Sinapate Dehydrodimers and Sinapate–Ferulate Heterodimers in Cereal Dietary Fiber

MIRKO BUNZEL,*,† JOHN RALPH,‡,§ HOON KIM,‡,§ FACHUANG LU,‡,§
SALLY A. RALPH,# JANE M. MARITA,‡,§ RONALD D. HATFIELD,‡ AND
HANS STEINHART†

Institute of Biochemistry and Food Chemistry, Department of Food Chemistry,
University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany; U.S. Dairy Forage Research
Center, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin 53706;
Department of Forestry, University of Wisconsin–Madison, Madison, Wisconsin 53706; and
U.S. Forest Products Laboratory, Forest Service, U.S. Department of Agriculture,
Madison, Wisconsin 53705

Two 8–8-coupled sinapic acid dehydrodimers and at least three sinapate–ferulate heterodimers have been identified as saponification products from different insoluble and soluble cereal grain dietary fibers. The two 8–8-disinapates were authenticated by comparison of their GC retention times and mass spectra with authentic dehydrodimers synthesized from methyl or ethyl sinapate using two different single-electron metal oxidant systems. The highest amounts (481 μg/g) were found in wild rice insoluble dietary fiber. Model reactions showed that it is unlikely that the dehydrodisinapates detected are artifacts formed from free sinapic acid during the saponification procedure. The dehydrodisinapates presumably derive from radical coupling of sinapate–polymer esters in the cell wall; the radical coupling origin is further confirmed by finding 8–8 and 8–5 (and possibly 8–*O*–4) sinapate–ferulate cross-products. Sinapates therefore appear to have an analogous role to ferulates in cross-linking polysaccharides in cereal grains and presumably grass cell walls in general.

INTRODUCTION

Grasses have substantial amounts of hydroxycinnamic acids intimately associated with the cell wall, as has been detailed in a number of reviews (*1–4*). Ferulate, in particular, has a significant role in cross-linking cell-wall polymers (*5–7*). Polysaccharide–polysaccharide cross-linking is effected by ferulate dimerization by either photochemical (*8*) or, more importantly, radical coupling reactions of ferulate–polysaccharide esters (*5*, *7*). The full range of ferulate cross-coupling products, not just the 5–5-coupled product, can now routinely be found in a variety of samples (*5, 7, 9–13*). Formation of dehydrodiferulates in the plant cell wall is thought to terminate the expansion of cell growth in grasses (*3*). Furthermore, dehydrodiferulates may play an important role in modifying the mechanical properties of cell walls (*9*) as well as in limiting polysaccharide degradation by exogenous enzymes (*14*) by acting as cross-links between polysaccharides. Dietary fiber cross-linking by dehydrodiferulates may affect the physicochemical behavior of the fraction and possibly also influence its physiological effects (*12*).

Although sinapic acid **1S** (X = H; **S**, sinapate moiety) (**Figure 1**) has been identified in plant extracts and can be released in small quantities from grass cell walls by base (*15–18*), it has not been determined if it acylates polysaccharides or other components. In 1969, the isolation and identification of thomasidioic acid (**5C3SS**; **SS**, disinapate compound, **Figure 1**) from aqueous extracts of the heartwood of elm (*Ulmus thomasii* Sarg.) were reported (*19*). Thomasidioic acid was described as a phenolic lignan but is an analogue of the cyclic form of the 8–8-coupled dehydrodiferulic acid **5C3FF** (**FF**, diferulate compound), which was more recently discovered as a plant component (*5*). In a study on the constituents of canola meal (*20, 21*) it was discovered that thomasidioic acid was formed when sinapic acid was air oxidized in dilute alkaline solutions. Due to this observation and the fact that isolated thomasidioic acid is optically inactive, Charlton and Lee (*22*) doubted that thomasidioic acid is a natural product. They suggested that thomasidioic acid was formed during the extraction procedure of the elm heartwood.

Here we describe the identification of alkali-releasable (ester-bound) thomasidioic acid in different insoluble (IDF) and soluble

