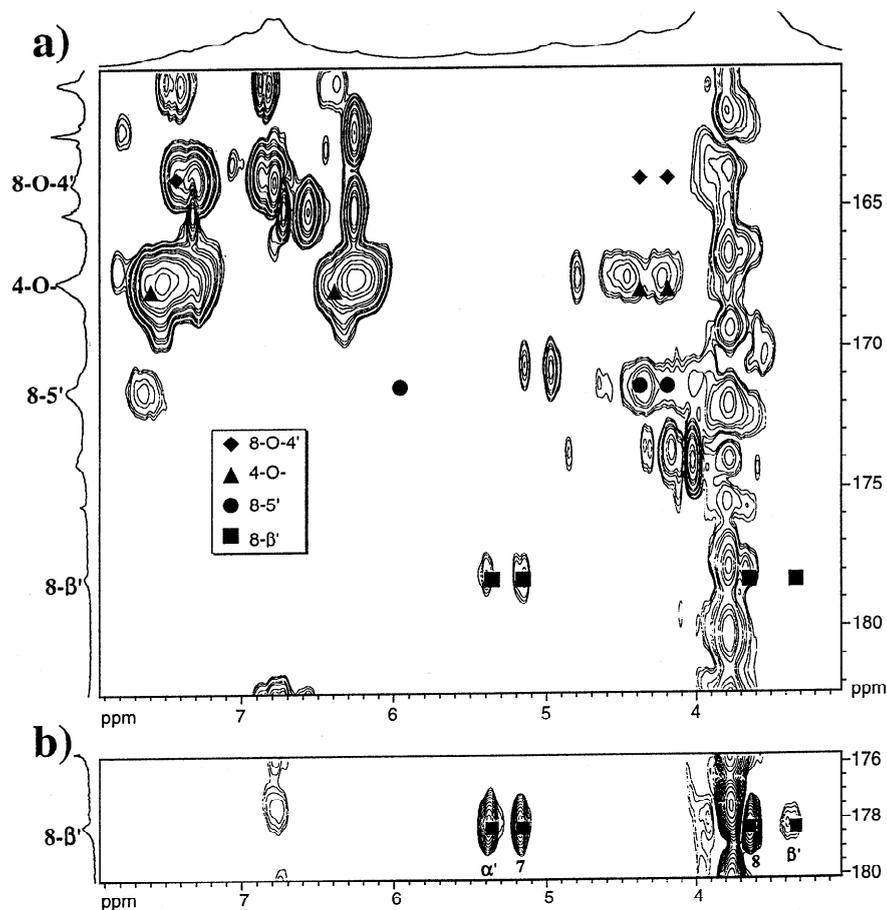


Figure 11. (a) Small section of a long-range ^{13}C - ^1H correlation (HMBC) spectrum of uniformly ^{13}C -enriched ryegrass LPC with an 80 ms long-range correlation delay showing just the carbonyl carbon region. Processing was with gaussian apodization in t_2 as a compromise for all correlations (LB = -40, GB = 0.35). Regions are labeled corresponding to regions in a prior synthetic lignin-FA-Ara polymer (Figure 10). Overlaid are the data for similar correlations in the FA-Ara DHP. (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 was employed in t_2 (LB = -40, GB = 0.4). Numbering convention relates to that in the original ferulate (7-9 in the sidechain) and the original hydroxycinnamyl alcohol (α' , β' , γ' in the sidechain). When describing a dimer by its linkage, *e.g.* 4-O- β' , the first character refers to the ferulate moiety and the primed character to the lignin moiety.

lignification must start at these sites). Similar mechanisms for lignin initiation had previously been proposed (6, 23, 103, 104).

All this seems to make more sense for plant growth and development. Now we have a mechanism whereby all accessible ferulate can become involved in cross-linking to lignin—there is no competition for quinone methides as in the ‘passive’ mechanism. Thus the plant has more direct control of this important process. Furthermore, it appears that the plant may utilize these ferulates to direct lignification to specific sites in the wall.

Further Evidence of ‘Active’ Mechanisms

A ferulate-coniferyl alcohol cross-coupling product **29a** released from grass straw was recently identified by Jacquet *et al.* (105). Such an observation already proves that radical mechanisms are in operation. Our group was humbled by not having thought to look for such a product. We had synthesized exactly these compounds earlier to provide NMR data (91), but had never considered that ‘lignification’ would finalize at the dimer stage. Since Jacquet’s finding, we have confirmed that cross-coupling dimeric products are releasable from grasses. These include both isomers of **29a**, **30a** and a ring-opened analog of **30a** and an analog of **28** without the hydroxymethyl group (106).

Additional evidence comes from observations pertaining to the lignification of feruloylated primary cell walls. A maize suspension culture system has been described (71, 76, 107) that serves as an excellent model for wall lignification. It is a primary wall system that has laid down cellulose and hemicelluloses; essentially no lignin is present but the arabinoxylans are feruloylated. The extent of feruloylation can be manipulated by using the phenylalanine ammonia lyase inhibitor 2-aminoindan-2-phosphonic acid (71). The walls also contain a complement of peroxidases. Thus a biomimetic lignification, utilizing the walls’ own peroxidases, can be effected by supplying lignin monomers and a source of hydrogen peroxide. The lignin produced in this manner is almost identical to native maize lignins as demonstrated by analytical thioacidolysis and NMR spectroscopy (75). It is assumed that slow diffusion of monomers into the wall containing the peroxidases is largely responsible for mimicking the natural process far better than traditional DHPs or even variants utilizing polysaccharide ‘templates’ and slow generation of lignin monomers (108, 109). It would be interesting to have similar analyses of Tanahashi and Higuchi’s high molecular weight lignins produced in dialysis tubes with and without added polysaccharides (110-113). When the feruloylated cell walls are lignified, the amount of ferulic acid releasable by room-temperature saponification drops to as low as 1/20th of its original level (71). That is, up to 95% of the ferulate has become incorporated into the lignin. But only *ca.* 40% of this incorporated ferulate is releasable by high temperature base treatment. We have shown that the only structures that release ferulate are the 4-*O*- α' - and the 4-*O*- β' -ethers **26** and **29** (71). Thus, even if the entire released component were from the passive α -ether products **26**, the upper limit on the passive incorporation mechanism would be 40%. However, the corollary is that at least 60% is incorporated *via* the active mechanism. This mechanism would of course be expected to produce 4-*O*- β' -ethers **29** and so the passive component must be significantly lower. We are currently working on methods utilizing acetyl bromide to unambiguously distinguish ‘active’ from ‘passive’ incorporation mechanisms and quantitate the partitioning between them (114).

