# Synthesis and identification of 2,5-bis-(4-hydroxy-3-methoxyphenyl)-tetrahydrofuran-3,4-dicarboxylic acid, an unanticipated ferulate 8–8-coupling product acylating cereal plant cell walls†

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A new product implicated in cereal grain polysaccharide cross-linking has been authenticated by independent synthesis. Saponification of cereal grain fiber releases the *RRRS/SSSR*-isomer of 2,5-bis-(4-hydroxy-3-methoxyphenyl)-tetrahydrofuran-3,4-dicarboxylic acid. The parent ester logically derives from 8–8-coupling of ferulate followed by water addition to one of the incipient quinone methide moieties and internal trapping of the other. The finding adds complexity to the analysis of plant cell wall cross-linking, but provides clues to important polysaccharide cross-linking pathways occurring *in planta.*

## Introduction

Hydroxycinnamates have important roles in plant cell wall cross-linking by linking polysaccharides to each other, to lignin, and to proteins.[1,2] Cross-linking is involved in terminating the expansion of the cell growth in grasses,[3,4] and in cell wall stiffening.[5] Important properties of forages and plant derived foods are attributed to cross-linked polysaccharides in the wall: limited forage degradability by ruminants,[6] thermal stability of cell adhesion and maintenance of crispness of plant-based foods after cooking,[7–9] gelling properties of sugar beet pectins and other food compounds,[10–12] and insolubility of cereal dietary fibers.[13] Hydroxycinnamates and their derivatives are also noted as bioactive compounds with antioxidative and antimutagenic properties.[14–16]

Ferulic acid, to a lesser extent *p*-coumaric acid, and probably sinapic acid acylate cell wall polysaccharides.[17–20] Mechanisms for cross-linking cell wall polymers involve photochemical dimerization,[21,22] or radical dehydrodimerization,[23–25] of these hydroxycinnamates. The dominant mechanism for cross-linking feruloylated polysaccharides is radical coupling, leading to dehydrodiferulates[24] and dehydrotriferulates.[26–29] Radical coupling of ferulates, *via* the action of wall-bound peroxidases, produces several regio-isomeric dehydrodiferulates with coupling occurring at their 4-O-, 5- or 8-carbons. After saponification, the dimeric products consist of 5–5-, 8–8-, 8–5-, 8–O–4- and 4–O–5-coupled dehydrodiferulic acids (DFAs) **5**, Fig. 1. The previously assumed DFAs were synthesized[24] and identified in different samples following alkaline hydrolysis.[5,9,13,24,30,31] Isolation and identification of dehydrodiferuloylated oligosaccharides following hydrolysis of the plant cell walls established the linkage of DFAs to polysaccharides.[32–34]

It can be assumed that the 5–5-DFA **5D** and 8–O–4-DFA **5A**, Fig. 1, moieties found in isolated dehydrodiferuloylated oligosaccharides, are present as structurally analogous esters in the plant, *i.e.* that the di-acid analyzed derives directly from saponification of its ester analog. It is likely that the minor 4–O–5-coupled DFA **5E** is also present as such. Furthermore, it is presumed that the cyclic form of the 8–5-coupled diferulate ester of **5B1** is the natural form of the 8–5-coupled diferulate; the open form **5B2** is produced under the alkaline saponification and GC-derivatization conditions used. The situation is more difficult to ascertain for the 8–8-coupled dehydrodimers (**5C1**) and (**5C3**). To date it remains unresolved whether they are natural or if they are formed during analysis from a common natural precursor. As indicated in earlier studies from our groups,[2,31,35] another 8–8-coupled DFA (8–8-THF–DFA, **5C2**) was preliminarily identified *via* mass spectral data, leading to an even more complex situation.

In this paper we describe the synthesis of one of the six diastereomers of compound **5C2** and its unambiguous identification and quantification in different cereal grains. Its significance to elucidating the nature of 8–8-coupled dehydrodiferulates in the plant is also discussed.