* Author to whom correspondence should be addressed [telephone +49-(0)40-42838-4379; fax +49-(0)40-42838-4342; e-mail Bunzel@lc.chemie.uni-hamburg.de].
† University of Hamburg.
‡ U.S. Dairy Forage Research Center.
§ University of Wisconsin–Madison.
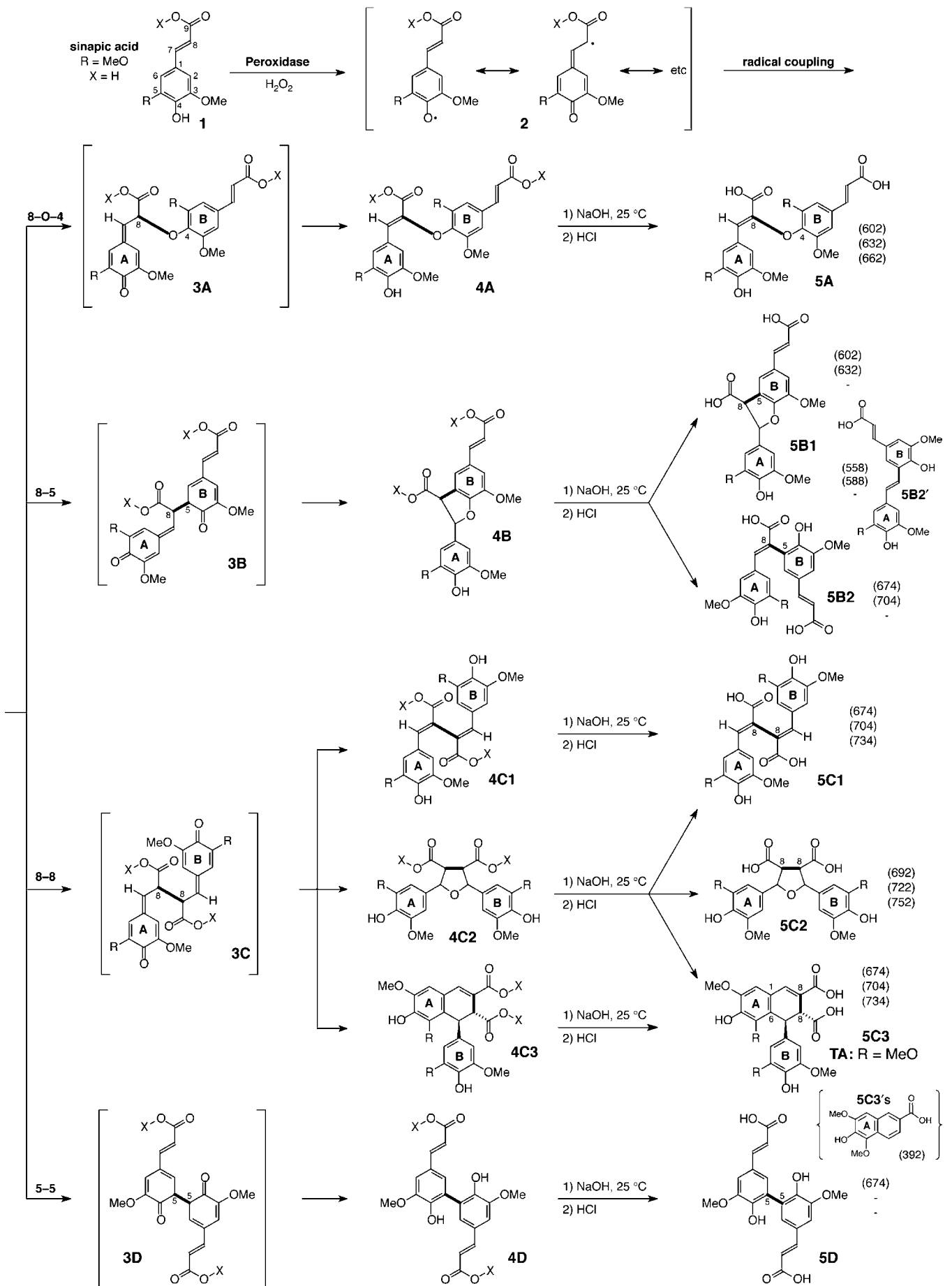# U.S. Forest Products Laboratory.

Figure 1. Radical dehydrodimerization of (cell-wall-bound) hydroxycinnamates or hetero-dehydrodimerization of sinapate and ferulate produces 8–*O*–4-, 8–5-, 8–8-, and 5–5-dehydrodimers via intermediates. Sinapic acid dehydrodimers **5C1SS** and **5C3SS** are released (following saponification) from several cereal grain dietary fibers. The actual form of the 8–8 products in the wall is unknown. With its extra 5-OMe, sinapate cannot make 5–5- or 8–5-dehydrodimers. The numbering scheme uses the convention relating to the monomer numbering (and not the IUPAC name, which is given under Materials and Methods for new compounds). The A-ring of **5C3SS** is numbered with the condensation between carbons A6 and B7 to be consistent with 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., **5B1SF** is from sinapate linked at its 8-position to ferulate at its 5-position. Columnar numbers in parentheses are nominal molecular masses (from GC-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.

dietary fibers (SDF) from cereal grains, as preliminarily disclosed by Ralph et al. (*23*). From different extraction studies, examination of the reactivity of model systems, the identification of a new dehydrodisinapic acid (the open form 8−8-coupled dehydrodisinapic acid isomer **5C1SS**), and the finding of sinapate−ferulate heterodimers, we suggest that dehydrodisinapic acids are natural, wall-bound, radical dimerization products and that sinapate functions analogously to ferulate in cell-wall cross-linking reactions.

MATERIALS AND METHODS

General. All samples were analyzed in triplicate. Heat-stable α-amylase Termamyl 120L (EC 3.2.1.1, from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase 2.4 L (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were from Novo Nordisk, Bagsvaerd, Denmark. Cellulose (SigmaCell, noncrystalline, highly purified) was obtained from Sigma, St. Louis, MO. NMR spectra of synthesized samples were run at 300 K on a Bruker AMX-360 instrument (Bruker, Rheinstetten, Germany). Samples were dissolved in [²H₆]acetone. Chemical shifts (δ) were referenced to the central solvent signals (¹H, δ 2.04; ¹³C, δ 29.8). *J* values are given in hertz. NMR assignments follow the numbering shown in **Figure 1**.

Plant Material. Whole grains of corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), spelt (*Triticum spelta* L.), rice (*Oryza sativa* L.), wild rice (*Zizania aquatica* L.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), oat (*Avena sativa* L.), and millet (*Panicum miliaceum* L.) were obtained from a local German supplier.

Preparation of Dietary Fiber. Preparation of cereal IDF and SDF was performed according to a preparative isolation procedure described previously (*12*). Briefly, milled sample material (10 g) was suspended in phosphate buffer (pH 6.0, 0.08 M, 300 mL), and α-amylase (750 μL) was added. Beakers were heated in a boiling water bath for 20 min. The pH was adjusted to 7.5, and samples were incubated with protease (300 μL) at 60 °C for 30 min. After the pH had been adjusted to 4.5, amyloglucosidase (350 μL) was added and the mixture was incubated at 60 °C for 30 min. The suspension was centrifuged, and the supernatant was kept for isolation of SDF. The residue was washed twice each with hot water (70 °C), 95% (v/v) ethanol, and acetone and finally dried in a vacuum oven to give IDF. Water washings were combined with the first supernatant for preparation of SDF. SDF was precipitated in 80% ethanol. After centrifugation, the residue was washed twice each with 78% (v/v) ethanol, 95% (v/v) ethanol, and acetone and was dried in a vacuum oven. For calculations, IDF and SDF were corrected for residual protein and ash contents.