Lignin-Polysaccharide Cross-linking by Diferulates

Diferulates, no longer just the 5-5'-coupled dimer, have already cross-linked polysaccharide chains. Each of them has at least one phenolic group free. Presumably, diferulates can be incorporated into lignins *via* ‘active’ (and perhaps ‘passive’) mechanisms too (Figure 12). Grabber’s work has provided significant

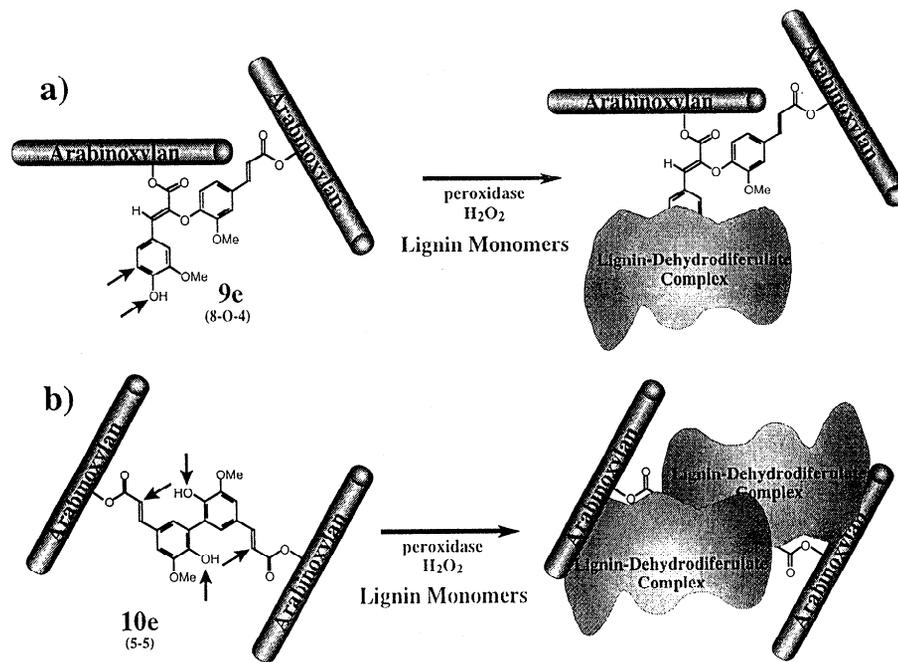


Figure 12. Ferulate dimers, already cross-linking polysaccharide chains, can also be incorporated into lignins via active mechanisms.

evidence that this is indeed the case. Just as ferulate release by room-temperature saponification was diminished by lignification, so was the release of all of the diferulates (75). A portion of the lignin-incorporated diferulates could be released by high temperature base treatment, the amounts depending on the particular diferulate concerned (76, 106).

Although not the most prevalent diferulate, the 5-5'-coupled structure has a special fascination. It is unique among the dimers of having the potential to couple with lignin (monomers) at both sidechain and phenolic positions (Figure 13). Since coupling at the sidechain 8-position still leaves the phenol remaining on that unit, there is the potential for 5-5'-coupled diferulate to couple to lignin molecule(s) at all four available positions, producing a multiple branching point and a very tightly coupled lignin-polysaccharide network (although 8-β'-coupled structures lose their connection to the polysaccharide—see structure 38, Figure 13 and structure 30, Figure 8). To understand how 5-5'-coupled diferulate 10 can be incorporated into lignins and, in particular, if it is possible to couple at the 8-positions, the strategically ¹³C-labeled 5-5'-coupled diferulate 10d was incorporated into a synthetic lignin DHP (115) (Figure 14). The DHP, even with the relatively modest loading of diferulate, had significantly different properties than normal DHPs. In particular, it was not soluble in 9:1 acetone-water for NMR spectroscopy. For NMR studies, the material was dissolved in DMSO-d₆ (2 parts) and diluted with acetone-d₆ (1 part) to lower the viscosity. ¹³C-NMR showed that the single 9-resonance in the diferulate incorporated into some 4 different groupings of resonances (vertical projection on Figure 15a) as was the case for ferulate monomers (Figure 15b). Again, long-range correlations from the labeled 9-carbon(s) were totally diagnostic (Figure 15a) particularly when compared to the corresponding ferulate monomer experiment (Figure 15b). Although the difference in solvent caused some significant proton shifts, it is clear that 8-O-4', 4-O-x'/5-x', 5-β', and 8-β' coupling has occurred. In fact, the incorporation profile is remarkably similar to that of ferulate. As with ferulate, the diferulate obviously reacts with lignin monomers to give 8-β' and 4-O-β' structures (lignin monomers invariably react at the β position in cross-coupling reactions) and dimers/oligomers to give 8-O-4' and 8-5' structures. Clearly, then, 5-5'-coupled diferulate is capable of radical coupling reactions with lignin monomers and dimers to give the range of expected structures. How extensively each molecule is cross-coupled (*i.e.* how many of the four possible coupling sites on the dimer are occupied) is not possible to determine.

