Journal of the Science of Food and Agriculture *J Sci Food Agric* **79**:403–407 (1999)

Review

Cell wall cross-linking by ferulates and diferulates in grasses†

Ronald D Hatfield,[1]* John Ralph[2] and John H Grabber[1]

[1] *US Dairy Forage Research Center, USDA-Agricultural Research Service, USA*
[2] *Department of Forestry, University of Wisconsin-Madison, Madison, WI 53706, USA*

Abstract: Ferulate polysaccharide esters in grasses enter into free-radical condensation reactions in the cell wall. By radical dimerisation of ferulates, polysaccharide–polysaccharide cross-linking is effected. A range of diferulate isomers are produced, not solely the 5-5′-coupled dimer which has been quantified historically. Both ferulates and diferulates enter lignification reactions and become intimately bound up with the lignin complex. Again, under-quantification is significant since it is not possible to release ferulate or diferulates from some of the structures. Overall, ferulates play a significant role in cell wall development and impact polysaccharide utilisation in grasses.
© 1999 Society of Chemical Industry

Keywords: cell walls; ferulate; diferulates; lignin; cross-linking; grasses

INTRODUCTION

The structural and functional roles of plant cell walls are controlled by the composition and organization of individual wall components. Cross-linking of cell wall components is expected to have a marked influence on numerous wall properties such as accessibility, extensibility, plasticity, digestibility, and adherence.[1] In grasses, ferulates play a pivotal role in this cross-linking process that controls wall organisation and structural integrity.

FERULATE ATTACHMENT TO POLYSACCHARIDES

Ferulic acid is shuttled into wall matrices attached to structural polysaccharides. Although grasses have the largest amounts, ferulates have also been found in spinach (*Spinacia oleracea* L) cell cultures,[2] sugar beets (*Beta vulgaris* L)[3] and, most recently, in pine (*Pinus pinaster*) hypocotyls[4] and water chestnuts (*Eleocharis dulcis*).[5] The attachment of ferulic acid has been carefully analysed in only a few plant species.[6] For grasses, the mode of attachment is via the acid group acylating the primary hydroxyl at the C5 position of α-L-arabinofuranosyl residues (Fig 1). Dicots contain ferulated pectic polysaccharides. Detailed structural work has been carried out on spinach culture cells[2] and sugar-beet pulp.[3] In both cases, feruloylation occurs on the arabinose or galactose side chains of pectic polysaccharides. Ferulate attachment in bamboo (*Phyllostahys edulis*) can also occur on secondary alcohol groups of xylose side-chain residues of xyloglucans.[7] If ferulates are attached to xyloglucans of dicots, there could be the potential for significant cross-linking — dicots have 25% xyloglucan as compared to 5% in grass primary walls.

FORMATION OF DEHYDRODIMERS

The presence of ferulates on specific types of polysaccharides provides a convenient and reliable method of cross-linking these polysaccharide chains.

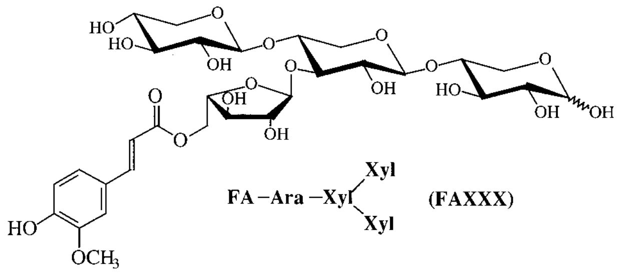

Figure 1. Ferulic acid is introduced into cell wall matrices via attachment to structural polysaccharides. In grasses ferulic acid is covalently attached via an ester linkage formed between the carboxylic acid group of ferulic acid and the primary alcohol on C5 carbon of arabinosyl side-chains of arabinoxylans. FAXXX is one of the diagnostic feruloylated saccharides released by polysaccharidase treatment.

---

† Based on a paper presented at Ferulate '98, IFR, Norwich, 8–11 July 1998.

