New Ether-Linked Ferulic Acid–Coniferyl Alcohol Dimers Identified in Grass Straws

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Grass cell walls are typified by ferulic esters linked to polysaccharides. In past research, these feruloylated esters have been repeatedly speculated to be cross-linking agents with lignins, via ether bonds. Whereas this hypothesis is strongly supported by degradative studies, model experiments, and NMR data, diagnostic fragments associating ferulic acid and lignin precursors, through an ether bond, have never been isolated from grass walls. This paper reports the isolation of such products by saponification of wheat and oat straws. New dimers associating ferulic acid to the β position of coniferyl alcohol are characterized by gas chromatography/mass spectrometry and authenticated by independently synthesized compounds. The biochemical implication is that ferulate esters are copolymerized with lignin precursors through oxidative coupling. These ferulate esters thereby provide points of growth for the polymer lignin, via ether bonds that anchor lignins to wall polysaccharides.

Keywords: *Grass cell walls; ferulic acid; lignin; ferulic acid bridge; ether-linked ferulic acid–coniferyl alcohol dimer*

INTRODUCTION

Grass cell walls are typified by the presence of ferulic and *p*-coumaric acids, linked to polysaccharides and/or lignins (Smith, 1955; Higuchi et al., 1967). As difunctional molecules with carboxylic and phenolic bonding sites, these acids and their dimers (formed by enzymic or light-induced radical coupling) provide a pathway for the cross-linking of wall polymers. Such a cross-linking can have a dramatic influence on the wall mechanical properties, extensibility, and biodegradability (Hartley, 1972; Fry, 1986; Ford and Harltey, 1988; Eraso and Hartley, 1990; Jung and Deetz, 1993).

Past research pointed out that ferulic acid is the predominant *p*-hydroxycinnamic acid esterified to grass polysaccharides (Lam et al., 1994). Feruloylated oligosaccharides were isolated by enzymic hydrolysis of grass cell walls and extensively characterized (Kato et al., 1983; Smith and Hartley, 1983; Kato et al., 1985; Mueller-Harvey et al., 1986; Hartley et al., 1990a; Ishii et al., 1990). Ferulate esters were shown to cross-link arabinoxylan chains through 5–5′ dimerization (Ishii, 1991). Recently, new dehydrodiferulate dimers were recovered from saponified grass cell walls (Ralph et al., 1994). These results together with appropriate model studies (Helm and Ralph, 1993 a,b) underscore the potential for ferulate esters to cross-link wall polysaccharides.

While there is substantial information on grass feruloylated carbohydrate esters, there is less and still indirect information on the attachment of ferulic acid to lignins. Scalbert et al. (1985) established that ferulic acid is associated with wheat lignin fractions through bonds that resist mild alkaline hydrolysis but are cleaved by acid hydrolysis. From these findings and from NMR signals assigned to ferulate ethers in grass lignins (Nimz et al., 1981; Scalbert et al., 1985), it was speculated that ferulic acid was associated both with lignins through acid-labile (i.e. ether) bonds and with polysaccharides through alkali-labile ester bonds. A sophisticated multistep strategy was recently developed to estimate such ferulic acid bridging units (Lam et al., 1990, 1992, 1994). This work suggested that all of the etherified ferulic acid was also ester-linked (Lam et al., 1994). In parallel, model studies provided important information. Ether-linked ferulic models that represent theorized lignin–ferulate ether condensation products were prepared (Helm and Ralph, 1992). In addition, ferulate esters were incorporated into synthetic lignins by peroxidase-assisted polymerization, via ether or carbon–carbon bonds (Ralph et al., 1992). Today, the hypothesis of ether bonds between lignins and ferulic acid is thus strongly supported by degradative studies, model experiments, and NMR data. However, a serious shortcoming of this hypothesis is that diagnostic fragments associating ferulic acid and lignin monomers through an ether bond have never been isolated from grass walls.

This paper reports the isolation of such products by alkaline treatment of wheat and oat straws. In this study, new dimers associating ferulic acid with the β position of lignin monomers are characterized by gas chromatography/mass spectrometry (GC/MS) of their trimethylsilyl (TMS) derivatives and authenticated with synthetic dimers. The biochemical implications of these new dimeric structures are discussed.

