

## Peroxidase-dependent cross-linking reactions of *p*-hydroxycinnamates in plant cell walls

John Ralph<sup>1,2,\*</sup>, Mirko Bunzel<sup>3</sup>, Jane M. Marita<sup>1,2</sup>, Ronald D. Hatfield<sup>1</sup>, Fachuang Lu<sup>1,2</sup>, Hoon Kim<sup>1,2</sup>, Paul F. Schatz<sup>1</sup>, John H. Grabber<sup>1</sup> & Hans Steinhart<sup>3</sup>

<sup>1</sup>*U.S. Dairy Forage Research Center, USDA-Agricultural Research Service, 1925 Linden Drive West, Madison, WI 53706, U.S.A.*; <sup>2</sup>*Department of Forestry, University of Wisconsin-Madison, Madison, WI 53706, U.S.A.*; <sup>3</sup>*Institute of Biochemistry and Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany*;  
\*Author for correspondence (Tel: (608) 264-5407; Fax: (608) 264-5147; E-mail: jralph@wisc.edu)

**Key words:** sinapate, sinapic acid, ferulate, ferulic acid, hydroxycinnamic acid, radical coupling, cell wall cross-linking, lignin, dehydrogenation, oxidative coupling, dehydrodimer, dehydrotrimer, radical

### Abstract

Peroxidases are heavily implicated in plant cell wall cross-linking reactions, altering the properties of the wall and impacting its utilization. Polysaccharide-polysaccharide cross-linking in grasses is achieved by dehydrodimerization of hydroxycinnamate-polysaccharide esters; a complex array of hydroxycinnamic acid dehydrodimers are released by saponification. Ferulates are the major cross-linking agents, but sinapate-ferulate cross-products have been discovered implicating sinapates in a similar role. New dehydrodimers have been authenticated, expanding our knowledge of the chemistry, role, and extent of cross-linking reactions. Ferulate dehydrotrimers have been discovered; whether these trimers truly cross-link three independent polysaccharide chains or only two remains to be determined. Hydroxycinnamates and their dehydrodimers also undergo radical coupling reactions with lignin monomers and possibly oligomers, resulting in lignin-polysaccharide cross-linking in the wall. Both polysaccharide-polysaccharide and lignin-polysaccharide cross-links inhibit the enzymatic hydrolysis of cell walls. The cross-linking process has particular relevance to plant physiology, human and animal nutrition and health, and food technology.

**Abbreviations:** CW – cell wall; DFA – dehydrodiferulic acid (or dehydrodiferulate in context); DSA – dehydrodisinapic acid; TFA – dehydrotriferulic acid; SA – sinapic acid (**1S**); TA – thomasidioic acid (**5C3SS**); IDF – insoluble dietary fiber; SDF – soluble dietary fiber; GC-MS – gas chromatography-mass spectrometry; NMR – nuclear magnetic resonance (spectroscopy).

### Introduction

Peroxidases are implicated in the biosynthesis of several components of the plant cell wall. Lignins, that are over-quoted as being the second most abundant terrestrial organic polymers, comprise some 10–30% of plant biomass. Peroxidases ostensibly provide the radical-generating capability for coupling each phenolic monomer into the complex lignin polymer.

This review is concerned with more subtle reactions that nevertheless have significant impacts on

plant cell wall biosynthesis and architecture. Low levels of cell wall cross-linking strengthens the walls (Monties, 1989; Bolwell, 1993). The same process negatively impacts economically important natural processes such as ruminant digestibility (Jung et al., 1993). Grasses have hydroxycinnamates intimately associated with their cell walls. Hydroxycinnamates are also apparent in cereal grains, where they may be responsible for some of the health benefits (Ferguson and Harris, 2003). They are recognized as being difunctional, but surpass their ability to simply attach

themselves to two different polymer chains (Ralph and Helm, 1993). Radical coupling reactions of hydroxycinnamates are crucial to a diverse array of important cell wall cross-linking mechanisms. Once the hydroxycinnamic acid acylates one polymer, usually a polysaccharide and presumably via a transferase enzyme, peroxidase is the key enzyme in promoting the following radical coupling pathways.

### Ferulate dehydrodimers cross-linking cell wall polysaccharides.

Ferulate is a well-known minor component of grass cell walls implicated in cell wall cross-linking, as previously reviewed (Bolwell, 1988; Fry and Miller, 1989; Hartley and Ford, 1989; Yamamoto et al., 1989; Jung and Ralph, 1990; Bolwell, 1993; Ralph and Helm, 1993; Ishii, 1997; Ralph et al., 1998b; Brett et al., 1999; Faulds and Williamson, 1999; Hatfield et al., 1999; Kroon and Williamson, 1999). Our involvement with ferulate dehydrodimers began from our curiosity that the only known dehydrodiferulate (DFA) was the 5–5-coupled DFA **4DFF** (Figure 1). Radical coupling of ferulates, necessary to produce the 5–5-coupled dehydrodimer **4D**, would be expected to produce other dehydrodimers by 8–5-, 8–8-, 8–O–4-, and 4–O–5-coupling products **4**, analogous to those observed for coniferyl alcohol during lignification. Indeed, *in vitro* coupling produced predominantly the 8–5-coupled dimer **4BFF** (Teutonico et al., 1991; Ralph et al., 1992, 1994a, 1998a; Chioccareta et al., 1993). Examination of the saponification products of cell walls from maize cell suspensions by GC-MS indicated that the 5–5-coupled dehydrodimer **5D** was only one of a whole class of DFAs (Ralph et al., 1994a; Grabber et al., 1995). Other researchers also suspected the multiplicity of diferulate products (van Huystee and Zheng, 1993; Stewart et al., 1994). We went through the lengthy process of synthesizing all the expected DFAs and confirmed that most of the compounds **5** could readily be found in extracts from a variety of saponified grasses, at levels up to 20-fold greater than the identified 5–5-diferulate (Ralph et al., 1994a, 1998b; Hatfield et al., 1999; MacAdam and Grabber, 2002). Diferulate, and therefore cell wall cross-linking, were seriously underestimated in previous studies where only the 5–5-DFA was quantified. Peroxidase-mediated coupling of ferulate into dehydrodimers in cell walls has been confirmed by adding hydrogen peroxide to primary maize walls (Grabber

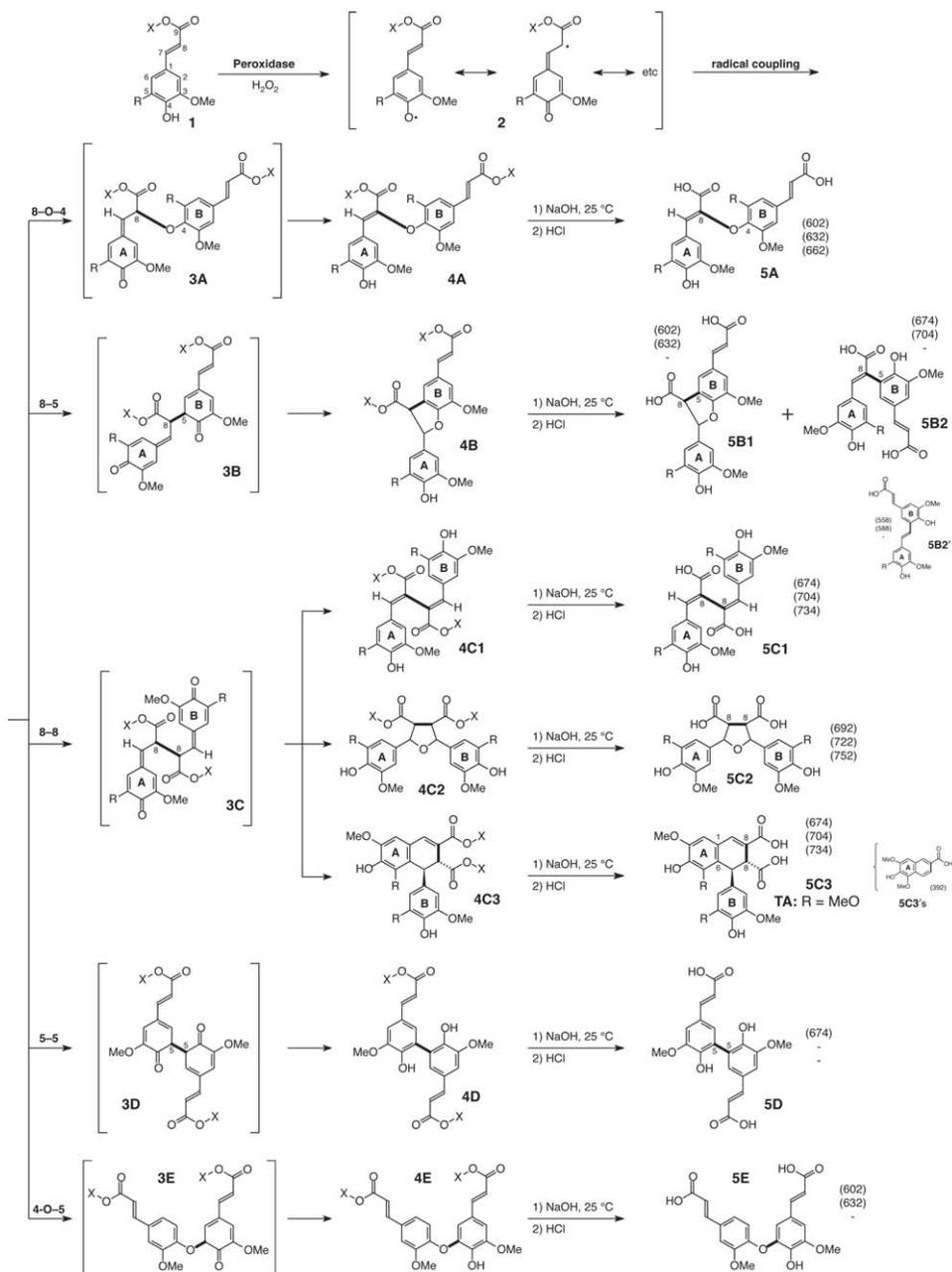
et al., 1995, 1998b). A range of ferulate dehydrodimers **5** are now also routinely found in a variety of non-graminaceous samples, including water chestnut (Parker and Waldron, 1995; Parr et al., 1996; Parker et al., 2003), sugar beet (Micard et al., 1997b; Oosterveld et al., 1997), and pine hypocotyls (Sánchez et al., 1996) where, interestingly, no 5–5-DFA was observed.

Mild acid or enzymatic hydrolyses of grasses allowed researchers to find ferulates attached to various saccharide residues, as reviewed (Ishii, 1997). The important isolation and structural elucidation of Xyl–Xyl–Ara–FA–(5–5)–FA–Ara–Xyl–Xyl (Xyl = xylose, Ara = arabinose, FA = ferulate) (Ishii, 1991), Ara–FA–(5–5)–FA–Ara and Ara–FA–(5–5)–FA–Ara–Xyl (Saulnier et al., 1999) provided structural evidence that ferulate dehydrodimerization was a mechanism to cross-link saccharide units and therefore presumably to cross-link the cell wall. However, molecular modeling approaches suggested that 5–5-DFA was unique in that it could be formed intramolecularly, i.e. by coupling of two ferulates on the same chain (see later in Figure 4) (Hatfield and Ralph, 1999). It is therefore ironic that the first dehydrodiferulic acid found and quantified for many years may not be fully involved in polysaccharide-polysaccharide cross-linking. Despite their higher concentrations in cell walls, finding 8–8-coupled diferulates acylating cell wall saccharides has presented a formidable challenge, but recently di-arabinosyl-8–O–4-diferulate, Ara–FA–(8–O–4)–FA–Ara, was isolated from maize bran (Allerdings et al., 2004).