* Correspondence to: Ronald D Hatfield, US Dairy Forage Research Center, USDA-ARS, 1925 Linden Drive West, Madison, WI 53707-1108, USA.

(*Received 9 July 1998; revised version received 1 September 1998; accepted 15 October 1998*)

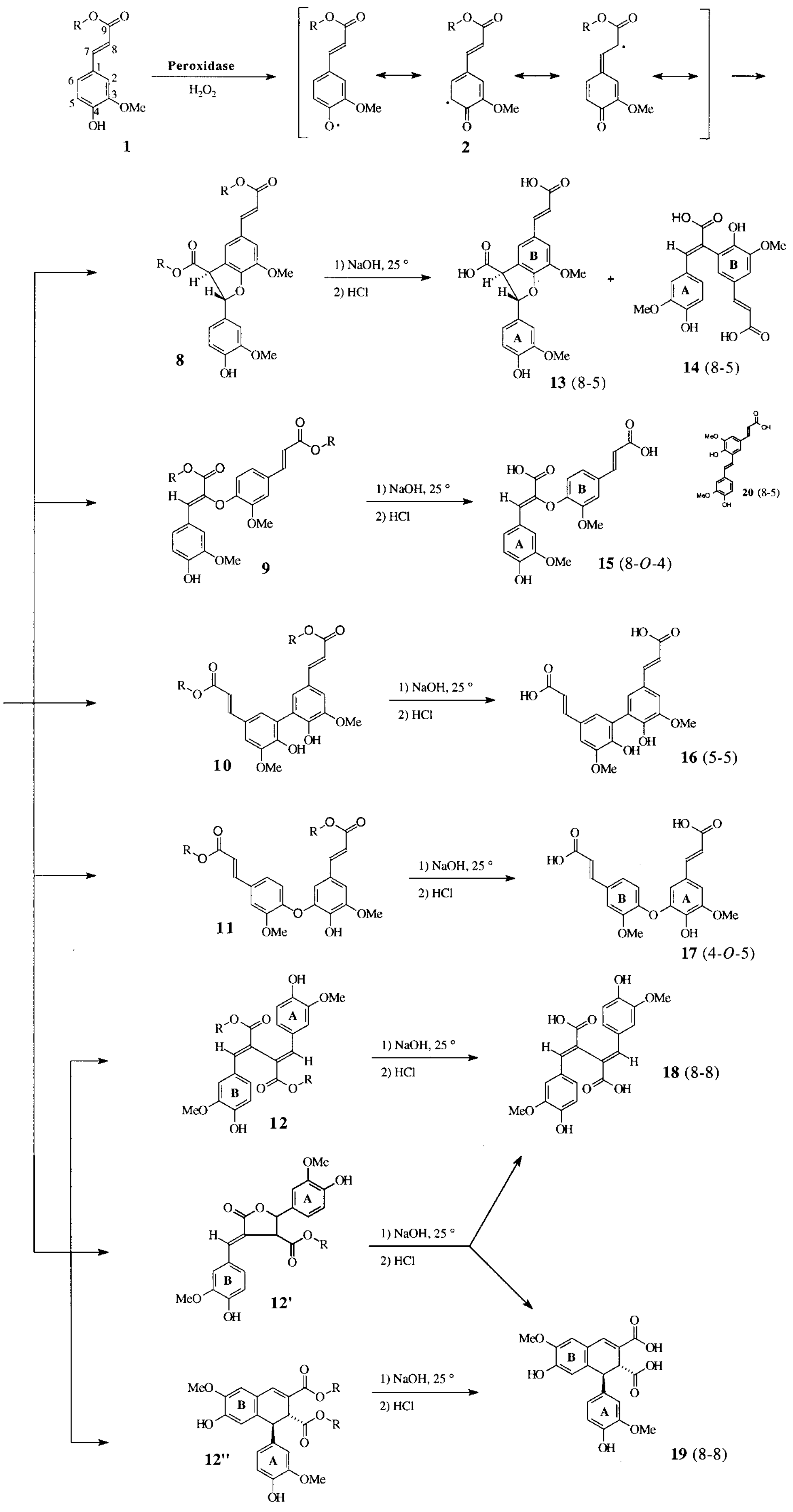

Figure 2. The general chemistry of dehydrodimer formation and saponification. Dimerisation of ferulate esters via phenoxy radical 2 gives rise to dehydrodiferulate esters 8–12. During chemical analysis esters 8–12 are saponified to dehydrodiferulic acids 13–19. Figure adapted from Ralph *et al* 1994 and number scheme used here corresponds to molecules described in the original figure.

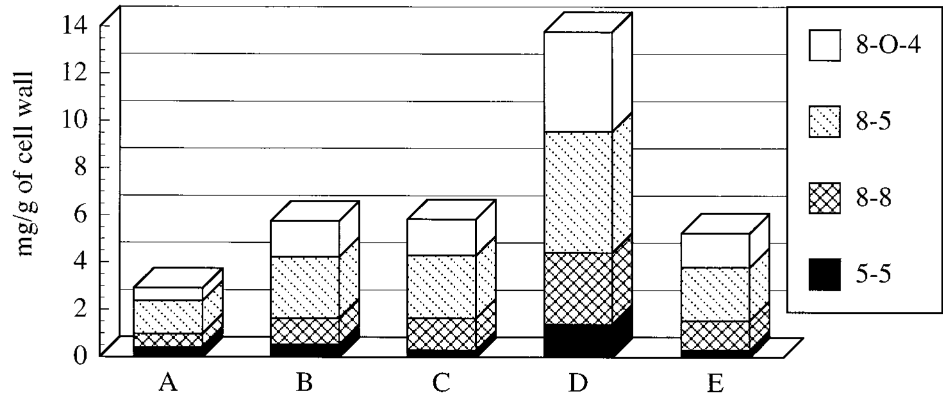

Figure 3. Concentration (mg g^{-1} cell wall material) of dehydrodiferulates derived from 5–5′-, 8–5′-, 8–8′-, and 8–*O*–4′-coupling reactions for various plant cell wall samples. Data adapted from Ref 12. (A) Suspension-cultured corn, (B) Cocksfoot parenchyma, (C) Cocksfoot sclerenchyma, (D) Switchgrass parenchyma, (E) Switchgrass sclerenchyma.

The formation of ferulate dimers is all that is required to covalently couple the two polysaccharides. There are two potential methods of covalently coupling ferulates; photochemical (2 + 2) cycloaddition produces cyclodimers,[8–11] whereas radical-mediated dimerisation produces a range of dehydrodimers (Fig 2).[12] Although wall polysaccharides, cross-linked through photochemical dimerisation of hydroxycinnamic acids, could lead to changes in wall structural integrity, the plant has little opportunity to exert control over the process either spatially or temporally. On the other hand, radical-mediated dimerisation is a mechanism that offers sufficient regulation to optimize the wall matrix cross-linking although the full importance of this was not realised until recently.