Additional Extraction Step of Some Wild Rice Insoluble Dietary Fiber. To ensure that dietary fibers are free of unbound sinapic acid and thomasidioic acid, wild rice IDF was exhaustively extracted with ethanol for 6 h and with acetone for 8 h using Soxhlet equipment. The combined extracts were evaporated, dissolved in dioxane, and divided into two parts. After evaporation and addition of internal standards, one part was directly trimethylsilylated as described below. The other part was saponified (2 M NaOH) under N₂ and protected from light for 4 h at room temperature, silylated, and analyzed by GC-MS and GC-FID for sinapic acid and thomasidioic acid. The extraction residue was dried at 55 °C for 5 h, weighed, and saponified as follows.

Saponification To Release Hydroxycinnamic Acids. Internal standards (IS) (*E,E*)-4-hydroxy-4′,5,5′-trimethoxy-3,3′-bicinnamic acid (5−5-Me-diferulic acid, the monomethyl ether of **5DFF**) (*12*) and *o*-coumaric acid dissolved in dioxane were added, and saponification with NaOH (2 M, 5 mL) was carried out protected from light for 18 h at room temperature. To avoid oxidative processes the NaOH solution was degassed by bubbling N₂ through for 30 min, and air in the headspace of the screw-cap tubes was exchanged with N₂. Samples were acidified with 0.95 mL of concentrated HCl (resulting pH <2) and extracted into diethyl ether (4 mL, three times). Extracts were combined, evaporated under a stream of filtered air, silylated, and analyzed by GC-MS and GC-FID.

Model Systems for the Saponification Process. To study sinapic acid dimerization during saponification, sinapic acid (200 μg) as well as ethyl sinapate **1S** (X = Et) (200 μg) were treated with 2 M NaOH under different conditions. Cellulose (50 mg) was used to mimic the polysaccharide matrix. 5−5-Me-diferulic acid and *o*-coumaric acid were added as internal standards. Two different saponification conditions were used: (1) nitrogen was bubbled through the NaOH solution for 30 min, and air in the headspace of the screw-cap tubes was exchanged by N₂ (conditions are identical with those used for saponification of dietary fiber samples); (2) air was bubbled through the NaOH solution for 30 min, and air in the headspace was not exchanged (to simulate conditions with the highest chance of artifactual sinapate to dehydrodisinapate conversion). Saponification was carried out protected from light for 18 h at room temperature. Saponification products were extracted as described above.

GC-MS and GC-FID. Dried extracts as well as synthesized dehydrodisinapic acids were trimethylsilylated by adding 10 μL of pyridine and 40 μL of BSTFA and heating for 30 min at 60 °C in sealed vials. Trimethylsilylated derivatives of phenolic acids were separated by GC [Thermoquest (Austin, TX) Trace 2000 GC] using a 25 m × 0.2 mm i.d., 0.33 μm film, DB-1 capillary column (J&W Scientific, Folsom, CA) and identified by their electron impact mass data collected on a Thermoquest QSC ion-trap MS. He (0.54 mL/min) was used as carrier gas. GC conditions were as follows: initial column temperature, 220 °C, held for 1 min, ramped at 4 °C/min to 248 °C, ramped at 30 °C/min to 300 °C, held for 40 min; injector temperature, 300 °C; split, 1/50 or 1/12 (determination of IDF or SDF, respectively). Quantitative determinations were carried out by GC (Hewlett-Packard 5980, Palo Alto, CA) using the same column and GC conditions and a flame ionization detector (detector temperature, 300 °C). He (0.4 mL/min) was used as carrier gas. For quantitative determinations of sinapate dimers 5−5-Me-DFS was used as internal standard. Response factors for sinapate dimers against 5−5-Me-DFS are 0.826 (compound **5C1SS**, see below) and 0.482 (compound **5C3SS**), respectively.

Synthesis of Sinapate Dehydrodimers. *Preparation of Compound 5C1SS [Noncyclic Form of 8−8-Coupled Dehydrodisinapic Acid−2,3-Bis(4-hydroxy-3,5-dimethoxybenzylidene)succinic Acid] (Figure 1).* To sinapic acid (5 g) in absolute ethanol (100 mL) was slowly added acetyl chloride (5 mL). The mixture was stirred for 16 h. TLC showed that the esterification was not complete, so further aliquots of ethanol and acetyl chloride were added and the mixture was stirred for another 16 h. The solution was concentrated on a rotary evaporator, and HCl (and other solvents) was removed by coevaporation several times with ethanol. The product, ethyl sinapate, was crystallized from MeOH. Ethyl sinapate **1S** (X = Et) (200 mg) was dissolved in pyridine (5 mL), and Mn(OAc)₃·2H₂O (255 mg) in pyridine (5 mL) was added over a few

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minutes. The reaction mixture was stirred for a further 5 min. Na_2SO_3 (solid, 120 mg) was added, and stirring of the mixture was continued for 10 min. To acetylate the phenols acetic anhydride (10 mL) was added. The mixture was stirred for 2 h. After evaporation of solvents on a rotary evaporator, the mixture was partitioned between CH_2Cl_2 and saturated aqueous NaCl. The organic layer was washed consecutively with 2 M HCl, 0.4 M $NaHCO_3$, and saturated aqueous NaCl, then dried over $MgSO_4$ and evaporated to dryness to provide 198 mg of product (yield 85%), which was crystallized from acetone/petroleum ether as red needles: mp 104 °C. Diacetate of compound **4C1SS** [X = Et, diethyl 2,3-bis(4-acetoxy-3,5-dimethoxybenzylidene)succinate]: ^1H NMR δ 1.11 (6H, t, $J = 7.1$, CH_3–CH$_2$–O), 2.20 (6H, s, OCOCH_3), 3.73 (12H, s, OMe), 4.14 (4H, q, $J = 7.1$, CH$_3$–CH_2–O), 6.90 (4H, s, H-2/6), 7.85 (2H, s, H-7); ^{13}C NMR δ 14.4 (*C*H$_3$–CH$_2$–O), 20.2 (OCO*C*H$_3$), 56.4 (OMe), 61.7 (CH$_3$-*C*H$_2$–O), 107.3 (C-2/6), 129.1 (C-8), 130.8 (C-4), 133.9 (C-1), 142.5 (C-7), 153.2 (C-3/5), 167.0 (C-9), 168.3 (O*C*OCH$_3$).