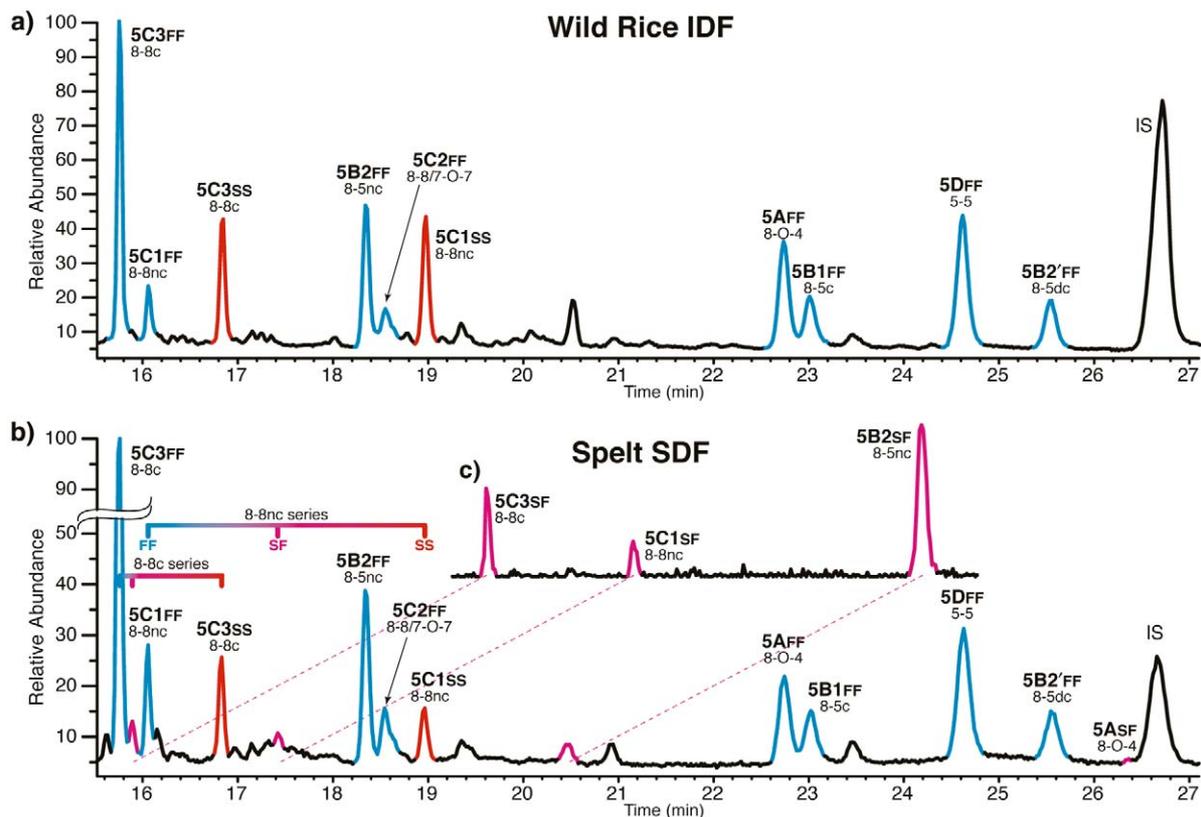
Findings relating to the dehydrodiferulic acids **5A**, **5B1**, **5B2**, **5B2'**, **5C1**, **5C3**, and **5D** have been reviewed (Ralph et al., 1998b; Hatfield et al., 1999), and will not be readdressed here. Instead we will cover the more recent discoveries and issues.

#### *Discovery of the 4–O–5-coupled ferulate dimer 5E*

The only dehydrodiferulic acid not found until recently was 4–O–5-DFA **5E**, Figure 1. It has now been found (in rather small amounts) following saponification of several insoluble cereal fibers (Bunzel et al., 2000, 2001). It was identified by comparison of its mass spectrum and its relative GLC retention time with that of the genuine compound, which was synthesized and authenticated by NMR. The relative retention time of 4–O–5-DFA **5E** against a new monomethylated 5–5-diferulate internal standard was 1.032 (Figure 2, although its concentration is too low to be



**Figure 1.** Radical dehydrodimerization of (cell-wall-bound) hydroxycinnamates (just ferulate (R=H) and sinapate (R=OMe) are shown here for simplicity) or hetero-dehydrodimerization of sinapate and ferulate produces 8-O-4-, 8-5-, 8-8-, and 5-5-dehydrodimers **4** via intermediates **3**. Hydroxycinnamic acid dehydrodimers **5** are released following saponification. With its extra 5-OMe, sinapate cannot make 5-5- nor 8-5-dehydrodimers. The numbering scheme uses the convention relating to the monomer numbering. The A-ring of **5C3** is numbered with the condensation between carbons A6 and B7 to be consistent between the sinapate and the corresponding ferulate dehydrodimer. The designation of units as arising from ferulate (F) and sinapate (S) in the dimers uses the A-ring first. e.g. **5B1<sub>sf</sub>** is from sinapate linked at its 8-position to ferulate at its 5-position. The F/S designators are only included in the accompanying text if there is any ambiguity. Thus in sections on diferulates, the FF descriptor is understood and is not used. Columnar numbers in parentheses are nominal molecular masses (for MS) of the diferulate (FF), the mixed (sf), and the disinapate (ss) compounds; where such compounds are not possible, the entry is a simple dash. e.g. 8-5-products cannot be ss. The bond formed by the radical coupling step is bolded. X = polysaccharide in the cell wall system, but also = Me, Et (and H for **1**, **2**) for synthetic compounds and precursors.



**Figure 2.** a) GLC-MS total-ion chromatogram of saponified extracts of wild rice insoluble dietary fiber showing ferulate (cyan) and sinapate (red) dehydromers (Bunzel et al., 2003b). b) GLC-MS total-ion-chromatogram of saponified extracts of spelt soluble dietary fiber showing analogous ferulate (cyan) and sinapate (red) dehydromers as well as the putative sinapate-ferulate crossed dimers (magenta). c) Selected-ion chromatogram ( $m/z$  704) revealing three of the potential **SF** crossed-dimers. Structures are from Figure 1. In the descriptors: c – cyclic form, nc – non-cyclic (open) form, dc = decarboxylated. Note: the 4–O–5-DFA **5E<sub>FF</sub>**, which elutes after the internal standard **IS**, was too small to be clearly seen in either of these chromatograms.

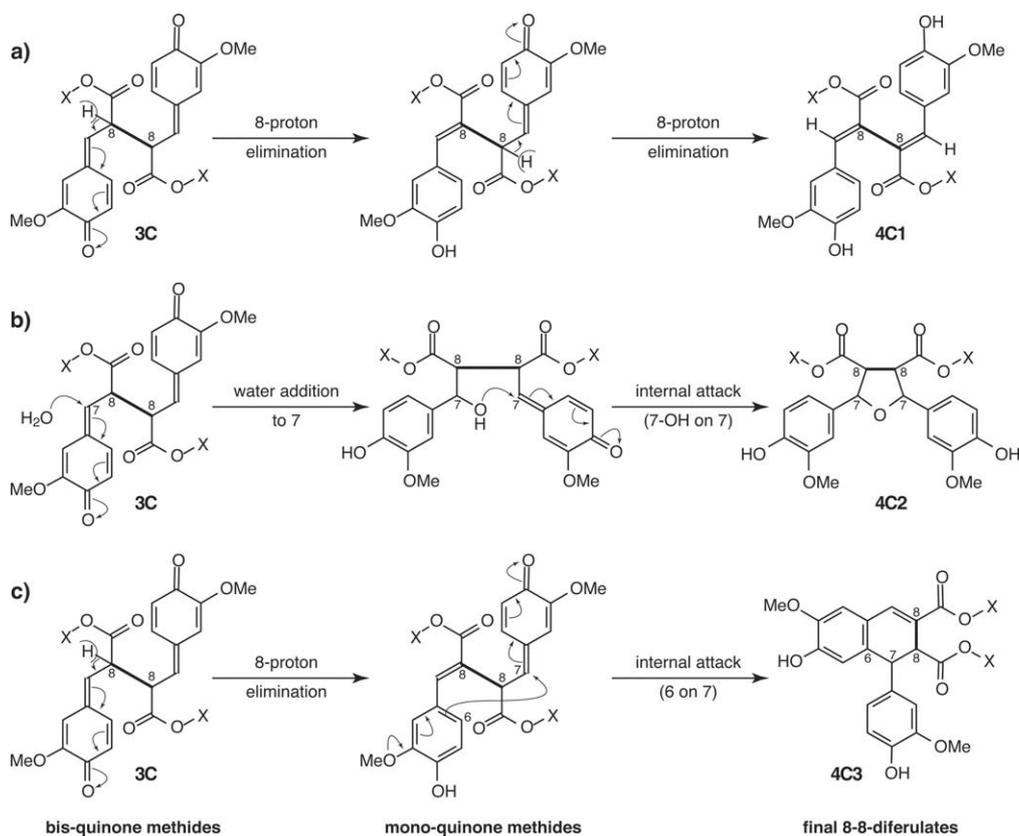
seen in these chromatograms). The amounts of 4–O–5-DFA **5E** were 33, 13, 10 and 8  $\mu\text{g/g}$  of cell wall (CW) in maize, spelt, wheat, and rice insoluble cereal fiber, respectively — approximately 70–100 times lower than the amounts of the sum of 8–5-coupled dehydrodiferulic acids **5B**. Recently, 4–O–5-DFA was also detected in suspension-cultured *Mentha* by GC-MS (Yang and Uchiyama, 2000a).

Finding the final coupling mode therefore provides evidence for the full range of possible ferulate radical coupling products in cereal grains, and presumably in other plant cell walls containing ferulates and diferulates. It also affirms the prevalence for coupling at ferulate's 8-position (to give the more predominant 8–5-, 8–8-, and 8–O–4-dimers), as also observed in ferulate cross-coupling into lignins (Ralph et al., 1992). The finding probably completes the spectrum of ferulate dehydromer coupling modes to be found

in plants; however, there is the possibility of 8–1-coupling analogous to the  $\beta$ -1-cross-coupling that occurs in lignification (Lundquist and Miksche, 1965; Harkin, 1967; Sarkanen and Ludwig, 1971), and with sinapate (Setälä et al., 1999). It also supports the concept of free-radical coupling of cell wall components independently of enzymes or proteins which might otherwise confer a strict regiochemical course, i.e. produce only a single DFA.

#### *Another 8–8-coupled diferulate*

Alkaline hydrolysates of grass cell walls also contain another previously unidentified peak. Mass spectrometric analysis suggested that it was the tetrahydrofuran DFA **5C2**, Figs 1–2 (Grabber et al., 2000, 2002; Ralph et al., 2000; Bunzel et al., 2003b). Strictly it is not a true dehydromer since an additional oxygen (from water) is incorporated, Figure 3b. Its higher



**Figure 3.** Mechanistic rationales for production of three possible 8–8-DFAs **4C** from the single bis-quinone methide intermediate **3C** (produced via radical coupling, Figure 1). a) Double 8-proton eliminations produce the non-cyclic DFA **4C1**. b) The new tetrahydrofuran DFA **4C2** requires water addition to one quinone methide moiety, followed by internal nucleophilic attack of the newly formed 7-OH on C7 of the other quinone methide moiety. c) Elimination of an 8-proton also affords an intermediate that can undergo internal nucleophilic attack of the aromatic ring C6 at C7 of the quinone methide to produce the cyclic DFA **4C3**; the methoxyl *para* to C6 is crucial (see mechanism). This is a particularly prevalent reaction for sinapate products because of the extra electron density in the ring from the additional methoxyl group. Note that the actual forms of diferulates **4C** in the wall remain contentious, but the three free acid analogs **5C** (Figure 1) are produced following saponification.

molecular mass is responsible for its being missed as a ferulate dehydrodimeric product previously. It is still formed via radical coupling, Figure 1, but the post-coupling steps are different from those in the previously identified DFAs, Figure 3b. As can be seen in the chromatogram in Figure 2, **5C2FF** is a substantial component that should also be quantified as resulting from 8–8-dimerization. There are six potential isomers of **5C2**, although there appears to be only one among the products from saponified plant cell walls. This may be due to isomerization to the most stable isomer during saponification. The compound has now been synthesized (Schatz, 2003, unpublished) and shown to be identical to the plant-derived dehydrodimer. Complete structural elucidation, determination of which of the six isomers it is by NMR, and amounts in cereal grains will shortly be reported. The finding of this

8–8-tetrahydrofuran DFA **5C2** adds to the confusion regarding exactly what form the dehydrodiferulate takes *in planta* (see below).