## Materials and methods

### General

Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacillus licheniformis*, 120 KNU $g^{-1}$), the protease Alcalase 2.4 L (EC

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† Electronic supplementary information (ESI) available: synthetic details of the early steps of the synthesis of compound **13** (and ultimately DFA **5C2**), along with the X-ray crystal structure data for compound **4C2-Ac**, are available. See DOI: 10.1039/b605918j

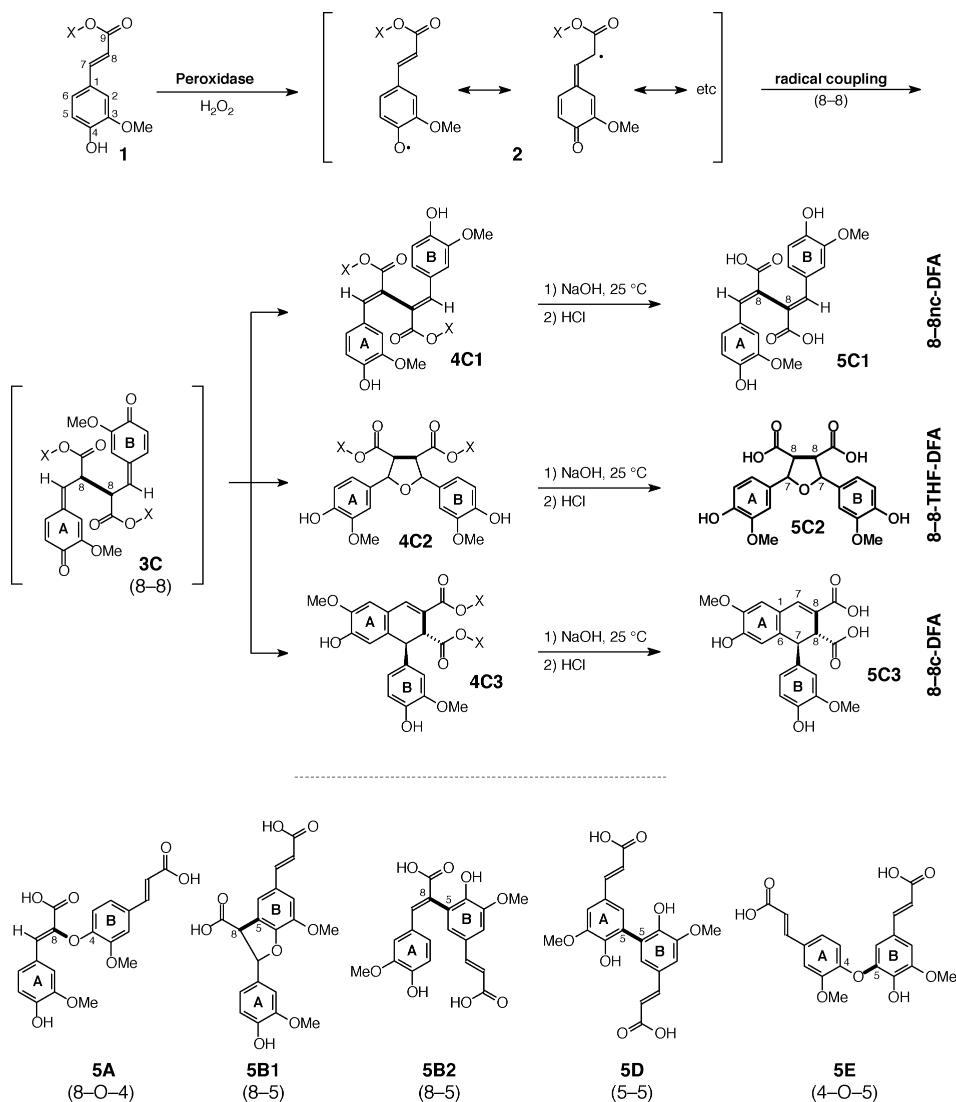


**Fig. 1** Numbering system and radical dehydrodimerization of (cell wall bound) ferulates. The bond formed by radical coupling is in bold. In synthetic compounds X represents the methyl or ethyl ester, in the cell wall X stands for arabinoxylans, arabinans or galactans.