For over 20 years, only one diferulate was identified, the 5–5′-coupled dehydrodiferulate, often referred to simply as 'diferulate'. In 1989, it was observed that 'no direct evidence has ever been obtained *in vivo* to prove that dehydrodiferulic acids are formed via free-radical coupling reactions'.[13] We were concerned with the limited nature of diferulate coupling reactions since it seemed unusual that molecules like ferulates would undergo oxidative free-radical coupling to form a single dimer. The best analogues are the monolignols, coniferyl and sinapyl alcohols, which undergo oxidative free radical coupling to produce a variety of dimers and higher oligomers with different inter-unit bonds.[14] From model studies using feruloylated arabinose (FA-Ara) and plant peroxidases we could not duplicate the observations cited in the literature. Ferulates were easily oxidised by the peroxidase, and the radical coupling products represented a range of dehydrodiferulates predicted from free-radical coupling chemistry (Fig 2). The 8–5′-dimer was always predominant while only trace amounts of the 5–5′ dimer were produced by a range of single-electron oxidants.[12,15–19] Producing the same results from a range of peroxidases isolated from maize walls ruled out the possibility of a specific peroxidase for 5–5′-diferulate formation.[20] Ferulate 5–5′-dimers may not be favoured structures and arise only under special conditions when the molecules are held in the proper spatial orientations. Analysis of extracts from saponified walls of grasses revealed the presence of the predicted range of dehydrodiferulate dimers (Fig 2).[12] The total quantity of dehydrodiferulates was up to 20 times the level of the 5–5′-dimer alone (Fig 3), indicating that past compositional work had severely underestimated the level of polysaccharide cross-linking in walls.

CROSS-LINKING POLYSACCHARIDES TO LIGNIN

The bifunctional nature of ferulic acid led to the natural speculation that ferulates may act as cross-links between specific polysaccharides and lignin.[21,22] High temperature alkaline hydrolysis released some of the ether linked ferulates, suggesting that hydroxycinnamic acids are bridging molecules between lignin and polysaccharides.[21] Iiyama *et al*,[23] using a novel analytical scheme, clearly demonstrated that ferulic acid that was etherified to lignin was also esterified to arabinoxylans, thus demonstrating that ferulates were indeed cross-linking lignin and polysaccharides. Their work also demonstrated that *p*-coumarates were not acting as cross-linking agents within grass walls. The positions of attachment of ferulates to lignin was not determined although they assumed that all ferulate etherification was at the α-carbon position of the propanoid side chains of lignin.[24–26] Such regiochemistry involves only nucleophilic attack of the phenol on a lignin intermediate quinone methide.[22] This is another reaction over which the plant can exert very little control. From extensive NMR analysis of ^{13}C-labelled ryegrass, it was evident that chance reactions via quinone methide intermediates were not the preferred method of ferulate incorporation into the growing lignin polymer.[27] In fact, ferulates are fully involved in the radical process of lignification and become intimately bound in with lignin units in a variety of structures for which they cannot be fully released.[16]

NUCLEATION SITES FOR LIGNIFICATION

Using NMR spectroscopy, ryegrass walls from plants grown in $^{13}CO_2$ ($\sim$15% ^{13}C) were analysed

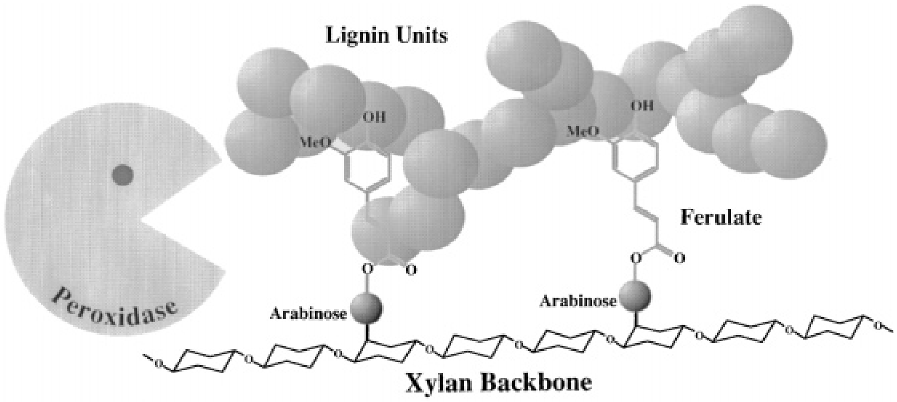

Figure 4. Schematic diagram of ferulate radical coupling to lignin, both coniferyl and sinapyl alcohol residues become linked to accessible ferulates. Ferulates act as nucleation sites for lignin formation.