#### *Elucidating the nature of in vivo dehydrodiferulates*

Although the nature (structure and bonding) of some of the dehydrodiferulates **4** is rather obvious from the products **5** released (Figure 1) and the known chemistry of model coupling reactions, the nature of others remains uncertain. For example, there is little doubt that the 8–5-coupled product is the cyclic phenylcoumaran product **4B** shown in Figure 1 (and not the opened product that might be suggested from the saponification product **5B2**) — it is the cyclic product **4B** that is produced from ferulate esters under a variety of single-electron oxidation conditions (Teutonico et al., 1991; Ralph et al., 1992, 1994a, 1998a; Chioccaro

et al., 1993; Wallace and Fry, 1995). The cyclic nature tells us that cyclization and internal trapping of the quinone methide **3B** occurs faster than elimination of the acidic 8-proton or of nucleophilic addition of water to the quinone methide **3B**. Furthermore, authentic 8–5-dehydrodiferulate **4B** (e.g., the diethyl ester) saponifies in the same way to yield both the ring-opened **5B2** and phenylcoumaran **5B1** forms as well as the decarboxylated product **5B2'** (Ralph et al., 1994a). Similarly the 8–*O*–4-dehydrodimer is likely to be in the form **4A** shown; acidic 8-proton elimination has proven to be faster than water addition in this case (Ralph et al., 1994a).

A significant DFA fraction is the 8–8-coupled DFAs – see for example Figure 2. However, their nature in the wall remains unknown. Yet it is essential to know what form the ester takes if serious esterase research is to be undertaken (Garcia-Conesa et al., 1999b; Kroon et al., 1999; Kroon, 2000), for example, or if antioxidant activity is to be determined. The Norwich group has examined the antioxidant activity of the 8–8-DFA **5C1** (Garcia-Conesa et al., 1999a), the 8–*O*–4-DFA **5A** (Garcia-Conesa et al., 1997a), as well as the 5–5-DFA **5D** and 8–5-DFA **5B1** (Garcia-Conesa et al., 1997b) that are released from the wall. But such studies would be inappropriate if the 8–8-DFAs actually all derive from the tetrahydrofuran ester **4C2** in the wall.

The initial diferulates disclosure (Ralph et al., 1994a) listed three possible esters, **4C1**, **4C3** (Figure 1) and another which no longer appears in our scheme. The missing compound (not shown) was suggested by reaction pathways observed in the synthesis of the 8–8-coupled DFAs. It is now considered a less likely product of ferulate dehydrodimerization and has not been seen in coupling products of ethyl ferulate (Lu, 2003, unpublished). The new tetrahydrofuran ester **4C2** has now been added in its place.

We have already established that esters of **4C1** and **4C3** yield only their corresponding acids **5C1** and **5C3**, respectively; for example, ester **4C1** does not produce acid **5C3** on saponification. Therefore, if these esters are in the wall, both forms need to be present to explain the saponification products. A more appealing situation that needed testing was that all three 8–8-DFAs result from a single ester precursor in the wall such as the tetrahydrofuran **4C2**. Saponification of synthesized **4C2** (X = Et) yielded essentially only the tetrahydrofuran **5C2**. We therefore conclude that 8–8-coupling must produce more than one product *in planta*, at least the three shown in Figures 1 and 3.

As with any chemical reaction which produces multiple products, the ratio of products is dependent on reaction conditions. Some hint as to what might be driving post-coupling reactions in the cell wall comes from more detailed recent unpublished examinations of ferulate coupling. [Partitioning among all the various coupling modes is also condition-dependent, but it is reasonable to assume that once radical coupling regiochemistry has been determined (e.g. as 8–8, for example) it is the various rearomatization possibilities that control the final product distribution]. Lu (2003, unpublished) has found that low pH favors the tetrahydrofuran product. This is a mechanistically reasonable observation. The intermediate quinone methide **3C**, produced directly following the 8–8-coupling step, will much more readily add water nucleophilically at low pH; such addition is protonation-dependent. At high pH, the elimination reactions are more likely. The anticipated pathways to the three products are detailed in Figure 3.

#### *Diferulates analysis issues*

Although HPLC methods have been used for qualitative and quantitative analysis of diferulates (Waldron et al., 1996), HPLC has not afforded the dispersion and resolution of GC. In addition, alkaline hydrolysates of plant materials produce a wide range of products that cannot be easily pre-fractionated. For example in grass stem samples, in addition to the diferulates from radical coupling of ferulates are the photochemical dimers (Hartley et al., 1990a, 1990b) and numerous ferulate-monoignol crossed dimers (see later). There are components still to be identified. Only cursory analyses were possible using HPLC; GC-FID and/or GC-MS were preferable if component detail is required. However, significant improvements in column technology suggest that HPLC methods may soon gain the upper hand. A semipreparative Sephadex LH-20 chromatography/HPLC method has been developed to isolate all of the DFAs for use as primary standards (Bunzel et al., 2004a).

The GC standard used for the original quantification of diferulates and most subsequent studies has been *o*-coumaric acid (Ralph et al., 1994a). Unfortunately this standard has neither ideal elution nor satisfactory response factors for the diferulates. A standard having better structural similarity was sought. Rather extensive surveys failed to unearth a satisfactory commercially available standard. The 5–5-coupled dimer which had been monomethylated became the

best alternative candidate, and this has since been used (Bunzel et al., 2000, 2001). The compound can be synthesized in pure form (Lu, 2004, unpublished) and will soon be available for researchers.

Also in progress is research aimed at improving the product distribution, the need for which is most readily apparent by considering the 8–5-DFA saponification products. In the wall the ester almost certainly exists in the cyclic form **4B**. However, three products **5B1**, **5B2**, and **5B2'** need final quantification. Since the only truly relevant data is the (total) amount of the 8–5-dehydrodimer in the wall, the quantities determined need to be summed, but the errors involved in determining three products, each with their own response factors, are much higher than for a single product. Furthermore, a simplified product spectrum would result in an easier application of HPLC methods that will be needed if higher oligomers (see below) are to be included in the analytical procedure.

#### *Levels of diferulates in cereal grain insoluble fiber*

Grain fiber is beneficial in human nutrition. The levels of DFAs in a range of cereal grain fibers were recently surveyed (Bunzel et al., 2001) where the following were found: maize 12.6, wheat 2.4, spelt 2.6, rice 4.0, wild rice 2.8, barley 3.7, rye 4.0, oat 3.6 and millet 5.7 mg/g of insoluble fiber. Very low levels were found in the soluble fiber fraction as might be anticipated. The high levels in maize make this an ideal secondary standard to check column performance and variations in the analyses over longer periods of time.

#### **Dehydrodisinapates and ferulate-sinapate cross-products**

Although sinapic acid (**SA**) **1S** (X=H, R=OMe) (Figure 1) has been identified in plant extracts, and can be released in small quantities from grass cell walls by base, it has not been determined if it acylates polysaccharides or other components. Recent identification of alkali-releasable (ester-bound) sinapate dehydrodimers in various insoluble (IDF) and soluble dietary fibers (SDF) from cereal grains suggests that dehydrodisinapic acids (DSAs) are natural, wall bound, radical dimerization products, and that sinapate functions analogously to ferulate in cell wall cross-linking reactions (Bunzel et al., 2003b) with the involvement of peroxidase.

#### *Identification and quantification of dehydrodisinapates in cereal grain dietary fibers*

As seen by the red peaks in Figure 2, two 8–8-DSAs, compounds **5C1SS** (the non-cyclic isomer) and **5C3SS** (**TA**, the cyclic isomer) of 8–8-coupled DSA, result following saponification of wild rice IDF (Bunzel et al., 2003b). The DSAs were authenticated by comparison of their mass spectra and their relative GLC retention times with those of the synthesized compounds. The 8–8-coupled DSAs are analogs of the corresponding DFAs, also seen in the same chromatograms, Figure 2. They were also detected in other cereal grains (wheat, spelt, corn, rice, rye, barley) but not in IDF from oats or millet, nor from SDF from oats, corn or millet. Compound **5C3'S**, which arises in synthesis during the saponification procedure of the dimethyl ester **4C3SS** (X = Me), was not detected. The dehydrodimer **5C2SS**, the analog of the recently identified diferulate tetrahydrofuran compound **5C2FF**, was also not detected. The sum of DSAs (where quantification of at least one compound was possible) in cereal IDF (wheat, spelt, rice) was about 1% of the total DFAs level. Wild rice IDF differed significantly from other cereal IDFs investigated: dehydrodisinapates amounted to 17% of the DFAs level with 355  $\mu\text{g g}^{-1}$  IDF of **5C1SS** and 126  $\mu\text{g g}^{-1}$  IDF of **5C3SS**. In SDF of wheat, spelt and rye the relative amounts of DSAs were a little higher than in the corresponding IDF at roughly 4–7% of the DFAs level. Contrary to the DSAs, the DFA level in wild rice SDF was below the determination limit (Bunzel et al., 2001).

GLC-MS-chromatograms were screened for 8–O–4-coupled DSAs **5A5S** by looking for typical masses expected from its structure by analogy with the DFA analog (Bunzel et al., 2001). The 8–O–4-DFA was not seen at any significant level. Sinapates are known to predominantly 8–8-couple (Wallis, 1973). Analogously, sinapic acid **SA** gives high yields of the 8–8-coupled dilactone (Freudenberg and Schraube, 1955), and sinapyl alcohol coupled under peroxidase- $\text{H}_2\text{O}_2$  gives some 91% syringaresinol, the 8–8-coupled product, and only about 9% 8–O–4-coupling (Tanahashi et al., 1976).

Considerable effort went into establishing that the disinapates were not air oxidation artifacts since the conversion of **SA** to thomasidioic acid (**TA**, identical to **5C3SS**) in the presence of oxygen had been described (Rubino et al., 1995, 1996). Only a small percentage of liberated **SA** may be converted to **TA**, but this can not explain the relatively high amounts

of **TA**, e.g. in wild rice IDF (126  $\mu\text{g TA/g IDF}$ , 518  $\mu\text{g SA/g IDF}$ ) (Bunzel et al., 2001). The identification of the non-cyclic isomer **5C1SS** provided the strongest indication that the DSAs occur naturally. Only the condensed structure **TA** results from the air oxidation mechanism described (Rubino et al., 1995, 1996; Charlton and Lee, 1997) and in our own experiments. The release of **5C1SS** from the fiber samples provides compelling evidence for the presence in the dietary fibers of sinapate esters and their resultant DSAs. The results from mild acidic and especially from enzymatic degradation of wild rice fiber with carbohydrases indicate that **SA** and the DSAs are attached to polysaccharides but a proof by isolation of defined SA-oligosaccharides has been elusive (Bunzel et al., 2002).

#### *Sinapate-ferulate crossed dehydrodimers*

Small amounts of sinapate-ferulate crossed dehydrodimers labeled **5C1SF**, **5C3SF**, **5B2SF** (see the magenta peaks in Figures 2b–c) could be detected in some cereal dietary fibers (IDF of wheat, spelt, rye, wild rice and SDF of wheat, spelt (Figure 2c), rice) (Bunzel et al., 2003b). The sinapate-ferulate crossed dehydrodimers (**SF**) were identified from their mass spectra.