3.4.21.62, from *Bacillus licheniformis*, 2.4 AU $g^{-1}$) and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU $g^{-1}$) were kindly donated by Novo Nordisk, Bagsvaerd, Denmark. NMR spectra were run at 300 K on a 360 MHz Bruker DRX-360 instrument (Bruker, Rheinstetten, Germany) using the standard array of 1D and 2D experiments. Samples were dissolved in acetone-$d_6$. Chemical shifts ($\delta$) were referenced to the central solvent signals ($^1$H, $\delta$ 2.04; $^{13}$C, $\delta$ 29.8). *J*-Values are given in Hz. NMR-assignments follow the numbering shown in Fig. 2. HPLC instrumentation was from Merck/Hitachi (Darmstadt, Germany) (L-6200 Intelligent pump, T-6300 Column thermostat) combined with a Waters 994 programmable photodiode array detector (Eschborn, Germany). HPLC-MS was performed using the HP Series 1100 instrumentation: autosampler G1313A, bin pump G1312A, degasser G1322A, mass spectrometer G1946A (ion-source: atmospheric pressure electro-spray ionization), Hewlett Packard (Waldbronn, Germany). GC-MS data, Fig. 3, was *via* instrumentation from Thermoquest (Austin, TX, USA): Trace 2000 GC, QSC ion-trap MS. GC-FID was performed on a Hewlett-Packard 5980 (Palo Alto, CA, USA) instrument. All fiber samples were analyzed in triplicate. Cellulose and oat spelt xylan used in the saponification of ester model **4C2** were from Sigma-Aldrich.

### Synthesis

Fig. 2 shows the synthetic route to the required isomer of compound **5C2**. All of the steps except the coupling step *vi* involve rather standard synthetic methods. Details of the early steps are given in the ESI.† Details of the crucial coupling reaction, and NMR data of the important products are given here.

**Dimethyl 2,5-bis-(4-benzyloxy-3-methoxyphenyl)-2,3-dihydrofuran-3,4-dicarboxylate 13.** Manganese(III) acetate dihydrate (0.75 g, 2.8 mmol) and 12 mL of acetic acid were placed into a 50 mL round-bottom flask equipped with a reflux condenser. To the stirred mixture was added a solution of methyl 4-benzyloxy-3-methoxybenzoylacetate **12** (0.40 g, 1.3 mmol) and methyl 3-(4′-benzyloxy-3′-methoxyphenyl)propenoate **9** (0.36 g, 1.2 mmol) in

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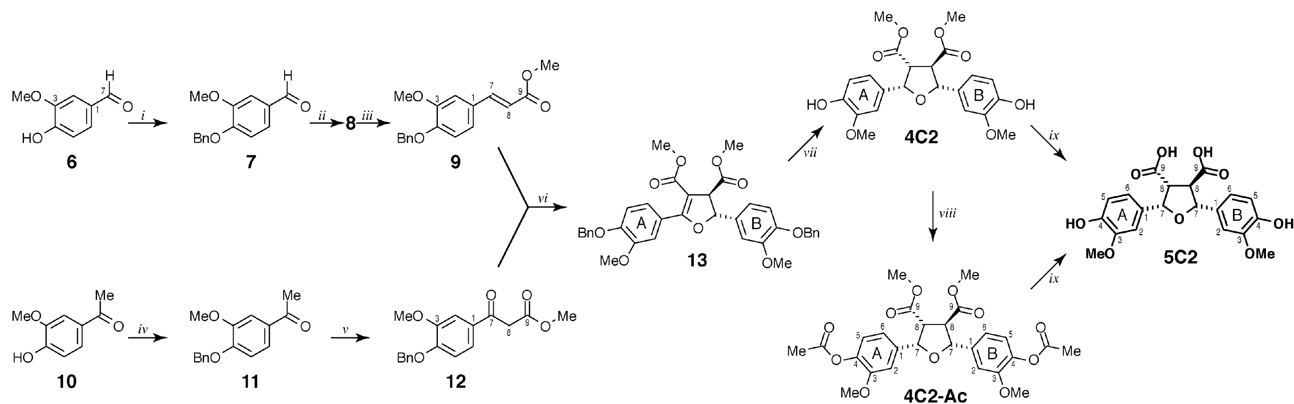