MATERIALS AND METHODS

Material. Mature and dried internodes of wheat (*Triticum aestivum* L. cv. Champlein) and oat (*Avena sativa*) were ground to pass a 1 mm sieve. The ground wheat straw was subsequently extracted with ethanol/toluene (1/2 v/v), ethanol, and water in a Soxhlet apparatus and then freeze-dried. This extractive-free straw was exhaustively permethylated with diazomethane as previously described (Lapierre et al., 1989).

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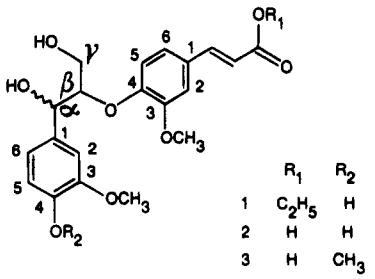

Figure 1. Structure of ether-linked ferulic acid–coniferyl alcohol dimers obtained by synthesis (**1**) or recovered by saponification of original (**2**) or permethylated (**3**) straws.

The oat ground straw was exhaustively washed with water and ethanol only.

Synthesis of Guaiacylglycerol β-Ethylferuloyl Ether. The synthesis of compound **1** (Figure 1), (*E*)-ethyl 4-[2-(4-hydroxy-3-methoxyphenyl)-2-hydroxy-1-(hydroxymethyl)ethyl]-ferulate, which mimics a theorized lignin–ferulate ether condensation product esterified at the ferulic carboxyl group, was performed according to a protocol adapted from Helm and Ralph (1992). This compound was prepared as a mixture of *erythro* and *threo* isomers and was accompanied by trace amounts of the *Z* analogues.

Isolation of Ether-Linked Hydroxycinnamic Acid Derivatives by Mild Alkaline Hydrolysis of Grass Straws. A 1 g portion of the ground straw (original or permethylated) was saponified for 2 h at 37 °C with 50 mL of 2 N aqueous NaOH under N_2, in the dark, with magnetic stirring. After vacuum-filtering (glass porosity 1) and washing (2 × 50 mL of water), the filtrate was adjusted to pH 3 with 6 M aqueous HCl, which caused the precipitation of lignin–carbohydrate complexes. This precipitate was discarded by centrifugation (1 h, 12000*g*, 4 °C) to restrict the formation of emulsions in subsequent extraction steps. The acidified supernatant was extracted with 4 × 100 mL of AcOEt/CH_2Cl_2 (1/1 v/v, spectroquality grade solvents). The combined organic extracts were dried over Na_2SO_4, and the solvent was removed under reduced pressure at 40 °C. This rough final residue containing the alkali-released phenolics was redissolved in 1 mL of AcOEt/CH_2Cl_2 mixture prior to silylation and subsequent GC/MS analysis. All analyses were done at least in triplicate. The model compound **1** was subjected to similar saponification and extraction.

Thin-Layer Chromatography (TLC). The various phenolics recovered by the previous procedure were spotted on TLC plates precoated with silica gel 60F 254 (particle size, 250 μm, Merck). The plates were developed with a toluene/acetone/acetic acid mixture (180/20/20 v/v/v). The compounds were detected as dark spots when illuminated under UV light. The hydroxycinnamic acid monomers (ferulic and *p*-coumaric acids with R_f values of 0.31 and 0.28) were separated from the hydroxycinnamic acid dimers with R_f values of 0.02–0.10. The dimer zone was scratched from the plate and eluted with AcOEt/CH_2Cl_2 mixture. The recovered organic extracts containing the dimers were washed with H_2O, dried over Na_2SO_4, and evaporated to dryness. The resulting dimer fraction was redissolved in 1 mL of AcOEt/CH_2Cl_2 mixture. An aliquot of this dimer fraction was irradiated for 4 h in a sealed quartz cell under UV A light to induce the $E \rightarrow Z$ photoisomerization of hydroxycinnamic derivatives.