and it was unambiguously demonstrated that ferulates (attached to C5 of arabinosyl units) do form covalent linkages to lignin monomers, both coniferyl and sinapyl alcohol residues.[27] Regiochemical characteristics of the bonding patterns (not only which molecules but also which carbons on the molecules are involved in the linkages) confirmed that ferulates on arabinoxylans do become covalently linked to lignin monomers by radical coupling reactions. Moreover, the types of linkages formed, in some cases, preclude their release from the wall by solvolytic techniques. Perhaps most important was the revelation that the types of bonds formed can only occur if ferulates are reacting strictly with monolignols (ie coniferyl or sinapyl alcohol monomers) and not with pre-formed lignin oligomers.[27] This means that one of the first events of wall lignification is the reaction with ferulates; therefore, ferulates are functioning as initiation sites or, more aptly, nucleation sites for the lignification process (Fig 4). Ferulate dimers are also incorporated into the newly forming lignins via the same radical coupling processes as ferulate monomers.[28,29] The positioning of ferulates within the wall may regulate lignin formation patterns and control cross-linking within wall matrices. Therefore, controlling the levels of total feruloylation should directly impact levels of cross-linking.

CONCLUSIONS

Ferulates have a more significant presence and role in grass cell wall development than initially realised.[29] The ability of ferulates in polysaccharide esters to dimerise by radical coupling processes to produce a variety of dehydrodimers effects strong polysaccharide – polysaccharide cross-linking. As the wall lignifies, ferulates and dehydrodiferulates become fully involved in radical cross-coupling reactions with lignin monomers to intimately incorporate ferulates into lignin, forming a strong polysaccharide – lignin cross-linked matrix. In the single grass species studied in sufficient detail, they appear to act as nucleation sites for lignification. The nature of the radical reactions means that ferulates and diferulates will never be fully released by any solvolytic method and will always be underestimated. Their roles in wall development are far greater than revealed by traditional analyses.

REFERENCES

1 Fry SC and Miller JC, Toward a working model of the growing plant cell wall. Phenolic cross-linking reactions in the primary cell walls of dicotyledons, in *Plant Cell Wall Polymers, Biogenesis and Biodegradation*, Ed by Lewis NG and Paice MG, ACS, Washington, DC, pp 33–46 (1989).

2 Fry SC, Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta* **157**:111–123 (1983).

3 Colquhoun IJ, Ralet M-C, Thibault J-F, Faulds CB and Williamson G, Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydr Res* **263**:243–256 (1994).

4 Sánchez M, Peña MJ, Revilla G and Zarra I, Changes in dehydrodiferulic acids and peroxidase activity against ferulic acid associated with cell walls during growth of *Pinus pinaster* hypocotyl. *Plant Physiol* **111**:941–946 (1996).

5 Parr AJ, Waldron KW, Ng A and Parker ML, The wall-bound phenolics of Chinese water chestnut. *J Sci Food Agric* **71**:501–507 (1996).

6 Ishii T, Structure and functions of feruloylated polysaccharides. *Plant Sci* **127**:111–127 (1997).

7 Ishii T and Hiroi T, Isolation and characterization of feruloylated arabinoxylan oligosaccharides from bamboo shoot cell walls. *Carbohydr Res* **196**:175–183 (1990).

8 Ford CW and Hartley RD, GC/MS characterization of cyclodimers from *p*-coumaric and ferulic acids by photodimerization – a possible factor influencing cell wall biodegradability. *J Sci Food Agric* **46**:301–310 (1989).

9 Ford CW and Hartley RD, Cyclodimers of *p*-coumaric and ferulic acids in the cell walls of tropical grasses. *J Sci Food Agric* **50**:29–43 (1990).

10 Hartley RD, Whatley FR and Harris PJ, 4,4′-Dihydroxytruxillic acid as a component of cell walls of *Lolium multiflorum*. *Phytochemistry* **27**:349–351 (1988).

11 Hartley RD, Morrison WH III, Balza F and Towers GHN, Substituted truxillic and truxinic acids in cell walls of Cynodon dactylon. *Phytochemistry* **29**:3699–3703 (1990).