**Fig. 2** Synthetic pathway for **5C2**: *i*: BnCl, KI, $K_2CO_3$, DMF; *ii*: malonic acid, $NH_4OAc$, microwave; *iii*: MeI, $K_2CO_3$, DMF; *iv*: BnCl, KI, $K_2CO_3$, DMF; *v*: $MeO_2CO$, NaH, THF; *vi*: $Mn(OAc)_3$, HOAc; *vii*: Pd/C, $H_2$, EtOH; *viii*: AcCl, pyridine; *ix*: 2 N NaOH.

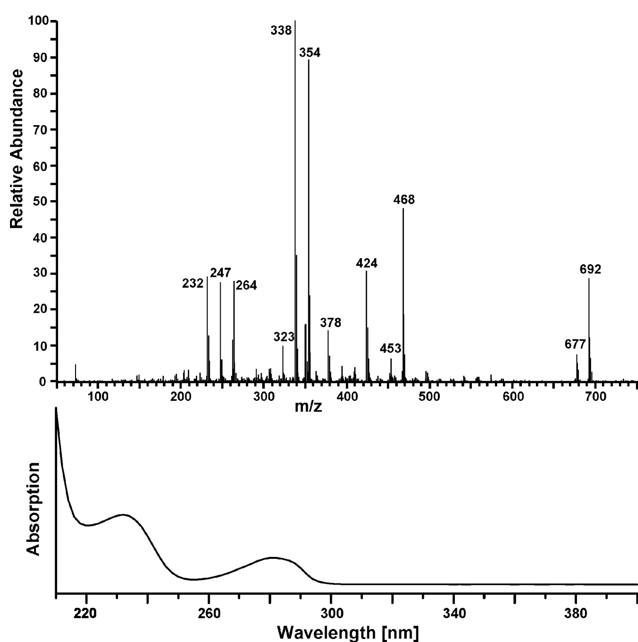


**Fig. 3** EI mass spectrum (top) and UV spectrum (bottom, in MeOH–1 mM aqueous trifluoroacetic acid) of **5C2**.

6 mL of acetic acid. The stirred mixture was heated with a hot water bath (75–80 °C) for 90 min. The mixture was dark brown at the beginning of the heating period, but became clear and light brown at the end of the heating period. The mixture was allowed to cool to room temperature and was then poured into cold water. A sticky solid formed. The mixture was extracted with 50 mL of ether. The organic phase was washed several times with 25 mL portions of sodium bicarbonate solution to remove the acetic acid. Evaporation of the ether yielded 0.65 g of a sticky solid. The residue was dissolved in approximately 2 mL of acetone. On standing overnight crystals formed. The liquid was decanted and the solid was recrystallized from methanol to afford 0.23 g of a white solid (32%), mp. 123–124 °C. $\delta_H$ 3.62 (3H, s, $CO_2CH_3$); 3.74 (3H, s, $CO_2CH_3$); 3.83 (3H, s, $OCH_3$); 3.86 (3H, s, $OCH_3$); 4.21 (1H, d, $J$ = 7.10 Hz, B8); 5.13 (2H, s, $OCH_2Ph$); 5.20 (2H, s, $OCH_2Ph$); 5.71 (1H, d, $J$ = 7.10 Hz, B7); 6.98 (1H, dd, $J$ = 2.10, 8.29 Hz, B6); 7.06 (1H, d, $J$ = 8.29 Hz, B5); 7.10 (1H, d, $J$ = 8.55 Hz, A5); 7.11 (1H, d, $J$ = 2.10 Hz, B2); 7.28–7.42 (6H, m, benzyl H3, H4, H5); 7.48 (2H, br d, $J$ = 7.2 Hz, benzyl H2, H6); 7.50 (2H, br d, $J$ = 7.0 Hz, benzyl H2, H6); 7.59 (1H, dd, $J$ = 2.10, 8.55 Hz, A6); 7.82 (1H, d, $J$ = 2.10 Hz, A2). $\delta_C$ 51.31 ($OCH_3$); 52.64 ($OCH_3$); 56.30 ($OCH_3$); 59.26 (B8); 71.18 ($OCH_2Ph$); 71.42 ($OCH_2Ph$); 85.50 (B7); 101.69 (A8); 110.72 (B2); 113.45 (A5); 114.65 (A2); 115.05 (B5); 118.88 (B6); 122.73 (A1); 123.87 (A6); 128.46 (benzyl C2, C6); 128.52 (benzyl C2, C6); 128.60 (benzyl C4); 128.71 (benzyl C4); 129.22 (benzyl C3, C5); 129.27 (benzyl C3, C5); 134.26 (B1); 138.07 (benzyl C1); 138.41 (benzyl C1); 149.69 (A3); 149.74 (B4); 151.19 (B3); 151.80 (A4); 165.17 (A9–C=O); 166.38 (A7); 173.55 (B9–C=O).