Preparation of Trimethylsilyl (TMS) Derivatives and GC/MS Analyses. The whole organic extracts from saponified straws, the original or photoisomerized dimer fractions isolated by TLC of the whole extracts, and AcOEt/CH_2Cl_2 solutions of compound **1**, before or after saponification, were subjected to trimethylsilylation as follows. About 20 μL of the sample organic solution was silylated with 100 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 10 μL of GC grade pyridine in a 200 μL silyl vial, at ambient temperature for 1 h. The GC/MS analysis was performed on a poly(dimethylsiloxane) capillary column (SPB1, Supelco, 30 m × 0.32 mm i.d.), working in the temperature program mode (from 110 to 260 °C, at +3 °C/min), with helium as the carrier gas (0.5 bar inlet pressure) and a moving needle type injector (250 °C). The gas chromatographic system was coupled to a quadrupole mass spectrometer operating in the electron impact mode (70 eV), with a source at 100 °C, an interface at 270 °C, and a 60–750 *m/z* scanning range. To further confirm the mass spectral assignments, deuterated trimethylsilyl (DTMS) derivatives of the dimer fractions were prepared by using deuterated bis-(trimethylsilyl)acetamide (DBSA; ISOTEC, St. Quentin, France) instead of BSTFA.

RESULTS AND DISCUSSION

GC/MS Characterization of TMS Cyclodimers and Dehydrodiferulic Acid Dimers Released from Grass Cell Walls by Mild Alkaline Hydrolysis. The saponification of wheat or oat extractive-free straw released various hydroxycinnamic derivatives. As expected, the predominant ones were (*E*)-*p*-coumaric acid (CA) and (*E*)-ferulic acid (FA), accompanied by their *Z* analogues. Besides these monomers, various hydroxycinnamic acid dimers could be observed in much lower amounts. These dimers were represented by a suite of cyclobutane dimers (CA–CA, CA–FA, and FA–FA) already described in the literature (Ford and Hartley, 1989, 1990a,b; Hartley et al., 1990b) and by some dehydrodiferulic acid dimers (DFA) (Ralph et al., 1994). The GC traces of the hydroxycinnamic acid dimers (Figure 2) indicated the large predominance of the cyclobutane type in these dimer mixtures. Besides the CA–CA, CA–FA, FA–FA, and DFA TMS derivatives, unknown compounds labeled X in Figure 2 could be observed, which are discussed in the next section.

The GC/MS analysis of the TMS hydroxycinnamic acid dimers was substantially facilitated by the prior TLC fractionation of the whole extract. This fractionation was performed to separate the two major monomers CA and FA from the series of minor dimers individually present in relative amounts considerably lower than the amount of CA or FA. By so doing, we could satisfactorily analyze the hydroxycinnamic dimer mixture by GC/MS without GC capillary column overload and MS source pollution by an excess of TMS monomers.

The GC profiles of the hydroxycinnamic acid dimers released by alkaline hydrolysis of wheat or oat straw and purified by TLC are shown in Figure 3. These profiles are very similar to those of the dimer region of the whole extracts (Figure 2). The TLC fractionation step thus does not introduce artifact dimers, only variations in the relative contents of the various dimers. To observe the diagnostic molecular ions of the TMS dimers, including the minor ones, large amounts of their complex mixture could be injected, in the absence of the TMS monomers. This dimer amount accounts for the lower resolution of the chromatograms shown in Figure 3, compared to those of Figure 2. The mass spectra of peaks 1–15 in Figure 3 are listed in Table 1. Many prominent peaks of the dimer mixture showed shoulders assignable to other cyclobutane isomers, not reported herein. Most of the cyclobutane CA–CA, CA–FA, and FA–FA dimers gave weak diagnostic molecular ions in the electron impact mode, at *m/z* 616, 646, and 676, respectively. The base peaks observed in their mass spectra corresponded to the monomer ions $CA(TMS)_2$ and $FA(TMS)_2$, at *m/z* 308 and 338, respectively (Ford and Hartley, 1989, 1990 a,b; Hartley et al., 1990). In these samples, we observed the preponderance of the truxillic type dimers formed by head to tail photodimerization of CA and FA acids. These truxillic dimers were characterized by weak but diagnostic fragments at *m/z*