The diacetate of **4C1SS** (X = Et) (80 mg) was dissolved in dioxane (5 mL), and degassed 2 M NaOH (20 mL) and additional dioxane (5 mL) were added. Deacetylation was carried out at room temperature for 16 h. The solution was acidified (pH 2) with 2 M HCl and partitioned between EtOAc and saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and evaporated. After degassed 2 M NaOH (20 mL) had been added, ester hydrolysis was carried out for 24 h at room temperature in the dark. This two-step saponification was necessary because the acetylated derivative of diethyl ester **4C1SS** (X = Et) was not completely dissolved in NaOH without dioxane and dissolved in dioxane/NaOH, but these conditions removed only the acetate groups. Following the saponification process, the solution was acidified with 2 M HCl and extracted into EtOAc. The organic layer was dried over $MgSO_4$, evaporated, and submitted to preparative silica-TLC in EtOAc/CHCl$_3$/formic acid (1:1:0.1) to afford compound **5C1SS** as a red solid (19 mg, yield 30%). The residual material still contained the ester, which was therefore surprisingly difficult to saponify. Compound **5C1SS**: ^1H NMR δ 3.75 (12H, s, OMe), 7.01 (4H, s, H-2/6), 7.85 (2H, s, H-7); ^{13}C NMR δ 56.5 (OMe), 108.8 (C-2/6), 126.4 (C-8), 126.6 (C-1), 138.8 (C-4), 142.6 (C-7), 148.5 (C-3/5), 168.6 (C-9); high-resolution MS, M$^+$ not sufficiently strong, seen in low-resolution spectrum; M$^+$ – H_2O 428.1103, $C_{22}H_{20}O_9$ requires M 428.1107; M$^+$ – H_2O – CO 400.1143, $C_{21}H_{20}O_8$ requires M 400.1158.

Preparation of **5C3SS** *[Cyclic Form of 8–8-Coupled Dehydrodisinapic Acid, Thomasidioic Acid, trans-7-Hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-6,8-dimethoxy-1,2-dihydronaphthalene-2,3-dicarboxylic Acid]* (**Figure 1**). Ahmed et al. (*24*) described the synthesis of thomasidioic acid in two steps starting from sinapic acid. Oxidation of methyl sinapate **1S** (X = Me) with ferric chloride in aqueous acetone gave the dimethyl ester of thomasidioic acid (**4C3SS**, X = Me) as a byproduct (*25*), whereas oxidative coupling of methyl sinapate in aqueous acetone at pH 4 with H_2O_2 and horseradish peroxidase gave the dimethyl ester of thomasidioic acid in 41% yield (*26*). Changing the oxidation conditions of the methyl sinapate reaction with ferric chloride gave the dimethyl ester of thomasidioic acid in good yields. To sinapic acid (5 g) in methanol (150 mL) was slowly added acetyl chloride (15 mL) over 15 min, and the mixture was stirred for 16 h, producing methyl sinapate as determined by TLC. Water (30 mL) and FeCl$_3$·6H$_2$O (45 g) were added, and the mixture was stirred continuously for another 48 h, by which time the methyl sinapate had been consumed, as indicated by TLC. After methanol was removed by evaporation under reduced pressure, the mixture was poured into EtOAc and washed with 3% HCl and saturated aqueous NH$_4$Cl. The EtOAc fraction was dried over $MgSO_4$ and evaporated. Crude products were applied to flash chromatography on silica gel in CHCl$_3$/EtOAc (5:1). The dimethyl ester, compound **4C3SS** (X = Me), was obtained in 52% yield along with ~5% of a minor isomer, which had a *cis* configuration between carbons B7 and B8. Compound **4C3SS** (X = Me, thomasidioic acid dimethyl ester): ^1H NMR δ 3.60 (3H, s, B-COOCH$_3$), 3.62 (3H, s, A5-OMe), 3.68 (6H, s, B3/5-OMe), 3.68 (3H, s, A-COOCH$_3$), 3.88 (3H, s, A3-OMe), 4.02 (1H, s, B H-8), 5.00 (1H, s, B H-7), 6.36 (2H, s, B H-2/6), 6.96 (1H, s, A H-2), 7.66 (1H, s, A H-7); ^{13}C NMR δ 40.3 (B C-7), 47.5 (B C-8), 51.9 (A-COO*C*H$_3$), 52.4 (B-COO*C*H$_3$), 56.58 (A3-OMe), 56.64 (B3/5-OMe), 60.4 (A5-OMe), 106.0 (B C-2/6), 109.0 (A C-2),

123.3 (A C-1), 123.9 (A C-8), 124.6 (A C-6), 134.2 (B C-1), 135.8 (B C-4), 138.4 (A C-7), 142.8 (A C-4), 146.4 (A C-5), 148.49 (B C-3/5), 148.59 (A C-3), 167.5 (A C-9), 172.8 (B C-9).

Compound **4C3SS** (X = Me) (100 mg) was dissolved in 2 M NaOH (20 mL) and hydrolyzed at room temperature for ~16 h. The solution was acidified with 2 M HCl to pH 2 and extracted into EtOAc. The organic layer was dried over $MgSO_4$, evaporated, and submitted to preparative silica TLC in EtOAc/CHCl$_3$/formic acid (2:1:0.1). EtOAc was used to extract products from the scraped-off TLC silica. The slower moving material was identified as compound **5C3SS** (56 mg, yield 60%). Compound **5C3SS** was crystallized from acetone/petroleum ether as gray granules, mp 230 °C. Compound **5C3SS**: ^1H NMR δ 3.40 (1H, d, $J = 1.3$, B H-8), 3.62 (3H, s, A5-OMe), 3.69 (6H, s, B3/5-OMe), 3.88 (3H, s, A3-OMe), 5.05 (1H, bs, B H-7), 6.40 (2H, s, B H-2/6), 6.95 (1H, s, A H-2), 7.68 (1H, s, A H-7); ^{13}C NMR δ 40.3 (B C-7), 47.3 (B C-8), 56.58 (A3-OMe), 56.64 (B3/5-OMe), 60.4 (A5-OMe), 106.1 (B C-2/6), 108.9 (A C-2), 124.07 (A C-1), 124.11 (A C-8), 125.0 (A C-6), 134.6 (B C-1), 135.7 (B C-4), 138.3 (A C-7), 142.7 (A C-4), 146.4 (A C-5), 148.45 (B C-3/5), 148.52 (A C-3), 168.6 (A C-9), 173.4 (B C-9); high-resolution MS, found M$^+$ 446.1219, $C_{22}H_{22}O_{10}$ requires M 446.1213.