The mass spectrum of **5C3SF** ( $m/z$  704) is analogous to those of **5C3SS** ( $m/z$  734) and the cyclic form of 8–8-coupled DFA **5C3FF** ( $m/z$  674) (Bunzel et al., 2001), showing the same diagnostic ions plus/minus  $m/z$  30. It is assumed that the ‘A-ring’, Figure 1, is syringyl since syringyl rings are more electron rich and therefore more capable of nucleophilic attack at carbon-7 of the ‘B’ moiety. However, the other regioisomer with a syringyl ring B) remains a potential product. The spectrum of the acyclic 8–8-cross-coupled dehydrodimer **5C1SF** similarly has characteristics of **5C1SS** and **5C1FF** (Bunzel et al., 2001). No analog of the ferulate tetrahydrofuran product **5C2FF** (in fact, neither **5C2SF** nor **5C2SS**) was detected. A small amount of the 8–O–4-cross-product **5ASF** was also detected (Figure 2b) with a mass spectrum (molecular ion,  $m/z$  632, not shown) analogous to the dehydrodiferulate analog **5AFF**.

Cross-coupling of sinapate with ferulate allows an extra possibility not possible in sinapate homodehydrodimerization, namely 8–5-coupling. The 8–5-coupled non-cyclic compound **5B2SF** is the major crossed dimer, Figure 2c. The saponification conditions may have been too severe for the esterified cell wall product **4B** since the cyclic phenylcou-

maran counterpart **5B1SF** was not detectable. However, the decarboxylated product **5B2'SF** was detectable at 31.1 min with a mass spectrum (not shown, molecular ion  $m/z$  588) analogous to the DFA counterpart.

These compounds represent a variation in the way the cell wall becomes cross-linked. The implications of the products are that both sinapates and ferulates are exported to the same regions of the walls (i.e. temporally and spatially in concert) and that they are capable of undergoing peroxidase-assisted coupling reactions with each other.

In general, when related monolignols are allowed to react, *in vitro*, by far the major products are homo-coupled products – it is difficult to find evidence of cross-coupling (Syrjanen and Brunow, 2000). However, when the supply of the phenolic radicals is limited, such as occurs during lignification, cross-coupling reactions (between a monomer and the growing polymer, for example) become dominant (Syrjanen and Brunow, 1998). In the case of hydroxycinnamates in cereal grain IDF, it may be more a matter of proximity. Even if a ferulate would preferentially react with another ferulate, the proximity of a sinapate with which to react during the radical lifetime may be sufficient to produce cross-coupling. Alternatively, some phenolics actually favor cross-coupling reactions. This is the case for cross-coupling between 5-hydroxyconiferyl alcohol (a novel monomer that builds up in plants deficient in an *O*-methyl transferase, COMT (Marita et al., 2001; Ralph et al., 2001a, b), in the monolignol biosynthetic pathway) and coniferyl alcohol — the major product *in vitro* is a cross-coupled product (Lu, 2003, unpublished).

#### **Hydroxycinnamate dehydrodimerization: Observations**

How could cross-coupling reactions get any more complex? It used to be so easy – photodimerization or 5–5-dehydrodimerization of ferulate were the only ways to cross-link wall polysaccharides. The single dehydrodimerization product **5D** was easy to measure. Now, there are more than 15 hydroxycinnamic acid dehydrodimers that need to be measured to comprehensively assess cell wall polysaccharide-polysaccharide cross-linking (although see below for the admonitions on the inability to measure such properties reliably in lignified walls). Of course there is also the possibility that other hydroxycinnamic acids are involved in

peroxidase-assisted cell wall cross-linking. To date, dehydrodimers involving, for example, caffeic acid or 5-hydroxyferulic acid, have not been found in grasses, nor have they been authenticated as acylating polysaccharides. However, indications for the presence of wall-bound dehydrodicaffeic acids in dicots were recently presented. Yang and Uchiyama saponified cell-walls of suspension cultured *Mentha* and determined trimethylsilylated phenolic acids by GC-MS. Selected-ion spectra ( $m/z$  790 and  $m/z$  718) showed eleven peaks, hinting at the presence of wall bound dehydrodicaffeic acids (Yang and Uchiyama, 2000a, b). Authentication of these dimers (e.g. full mass spectra, comparison with synthesized standard compounds) is required.

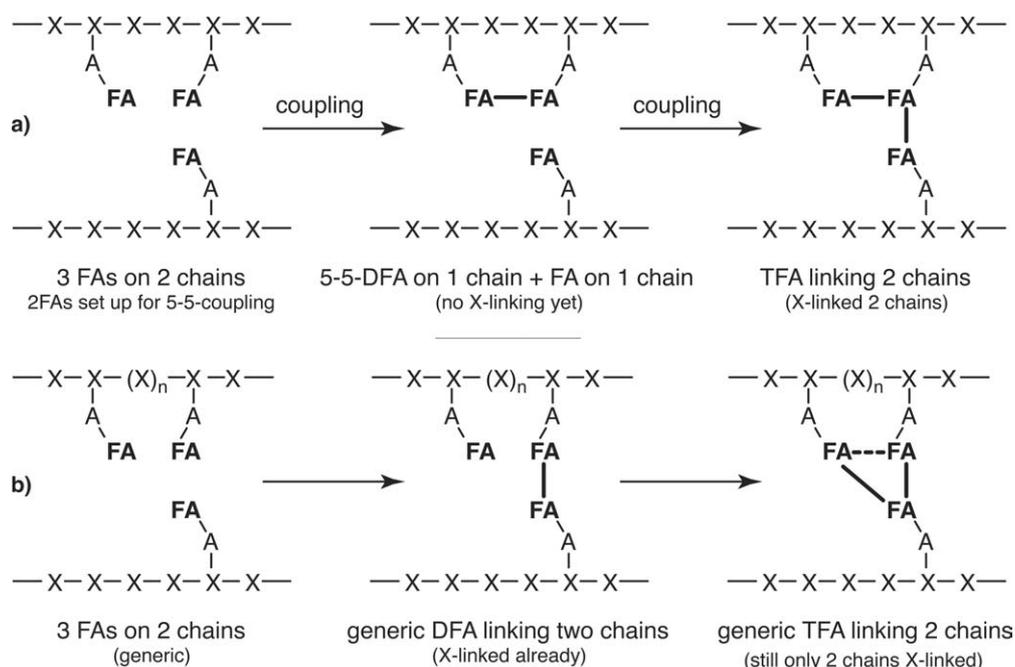
Obviously, dehydrodimerization reactions are important factors in cell wall development and must impact the properties of the wall. DFAs have been found in many types of plants and plant tissues as reviewed above. The Norwich group described their influence on texture, and ascribed the crunchiness of water chestnuts, even after cooking, to DFAs (Parker and Waldron, 1995; Parr et al., 1996), especially to 8-8-DFAs (Parker et al., 2003). The discoveries have also lead to some important new applications. For example, sugar beet pulp was found to be particularly rich in ferulates and DFAs (Rombouts and Thibault, 1986; Ralet et al., 1994b; Micard et al., 1997b; Oosterveld et al., 1997; Saulnier and Thibault, 1999); manipulating the levels of ferulate-mediated polysaccharide-polysaccharide cross-linking by treating the pulp with peroxidase and dilute hydrogen peroxide changed the gelling properties of the various beet pulp fractions, expanding food uses (Rouau et al., 1987; Faulds and Williamson, 1992; Micard et al., 1997a; Oosterveld et al., 1997, 2000; Garcia-Conesa et al., 1999b; Levigne et al., 2002). Fungal esterases were able to partially destroy the cross-linking on simple soluble substrates (Faulds and Williamson, 1992; Ralet et al., 1994a; Garcia-Conesa et al., 1999b; Kroon et al., 1999; Kroon, 2000), but not on cell walls. Whether esterases are useful without the action of carbohydrases to first break down the wall is less clear. In studies aimed at improving cell wall digestibility in ruminant feeds is had been previously shown that even low-level cross-linking achieved by ferulate dehydrodimerization or lignification was responsible for decreased wall degradability (Grabber et al., 1996, 1998a, c). Fairly potent antioxidant activities of ferulates and their dimers were revealed (Garcia-Conesa et al., 1997a, b; Garcia-Conesa et al., 1999a; Kroon

and Williamson, 1999). Diferulates bound to cell walls can not be absorbed by humans but it was shown that appropriate esterases are present in the small intestine and in the colon (Andreasen et al., 2001a, b). A recent study also shows the ability of Caco-2 cells to de-esterify DFA-diesteres (Kern et al., 2003).

### **Ferulate dehydrotrimers (TFAs) cross-linking cell walls?**

Cell wall polysaccharide cross-linking does indeed appear to be even more complex than has been described above. Fry has presented evidence suggesting that ferulate trimers (TFAs) and even higher oligomers might be biosynthesized in maize cell walls (Fry et al., 2000). He suggests that it may be necessary to consider the formation of cross-links during polysaccharide biosynthesis and before their export to the cell wall, a significant departure from current theories. Currently it is held that feruloylated polysaccharides are biosynthesized in, for example, the Golgi, and are exported to the cell wall where laccase-O<sub>2</sub> or peroxidase-H<sub>2</sub>O<sub>2</sub> systems cause the oxidative coupling that cross-links the polysaccharides. During the early studies we were skeptical about the possibility of TFAs since their formation requires that three ferulates, encumbered by rather unwieldy polysaccharide chains, would need to converge in a limited space. Ferulates near the ends of polysaccharide chains might be able to do this. It would help to explain why, when cell walls are submitted to oxidative conditions (or allowed to mature), so producing more dimers (and depleting the monomer), the mass balance is always short (Grabber et al., 1995; Oosterveld et al., 1997), i.e. the total ferulate level (ferulates plus DFAs) at the end of the processes is lower than that at the beginning. In part this can be due to the fact that DFAs quantification is far from perfect, with so many products to quantify and sum.

A likely scenario for dehydrotrimerization comes from extending prior modeling work showing that 5-5-coupled dehydrodiferulate, and only that DFA, can form intramolecularly in arabinoxylans (Hatfield and Ralph, 1999), i.e. on the same polysaccharide chain. The ferulates need to be attached (via arabinose, as they almost universally are in grasses) to the xylan backbone on xylose units that are separated as shown in Figure 4a. It suggests that this may even be the reason why 5-5-DFA is so prevalent in grass cell walls, yet is formed only in low yield by *in vitro* coupling reactions (which is a better mimic for inter-



*Figure 4.* Schematic presentation explaining how ferulate trimers might be formed in plant cell walls, and how they may only cross-link two polysaccharide chains, not three. a) As previously demonstrated (Hatfield & Ralph, 1999), only 5-5-DFA can be formed intramolecularly, i.e. by ferulates on the same polysaccharide chain, if they are appropriately situated on the chain (as shown). Cross-coupling of the 5-5-DFA with a ferulate on a neighboring chain can then produce a dehydrotrimer (TFA) that cross-links the two polysaccharide chains. Given the disproportionately high level of 5-5-DFAs in grass stem and cereal grain cell walls, this seems likely. b) We know nothing yet about the possibility of DFAs cross-linking two polysaccharide chains and being able to cross-couple with another (differently situated,  $n = 0, 1, 2, \dots$ ) ferulate on one of the chains to produce TFAs.

molecular reactions, i.e. between two polysaccharide chains). Then a 5-5-DFA unit on one polysaccharide chain could reasonably cross-couple with a ferulate on a second chain, forming a TFA unit, but cross-linking only two polysaccharide chains, Figure 4a. It would provide an explanation for trimerization that can be achieved in the cell wall, as an alternative to Fry's theory (Fry et al., 2000) for creating the cross-links prior to their export to the wall. Of course, cross-linking of three independent feruloylated polysaccharide chains also remains in contention.