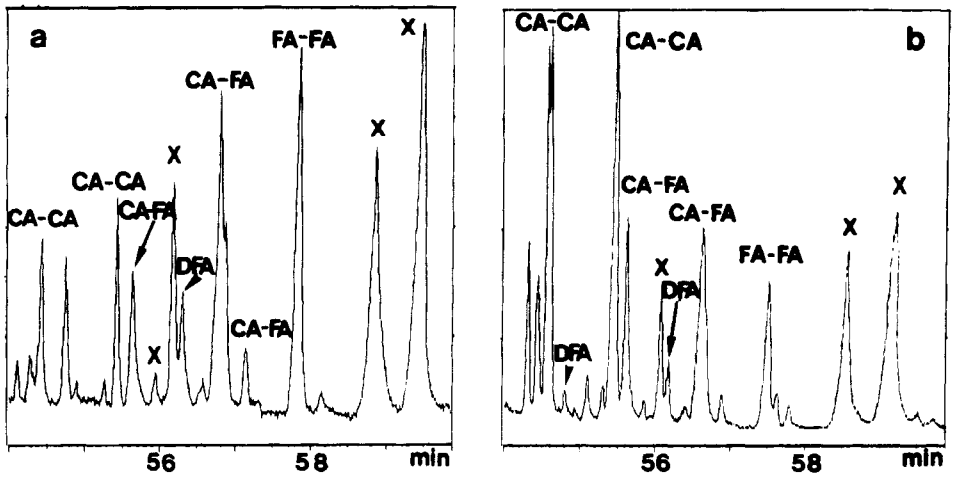

Figure 2. Dimer regions of total ion chromatograms from GC/MS of saponified extracts from (a) wheat and (b) oat straws. Peaks labeled CA–CA, CA–FA, and FA–FA are assigned to cyclodimers on the basis of their mass spectra and literature data (Ford and Hartley, 1990b; Hartley et al., 1990b). Peaks labeled DFA are similarly assigned to dehydrodiferulic acid dimers. Peaks labeled X correspond to new ferulic acid–coniferyl alcohol dimers.

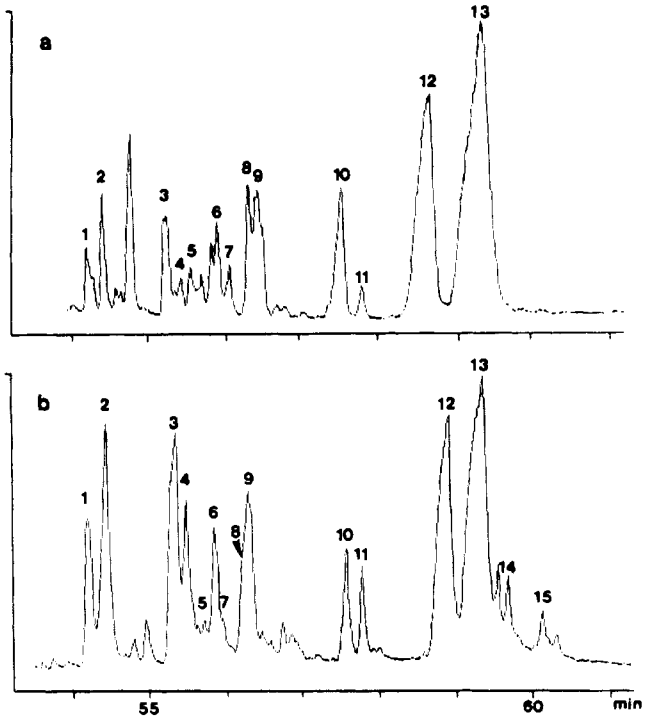

Figure 3. Total ion chromatograms from the GC/MS of the dimers recovered after saponification of (a) wheat and (b) oat straws and subsequent TLC fractionation of the extracts. Numbers refer to mass spectral identifications of Table 1.

381, 411, and 441 (Table 1) resulting from the symmetrical opening of the cyclobutane ring and subsequent McLafferty rearrangement (Hartley et al., 1990). Minor peaks could be identified as TMS dehydrodiferulate dimers.