One unique feature of incorporation of the 5-5'-coupled dimer into lignins is the ability to form dibenzodioxocins 35 (115). Such 8-membered ring structures have recently been identified in lignin itself and are strikingly predominant (98, 99). Normally, incorporation of each of the diferulate's ferulate moiety into lignin would be considered to be two independent events. This unique structure allows a sequential incorporation of both moieties (Figure 14). If one or both moieties couple first at the 8-position, we assume that NMR spectroscopic data from existing models would be appropriate. However, if the ferulate dimer itself reacts directly at the β position (and cyclizes at α) of a coniferyl alcohol radical, the product is likely to exhibit significantly different NMR spectroscopic data. In an effort to see if such diferulate structures 35 are likely to make important contributions, models were prepared to obtain the NMR data required to find them in the DHP (115). The most direct way to synthesize these models was *via* radical-coupling methods. Thus, the 5-5'-diferulate 10b or 10c was reacted with an excess of coniferyl alcohol using Ag₂O as a single-electron oxidant. The purified yields of the required products were low (*ca.* 15-20% based on diferulate 10) but the single-step preparation was significantly easier than the multi-step approach that did not use a radical coupling step. The data for these compounds agreed closely with those reported from similar compounds by Karhunen *et al.* (99). The spectra were fully consistent with the dibenzodioxocin structures; the HMQC, HMBC, and HMQC-TOCSY experiments were the most diagnostic. In particular, in the long-range ¹³C-¹H correlation

experiment (HMBC, not shown), H α correlated nicely with an aromatic 4-*O*-carbon. This three-bond coupling correlation establishes that the α -*O*-4' bond has formed.

At first the data from models dissolved in acetone and the DHP, which was in 2:1 DMSO-acetone, seemed to have little in common. However, the solvent shift afforded by the DMSO was remarkable. When models were dissolved in 2:1 DMSO-acetone, the shifts coincided directly with the corresponding shifts in the polymeric DHP (115). As seen in Figure 16, the dibenzodioxocin structure is clearly identified in both the HMQC and, more diagnostically, in the HMQC-TOCSY. The HMBC spectrum (not shown) is also consistent; H α correlates with H β , A1, A2, A6, and C4.

Advances in NMR Spectroscopy for Detailing Plant Chemistry

As an aside, the NMR studies described in this chapter were all performed on what is now considered older generation instrumentation that is not fully digital, and without the use of pulsed field gradients. It was also conducted at 360 MHz, a quite adequate field strength but one far below the 600, 750 and now even 800 MHz instruments that are available. The differences in spectra obtained on these two generations of machines is quite spectacular (Figure 17). Even ignoring the obvious dispersion gain from the 750 MHz instrument, the freedom from T₁-noise artifacts in the digital/gradient system (bottom spectrum) is striking. Peaks close to the methoxyl region are no longer ambiguous. In addition the flat baseplanes and improved sensitivity allow us to look more closely down to the noise level to reveal many more potentially valuable correlations. The only drawback is that we now have a great many more assignments to make in this spectrum! The expectation is that a great many more questions will succumb to unambiguous answers *via* spectra such as these.

Conclusions

In past primary publications on ferulates and their cross-linking with other ferulates and with lignin, two misconceptions have been perpetuated. First, the only dehydrodiferulate reported was the 5-5'-coupled dimer. It is certainly not the major dehydrodimer in any plant sample recently analyzed but, fortuitously, may be one of the more important in effecting tight lignin-polysaccharide cross-linking due to the number of coupling sites available to it. Second, it has frequently been reported that the only cross-linking structure to lignin is an α -ether or benzyl aryl ether. Such ethers can be produced by the attack of the phenolic hydroxyl (in ferulate/diferulate) on lignin quinone methides, which result from the coupling of a lignin monomer with another monomer or a lignin oligomer. Adherence solely to such a mechanism has a number of significant drawbacks. It does not permit ferulate/diferulate access to radical mechanisms that are going on all around it and producing the very quinone methides with which they must react. In the case of the 5-5'-coupled diferulate, nucleophilic attack by the sterically hindered 4-phenol is likely to be a particularly poor reaction. In practical terms, it has the unfortunate consequence that quantitation of ferulates and diferulates (by their hydrolytic release) is assumed to reflect their involvement in cross-linking. As is hopefully abundantly clear from this chapter, ferulates and diferulates readily undergo radical coupling reactions and cross-react with lignin monomers by radical coupling mechanisms; whether they cross-couple with lignin oligomers *in vivo* has yet to be investigated—certainly in ryegrass there is evidence only for coupling with lignin monomers suggesting a nucleation-site role for ferulates and diferulates in lignification (90). Since some of the structures that ferulates/diferulates are involved in cannot be hydrolytically cleaved, current 'quantitation' methods significantly under-estimate the importance of these species. What is clear is that ferulates and diferulates are more abundant components in grass cell walls than previously recognized and have powerful roles in effecting polysaccharide-polysaccharide and lignin-polysaccharide cross-linking. The 5-5'-

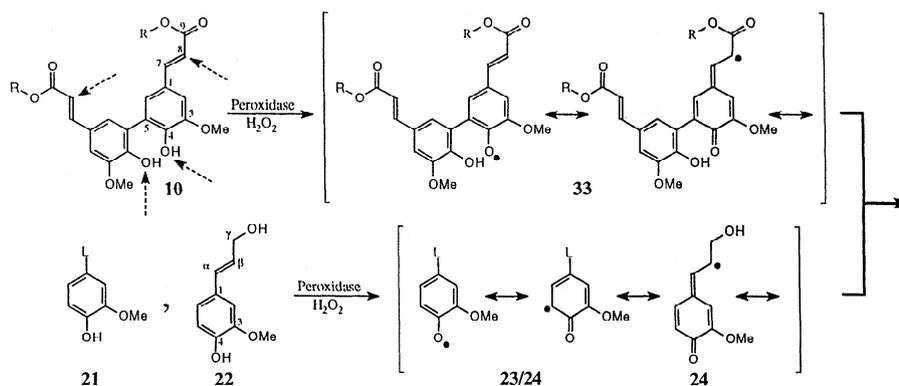


Figure 13. Peroxidase/H₂O₂ produces radical **33** from diferulate **10**, and radicals **23/24** from coniferyl alcohol **22** and pre-formed lignin dimers or oligomers **21**. The diferulate radical **33** cross-couples with a coniferyl alcohol radical, the latter invariably reacting at the β -position; initial 8-O-4'-coupling leads to dibenzodioxocin **35**, 8- β -coupling to the furofuranoid 'monoepoxylignanolate' (*102*, *116*) **38**. Note that 8- β -coupling results in a loss of connectivity with the polysaccharide moiety, (structure **38**). Cross-coupling with a lignin oligomer can afford products from 8-coupling of the ferulate moiety; 8-5'-coupling leads to phenylcoumaran **40**, and 8-O-4'-coupling to styryl aryl ether **42**. Dashed arrows on the diferulate **10** and final products **35**, **38**, **40** and **42**, identify sites at which radical coupling reactions are possible.