#### *What form would a ferulate dehydrotrimer take?*

Assuming firstly that the dehydrotrimer should contain a 5-5-DFA unit (since this is the only dehydrodimer that can apparently be formed on a single polysaccharide chain), the subsequent coupling reaction with the next ferulate can proceed in two ways, Figure 5 (if we neglect the possibility of 4-*O*-5-coupling, which is found to be extremely minor with ferulate). a) Coupling at the 5-5-diferulate's 4-*O*-position with the ferulate's 8-position (Figure 5a), i.e.

8-*O*-4-coupling, would most likely give the dibenzodioxocin product **6A** following internal trapping of the resultant quinone methide. Analogous 8-membered ring dibenzodioxocins were only recently identified as major components in lignins (Karhunen et al., 1995a, b). Coupling at one of the 5-5-diferulate's sidechain 8-positions could be with either the 4-*O*-, 5-, or 8-position of ferulate, leading to three possible products **6B**, **6C**, or **6D** (Figure 5b), only one of which (**6B**) is shown explicitly.

#### *The first isolated dehydrotriferulate*

Two groups have recently independently isolated and characterized a TFA from maize bran (Bunzel et al., 2003a; Rouau et al., 2003). The compound contains a 5-5-linked DFA moiety, and is compound **6BFFF** (Figure 5b). NMR spectra were unambiguous, and both groups deduced the same structure (although some of the NMR assignments appear to differ). Its isolation and identification is important as it extends the role of ferulates in cell wall cross-linking.

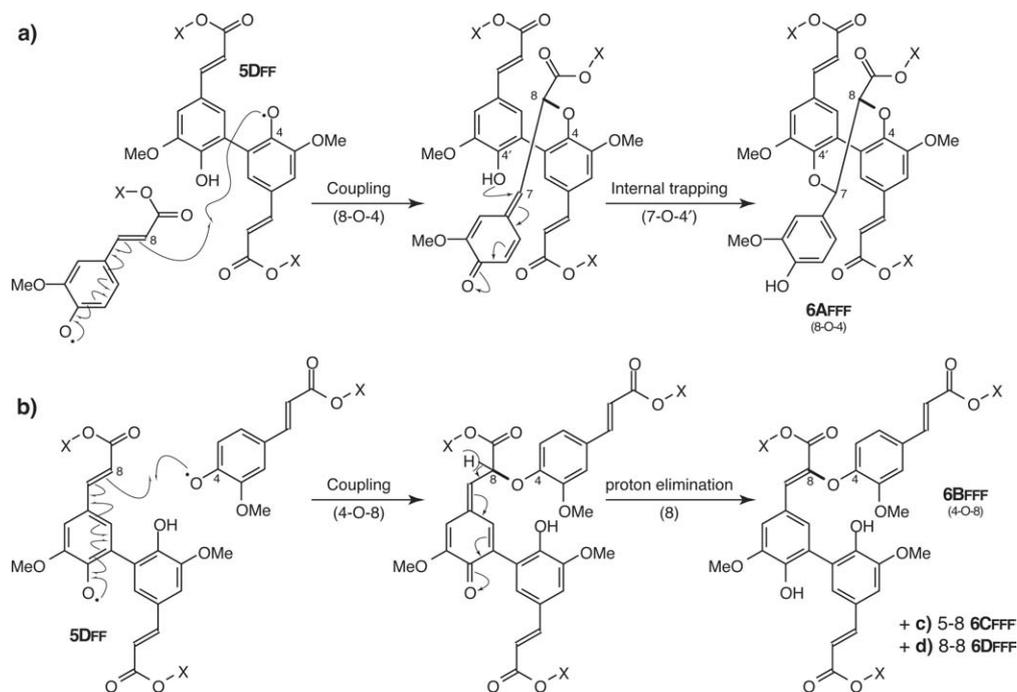
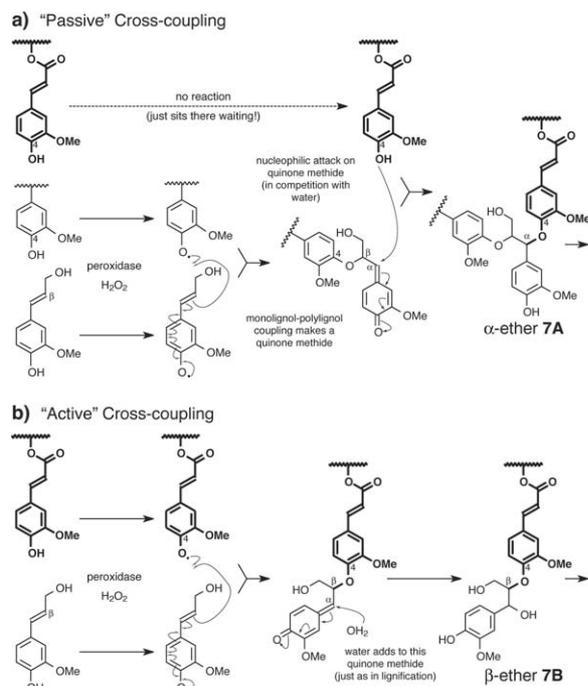


Figure 5. Two possible routes toward ferulate dehydrotrimers **6** from 5-5-DFA **5D** plus ferulate. a) Coupling at the ferulate's 8-position with a DFA 4-*O*-position would lead to the novel 8-*O*-4-dibenzodioxocin structure **6AFFF**, an analog of the dibenzodioxocin structures found in lignins. b) Coupling of a ferulate with 5-5-DFA at its sidechain 8-position can lead to 3 products, the 8-8-, 4-*O*-8-, and 5-8-coupled dehydrotrimers (the ferulate coupling is specified first); the mechanism for the 4-*O*-8-coupled product **6BFFF** is shown.

#### Other dehydrotriferulates

Detailed examinations of cereal grain hydroxycinnamates reveal the presence of other TFAs. In a combinatorial sense, there is the possibility of four (neglecting 4-*O*-5-coupling which appears from the dehydrodimer data to be minor) homo-trimers (8-*O*-4/8-*O*-4, 8-5/8-5, 8-8/8-8, 5-5/5-5), but also many hetero-trimers — note that the linkages can be with two different regiochemistries in most cases (e.g. from left to right) 8-*O*-4/8-5 or 8-5/8-*O*-4) and in the case of 8-8-DFAs **4C** which are unsymmetrical and retain both phenolic-OH groups, there are two options for each type of coupling, one on each ring. Thus pre-formed 8-8-DFA **4C3**, for example, can in principle cross-couple with a further ferulate to produce two different 8-*O*-4/8-8-TFAs, two different 8-5/8-8-TFAs, and two different 5-5/8-8-TFAs. There appear to be at least 19 possible TFAs to consider. We have already isolated and identified two more, an 8-*O*-4/8-*O*-4- and an 8-*O*-4/8-8-TFA (Funk et al., 2004). Since neither of these contains a 5-5-DFA moiety, forming dehydrotrimers only from intramolecularly-formed DFAs (of which only 5-5-DFA is possible) is an idea that must be reevaluated.

The key question to answer in future research will be whether any of these dehydrotrimers implicate the cross-linking of 3 independent polysaccharide chains. The modeling studies that were done to determine whether intramolecular coupling was possible did not look at the possibilities of 'back-crossing' to another ferulate on the same chain. i.e. There is the possibility that ferulates, not necessarily separated as in Figure 4a, see Figure 4b ( $n = 0, 1, 2, \dots$ ), on two polysaccharide chains may couple, then another ferulate on one chain could cross-couple with either of the two ferulate moieties already involved in a diferulate unit, Figure 4b. We are actively pursuing the isolation and identification of more TFAs to provide insight into this complex phenomenon. It is, however, going to take some extraordinary experiments to unambiguously determine the important question: do any of these TFAs implicate cross-coupling of three polysaccharide chains, or just two? And are higher oligomers to be found? In principle, two 5-5-DFAs on two polysaccharide chains might approach sufficiently closely to cross-couple to form a ferulate dehydro-tetramer but still only cross-link two polysaccharide



**Figure 6.** Two mechanisms for etherification of hydroxycinnamates (illustrated with ferulate) during lignification. a) The 'passive' mechanism in which ferulate sits around during the radical coupling and opportunistically adds to quinone methide intermediates produced during lignification, leads to  $\alpha$ -ferulate ethers **7A**. b) 'Active' radical coupling mechanisms directly couple ferulate with, e.g., a monolignol, resulting in  $\beta$ -ferulate ethers **7B**.

chains. More extensive cross-linking involving higher oligomers may also be possible.

### Lignin-polysaccharide cross-linking via hydroxycinnamates

Another area of cell wall cross-linking via similar peroxidase-mediated cross-linking mechanisms occurs during lignification, particularly in grasses. Lignification is a radical-coupling process mediated by peroxidases; being a solely chemical process, the polymerization is not controlled by enzymes or proteins and no exact primary structure is therefore stipulated – it is essentially a combinatorial process like the coupling of the ferulates themselves (to give the various DFAs) (Ralph et al., 2004). As a result, any phenolic present in the lignifying zone has the potential to incorporate into the lignification process, subject to its ability to form a radical and its compatibility with the other phenolics undergoing radical (cross-)coupling reactions. Ferulates are compatible

with the mono- and oligolignols, enter the lignin by the same types of radical coupling reactions that typify lignification, and become integral to the structure, only being partially releasable by known cleavage methods. Since ferulates are tethered to polysaccharides, the result is the cross-linking of the two diverse polymers, polysaccharides and lignin. Ferulates are not unique; their dehydromers, the DFAs, are also compatible phenolics (Quideau and Ralph, 1997). Incorporation of DFAs into lignins can result in more extensive cell wall cross-linking, between lignins and multiple polysaccharide chains.

It is now well established that cross-coupling of the ferulates and dehydromers with lignin monomers (and perhaps oligomers) is a mechanism for cross-linking lignins and polysaccharides in grasses, as reviewed (Ralph et al., 1998b; Hatfield et al., 1999). It is too early yet to tell whether dehydrosinapates can also enter into lignification, but it seems likely. Lignin-polysaccharide cross-linking has already been reviewed several times, so only a few topics will be added here.

### Incorporation of ferulate dehydromers into lignins

Cross-linking of lignins and polysaccharides via ferulates has been well studied. However, the DFAs also enter into radical cross-coupling reactions during lignification, becoming intimately incorporated into the polymer (Quideau and Ralph, 1997; Grabber et al., 2000). During lignification, ferulate and 5–5-coupled DFA copolymerized more rapidly and formed fewer ether-linked structures with coniferyl alcohol than 8–5-, 8–O–4- and 8–8-DFAs. The potential incorporation of most ferulates and diferulates into lignin exceeded 90%. As a result, xylans in grasses become extensively cross-linked by ferulate dehydromerization and incorporation into lignins, but only a small and variable proportion of these cross-links is measurable, since solvolytic cleavage does not release analyzable products.

### Mechanisms for lignin-polysaccharide cross-linking via hydroxycinnamates

There are two divergent mechanisms, Figure 6, by which polysaccharides can become cross-linked to phenolics (notably lignin) during plant cell wall growth and development (Ralph and Helm, 1993). The only mechanism considered early on was the nucleophilic addition of the ferulate phenols to quinone methides (Yamamoto et al., 1989; Iiyama et al., 1990;

Lam et al., 1992); reactive quinone methide intermediates are produced during coupling of a monolignol at its  $\beta$ -position with a growing lignin oligomer at its 4-*O*-position, Figure 6a. We described this mechanism as ‘opportunistic’ or ‘passive’ (Ralph and Helm, 1993). It can occur, and there is evidence for such reactions *in vitro* (Scalbert et al., 1986), but it requires that the ferulate compete with other phenols which don’t add extensively in lignins as evidenced by the paucity of non-cyclic  $\alpha$ -ethers in lignins (Ede and Kilpeläinen, 1995) and the more prevalent water which adds to at least the vast majority of such quinone methides. To us it seemed to be a strange mechanism for a process as important to the plant as cell wall cross-linking. This became even more of an issue once it was realized that ferulates underwent radical coupling with other ferulates to make the dehydrodiferulates that cross-linked polysaccharides (see above sections). Why then should ferulates undergo radical coupling with each other, but be denied the same type of reaction with lignin monomers and oligomers? Evidence that ferulates were cross-coupling by radical mechanisms, Figure 6b, was presented (Ralph et al., 1992, 1995). The research revealed other details which suggested that ferulates might be acting as nucleation sites from which lignification began (Ralph et al., 1992, 1995; Quideau and Ralph, 1997; Grabber et al., 2002). Incidentally, Harris’ group revealed that ferulates are present in gymnosperm cell walls as well (Carnachan and Harris, 2000). Ferulates should therefore not be ruled out as explanations for the beautiful pictures showing apparent nucleation behavior in softwood walls that are beginning to lignify (Donaldson, 1994, 2001; Terashima et al., 2004).