GC/MS Identification of New Dimers Released from Grass Cell Walls by Mild Alkaline Hydrolysis. Besides the peaks assigned to TMS cyclobutane and dehydrodiferulate dimers, the GC/MS profiles obtained from wheat and oat samples revealed the presence of an additional and prominent pair of peaks, with higher retention times (quoted as X in Figure 2 and as 12–13 in Figure 3). This chromatographic doublet was systematically observed for a series of independent experiments repeated over a 1 year period, with these wheat and oat samples. The two mass spectra of these two peaks were essentially identical, which suggests that they are isomers. In addition, the presence of a TMS ferulic unit in these unknown isomers was evidenced from the diagnostic ferulic monomer ion $FA(TMS)_2$, at *m/z* 338. Informative fragments at *m/z* 297 (base peak) and at *m/z* 103 could be assigned to the TMS benzylic ion G-$CHOTMS^+$ (with G = guaiacyl or 4-hydroxy-3-methoxyphenyl ring) and TMS hydroxymethyl ion CH_2-$OTMS^+$, respectively. This mass spectral information suggested that the unknown compounds X were the *erythro* and *threo* TMS isomers of compound **2** (Figure 1) that represents a β-O-4′ condensation product obtained by radical coupling of (*E*)-ferulic acid and coniferyl alcohol, the precursor of lignin G units. The presence of an *E* double bond in these isomers was supported by UV irradiation experiments. This irradiation caused a relative decrease of the unknown pair of peaks and a concomitant increase of another pair present in lower amount in the nonirradiated sample and having shorter GC retention time and similar but not identical mass spectra (peaks 6 and 8 in Figure 3). Such behavior suggested a light-induced $E \rightarrow Z$ isomerization. Authentication of structure **2** was afforded by synthesis of the appropriate ethyl ester **1**. When subjected to the same alkaline hydrolysis, extraction, silylation, and GC/MS steps as the grass samples, the authentic compound **1** quantitatively provided the TMS isomers **2**, with identical GC retention times and mass spectra. Conversely, the ethylation of the grass sample extract by ethanol in the presence of a catalytic amount of acid at 60 °C afforded compound **1**. When saponification was run from CH_2N_2 permethylated straw, isomers **2** were no longer observed, but their analogues **3** (Figure 1), methylated at the phenolic group of the guaiacyl ring, were. This result means that, in the original grass cell walls, structures giving rise to isomers **2** are esterified at the carboxylic group of the ferulic unit and are free at the phenolic group of the guaiacyl unit. The speculative formation of the main fragments of TMS or DTMS compound **2**, when subjected to electron impact, is given in Figure 4. Not unexpectedly, many of these fragments arise by breakdown of the β-O-4′ bond.

Among the phenolics released from the oat sample, we could observe the syringyl S (4-hydroxy-3,5-dimethoxyphenyl) and *p*-hydroxyphenyl H analogues of the guaiacyl G isomers **2**. These dimers, corresponding to peaks 14–15 and to peak 11 (Figure 3 and Table 1), originate from the radical coupling of ferulic acid and

Table 1. Abbreviated Mass Spectra (Electron Impact at 70 eV) of the TMS Phenolic Dimers Recovered after Saponification of Wheat or Oat Straw Samples and Subsequent TLC Fractionation of the Extracts