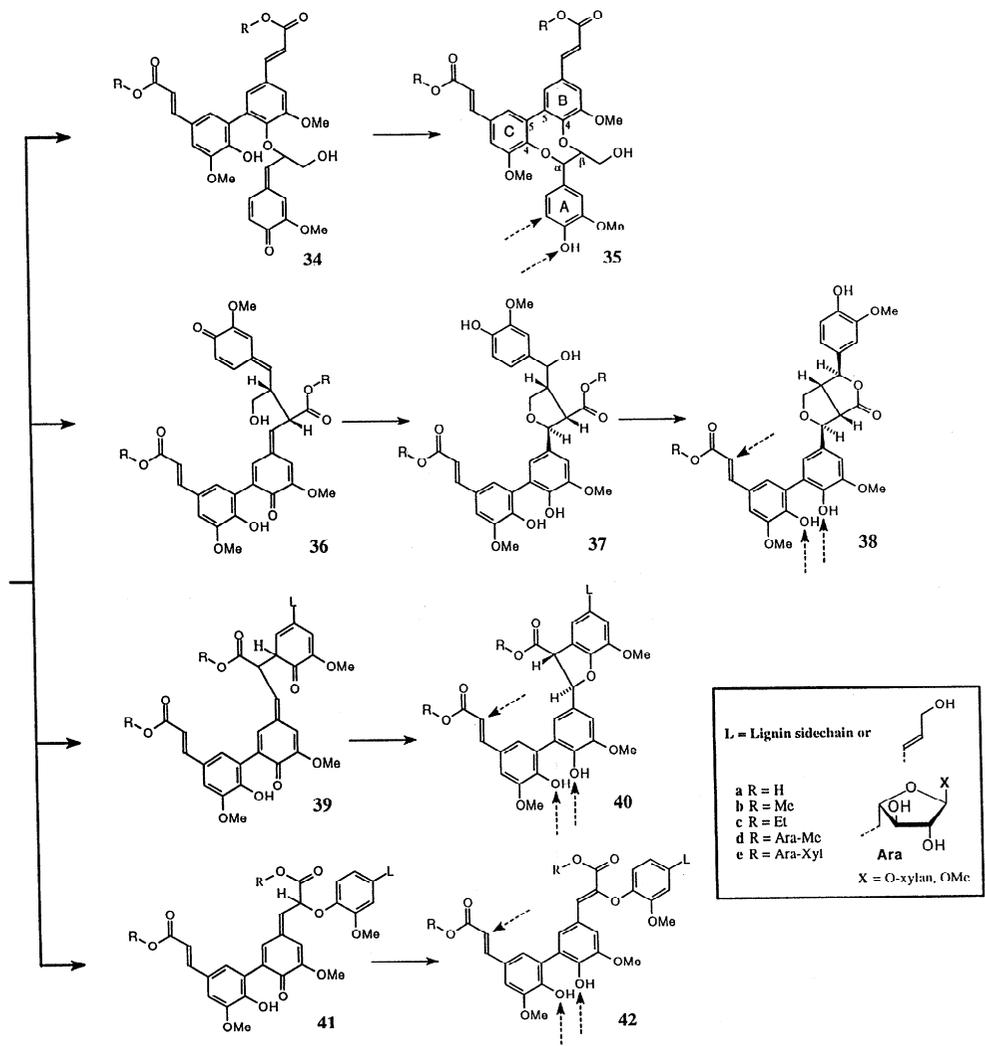


Figure 13. *Continued.*

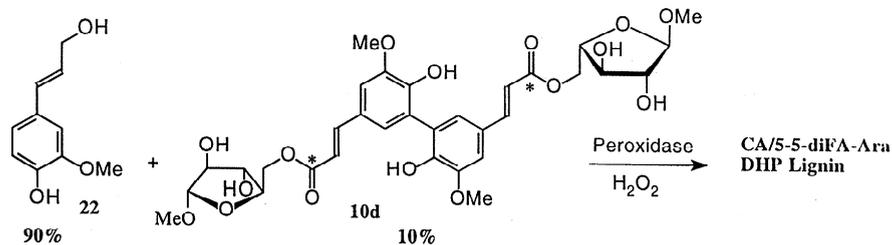


Figure 14. Preparation of 5,5'-diFA-Ara/coniferyl alcohol copolymer DHP. The diFA-Ara is strategically ^{13}C -labeled.