The faster moving fraction (14 mg, yield 27%) was identified by NMR and GC-MS as compound **5C3'S** (6-hydroxy-5,7-dimethoxy-naphthalene-2-carboxylic acid). Compound **5C3'S**: ^1H NMR δ 3.99 (3H, s, A5-OMe), 4.01 (3H, s, A3-OMe), 7.30 (1H, s, A H-2), 7.91 (1H, dd, $J = 8.7$, 1.6, B H-8), 8.00 (1H, d, $J = 8.7$, B H-7), 8.48 (1H, d, $J = 1.6$, A H-7); ^{13}C NMR δ 56.4 (A3-OMe), 60.9 (A5-OMe), 104.1 (A C-2), 121.6 (B C-7), 124.2 (B C-8), 126.8 (A C-4), 127.8 (A C-6), 128.2 (A C-8), 130.6 (A C-7), 141.05 (A C-5), 141.11 (A C-1), 150.8 (A C-3), 168.1 (A C-9); high-resolution MS, found M$^+$ 248.0692, $C_{13}H_{12}O_5$ requires M 248.0685. Methods for the synthesis of compound **5C3'S** described in the literature (*27, 28*) are time-consuming, requiring five or six steps. To produce small amounts, the procedure described here seems to be more convenient.

RESULTS AND DISCUSSION

Possible Pathways for Dimerization of Sinapate Esters. Along with ferulic acid, the main hydroxycinnamic acid in the plant cell wall, alkali-extractable *p*-coumaric acid and sinapic acid [**1S** (X = H), **Figure 1**] have been identified from grasses (*1–4, 18*). *p*-Coumaric acid may cross-link cell-wall polymers by [2+2]-cyclodimerization; radical coupling products have not been identified (*29*). Radical dehydrodimerization of sinapate esters **1S** can produce only two primary products, 8–*O*–4- and 8–8-dehydrodimers (**Figure 1**). Ferulates additionally produce 8–5-, 5–5-, and 4–*O*–5-coupled products (*5*). Following the radical coupling step, which produces intermediates **3** (quinone methides for the 8-coupled dimers **3A**–**3C**), re-aromatization is achieved during subsequent reactions. For the 8–*O*–4-coupled intermediate **3A** re-aromatization is via elimination of the acidic 8-proton (*5*). 8–5-Coupling leads to the formation of phenylcoumaran structures **4B**; with no 5-position available for coupling, there is obviously no disinapate 8–5-dehydrodimer, although sinapate–ferulate cross-coupling can give 8–5-coupled products. Saponification of the phenylcoumaran ester **4B** gives variable partitioning between the phenylcoumaran acid **5B1** and the eliminated open form **5B2**. As with ferulate dimerization, the pathway from the 8–8-coupled product is less clear. Elimination of both 8-protons from quinone methide **3C** can occur to produce the conjugated noncyclic product **4C1**. However, this cannot be the only path occurring in vivo; such a product cannot give rise to the observed cyclic 8–8-product **5C3** or the more recently discovered diferulate tetrahydrofuran **5C2FF** involving water addition to the quinone methide (*23*). The finding of the cyclic product **5C3SS**, complementing its ferulate analogue **5C3FF**, implies that either **4C2** or **4C3** (or both, or possibly an alternate intermediate) are the in vivo products. As is the case with ferulates, the nature of the 8–8-
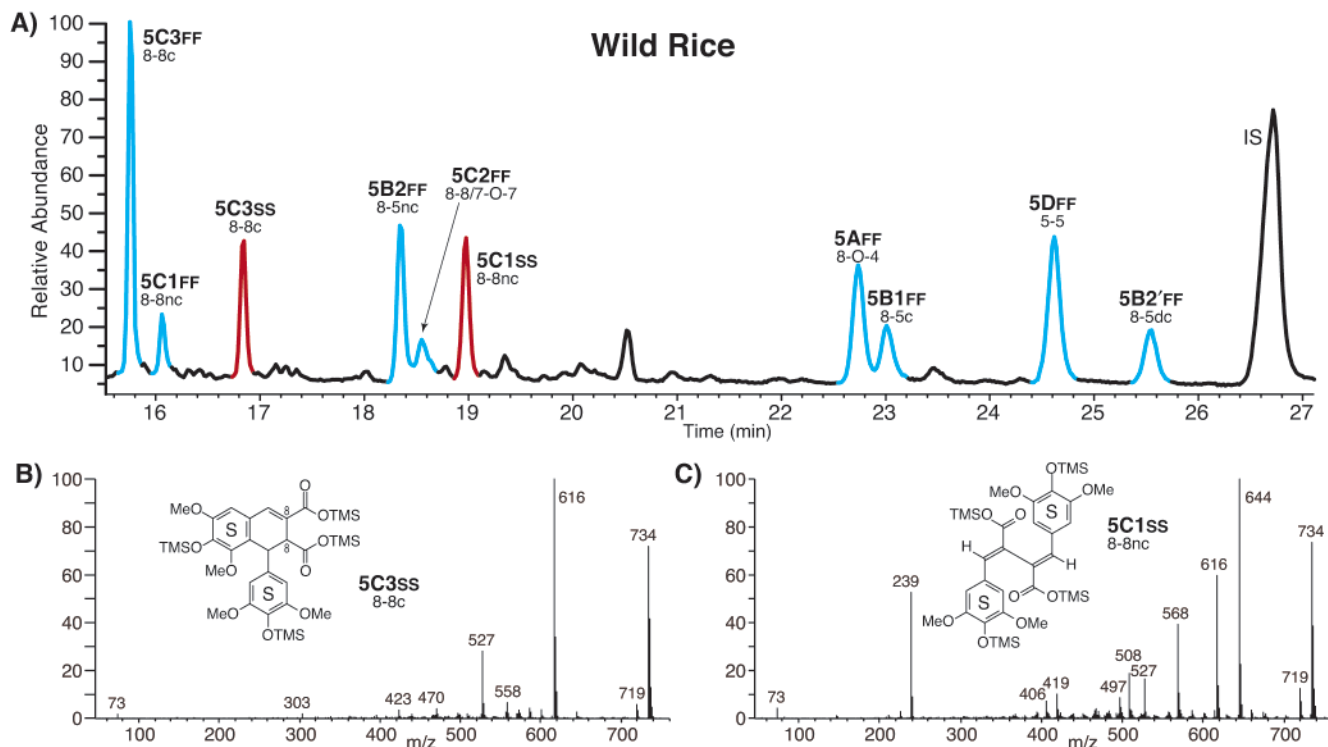
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Figure 2. (A) GC-MS total-ion chromatogram of saponified extracts of wild rice IDF showing ferulate dehydrodimers (cyan) and the two new sinapate dehydrodimers **5C1SS** and **5C3SS** (red), as well as (B, C) their mass spectra. Structures are from **Figure 1**. c, cyclic form; nc, noncyclic (open) form; dc, decarboxylated form.