peak[a] and tentative assignment	M^+, (*m/z* %)	other ions, (*m/z* %)
1, CA–CA	616 (1)	601 (3), 498 (3), 381 (8), 356 (20), 308 (100), 293 (36), 249 (14), 245 (23), 219 (33) 203 (3), 192 (3), 191 (2), 179 (9), 163 (4), 147 (10), 133 (4), 115 (3), 73 (81)
2, CA–CA	616 (1)	601 (4), 498 (2), 381 (10), 356 (5), 308 (100), 293 (65), 249 (20), 245 (17), 219 (59), 203 (6), 192 (4), 191 (4), 179 (13), 147 (10), 133 (3), 115 (3), 73 (94)
3, CA–CA	616 (0.5)	601 (1), 526 (1), 498 (1), 381 (10), 308 (100), 293 (74), 249 (24), 219 (65), 203 (6), 192 (4), 191 (4), 179 (14), 147 (8), 133 (4), 115 (4), 73 (96)
4, CA–FA	ND[b]	631 (2), 386 (6), 381 (3), 338 (100), 323 (10), 293 (20), 249 (17), 245 (17), 219 (19), 203 (3), 179 (6), 147 (11), 115 (2), 73 (64)
5, CA–FA	646 (10)	338 (83), 323 (22), 308 (30), 293 (41), 249 (43), 219 (36), 203 (7), 179 (11), 147 (11), 133 (7), 115 (8), 73 (100)
6, ferulic acid–coniferyl alcohol ether	678 (8)	663 (4), 588 (6), 498 (6), 471 (5), 439 (2), 365 (10), 340 (3), 338 (4), 297 (77), 275 (5), 239 (6), 235 (25), 223 (30), 222 (26), 209 (13), 194 (8), 193 (5), 179 (4), 147 (6), 73 (100)
7, DFA	674 (9)	659 (8.4), 556 (37.8), 467 (18.7), 410 (3.4), 380 (4), 147 (7), 73 (100)
8, ferulic acid–coniferyl alcohol ether	678 (6)	663 (4), 598 (8), 498 (6), 474 (5), 365 (9), 338 (22), 297 (66), 275 (5), 249 (6), 235 (31), 223 (34), 222 (26), 209 (11), 194 (7), 193 (5), 179 (4), 147 (10), 73 (100)
9, CA–FA	646 (3)	631 (3), 411 (1), 381 (2), 338 (100), 323 (13), 308 (19), 293 (23), 249 (24), 219 (24), 179 (7), 147 (5), 133 (3), 115 (3), 73 (63)
10, FA–FA	676 (2)	661 (4), 586 (1), 441 (1), 411 (2), 338 (100), 323 (21), 308 (14), 293 (9), 249 (21), 219 (10), 209 (4), 192 (3), 179 (5), 147 (8), 133 (3), 115 (3), 73 (46)
11, ferulic acid *p*-hydroxycinnamyl alcohol ether	648 (3)	633 (3), 382 (3), 367 (1), 351 (6), 338 (15), 323 (5), 293 (10), 267 (100), 249 (3), 223 (2), 219 (4), 205 (5), 179 (21), 147 (13), 103 (13), 73 (90)
12, 13, ferulic acid–coniferyl alcohol ether	678 (5)	663 (3), 412 (3), 397 (1), 351 (4), 340 (3), 338 (9), 323 (15), 308 (3), 297 (100), 293 (5), 265 (4), 263 (3), 249 (4), 235 (3), 223 (56), 219 (5), 209 (15), 193 (4), 179 (3), 147 (13), 103 (9), 73 (83)
14, 15, ferulic acid–sinapyl alcohol ether	ND	693 (3), 442 (2), 427 (1), 412 (2), 370 (3), 354 (10), 340 (3), 338 (5), 327 (100), 297 (14), 265 (2), 253 (2), 249 (3), 239 (9), 223 (3), 219 (5), 177 (2), 147 (9), 103 (11), 73 (71)

[a] Numbers refer to peak numbering of Figure 3. [b] ND, not detected.