**Dimethyl 2,5-bis-(4-hydroxy-3-methoxyphenyl)-tetrahydrofuran-3,4-dicarboxylate 4C2.** Prepared in essentially quantitative yield by debenzylation and hydrogenation of the double bond using 10% Pd on carbon (95% EtOH, RT, $H_2$-balloon, 24 h). The NMR spectrum indicated that the major product was the *cis–trans–trans*-isomer (approx. 96%) and the minor product was the *cis–cis–trans*-product. $\delta_H$ 3.21 (3H, s, $CO_2CH_3$); 3.66 (3H, s, $CO_2CH_3$); 3.84 (3H, s, $OCH_3$); 3.89 (3H, s, $OCH_3$); 3.60 (1H, dd, $J$ = 6.31, 8.55 Hz, B8); 3.78 (1H, dd, $J$ = 6.31, 8.29 Hz, A8); 4.95 (1H, d, $J$ = 8.55 Hz, B7); 5.27 (1H, d, $J$ = 8.29 Hz, A7); 6.80 (1H, d, $J$ = 8.02 Hz, A5); 6.85 (1H, d, $J$ = 8.02 Hz, B5); 6.88 (1H, dd, $J$ = 1.97, 8.02 Hz, A6); 7.02 (1H, d, $J$ = 1.97 Hz, A2); 7.04 (1H, dd, $J$ = 1.97, 8.02 Hz, B6); 7.27 (1H, d, $J$ = 1.97 Hz, B2); 7.53 (1H, s, phenol), 7.59 (1H, s, phenol). $\delta_C$ 51.84 ($OCH_3$); 52.37 ($OCH_3$); 55.43 (A8); 55.83 (B8); 56.21 ($OCH_3$); 56.27 ($OCH_3$); 83.04 (A7); 84.14 (B7); 111.25 (A2); 111.33 (B2); 115.24 (A5); 115.50 (B5); 120.48 (A6); 120.71 (B6); 130.08 (A1); 132.22 (B1); 147.26 (A4); 147.54 (B4); 147.86 (A3); 148.32 (B3); 172.45 (A9–C=O); 172.85 (B9–C=O).

**Dimethyl 2,5-bis-(4-acetoxy-3-methoxyphenyl)-tetrahydrofuran-3,4-dicarboxylate 4C2-Ac.** This isomeric mixture above was used to prepare the diacetate using pyridine–acetyl chloride (82% yield as a white solid). The solid was recrystallized from methanol to yield **4C2-Ac** as colorless prisms, mp. 137–140 °C. $\delta_H$ 2.22 (3H, s, Ac–$CH_3$); 2.24 (3H, s, Ac–$CH_3$); 3.19 (3H, s, $CO_2CH_3$); 3.70 (3H, s, $CO_2CH_3$); 3.64 (1H, dd, $J$ = 5.39, 8.42 Hz, B8); 3.82 (3H, s, $OCH_3$); 3.86 (1H, dd, $J$ = 5.39, 8.16 Hz, A8); 3.88 (3H, s, $OCH_3$); 5.10 (1H, d, $J$ = 8.42 Hz, B7); 5.39 (1H, d, $J$ = 8.16 Hz, A7); 7.02 (2H, m, A5 + A6); 7.08 (1H, d, $J$ = 8.16 Hz, B5); 7.18 (1H, bs, A2); 7.20 (1H, dd, $J$ = 1.84, 8.16 Hz, B6); 7.44 (1H, d, $J$ = 1.84 Hz, B2).