Table 1. Amounts of 8–8-Coupled Sinapic Acid Dehydrodimers (**5C3SS** and **5C1SS**) with Corresponding Standard Deviations (SD) (*n* = 3) Released by Saponification from Soluble and Insoluble Cereal Grain Dietary Fiber Fractions

	μg/g of IDF				μg/g of SDF			
	5C3SS	SD	5C1SS	SD	5C3SS	SD	5C1SS	SD
	Insoluble Dietary Fiber				Soluble Dietary Fiber			
wheat	33	4	+[a]		8	1	4	1
spelt	16	1	+		15	6	tr[b]	
rye	tr		nd[c]		3	1	tr	
barley	tr		nd		tr		nd	
oat	nd		nd		nd		nd	
corn	tr		nd		nd		nd	
millet	nd		nd		nd		nd	
rice	44	10	nd		tr		nd	
wild rice	126	16	355	49	3	1	2	1

[a] +, compound identified but not possible to quantify due to peak impurities. [b] tr, traces, compounds identified by GC-MS, but levels below their determination limit by GC-FID of ~2 μg/g. [c] nd, not detected.

coupled product in vivo remains to be elucidated. Finally, 5–5-coupling is only possible between ferulates to yield, after saponification, **5DFF**.

Identification and Quantification of Dehydrodisinapates in Cereal Grain Dietary Fibers. As seen in **Figure 2**, two 8–8-dehydrodimers, compounds **5C1SS** (the noncyclic isomer) and **5C3SS** (thomasidioic acid, the cyclic isomer) of 8–8-coupled dehydrodisinapic acid, result following saponification of wild rice IDF. The dehydrodisinapates were authenticated by comparison of their mass spectra (**Figure 2**) and their relative GC retention times with those of the synthesized compounds. The 8–8-coupled dehydrodisinapates are analogues of the corresponding ferulate dimers (*5*). They were also detected in other cereal grains (**Table 1**) but not in IDF from oats or millet

or from SDF from oats, corn, or millet (**Table 1**). Furthermore, compound **5C3′S**, which arises in synthesis during the saponification step of the dimethyl ester **4C3SS** (X = Me), was generally not detected. The compound **5C2SS**, an analogue of the recently identified diferulate tetrahydrofuran compound **5C2FF**, was not detected. Quantification (**Table 1**) was carried out using internal standards. Although identified unequivocally, quantification of compound **5C1SS** was not carried out in IDF of wheat and spelt due to some peak impurities recognized by GC-MS. The highest amounts of dehydrodisinapates were detected in wild rice IDF, with 355 μg/g IDF of **5C1SS** and 126 μg/g IDF of **5C3SS**. Interestingly, wild rice IDF is the only sample from which higher amounts of **5C1SS** than of **5C3SS** were detected. Although the saponification procedure was carried out identically, other dietary fibers showed higher amounts of **5C3SS** compared to **5C1SS**. As shown in **Table 1**, amounts of dehydrodisinapic acids in some cereal dietary fibers are substantial, but their amounts are rather low in comparison to dehydrodiferulates (*12*). The sum of dehydrodisinapic acids, where quantification of at least one compound was possible, in cereal IDF (wheat, spelt, and rice) is ~1% of the total dehydrodiferulates level. Wild rice IDF differs significantly from other cereal IDFs investigated: dehydrodisinapates amount to 17% of the dehydrodiferulates level. In wild rice IDF the disinapate/sinapate ratio (~40%) is slightly lower than the diferulate/ferulate ratio at ~60%. In SDF of wheat, spelt, and rye the relative amounts of dehydrodisinapates are a little higher than in the corresponding IDF at roughly 4–7% of the dehydrodiferulates level. Contrary to the dehydrodisinapates, the dehydrodiferulate level in wild rice SDF was below the determination limit (*12*).