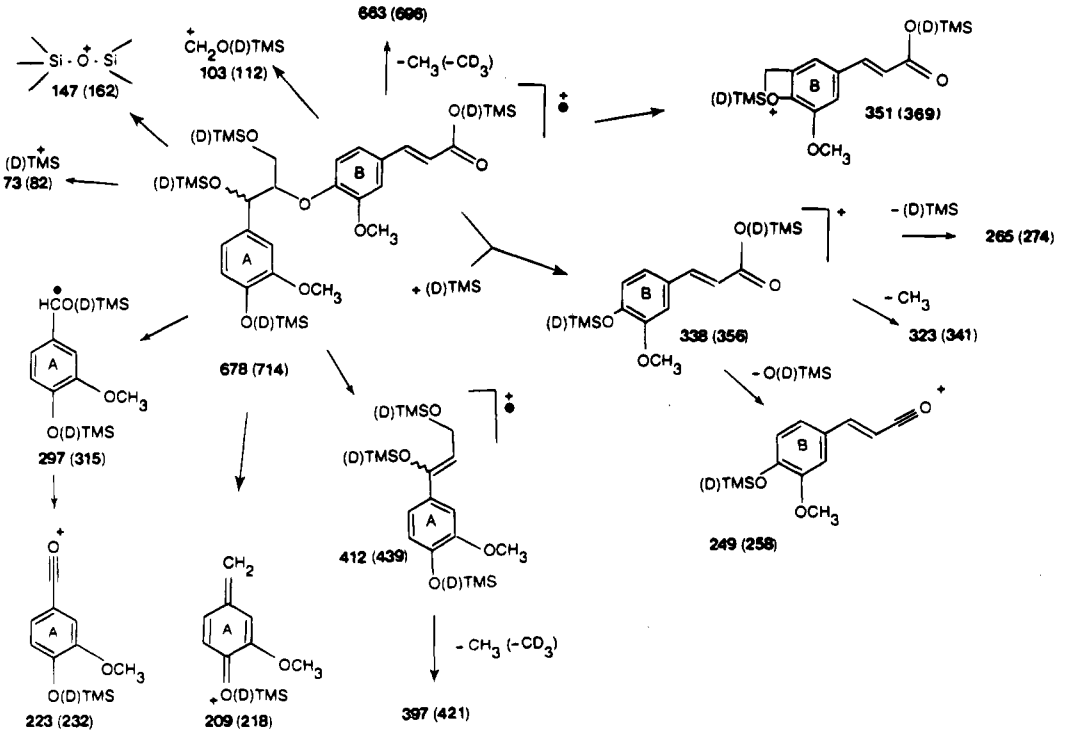

Figure 4. Proposed mass spectral fragmentation pattern for the TMS isomers **2** (Figure 1). The values below the structures represent the nominal masses for the TMS (or DTMS in parentheses) derivatives.

sinapyl or *p*-hydroxycinnamyl alcohol, the precursor of S or H lignin units, respectively. The first isomer of the H doublet was eluted as a leading shoulder of FA–FA peak 10. These S and H TMS compounds showed fragmentation patterns similar to those of their G analogues **2**. However, the S isomers gave only [M^+ –

CH_3], at *m/z* 693, and not M^+ (Table 1). These S and H isomers were present as trace components in the wheat sample. Interestingly, we could not observe analogous dimers associating *p*-coumaric acid and G, S, or H units in these grass samples.

It is surprising that these ferulic units ether-linked at Cβ of cinnamyl alcohols have not been identified previously. To the best of our knowledge, the only ferulic acid–coniferyl alcohol adducts released by saponification of grass walls were tentatively identified as photochemically derived cyclodimers (Ford and Hartley, 1990b). It is, however, noteworthy that new ferulic acid dehydrodimers have been similarly evidenced from saponified grass walls in very recent studies (Ralph et al., 1994). A complete description of the various substitution patterns of ferulic acid in the grass cell walls might thus be hampered by analytical difficulties met in their GC/MS evaluation, which further emphasizes the need for sound analytical methodology (Ralph et al., 1994).

Conclusion. In this study, we isolated and authenticated new dimers formed by radical coupling of ferulic acid and coniferyl alcohol. In past research, the ferulate esters associated with grass arabinoxylans have been speculated to be ether-linked either to the α position of lignin side chains, via opportunistic quinone methide trapping, or to their β position, via radical coupling (Ralph et al., 1992; Lam et al., 1994). The latter mechanism has been definitely established herein, while the former is still speculative. From a biosynthetic point of view, this means that ferulate esters are oxidatively copolymerized with lignin precursors. Such a radical coupling should occur to an appreciable extent, as the new G dimers described herein were recovered in amounts that seem to approximate those of the cyclobutane dimers, on the basis of the peak relative areas (Figures 2 and 3). The recovery of these G dimers, and of their H and S analogues, by saponification of grass walls, further confirms that ferulic esters provide points of growth for the polymer lignin, via ether (and probably other) linkages which anchor lignins to cell wall polysaccharides. Quantitative GC/MS or HPLC determination of these new ferulic acid–coniferyl alcohol dimers, and of their S and H analogues, from grass straws or from other grass samples, will be attempted in the near future to further delineate the role of ferulic acid in the architecture of grass cell walls.

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