12 Ralph J, Quideau S, Grabber JH and Hatfield RD, Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J Chem Soc Perkin Trans 1* 3485–3498 (1994).

13 Yamamoto E, Bokelman GH and Lewis NG, Phenylpropanoid metabolism in cell walls. An overview, in *Plant Cell Wall Polymers, Biogenesis and Biodegradation*, Ed by Lewis NG and Paice MG. ACS, Washington, DC, pp 68–88 (1989).

14 Harkin JM, Lignin – a natural polymeric product of phenol oxidation, in *Oxidative Coupling of Phenols*, Ed by Taylor WI and Battersby AR, Marcel Dekker, New York, pp 243–321 (1967).

15 Chioccara F, Poli S, Rindone B, Pilati T, Brunow G, Pietikainen P and Setala H, Regio- and diastereoselective synthesis of dimeric lignans using oxidative coupling. *Acta Chem Scand* **47**:610–616 (1993).

16 Ralph J, Helm RF, Quideau S and Hatfield RD, Lignin–feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *J Chem Soc Perkin Trans 1* 2961–2969 (1992).

17 Ralph J, Garcia-Conesa MT and Williamson G, Simple preparation of 8-5-coupled diferulate. *J Agric Food Chem* **46**:2531–2532 (1998).

18 Teutonico RA, Dudley MW, Orr JD, Lynn DG and Binns AN, Activity and accumulation of cell division-promoting phenolics in tobacco tissue cultures. *Plant Physiol* **97**:288–297 (1991).

19 Wallace G and Fry SC, *In vitro* peroxidase-catalyzed oxidation of ferulic acid esters. *Phytochemistry* **39**:1293–1299 (1995).

20 Hatfield RD, Jones BA, Grabber JH and Ralph J, Formation of dehydrodiferulates by peroxidases. *Plant Physiol* (Suppl) **108**:A641 (1995).

21 Scalbert A, Monties B, Lallemand JY, Guittet E and Rolando C, Ether linkage between phenolic acids and lignin fractions from wheat straw. *Phytochemistry* **24**:1359–1362 (1985).

22 Scalbert A, Monties B, Rolando C and Sierra-Escudero A, Formation of ether linkage between phenolic acids and Gramineae lignin: a possible mechanism involving quinone methides. *Holzforschung* **40**:191–195 (1986).

23 Iiyama K, Lam TBT and Stone BA, Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry* **29**:733–737 (1990).

24 Iiyama K, Lam TBT, Meikle PJ, Ng K, Rhodes D and Stone BA, Cell wall biosynthesis and its regulation, in *Forage Cell Wall Structure and Digestibility*, Ed by Jung HG, Buxton DR, Hatfield RD and Ralph J. ASA-CSSA-SSSA, Madison, pp 621–683 (1993).

25 Lam TBT, Iiyama K and Stone BA, Cinnamic acid bridges between cell wall polymers in wheat and *Phalaris* internodes. *Phytochemistry* **31**:1179–1183 (1992).

26 Lam TBT, Iiyama K and Stone BA, Determination of etherified hydroxycinnamic acids in cell walls of grasses. *Phytochemistry* **36**:773–775 (1994).

27 Ralph J, Grabber JH and Hatfield RD, Lignin–ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr Res* **275**:167–178 (1995).

28 Quideau S and Ralph J, Lignin–ferulate cross-links in grasses. Part 4. Incorporation of 5-5-coupled diferulate into lignin. *J Chem Soc Perkin Trans 1* 2351–2358 (1997).

29 Ralph J, Hatfield RD, Grabber JH, Jung HG, Quideau S and Helm RF, Cell wall cross-linking in grasses by ferulates and diferulates, in *Lignin and Lignan Biosynthesis*, Ed by Lewis NG and Sarkanen S. American Chemical Society, Washington, DC, pp 209–236 (1998).