$\delta_C$ 20.44 (Ac–$CH_3$); 20.48 (Ac–$CH_3$); 51.95 ($OCH_3$); 52.54 ($OCH_3$); 55.43 (A8); 55.83 (B8); 56.20 ($OCH_3$); 56.26 ($OCH_3$); 82.99 (A7); 83.76 (B7); 111.85 (A2); 112.14 (B2); 119.55 (A6); 119.90 (B6); 123.18 (A5); 123.51 (B5); 137.27 (A1); 139.77 (B1); 140.66 (B4); 140.86 (A4); 151.83 (A3); 152.31 (B3); 168.97 (acetate), 172.37 (A9–C=O); 172.62 (B9–C=O).

**2,5-Bis-(4-hydroxy-3-methoxyphenyl)-tetrahydrofuran-3,4-dicarboxylic acid 5C2.** Saponification (2 N NaOH, 18 h) of **4C2-Ac** produced **5C2** as a colorless foam (54%). $\delta_H$ 3.81 (3H, s, $OCH_3$); 3.87 (3H, s, $OCH_3$); 3.61 (1H, dd, $J$ = 6.58, 8.68 Hz, B8); 3.80 (1H, dd, $J$ = 6.58, 8.55 Hz, A8); 4.98 (1H, d, $J$ = 8.68 Hz, B7); 5.31 (1H, d, $J$ = 8.55 Hz, A7); 6.77 (1H, d, $J$ = 8.16 Hz, A5); 6.84 (1H, d, $J$ = 8.16 Hz, B5); 6.94 (1H, dd, $J$ = 1.97, 8.16 Hz, A6); 7.06 (1H, dd, $J$ = 1.97, 8.16 Hz, B6); 7.09 (1H, d, $J$ = 1.97 Hz, A2); 7.31 (1H, d, $J$ = 1.97 Hz, B2). $\delta_C$ 55.39 (A8); 55.99 (B8); 56.13 ($OCH_3$); 56.17 ($OCH_3$); 82.91 (A7); 84.13 (B7); 111.48 (B2); 111.51 (A2); 115.16 (A5); 115.42 (B5); 120.75 (A6); 120.80 (B6); 130.45 (A1); 132.43 (B1); 147.14 (A4); 147.44 (B4); 147.75 (A3); 148.26 (B3); 172.86 (A9–C=O); 173.42 (B9–C=O).

### X-Ray crystal structure determination of 4C2-Ac

A colorless crystal with approximate dimensions 0.43 × 0.32 × 0.26 mm$^3$ was selected for crystal structure determination on a Bruker CCD-1000 diffractometer with Mo $K_\alpha$ ($\lambda$ = 0.71073 Å) radiation and a diffractometer to crystal distance of 4.9 cm. Crystallographic data (excluding structure factors) for structure **4C2-Ac**, Fig. 4, in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 603905.‡ The compound and crystal had the following properties and collection conditions: empirical formula, $C_{26}H_{28}O_{11}$; formula weight, 516.48; crystal system, orthorhombic; unit cell dimensions $a = 7.3722(6)$, $b = 25.899(2)$, $c = 26.760(2)$ Å, $\alpha = \beta = \gamma = 90°$; unit cell volume, 5109.4(7) Å$^3$; temperature 173(2) K; space group, *Pbca*; number of formula units in unit cell, 8; linear absorption coefficient 0.106 mm$^{-1}$; number of reflections collected, 39503; number of independent reflections, 5229 [$R$(int) = 0.0350]; final $R$ indices [$I > 2\sigma(I)$], $R1 = 0.0453$, $wR2 = 0.1220$; $R$ indices (all data), $R1 = 0.0514$, $wR2 = 0.1280$.