GC-MS chromatograms were screened for 8–*O*–4-coupled dehydrodisinapates, **5ASS**, by looking for typical masses expected from its structure by analogy with the dehydrodiferulic
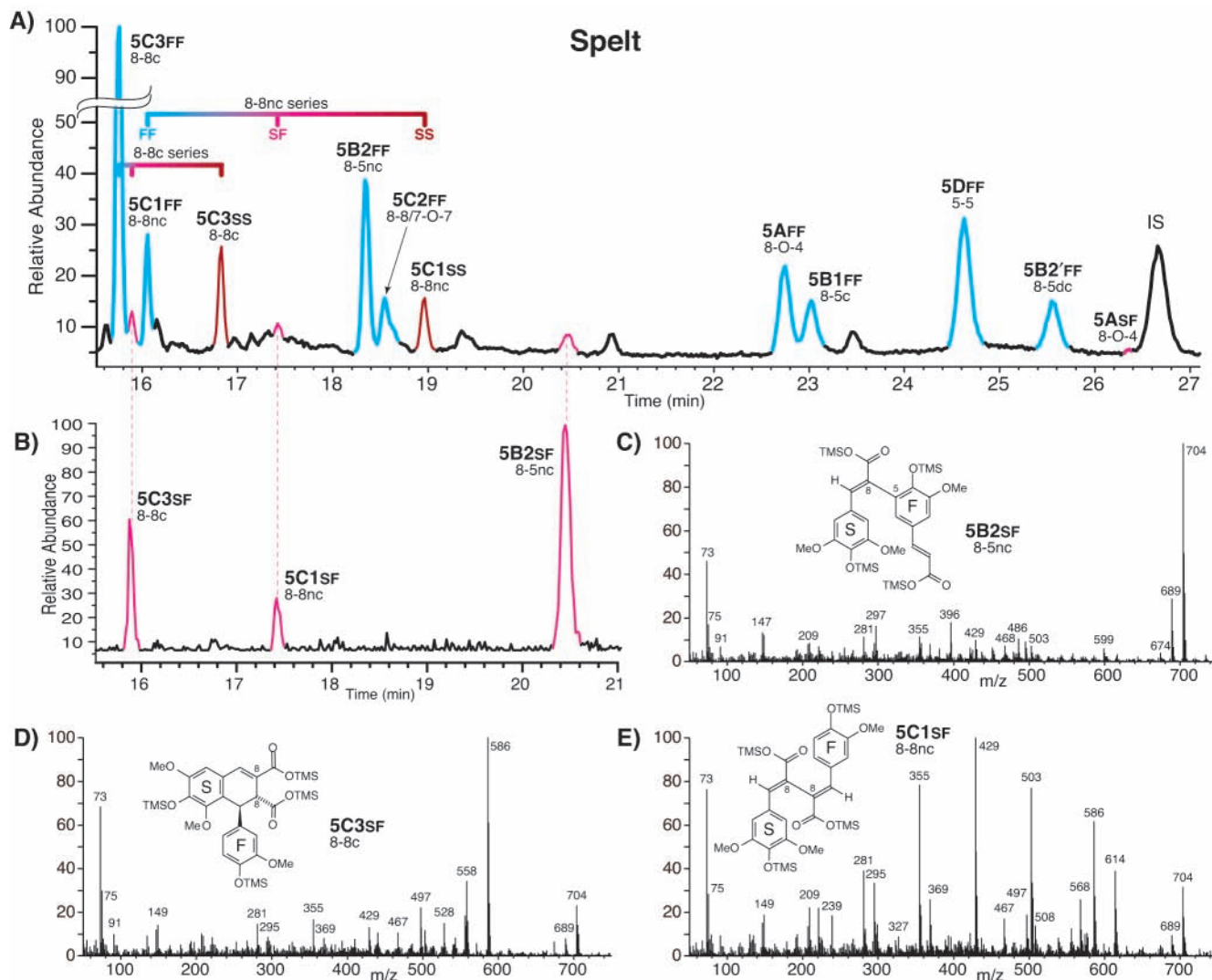
Figure 3. (A) GC-MS total-ion chromatogram of saponified extracts of spelt SDF showing ferulate (cyan) and sinapate (red) dehydrodimers as well as the sinapate–ferulate crossed dimers (magenta). (B) Selected-ion chromatogram (*m/z* 704) revealing three of the SF cross-dimers. (C–E) Mass spectra of sinapate–ferulate cross products. Structures are from **Figure 1**. c, cyclic form; nc, noncyclic (open) form; dc, decarboxylated form.

acid analogue (*12*). The 8−*O*−4-dimer was not seen at any significant level. Sinapates are known to predominantly 8−8-coupled (*25*). Analogously, sinapic acid gives high yields of the 8−8-coupled dilactone (*30*), and sinapyl alcohol coupled under peroxidase−H_2O_2 gives some 95% syringaresinol, the 8−8-coupled product, and only ∼5% 8−*O*−4-coupling (*31*).

Dehydrodisinapic Acids—Natural Products or Air Oxidation Artifacts from Sinapic Acid? In 1968−1969 three papers were published about the occurrence of lignans in wood of the elm *U. thomasii* Sarg. (*19*, *32*, *33*). Besides thomasic acid, racemic lyoniresinol and (+)-lyoniresinol-2α-*O*-rhamnoside, thomasidioic acid (**5C3SS**), and 6-hydroxy-5,7-dimethoxynaphthalene-2-carboxylic acid (compound **5C3′S**) were isolated and identified from an aqueous extract of the heartwood. Rubino et al. (*20*, *21*) described the conversion of sinapic acid to thomasidioic acid in the presence of oxygen predominantly under alkaline conditions, but on a smaller scale also under neutral conditions. Charlton and Lee (*22*) also noted the conversion of thomasidioic acid to compound **5C3′S** under strong alkaline conditions (pH 13) in the presence of oxygen, an observation we exploited for synthesizing compound **5C3′S**. The air oxidation process of sinapic acid caused Charlton and Lee (*22*) to

question whether thomasidioic acid and compound **5C3′S** are really natural products.