Additional evidence came from the isolation of a coniferyl alcohol- $\beta$ -*O*-4-ferulate dimer **7B** (Figure 6b) isolated from wheat and oat straw (Jacquet et al., 1995). A number of ferulate-monolignol (primarily coniferyl alcohol) cross-products have now been identified from grass cell wall samples (Grabber et al., 2002; Bunzel et al., 2004b). These crossed dimers appear to be relatively prominent (at levels comparable to some of the ferulate dimers) in some samples, notably rye fibers. Their structures are being steadily authenticated by independent synthesis. Screening for the presence of ferulate-coniferyl alcohol cross-products in different cereal grain fibers has been presented (Bunzel et al., 2004b).

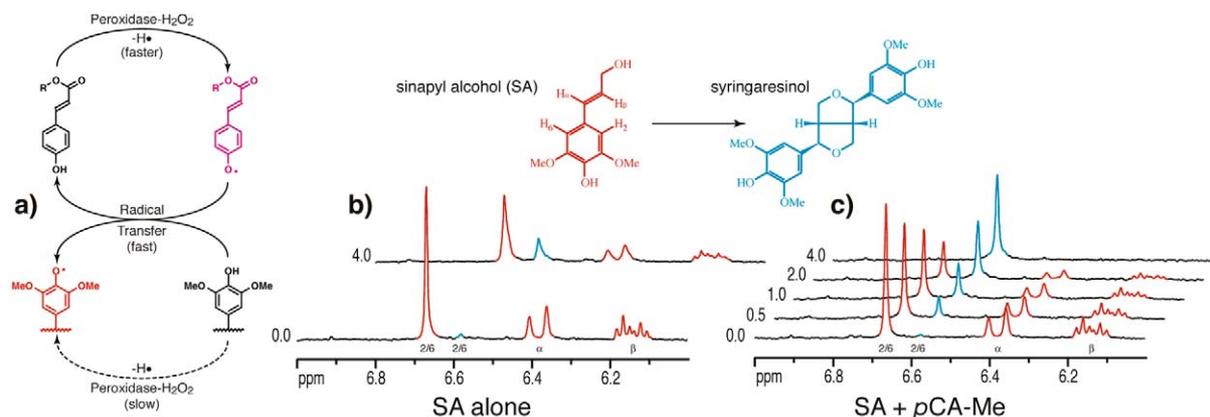
The evidence was therefore compelling that the ferulate ethers (that could be released by high temperature saponification) were therefore  $\beta$ -ethers, pro-

duced by what we termed ‘active’ radical coupling mechanisms, i.e. reactions over which the plant has more control in ensuring cross-linking would indeed take place (Ralph et al., 1998b). However, one of the major proponents of  $\alpha$ -ethers recently described ‘evidence’ that ferulates were largely attached as  $\alpha$ -ethers (benzyl ethers), not  $\beta$ -ethers in a paper entitled ‘Bonding of hydroxycinnamic acids to lignin: ferulic and *p*-coumaric acids are predominantly linked at the benzyl position of lignin, not the beta-position, in grass cell walls’ (Lam et al., 2001). The methods used have significant shortcomings. Our (unpublished) model studies show that  $\alpha$ -keto- $\beta$ -ethers (which are produced in the 2,3-dichloro-5,6-dicyanoquinone oxidation step used) will cleave under low-temperature base conditions if, for example, oxidants are present (including the peroxides in some ether solvents). This has been reported in other contexts previously (Aoyagi et al., 1975). Their  $\alpha$ -hydroxy-counterparts (before DDQ oxidation) will not cleave. The best way to address the  $\alpha$ - vs  $\beta$ -ferulate ether dilemma is to develop an independent method that will unambiguously resolve this issue and allow a quantification of  $\alpha$ - vs  $\beta$ -ferulate ethers. Some ideas are undergoing evaluation at this time.

Improved methods for determining etherified from esterified-only hydroxycinnamates are also required to resolve a problem with current methods which determine etherified hydroxycinnamate monomers by a subtraction method from two independent analyses. It is necessary to unambiguously answer the conundrum of whether *p*-coumarates are truly etherified in the cell wall. Finally, the envisioned method needs to be amenable to analysis of the DFAs, which is an even bigger nightmare currently.

#### *A role for p-coumarates in lignification*

Little has been said above about the hydroxycinnamate *p*-coumarate, the ‘methoxyl-less analog of **1**’, Figure 1. This is because *p*-coumarates in plant cell walls do not undergo radical coupling reactions. *p*-Coumarates acylate arabinoxylans in grass cell walls, just as ferulates do, but at a much lower level (Mueller-Harvey et al., 1986). They do dimerize photochemically, potentially cross-linking the polysaccharides (Ford and Hartley, 1989). *p*-Coumarates are more prominently found acylating the  $\gamma$ -hydroxyls of lignin sidechains (Shimada et al., 1971; Ralph et al., 1994b). All evidence suggests that their presence on the lignin polymer results from the incorporation of acylated



**Figure 7.** a) An important role for *p*-coumarates — radical transfer to sinapyl alcohol and syringyl units to facilitate lignification (Takahama & Oniki, 1994; Takahama et al., 1996; Hatfield et al., 1997). Radical transfer may be a reason why *p*-coumarates do not become significantly etherified during lignification (as do ferulates). b) and c) partial proton NMR spectra revealing the time-course of sinapyl alcohol dehydrodimerization in the presence of peroxidase and H<sub>2</sub>O<sub>2</sub> alone (b) and in the presence of added *p*-coumarate (0.01 equivalents, c) (Hatfield et al., 1997). After 4 h, sinapyl alcohol (red) has only partially dehydrodimerized to syringaresinol (cyan) in the absence of methyl *p*-coumarate, but has completely reacted in its presence, implicating radical transfer from *p*-coumarate to sinapyl alcohol.

monolignols, particularly sinapyl *p*-coumarate, as ‘monomers’ of lignification (Ralph et al., 1994b). Evidence for kenaf’s utilization of sinapyl acetate as an analogous acylated monolignol has been presented (Lu and Ralph, 2002), and similar findings for sinapyl *p*-hydroxybenzoate in aspen and palms will be presented this year.

So why do *p*-coumarates, unlike ferulates, not enter into radical coupling reactions? On their own *in vitro*, or under conditions where radical generation capability is not limiting, *p*-coumarates will undergo radical coupling. But there is no evidence that they do so in the wall. *p*-Coumarate esters are essentially all un-etherified; the *p*-coumarates acylating lignins are all free-phenolic terminal groups (Ralph et al., 1994b). The reason appears to be due to radical transfer reactions. Although *p*-coumarates interact more rapidly with most peroxidases to generate radicals, they quickly undergo radical transfer reactions with other phenolics, producing more stable radicals, Figure 7a.

The radical transfer idea was first introduced by Takahama et al. (Takahama and Oniki, 1994; Takahama et al., 1996). We have been able to demonstrate the concept (Hatfield et al., 1997), as illustrated in Figures 7b–c. When sinapyl alcohol is in solution with low concentrations of peroxidase and H<sub>2</sub>O<sub>2</sub>, its dehydrodimerization to syringaresinol is slow; most of the sinapyl alcohol remains after 4 h in the experiment shown. However, if just 0.01 equivalents of methyl *p*-coumarate are added to the same system, the sinapyl alcohol is totally converted to syringares-

inol in under 4 h. The logical conclusions are: (1) *p*-Coumarate is oxidized (to its radical) by peroxidase more rapidly than sinapyl alcohol; (2) Radical transfer from *p*-coumarate to sinapyl alcohol occurs, allowing sinapyl alcohol to more rapidly dehydrodimerize. The *p*-coumarate is acting like an ‘oxidation catalyst’ for sinapyl alcohol dehydrodimerization. Whether this facilitation of sinapyl alcohol oxidation is a primary role for *p*-coumarates in grasses (and analogously, *p*-hydroxybenzoates in aspen, poplar, willow, and palms) is unknown. The mechanism would however allow ready polymerization of sinapyl alcohol into lignins even in regions in which the available peroxidases don’t efficiently oxidize sinapyl alcohol directly. Peroxidases we have surveyed in grasses have all utilized sinapyl alcohol poorly as a substrate, but efficiently oxidized *p*-coumarate. In recent studies, the formation of syringyl-rich lignins in suspension-cultured maize cell walls was increased if they were lignified with moderate amounts (~100 g/kg) of a monolignol mixture that included sinapyl *p*-coumarate (Grabber, 2004).

## Conclusions

Dehydrodiferulates continue to emerge in varied roles as significant components of many plant fibers and are implicated in aspects of human and animal health as well as for their properties in limiting cell wall digestibility by ruminants. New dehydrodimers and various

crossed-‘dimers’ continue to be found, implicating further pathways in peroxidase-assisted cross-linking reactions in the cell wall. Dehydrotriferulates have recently been discovered, indicating more extensive roles for peroxidase-assisted polysaccharide cross-linking. Significant issues remain for separating and quantifying these important components and in understanding the mechanistic details. Ultimately, researchers will evaluate the impact these diverse products of cell wall cross-linking have on physiological processes, including ruminant and human nutrition.

## Acknowledgements

This work was supported in part by funding through the USDA National Research Initiatives (# 2003-01246), by the Deutsche Forschungsgemeinschaft, and the H. Wilhelm Schaumann-Stiftung.