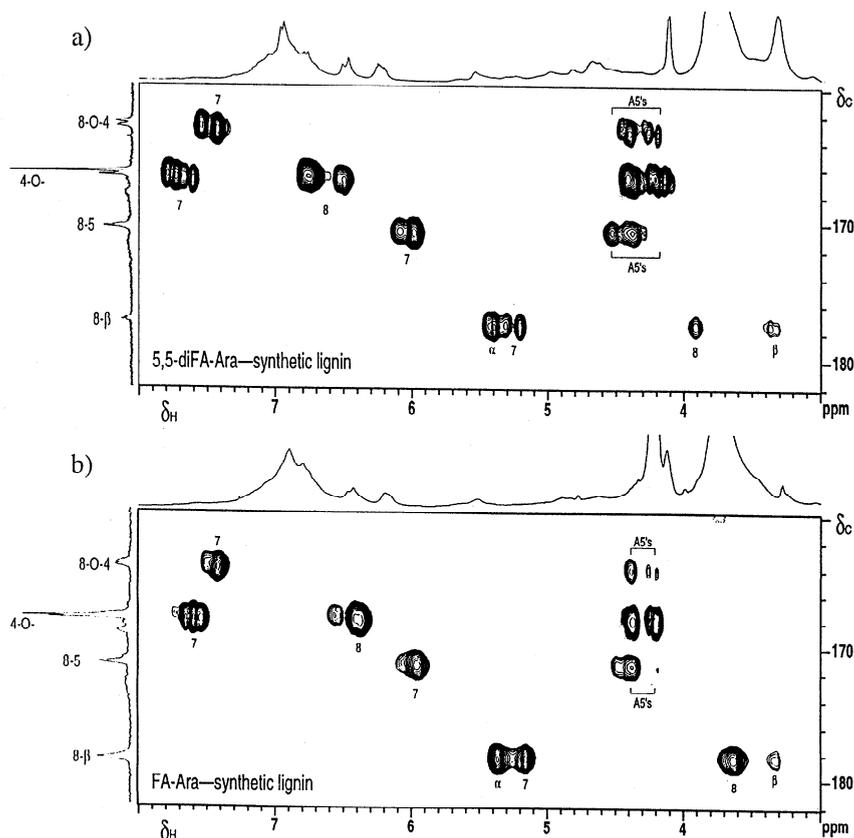


Figure 15. Partial HMBC spectra (carbonyl carbon region only) showing correlations of C-9 with protons within 3-bonds. The concurrent matching of several (up to 5) carbon and proton chemical shift data makes the assignments essentially unambiguous. The 5,5'-diFA-Ara DHP has some solvent-induced shifts caused by the DMSO. Nevertheless, as in the ferulate, clear evidence is seen for 8-O-4', 4-O-x', 8-5' and 8- β ' cross-coupling structures. In fact, the incorporation profiles are remarkably similar.

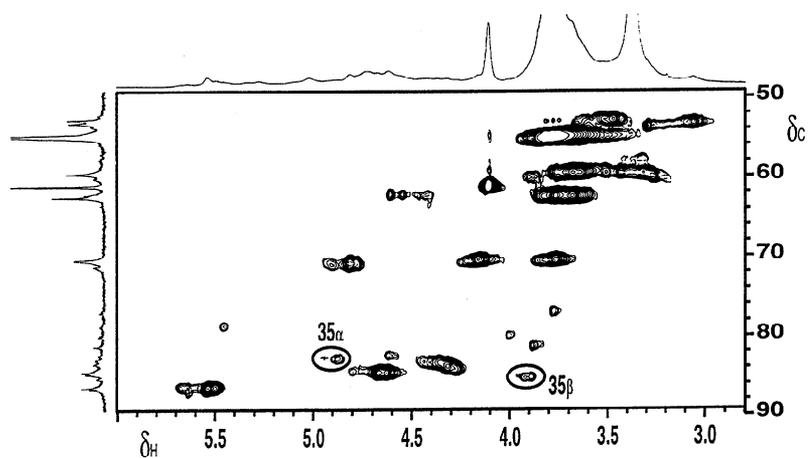
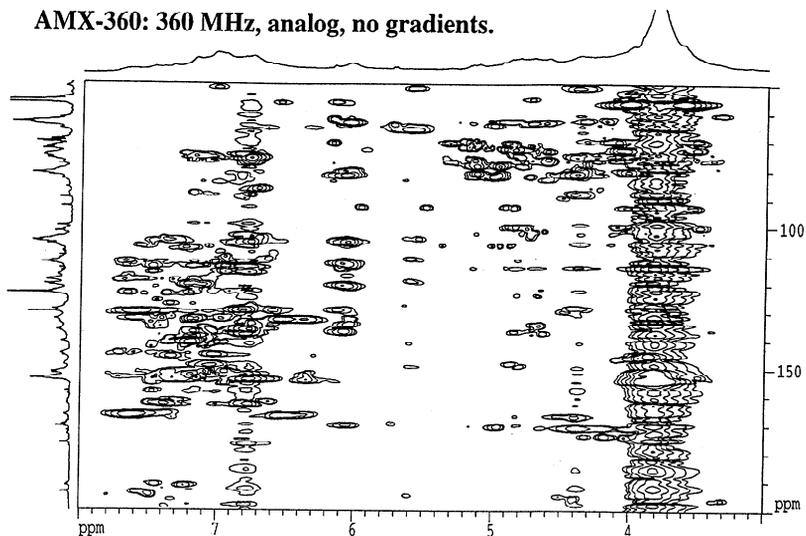


Figure 16. Partial (sidechain region) HMQC spectrum showing clear evidence for the dibenzodioxocin structures **35**. Correlations from models **35b/c** are shown as x's. HMBC and HMQC-TOCSY spectra (not shown) provided further confirmatory evidence by correctly correlating other resonances.

AMX-360: 360 MHz, analog, no gradients.



DMX-750: 750 MHz, digital, gradients.