Sinapic acid is a common constituent of many plants. Dietary fibers investigated are washed with water, ethanol, and acetone after their isolation. One can imagine that thomasidioic acid could be formed during dietary fiber preparation from free sinapic acid and that identified thomasidioic acid is just an extraction residue after washing. This is not the case as we showed by exhaustive extraction of wild rice IDF with ethanol and acetone. Exhaustively extracted wild rice IDF and normally isolated wild rice IDF showed quantitatively comparable amounts of thomasidioic acid **5C3SS** and compound **5C1SS** following saponification. Neither thomasidioic acid nor sinapic acid was detected in the combined ethanol/acetone extracts. Furthermore, model reactions showed that sinapate esters will hydrolyze with only minor levels of contaminating artifacts under the conditions used in this study for the saponification of dietary fibers. As the conversion of free sinapic acid to thomasidioic acid depends on oxygen, two different saponification conditions were tested on both ethyl sinapate and free sinapic acid: (1) nitrogen was bubbled through the NaOH solution for 30 min, and air in the headspace of the screw-cap tubes was exchanged with N_2 ("normal" saponification condi-

tions); (2) air was bubbled through the NaOH solution for 30 min, and air in the headspace was not exchanged ("oxygen" saponification conditions). In accordance with the saponification process of dietary fiber samples, screw-cap tubes were closed and the mixture was neither shaken nor stirred. Under normal saponification conditions 2.8% of initial sinapic acid dimerized to thomasidioic acid and 2.8% of hydrolyzable sinapic acid (from ethyl sinapate) was converted to thomasidioic acid. Oxygen conditions converted 5.1% of sinapic acid to thomasidioic acid and 2.5% of hydrolyzable sinapic acid from ethyl sinapate were dimerized. Under normal conditions compound **5C3′S** was not detected, and oxygen conditions produced only trace amounts of it. These results show that a small percentage of liberated sinapic acid may be converted to thomasidioic acid, but this cannot explain the relatively high amounts of thomasidioic acid, for example, in wild rice IDF (126 μg of thomasidioic acid/g of IDF, 445 μg of sinapic acid/g of IDF).

The identification of the noncyclic isomer **5C1SS** provides a strong indication that the dehydrodisinapates are naturally occurring. Only the condensed structure of thomasidioic acid results from the air oxidation mechanism described by Rubino et al. (*20, 21*) and Charlton and Lee (*22*) and in our own experiments. The release here of **5C1SS** from the fiber samples provides compelling evidence for the presence in the dietary fibers of sinapate esters and their resultant dimers.

Sinapate−Ferulate Crossed Dehydrodimers. The major dimeric components in these cereal dietary fiber fractions, as was described more fully elsewhere (*12*), were the dehydrodimers from ferulates. As shown above, dehydrodimers from sinapates are significant in some cereal grains. More minor were "dimers" resulting from cross-coupling of ferulate and coniferyl alcohol (unpublished results). Small amounts of sinapate−ferulate crossed dehydrodimers could be detected here in some cereal dietary fibers [IDF of wheat, spelt, rye, and wild rice and SDF of wheat, spelt (**Figure 3A**), and rice]. The sinapate−ferulate crossed dehydrodimers were identified from their mass spectra, by their fragmentation similarity with their diferulate, and, when possible, disinapate analogues but have not been unambiguously validated.

The mass spectrum of **5C3SF** (**SF**, mixed sinapate−ferulate compound) (*m/z* 704, **Figure 3D**) is analogous to the spectra of **5C3SS** (*m/z* 734, **Figure 2B**) and the cyclic form of 8−8-coupled dehydrodiferulic acid **5C3FF** (*m/z* 674) (*12*), showing the same diagnostic ions plus/minus *m/z* 30. It is assumed that the "A-ring", **Figure 1**, is syringyl because 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 noncyclic 8−8-coupled crossed dimer **5C1SF** (**Figure 3E**) similarly has characteristics of **5C1SS** (**Figure 2C**) and **5C1FF** (*12*). No analogue of the ferulate tetrahydrofuran product **5C2FF** (i.e., neither **5C2SF** nor **5C2SS**) was detected. A small amount of the 8−*O*−4-cross-product **5ASF** was also detected at 26.4 min (**Figure 3A**) with a mass spectrum (M⁺, *m/z* 632, not shown) analogous to the dehydrodiferulate analogue **5AFF** (*12*).

Cross-coupling of sinapate with ferulate allows an extra possibility not possible with sinapate homo-dehydrodimerization, namely, 8−5-coupling. The tentatively identified 8−5-coupled noncyclic compound **5B2SF** is the major crossed dimer; its mass spectrum (M⁺, *m/z* 704), **Figure 3C**, is dominated by the high-mass peaks just as its ferulate analogue **5B2FF** (*12*). Apparently the saponification conditions are a little severe for the esterified cell-wall product **4B** because the cyclic phenylcoumaran

counterpart **5B1SF** was not detectable. However, the decarboxylated product **5B2′SF** was detectable at 31.1 min, with a mass spectrum (not shown, M⁺, *m/z* 588) analogous to that of the diferulate counterpart (*12*).

The cross-coupling products are considerably more evident in the spelt sample (**Figure 3A**) than in the wild rice (**Figure 2A**), which has a higher proportion of sinapate versus ferulate dehydrodimers. Presumably there are either spatial or temporal mechanisms occurring in the wall, or perhaps in the organelles that form the sinapate esters, that limit cross-coupling reactions in the wild rice. In a purely chemical sense, sinapates may have a higher propensity for homocoupling reactions, as do their alcohol analogues in lignification; homocoupling products are usually the prevalent products in laboratory reactions attempting to produce cross-coupled dehydrodimers.

Sinapates in some cereal grains, like ferulates in all grasses, dimerize via radical coupling reactions to produce sinapate dehydrodimers. Following saponification, two 8−8-dehydrodisinapic acids are released and can be detected by GC-MS in the dimer region where ferulate−ferulate-dehydrodimers and ferulate−monolignol crossed products also elute. The levels found, and the presence of noncyclic 8−8-dehydrodisinapate, provide evidence that dehydrodisinapates are not simply artifacts derived from sinapate during the saponification. Sinapate−ferulate cross-products from 8−8-, 8−5-, and possibly 8−*O*−4-coupling reactions were also observed. The findings suggest that oxidative dehydrodimerization of available polysaccharide hydroxycinnamate esters is a general mechanism for cross-linking plant cell wall polysaccharides.

ABBREVIATIONS USED

IDF, insoluble dietary fiber; SDF, soluble dietary fiber; IS, internal standard; 5−5-Me-diferulic acid, (*E,E*)-4-hydroxy-4′,5,5′-trimethoxy-3,3′-bicinnamic acid; **F**, ferulate moiety; **S**, sinapate moiety; **FF**, diferulate compound; **SF**, mixed sinapate−ferulate compound; **SS**, disinapate compound.

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