## References

- Allerdings E, Ralph J, Schatz P, Gniechwitz D, Steinhart H & Bunzel M (2004) Isolation and structural identification of diabinosyl 8-O-4-dehydrodiferulate from maize bran insoluble fibre. *Phytochem.*: submitted.
- Andreasen MF, Kroon PA, Williamson G & Garcia-Conesa MT (2001a) Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radical Biol. Med.* 31(3): 304–314.
- Andreasen MF, Kroon PA, Williamson G & Garcia-Conesa MT (2001b) Esterase activity able to hydrolyze dietary antioxidant hydroxycinnamates is distributed along the intestine of mammals. *J. Agric. Food Chem.* 49: 5679–5684.
- Aoyagi T, Hosoya S & Nakano J (1975) New reaction site in lignins during the oxygen-alkali treatment. *Mokuzai Gakkaishi* 21(9): 532–534.
- Bolwell GP (1988) Synthesis of cell wall components: aspects of control. *Phytochem.* 27(5): 1235–1253.
- Bolwell GP (1993) Dynamic aspects of the plant extracellular matrix. *Int. Rev. Cytol* 146: 261–324.
- Brett CT, Wende G, Smith AC & Waldron KW (1999) Biosynthesis of cell-wall ferulate and diferulates. *J. Sci. Food Agric.* 79(3): 421–424.
- Bunzel M, Funk C & Steinhart H (2004a) Semipreparative isolation of dehydrodiferulic and dehydrotriferulic acids as standard substances from maize bran. *J. Sep. Sci.* 27: in press. DOI: 10.1002/jssc.200301703.
- Bunzel M, Ralph J, Marita JM & Steinhart H (2000) Identification of 4-O-5'-coupled diferulic acid from insoluble cereal fiber. *J. Agric. Food Chem.* 48(8): 3166–3169.
- Bunzel M, Ralph J, Funk C & Steinhart H (2003a) Isolation and identification of a ferulic acid dehydrotrimer from saponified maize bran insoluble fiber. *Eur. Food Res. Technol.* 217(2): 128–133.
- Bunzel M, Ralph J, Marita JM, Hatfield RD & Steinhart H (2001) Diferulates as structural components in soluble and insoluble dietary fibre. *J. Sci. Food Agric.* 81(7): 653–660.
- Bunzel M, Allerdings E, Sinwell V, Ralph J & Steinhart H (2002) Cell wall hydroxycinnamates in wild rice (*Zizania aquatica* L.) insoluble dietary fibre. *Eur. Food Res. Technol.* 214(6): 482–488.
- Bunzel M, Ralph J, Kim H, Hatfield RD & Steinhart H (2004b) Are cereal grains lignified? *J. Agric. Food Chem.*: in press.
- Bunzel M, Ralph J, Kim H, Lu F, Ralph SA, Marita JM, Hatfield RD & Steinhart H (2003b) Sinapate dehydrodimers and sinapate-ferulate heterodimers in cereal dietary fiber. *J. Agric. Food Chem.* 51(5): 1427–1434.
- Carnachan SM & Harris PJ (2000) Ferulic acid is bound to the primary cell walls of all gymnosperm families. *Biochem. Systematics and Ecol.* 28: 865–879.
- Charlton JL & Lee KA (1997) Thomasidic acid and 6-hydroxy-5,7-dimethoxy-2-naphthoic acid: Are they really natural products? *Tetrahedron Lett.* 38(42): 7311–7312.
- Chioccare F, Poli S, Rindone B, Pilati T, Brunow G, Pietikainen P & Setälä H (1993) Regio- and diastereoselective synthesis of dimeric lignans using oxidative coupling. *Acta Chem. Scand.* 47(6): 610–616.
- Donaldson LA (1994) Mechanical constraints on lignin deposition during lignification. *Wood Sci. Technol.* 28(2): 111–118.
- Donaldson LA (2001) Lignification and lignin topochemistry – an ultrastructural view. *Phytochem.* 57(6): 859–873.
- Ede RM & Kilpeläinen I (1995) Homo- and hetero-nuclear 2D NMR techniques: unambiguous structural probes for non-cyclic benzyl aryl ethers in soluble lignin samples. *Res. Chem. Intermediates* 21(3–5): 313–328.
- Faulds CB & Williamson G (1992) Ferulic acid release from plant polysaccharides by specific esterases. *Prog. Biotechnol.* 7: 419–422.
- Faulds CB & Williamson G (1999) The role of hydroxycinnamates in the plant cell wall. *J. Sci. Food Agric.* 79(3): 393–395.
- Ferguson LR & Harris PJ (2003) The dietary fibre debate: more food for thought. *Lancet* 361: 1487–1488.
- Ford CW & Hartley RD (1989) 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(3): 301–310.
- Freudenberg K & Schraube H (1955) Synthese des Syringaresinols und Versuche mit Sinapinalkohol. *Chem. Ber.* 88(1): 16–23.
- Fry SC & Miller JC (1989) Toward a Working Model of the Growing Plant Cell Wall. Phenolic Cross-linking reactions in the primary cell walls of dicotyledons. In: Lewis NG & Paice MG, (ed), *Plant Cell Wall Polymers, Biogenesis and Biodegradation*, Vol 399, Amer. Chem. Soc. Symp. Ser. (pp. 33–46). Amer. Chem. Soc., Washington, DC.
- Fry SC, Willis SC & Paterson AEJ (2000) Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. *Planta* 211(5): 679–692.
- Funk C, Ralph J, Steinhart H & Bunzel M (2004) Isolation and structural characterisation of 8-O-4/8-O-4- and 8-O-4/8-O-coupled dehydrotriferulic acids from maize bran. *Phytochem.*: submitted.
- Garcia-Conesa MT, Plumb GW, Waldron KW, Ralph J & Williamson G (1997a) Ferulic acid dehydrodimers from wheat bran: isolation, purification and antioxidant properties of 8-O-4-diferulic acid. *Redox Rep.* 3(5–6): 319–323.
- Garcia-Conesa MT, Plumb GW, Kroon PA, Wallace G & Williamson G (1997b) Antioxidant properties of ferulic acid dimers. *Redox Rep.* 3(4): 239–244.
- Garcia-Conesa MT, Wilson PD, Plumb GW, Ralph J & Williamson G (1999a) Antioxidant properties of 4,4'-dihydroxy-

- 3,3'-dimethoxy- $\beta$ , $\beta'$ -bicycinnamic acid (8-8-diferulate non-cyclic form). *J. Sci. Food Agric.* 79(3): 379–384.
- Garcia-Conesa MT, Kroon P, Ralph J, Mellon FA, Colquhoun IJ, Saulnier L, Thibault J-F & Williamson G (1999b) A cinnamoyl esterase from *Aspergillus niger* can break plant cell wall cross-links without release of free diferulic acids. *European J. Biochem.* 266(2): 644–652.
- Grabber JH (2004) How do lignin composition, structure, and cross-linking impact degradability? A review of cell wall model studies. *Crop Sci.* 44: in press.
- Grabber JH, Hatfield RD & Ralph J (1998a) Diferulate cross-links impede the enzymatic degradation of nonlignified maize walls. *J. Sci. Food Agric.* 77(2): 193–200.
- Grabber JH, Ralph J & Hatfield RD (1998b) Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. *J. Agric. Food Chem.* 46(7): 2609–2614.
- Grabber JH, Ralph J & Hatfield RD (1998c) Modeling lignification in grasses with monolignol dehydropolymerisate-cell wall complexes. In: Lewis NG & Sarkanen S, (ed), *Lignin and Lignan Biosynthesis*, Vol 697, Amer. Chem. Soc. Symp. Ser. (pp. 163–171). American Chemical Society, Washington, DC.
- Grabber JH, Ralph J & Hatfield RD (2000) Cross-linking of maize walls by ferulate dimerization and incorporation into lignin. *J. Agric. Food Chem.* 48(12): 6106–6113.
- Grabber JH, Ralph J & Hatfield RD (2002) Model studies of ferulate-coniferyl alcohol cross-product formation in primary maize walls: implications for lignification in grasses. *J. Agric. Food Chem.* 50(21): 6008–6016.
- Grabber JH, Hatfield RD, Ralph J, Zon J & Amrhein N (1995) Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochem.* 40(4): 1077–1082.
- Grabber JH, Ralph J, Hatfield RD, Quideau S, Kuster T & Pell AN (1996) Dehydrogenation polymer-cell wall complexes as a model for lignified grass walls. *J. Agric. Food Chem.* 44(6): 1453–1459.
- Harkin JM (1967) Lignin – a natural polymeric product of phenol oxidation. In: Taylor WI & Battersby AR, (ed), *Oxidative Coupling of Phenols*, (pp. 243–321). Marcel Dekker, New York.
- Hartley RD & Ford CW (1989) Phenolic constituents in plant cell walls and wall biodegradability. In: Lewis NG & Paice MG, (ed), *Plant Cell Wall Polymers, Biogenesis and Biodegradation*, Vol 399, Amer. Chem. Soc. Symp. Ser. (pp. 137–145). Amer. Chem. Soc., Washington, DC.
- Hartley RD, Morrison WH, III, Balza F & Towers GHN (1990a) Substituted truxillic and truxinic acids in cell walls of *Cynodon dactylon*. *Phytochem.* 29(12): 3699–3703.
- Hartley RD, Morrison WH, III, Himmelsbach DS & Borneman WS (1990b) Cross-linking of cell wall phenolic arabinoxylans in graminaceous plants. *Phytochem.* 29(12): 3705–3709.
- Hatfield RD & Ralph J (1999) Modeling the feasibility of intramolecular dehydrodiferulate formation in grass walls. *J. Sci. Food Agric.* 79(3): 425–427.
- Hatfield RD, Grabber J & Ralph J (1997) A potential role of sinapyl *p*-coumarate in grass lignin formation. In: Proceedings of the Annual Meeting of the American Society of Plant Physiologists. Vol Plant Physiol. 114 (pp. 346). Vancouver, British Columbia. American Soc. Plant Physiologists.
- Hatfield RD, Ralph J & Grabber JH (1999) Cell wall cross-linking by ferulates and diferulates in grasses. *J. Sci. Food Agric.* 79(3): 403–407.
- Iiyama K, Lam TBT & Stone BA (1990) Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochem.* 29(3): 733–737.
- Ishii T (1991) Isolation and characterization of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell-walls. *Carbohydr. Res.* 219: 15–22.
- Ishii T (1997) Structure and functions of feruloylated polysaccharides. *Plant Sci.* 127(2): 111–127.
- Jacquet G, Pollet B, Lapiere C, Mhamdi F & Rolando C (1995) New ether-linked ferulic acid-coniferyl alcohol dimers identified in grass straws. *J. Agric. Food Chem.* 43(10): 2746–2751.
- Jung HG & Ralph J (1990) Phenolic-carbohydrate complex in plant cell walls and their effect on lignocellulose utilization. In: Akin DE, Ljungdahl LG, Wilson JR & Harris PJ, (ed), *Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants*, (pp. 173–182). Elsevier, New York.
- Jung HG, Buxton DR, Hatfield RD & Ralph J (ed). (1993) *Forage Cell Wall Structure and Digestibility*. Am. Soc. Agronomy, Crop Sci. Soc. Am., Soil Soc. Am., Madison.
- Karhunen P, Rummakko P, Sipilä J, Brunow G & Kilpeläinen I (1995a) The formation of dibenzodioxocin structures by oxidative coupling. A model reaction for lignin biosynthesis. *Tetrahedron Lett.* 36(25): 4501–4504.
- Karhunen P, Rummakko P, Sipilä J, Brunow G & Kilpeläinen I (1995b) Dibenzodioxocins; a novel type of linkage in softwood lignins. *Tetrahedron Lett.* 36(1): 169–170.
- Kern SM, Bennet RN, Needs PW, Mellon FA, Kroon PA & Garcia-Conesa MT (2003) Characterization of metabolites of hydroxycinnamates in the in vitro model of human small intestinal epithelium Caco-2 cells. *J. Agric. Food Chem.* 51: 7884–7891.
- Kroon PA (2000) What role for feruloyl esterases today? *Polyphenols Actualités* 19: 4–5.
- Kroon PA & Williamson G (1999) Hydroxycinnamates in plants and food: current and future perspectives. *J. Sci. Food Agric.* 79(3): 355–361.
- Kroon PA, Garcia-Conesa MT, Fillingham IJ, Hazlewood GP & Williamson G (1999) Release of ferulic acid dehydrodimers from plant cell walls by feruloyl esterases. *J. Sci. Food Agric.* 79(3): 428–434.
- Lam TBT, Iiyama K & Stone BA (1992) Changes in phenolic acids from internode walls of wheat and *Phalaris* during maturation. *Phytochem.* 31(8): 2655–2658.
- Lam TBT, Kadoya K & Iiyama K (2001) Bonding of hydroxycinnamic acids to lignin: ferulic and *p*-coumaric acids are predominantly linked at the benzyl position of lignin, not the beta-position, in grass cell walls. *Phytochem.* 57(6): 987–992.
- Levigne S, Ralet M-C & Thibault J-F (2002) Characterisation of pectins from fresh sugar beet under different conditions using an experimental design. *Carbohydr. Polym.* 49: 145–153.
- Lu F & Ralph J (2002) Preliminary evidence for sinapyl acetate as a lignin monomer in kenaf. *J. Chem. Soc., Chem. Commun.* (1): 90–91.
- Lundquist K & Miksche GE (1965) Nachweis eines neuen Verknüpfungsprinzips von Guajacylpropaneinheiten im Fichtenlignin. *Tetrahedron Lett.* (25): 2131–2136.
- MacAdam JW & Grabber JH (2002) Relationship of growth cessation with the formation of diferulate cross-links and *p*-coumaroylated lignins in tall fescue leaf blades. *Planta* 215(5): 785–793.
- Marita JM, Ralph J, Lapiere C, Jouanin L & Boerjan W (2001) NMR characterization of lignins from transgenic poplars with suppressed caffeic acid *O*-methyltransferase activity. *J. Chem. Soc., Perkin Trans. 1* (22): 2939–2945.
- Micard V, Renard CMGC & Thibault JF (1997a) Influence of pre-treatments on enzymic degradation of a cellulose-rich residue from sugar-beet pulp. *Food Sci. Technol.* 30: 284–291.