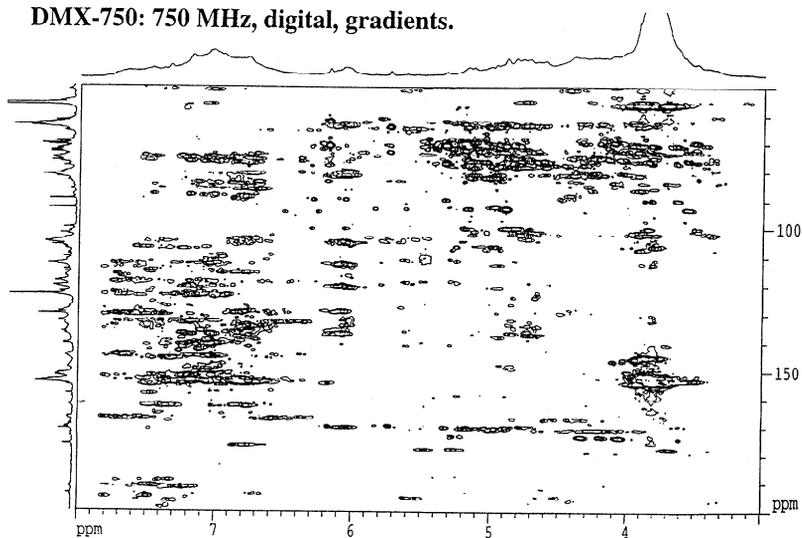


Figure 17. Comparison of HMBc spectra of labeled ryegrass lignins at different field strengths with and without gradients and digital acquisition. Top spectrum: AMX-360, non-digital, without gradients, 300 mg, 60 h. Bottom spectrum: DMX-750, fully digital, with gradients, 122 mg, 24 h. Note particularly the absence of so-called T₁-noise artifacts obfuscating the methoxyl region in the top spectrum (*ca.* 4 ppm on the proton scale), and the richness of detectable peaks due to the ability of approaching the flat base-plane more closely (bottom spectrum), and the enhanced dispersion resulting from the MHz difference.

dimer is particularly suited to this role, and the demonstration of the ability of such structures to form dibenzodioxocins during lignification is significant

Ferulate-mediated cross-linking of cell wall components is likely to be found to be increasingly important as more aspects of plant growth and development are related to this process. Although the chemistry is now becoming more clearly defined, a great deal remains unknown about the biochemistry, genetics, and regulation/control of these processes (1). With respect to genetic manipulation in grass plants for improved cell wall degradability, we suggest that current approaches based on lignin manipulation are likely to be less efficacious than an attack on the very mechanism that strongly limits degradability, *i.e.* lignin-polysaccharide cross-linking effected by ferulates. That is not to say that such approaches will be successful. This cross-linking process appears to be rather essential to the plant and it is conceivable that ferulate levels cannot be fully down-regulated without a severe impact on plant viability. However, woody plants make their cell walls without cross-linking mechanisms mediated by ferulate. The exploitation of genetic options for modifying ferulate-mediated cross-linking could prove to be extremely useful.

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Literature Cited

1. Bolwell, G. P. *Int. Rev. Cytol.* **1993**, *146*, 261-324.
2. Monties, B. L. In *Methods in Plant Biochemistry*; Harborne, J., Ed.; Academic Press: London, 1989; Vol. 1, pp 113-157.
3. Taiz, L. *Ann. Rev. Plant Physiol.* **1984**, *35*, 585.
4. Fry, S. C. *Phytochemistry* **1984**, *23*, 59-64.
5. Fry, S. C.; Miller, J. C. *ACS Symp. Ser.* **1989**, *399*, 33-46.
6. Yamamoto, E.; Bokelman, G. H.; Lewis, N. G. *ACS Symp. Ser.* **1989**, *399*, 68-88.
7. Hartley, R. D.; Ford, C. W. *ACS Symp. Ser.* **1989**, *399*, 137-145.
8. 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, NY, 1990; pp 173-182.
9. 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.
10. Bolwell, G. P. *Phytochemistry* **1988**, *27*, 1235-1253.
11. *211th ACS Natl. Mtg. Book of Abstracts* **1996**, *1*, abstr. Cell 51-57, 73-79
12. Monties, B. In *Proc. 6th Internatl. Symp. Wood Pulp. Chem.* **1991**, *1*, 113-121.
13. Kato, A.; Azuma, J.; Koshijima, T. *Chem. Lett.* **1983**, 137-140.
14. Kato, Y.; Nevins, D. J. *Carbohydr. Res.* **1985**, *137*, 139-150.
15. Kato, A.; Azuma, J.; Koshijima, T. *Agric. Biol. Chem.* **1987**, *51*, 1691-1693.