- Micard V, Grabber JH, Ralph J, Renard CMGC & Thibault J-F (1997b) Dehydrodiferulic acids from sugar-beet pulp. *Phytochem.* 44(7): 1365–1368.
- Monties BL (1989) Lignins. In: Harborne J, (ed), *Methods in Plant Biochemistry*, Vol 1 (pp. 113–157). Academic Press, London.
- Mueller-Harvey I, Hartley RD, Harris PJ & Curzon EH (1986) Linkage of *p*-coumaryl and feruloyl groups to cell wall polysaccharides of barley straw. *Carbohydr. Res.* 148: 71–85.
- Oosterveld A, Beldman G & Voragen AGJ (2000) Oxidative cross-linking of pectic polysaccharides from sugar beet pulp. *Carbohydr. Res.* 328(2): 199–207.
- Oosterveld A, Grabber JH, Beldman G, Ralph J & Voragen AGJ (1997) Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydr. Res.* 300(2): 179–181.
- Parker CC, Parker ML, Smith AC & Waldron KW (2003) Thermal stability of texture in Chinese water chestnut may be dependent on 8,8'-diferulic acid (aryltetralyn form). *J. Agric. Food Chem.* 51: 2034–2039.
- Parker ML & Waldron KW (1995) Texture of Chinese water chestnut – involvement of cell-wall phenolics. *J. Sci. Food Agric.* 68(3): 337–346.
- Parr AJ, Waldron KW, Ng A & Parker ML (1996) The wall-bound phenolics of Chinese water chestnut. *J. Sci. Food Agric.* 71: 501–507.
- Quideau S & Ralph J (1997) Lignin-ferulate cross-links in grasses. Part 4. Incorporation of 5–5-coupled diferulate into lignin. *J. Chem. Soc., Perkin Trans. 1* (16): 2351–2358.
- Ralet M-C, Faulds CB, Williamson G & Thibault J-F (1994a) Degradation of feruloylated oligosaccharides from sugar-beet pulp and wheat bran by ferulic acid esterases from *Aspergillus niger*. *Carbohydr. Res.* 263: 257–269.
- Ralet M-C, Thibault J-F, Faulds CB & Williamson G (1994b) Isolation and purification of feruloylated oligosaccharides from cell walls of sugar-beet pulp. *Carbohydr. Res.* 263: 227–241.
- Ralph J & Helm RF (1993) Lignin/hydroxycinnamic acid/polysaccharide complexes: Synthetic models for regiochemical characterization. In: Jung HG, Buxton DR, Hatfield RD & Ralph J, (ed), *Forage Cell Wall Structure and Digestibility*, (pp. 201–246). ASA-CSSA-SSSA, Madison, WI.
- Ralph J, Grabber JH & Hatfield RD (1995) Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.* 275(1): 167–178.
- Ralph J, Garcia-Conesa MT & Williamson G (1998a) Simple preparation of 8-5-coupled diferulate. *J. Ag. Food Chem.* 46(7): 2531–2532.
- Ralph J, Helm RF, Quideau S & Hatfield RD (1992) Lignin-feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *J. Chem. Soc., Perkin Trans. 1* (21): 2961–2969.
- Ralph J, Quideau S, Grabber JH & Hatfield RD (1994a) Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J. Chem. Soc., Perkin Trans. 1* (23): 3485–3498.
- Ralph J, Hatfield RD, Quideau S, Helm RF, Grabber JH & Jung H-JG (1994b) Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Amer. Chem. Soc.* 116(21): 9448–9456.
- Ralph J, Hatfield RD, Grabber JH, Jung HG, Quideau S & Helm RF (1998b) Cell wall cross-linking in grasses by ferulates and diferulates. In: Lewis NG & Sarkanen S, (ed), *Lignin and Lignan Biosynthesis*, Vol 697, Amer. Chem. Soc. Symp. Ser. (pp. 209–236). American Chemical Society, Washington, DC.
- Ralph J, Lapierre C, Lu F, Marita JM, Pilate G, Van Doorselaere J, Boerjan W & Jouanin L (2001a) NMR evidence for benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into lignins of *O*-methyl-transferase-deficient poplars. *J. Agric. Food Chem.* 49(1): 86–91.
- Ralph J, Bunzel M, Marita JM, Hatfield RD, Lu F, Kim H, Grabber JH, Ralph SA, Jimenez-Monteon G & Steinhart H (2000) Diferulates analysis: new diferulates and disinapates in insoluble cereal fibre. *Polyphénols Actualités* (19): 13–17.
- Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, Marita JM, Hatfield RD, Ralph SA, Christensen JH & Boerjan W (2004) Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochem. Reviews*: 3: 29–60.
- Ralph J, Lapierre C, Marita J, Kim H, Lu F, Hatfield RD, Ralph SA, Chapple C, Franke R, Hemm MR, Van Doorselaere J, Sederoff RR, O'Malley DM, Scott JT, MacKay JJ, Yahiaoui N, Boudet A-M, Pean M, Pilate G, Jouanin L & Boerjan W (2001b) Elucidation of new structures in lignins of CAD- and COMT-deficient plants by NMR. *Phytochem.* 57(6): 993–1003.
- Rombouts FM & Thibault JF (1986) Feruloylated pectic substances from sugar-beet pulp. *Carbohydr. Res.* 154: 177–187.
- Rouau X, Bertin C & Thibault JF (1987) Characterization and enzymic degradation of sugar beet fiber. *Food Hydrocolloids* 1(5–6): 439–443.
- Rouau X, Cheynier V, Surget A, Gloux D, Barron C, Meuded E, Louis-Montero J & Criton M (2003) A dehydrodimer of ferulic acid from maize bran. *Phytochem.* 63: 899–903.
- Rubino MI, Arntfield SD & Charlton JL (1995) Conversion of phenolics to lignans: Sinapic acid to thomasidic acid. *J. Amer. Oil Chem. Soc.* 72(12): 1465–1470.
- Rubino MI, Arntfield SD & Charlton JL (1996) Evaluation of alkaline conversion of sinapic acid to thomasidic acid. *J. Agric. Food Chem.* 44(6): 1399–1402.
- Sánchez M, Peña MJ, Revilla G & Zarra I (1996) 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.
- Sarkanen KV & Ludwig CH (1971) *Lignins, Occurrence, Formation, Structure and Reactions*. Wiley-Interscience, New York.
- Saulnier L & Thibault J-F (1999) Ferulic acid and diferulic acid as components of sugar-beet pectins and maize heteroxylans. *J. Sci. Food Agric.* 79: 396–402.
- Saulnier L, Crepeau MJ, Lahaye M, Thibault JF, Garcia-Conesa MT, Kroon PA & Williamson G (1999) Isolation and structural determination of two 5,5'-diferuloyl oligosaccharides indicate that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates. *Carbohydr. Res.* 320(1–2): 82–92.
- Scalbert A, Monties B, Rolando C & Sierra-Escudero A (1986) Formation of ether linkage between phenolic acids and Gramineae lignin: a possible mechanism involving quinone methides. *Holzforschung* 40(3): 191–195.
- Setälä H, Pajunen A, Rummakko P, Sipilä J & Brunow G (1999) A novel type of spiro compound formed by oxidative cross-coupling of methyl sinapate with a syringyl lignin model compound. A model system for the  $\beta$ -1 pathway in lignin biosynthesis. *J. Chem. Soc., Perkin Trans. 1* (4): 461–464.
- Shimada M, Fukuzuka T & Higuchi T (1971) Ester linkages of *p*-coumaric acid in bamboo and grass lignins. *Tappi* 54(1): 72–78.
- Stewart D, Robertson GW & Morrison IM (1994) Phenolic acid dimers in the cell walls of barley. *Biol. Mass Spec.* 23: 71–74.
- Syrjanen K & Brunow G (1998) Oxidative cross coupling of *p*-hydroxycinnamic alcohols with dimeric arylglycerol  $\beta$ -aryl ether lignin model compounds. The effect of oxidation potentials. *J. Chem. Soc. Perkin Trans. 1* (20): 3425–3429.

- Syrjanen K & Brunow G (2000) Regioselectivity in lignin biosynthesis. The influence of dimerization and cross-coupling. *J. Chem. Soc. Perkin Trans. 1* (2): 183–187.
- Takahama U & Oniki T (1994) Effects of ascorbate on the oxidation of derivatives of hydroxycinnamic acid and the mechanism of oxidation of sinapic acid by cell wall-bound peroxidases. *Plant Cell Physiol.* 35(4): 593–600.
- Takahama U, Oniki T & Shimokawa H (1996) A Possible Mechanism for the Oxidation of Sinapyl Alcohol by Peroxidase-Dependent Reactions in the Apoplast: Enhancement of the Oxidation by Hydroxycinnamic Acids and Components of the Apoplast. *Plant Cell Physiol.* 37(4): 499–504.
- Tanahashi M, Takeuchi H & Higuchi T (1976) Dehydrogenative polymerization of 3,5-disubstituted *p*-coumaryl alcohols. *Wood Res.* 61: 44–53.
- Terashima N, Awano T, Takabe K & Yoshida M (2004) Formation of macromolecular lignin in ginkgo xylem cell walls as observed by electron microscopy. *Comptes Rend. Biologies* 327(6): in press.
- Teutonico RA, Dudley MW, Orr JD, Lynn DG & Binns AN (1991) Activity and accumulation of cell division-promoting phenolics in tobacco tissue cultures. *Plant Physiol.* 97(1): 288–297.
- van Huystee RB & Zheng X (1993) Cationic peanut peroxidase and the oxidation of ferulic acid. *Phytochem.* 34(4): 933–939.
- Waldron KW, Parr AJ, Ng A & Ralph J (1996) Cell-wall-esterified phenolic monomers and dimers: identification and quantification by reverse-phase HPLC and diode-array detection. *Phytochem. Anal.* 7(6): 305–312.
- Wallace G & Fry SC (1995) In vitro peroxidase-catalyzed oxidation of ferulic acid esters. *Phytochem.* 39(6): 1293–1299.
- Wallis AFA (1973) Oxidative dimerization of methyl (*E*)-sinapate. *Aust. J. Chem.* 26: 1571–1576.
- Yamamoto E, Bokelman GH & Lewis NG (1989) Phenylpropanoid metabolism in cell walls. An overview. In: Lewis NG & Paice MG, (ed), *Plant Cell Wall Polymers. Biogenesis and Biodegradation*, Vol 399, Amer. Chem. Soc. Symp. Ser. (pp. 68–88). Amer. Chem. Soc., Washington, DC.
- Yang J-G & Uchiyama T (2000a) Hydroxycinnamic acids and their dimers involved in the cessation of cell elongation in *Mentha* suspension culture. *Biosci. Biotechnol. Biochem.* 64(8): 1572–1579.
- Yang J-G & Uchiyama T (2000b) Dehydrodimers of caffeic acid in the cell walls of suspension-cultured *Mentha*. *Biosci. Biotechnol. Biochem.* 64(4): 862–864.