16. Smith, M. M.; Hartley, R. D. *Carbohydr. Res.* **1983**, *118*, 65-80.
17. Ishii, T.; Hiroi, T. *Carbohydr. Res.* **1990**, *196*, 175-183.
18. Ishii, T. *Phytochemistry* **1991**, *30*, 2317-2320.
19. Hartley, R. D.; Morrison, W. H., III; Himmelsbach, D. S.; Borneman, W. S. *Phytochemistry* **1990**, *29*, 3705-3709.
20. Mucller-Harvey, I.; Hartley, R. D.; Harris, P. J.; Curzon, E. H. *Carbohydr. Res.* **1986**, *148*, 71-85.
21. Chesson, A.; Gordon, A. H.; Lomax, J. A. *J. Sci. Food Agric.* **1983**, *34*, 1330-1340.
22. Nishitani, K.; Nevins, D. J. *Plant Physiol.* **1989**, *91*, 242-248.
23. 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.
24. Bacic, A.; Harris, P. J.; Stone, B. A. In *The Biochemistry of Plants*; Academic Press Inc.: New York, NY, 1988; Vol. 14, pp 297-371.
25. Ishii, T.; Hiroi, T.; Thomas, J. R. *Phytochemistry* **1990**, *29*, 1999-2003.
26. Ishii, T.; Hiroi, T. *Carbohydr. Res.* **1990**, *206*, 297-310.
27. Wende, G.; Fry, S. C. *Phytochemistry* **1997**, *44*, 1011-1018.
28. Wende, G.; Fry, S. C. *Phytochemistry* **1997**, *44*, 1019-1030.
29. Saulnier, L.; Vigouroux, J.; Thibault, J.-F. *Carbohydr. Res.* **1995**, *272*, 241-253.
30. Himmelsbach, D. S.; Hartley, R. D.; Bornemann, W. S.; Poppe, L.; van Halbeek, H. *Magn. Reson. Chem.* **1994**, *32*, 158-165.
31. Ishii, T. *Plant Cell Physiol.* **1994**, *35*, 701-704.
32. Wende, G.; Fry, S. C. *Phytochemistry*, **1997**, *45*, 1123-1129.
33. Fry, S. C. *Biochem. J.* **1982**, *203*, 493-504.
34. Ford, C. W.; Hartley, R. D. *J. Sci. Food Agric.* **1990**, *50*, 29-43.
35. Hartley, R. D.; Morrison, W. H., III; Balza, F.; Towers, G. H. N. *Phytochemistry* **1990**, *29*, 3699-703.
36. Hartley, R. D.; Morrison, W. H., III. *J. Sci. Food Agric.* **1991**, *55*, 365-375.
37. Hartley, R. D.; Whatley, F. R.; Harris, P. J. *Phytochemistry* **1988**, *27*, 349-351.
38. Ford, C. W.; Hartley, R. D. *J. Sci. Food Agric.* **1989**, *46*, 301-310.
39. Markwalder, H. U.; Neukom, H. *Phytochemistry* **1976**, *15*, 836-7.
40. Hartley, R. D.; Jones, E. C. *Phytochemistry* **1976**, *15*, 1157-1160.
41. Hartley, R. D.; Jones, E. C. *Phytochemistry* **1977**, *16*, 1531-1534.
42. Harris, P. J.; Hartley, R. D.; Lowry, K. H. *J. Sci. Food Agric.* **1980**, *31*, 959-62.
43. Kamisaka, S.; Takeda, S.; Takahashi, K.; Shibata, K. *Physiol. Plant.* **1990**, *78*, 1-7.
44. Tan, K.-S.; Hoson, T.; Masuda, Y.; Kamisaka, S. *Physiol. Plant.* **1991**, *83*, 397-403.
45. Tan, K.-S.; Hoson, T.; Masuda, Y.; Kamisaka, S. *Plant Cell Physiol.* **1992**, *33*, 103-108.
46. Eraso, F.; Hartley, R. D. *J. Sci. Food Agric.* **1990**, *51*, 163-70.
47. Ishii, T. *Carbohydr. Res.* **1991**, *219*, 15-22.
48. Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3485-3498.
49. Ralph, J.; Helm, R. F.; Quideau, S.; Hatfield, R. D. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2961-2969.
50. Teutonico, R. A.; Dudley, M. W.; Orr, J. D.; Lynn, D. G.; Binns, A. N. *Plant Physiol.* **1991**, *97*, 288-297.
51. Chioccare, F.; Poli, S.; Rindone, B.; Pilati, T.; Brunow, G.; Pietikäinen, P.; Setälä, H. *Acta Chem. Scand.* **1993**, *47*, 610-616.
52. Wallace, G.; Fry, S. C. *Phytochemistry* **1995**, *39*, 1293-9.
53. Harkin, J. M. In *Oxidative Coupling of Phenols*; Taylor, W. I., Battersby, A. R., Eds.; Marcel Dekker: New York, NY, 1967, pp 243-321.
54. Harkin, J. M. In *Chemistry and Biochemistry of Herbage*; Butler, G. W., Ed.; Academic Press: London, 1973; Vol. 1, pp 323-373.
55. Freudenberg, K. *Nature* **1959**, *183*, 1152-1155.

56. Davin, L. B.; Lewis, N. G. In *Phenolic Metabolism in Plants*; Stafford, H. A., Ibrahim, R. K., Eds.; Plenum Press: New York, NY, 1992; pp 325-375.
57. Quideau, S.; Ralph, J. *Holzforschung* **1994**, *48*, 12-22.
58. Landucci, L. L. *J. Wood Chem. Technol.* **1995**, *15*, 349-368.
59. van Huystee, R. B.; Zheng, X. *Phytochemistry* **1993**, *34*, 933-939.
60. Stewart, D.; Robertson, G. W.; Morrison, I. M. *Biological Mass Spectrometry* **1994**, *23*, 71-74.
61. Waldron, K. W.; Parr, A. J.; Ng, A.; Ralph, J. *Phytochemical Analysis* **1996**, *7*, 305-312.
62. Cohen, M. D.; Schmidt, G. M. J.; Sonntag, F. I. *J. Chem. Soc.* **1964**, 2000-2013.
63. Parr, A. J.; Waldron, K. W.; Ng, A.; Parker, M. L. *J. Sci. Food Agric.* **1996**, *71*, 501-507.
64. Waldron, K. W. *Biotechnology and Biological Sciences Research Council Business* **1996**, *April*, 10-11.
65. Sánchez, M.; Peña, M. J.; Revilla, G.; Zarra, I. *Plant Physiol.* **1996**, *111*, 941-946.
66. Shibuya, N. *Phytochemistry* **1984**, *23*, 2233-7.
67. Kamisaka, S.; Takeda, S.; Takahashi, K.; Shibata, K. *Physiol. Plant.* **1990**, *78*, 1-7.
68. Wende, G. **1997**, unpublished.
69. Micard, V.; Grabber, J. H.; Ralph, J.; Renard, C. M. G. C.; Thibault, J.-F. *Phytochemistry* **1997**, *44*, 1365-1368.
70. Oosterveld, A.; Grabber, J. H.; Beldman, G.; Ralph, J.; Voragen, A. G. J. *Carbohydr. Res.* **1997**, *300*, 179-182.
71. Grabber, J. H.; Hatfield, R. D.; Ralph, J.; Zon, J.; Amrhein, N. *Phytochemistry* **1995**, *40*, 1077-1082.
72. Jung, H. G.; Valdez, F. R.; Abad, A. R.; Blanchette, R. A.; Hatfield, R. D. *J. Anim. Sci.* **1992**, *70*, 1928-1935.
73. Jung, H. G.; Valdez, F. R.; Hatfield, R. D.; Blanchette, R. A. *J. Sci. Food Agric.* **1992**, *58*, 347-355.
74. Jung, H. G.; Ralph, J.; Hatfield, R. D. *J. Sci. Food Agric.* **1991**, *56*, 469-478.
75. Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S.; Kuster, T.; Pell, A. N. *J. Ag. Food Chem.* **1996**, *44*, 1453-1459.
76. Grabber, J. H.; Ralph, J.; Hatfield, R. D. *ACS Symp. Ser.* **1997**, see *Chapter 12*, this volume.
77. Grabber, J. H.; Ralph, J.; Hatfield, R. D., in preparation for *J. Sci. Food Agric.*
78. Iiyama, K.; Lam, T. B. T.; Stone, B. A. *Phytochemistry* **1990**, *29*, 733-737.
79. Iiyama, K.; Kasuya, N.; Lam, T. B. T.; Stone, B. A. *J. Sci. Food Agric.* **1991**, *56*, 551-560.
80. Lam, T. B. T.; Iiyama, K.; Stone, B. A. *Phytochemistry* **1992**, *31*, 1179-1183.
81. Lam, T. B. T.; Iiyama, K.; Stone, B. A. *Phytochemistry* **1992**, *31*, 2655-2658.
82. Jung, H. G.; Vogel, K. P. *J. Sci. Food Agric.* **1992**, *59*, 169-176.
83. Jung, H.-J. G.; Buxton, D. R. *J. Sci. Food Agric.* **1994**, *66*, 313-322.
84. Deetz, D. A.; Jung, H.-J. G.; Buxton, D. R. *Crop Sci.* **1996**, *36*, 383-388.
85. Scalbert, A.; Monties, B.; Lallemand, J. Y.; Guittet, E.; Rolando, C. *Phytochemistry* **1985**, *24*, 1359-1362.
86. Scalbert, A.; Monties, B.; Rolando, C.; Sierra-Escudero, A. *Holzforschung* **1986**, *40*, 191-195.
87. Sharma, A.; Brillouet, J.-M.; Scalbert, A.; Monties, B. *Agronomie* **1986**, *6*, 265-271.
88. Lam, T. B. T.; Iiyama, K.; Stone, B. In *1991 International Symposium on Forage Cell Wall Structure and Digestibility*; Madison, WI, 1991; p A4.
89. Lam, T. B. T.; Iiyama, K.; Stone, B. *Proc. 6th Internatl. Symp. Wood Pulp. Chem.* **1991**, *2*, 29-33.
90. Ralph, J.; Grabber, J. H.; Hatfield, R. D. *Carbohydr. Res.* **1995**, *275*, 167-178.
91. Helm, R. F.; Ralph, J. *J. Agric. Food Chem.* **1992**, *40*, 2167-2175.
92. Sipilä, J.; Brunow, G. *Holzforschung* **1991**, *45*, 9-14.

93. Sipilä, J.; Brunow, G. *Holzforschung* **1991**, *45*, 275-278.
94. Sipilä, J.; Brunow, G. *Holzforschung* **1991**, *45*, 3-7.
95. Adler, E. *Wood Sci. Technol.* **1977**, *11*, 169-218.
96. Ede, R. M.; Kilpeläinen, I. *Res. Chem. Intermediates* **1995**, *21*, 313-328.
97. Ede, R. M. In *Proc. 8th Internatl. Symp. Wood Pulp. Chem.* **1995**, *1*, 487-494.
98. Karhunen, P.; Rummakko, P.; Sipilä, J.; Brunow, G.; Kilpeläinen, I. *Tetrahedron Lett.* **1995**, *36*, 169-170.
99. Karhunen, P.; Rummakko, P.; Sipilä, J.; Brunow, G.; Kilpeläinen, I. *Tetrahedron. Lett.* **1995**, *36*, 4501-4504.
100. Hatfield, R. D.; Helm, R. F.; Ralph, J. *Anal. Biochem.* **1991**, *194*, 25-33.
101. Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093-2094.
102. Ralph, J.; Helm, R. F.; Quideau, S. *J. Chem. Soc., Perkin Trans. 1*, **1992**, 2971-2980.
103. Delmer, D. P.; Stone, B. A. In *The Biochemistry of Plants*; Academic Press Inc.: New York, NY, 1988; Vol. 14, pp 373-420.
104. Fry, S. C. In *Oxford Surveys of Plant Molecular and Cell Biology*; Milflin, B. J., Ed.; Oxford Univ. Press: New York, NY, 1985; Vol. 2, pp 1-42.
105. Jacquet, G.; Pollet, B.; Lapierre, C.; Mhamdi, F.; Rolando, C. *J. Agric. Food Chem.* **1995**, *43*, 2746-51.
106. Grabber, J. H.; Ralph, J., in preparation for *Phytochemistry*.
107. Grabber, J. H.; Ralph, J.; Hatfield, R. D.; Quideau, S.; Kuster, T. A. *211th ACS Natl. Mtg. Book of Abstracts* **1996**, *1*, abstr. Cell 20.
108. Terashima, N.; Atalla, R. H.; Ralph, S. A.; Landucci, L. L.; Lapierre, C.; Monties, B. *Holzforschung* **1996**, *50*, 9-14.
109. Terashima, N.; Atalla, R. H.; Ralph, S. A.; Landucci, L. L.; Lapierre, C.; Monties, B. *Holzforschung* **1995**, *49*, 521-527.
110. Tanahashi, M.; Higuchi, T. *Mokuzai Gakkaishi* **1990**, *36*, 424-428.
111. Tanahashi, M.; Aoki, T.; Higuchi, T. *Mokuzai gakkaishi* **1981**, *27*, 116-124.
112. Tanahashi, M.; Higuchi, T. *Wood Res.* **1981**, *67*, 29-42.
113. Tanahashi, M.; Aoki, T.; Higuchi, T. *Holzforschung* **1982**, *36*, 117-122.
114. Lu, F.; Ralph, J. *ACS Symp. Ser.* **1997**, see *Chapter 20*, this volume.
115. Quideau, S.; Ralph, J., submitted to *J. Chem. Soc., Perkin Trans. 1*.
116. Weinges, K.; Nader, F.; Künstler, K. In *Chemistry of Lignans*; Rao, C. B. S., Ed.; Andhra University Press: Visakhapatnam, India, 1978; pp 1-37.