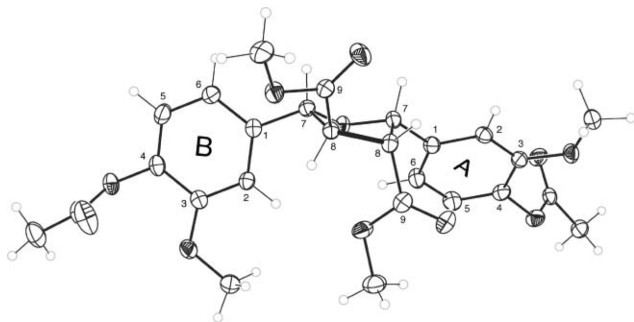


**Fig. 4** 3D Structure of compound **4C2-Ac** (Fig. 2) from the X-ray crystal structure, demonstrating the assigned *cis–trans–trans*-stereochemistry. The 8–8-bond formed (*in planta*) *via* the radical coupling step is in bold; in the ESI, this bond and the two original ferulates are color-differentiated. The structure is numbered to be consistent with the structures in Fig. 2; full numbering for the crystal structure coordinates is provided in the ESI.†

### Identification of 8–8-THF–DFA in cereal grain fibers

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 local German suppliers. Botanical origin was confirmed by the suppliers.

Preparation of whole grain insoluble and soluble cereal fibers was performed according to an enzymatic, preparative isolation procedure described previously.[13] This procedure was developed to use 10 g of sample material and involves the application of a sequence of heat stable α-amylase, protease and amyloglucosidase. For calculations, insoluble and soluble fibers were corrected for residual protein and ash contents.

For the identification and quantification of 8–8-THF–DFA **5C2**, the internal standard ($E$,$E$)-4-hydroxy-4′,5,5′-trimethoxy-3,3′-bicinnamic acid (5–5-Me–DFA, the monomethyl ether of **5D**)[13] dissolved in dioxane was added to the insoluble (40–90 mg) or soluble (60–120 mg) fiber samples weighed into a screw-cap tube, 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 $N_2$ through for 30 min, and air in the headspace of the screw-cap tubes was exchanged with $N_2$. Samples were acidified with 0.95 mL 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 as previously described.[13] Some samples were also analyzed as described above but spiked following the saponification process with small amounts of the synthesized 8–8-THF–DFA **5C2**. Relative retention times of **5C2** against the internal standard 5–5-Me–DFA: GC-MS-instrumentation: 0.691, GC-FID-instrumentation: 0.683.

To check if the saponification procedure releases only 8–8-THF–DFA **5C2** or if additional compounds are formed, the following experiments were performed. The synthesized dimethyl ester of **5C2** (*i.e.* **4C2**, 300 µg) was added to a mixture of 45 mg xylan and 30 mg cellulose simulating a typical matrix composition of cereal insoluble fiber. In addition, the dimethyl ester of **5C2** was added to 75 mg cellulose. Both reactions were carried out in triplicate. To monitor interfering peaks from the carbohydrate matrix, a mixture consisting of xylan (45 mg) and cellulose (30 mg) was also saponified. Saponification and extraction was carried out as described above. The extract was used for RP-HPLC analysis with UV and MS detection. HPLC analysis was performed on a Luna 5 µ 250 × 4.6 mm phenyl–hexyl column (Phenomenex, Aschaffenburg, Germany) at 45 °C, maintaining a flow rate of 1 mL min$^{-1}$. The following gradient was used (eluent A: MeOH, eluent B: acetonitrile, eluent C: 1 mM aqueous trifluoroacetic acid): initially A 27%, B 4%, C 69 %, held isocratically for 7 min, linear over 11 min to A 29%, B 6%, C 65%, linear over 5 min to A 20%, B 70%, C 10%, held isocratically for 5 min, following an equilibration step. Extracts were partially also used for GC-MS investigations using the conditions described above.

‡ CCDC reference numbers 603905. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b605918j


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## Results and discussion

### Synthesis

Since the initial identification of compound **5C2** was based on mass spectral data (see Fig. 3 for EI mass spectrum of trimethylsilylated **5C2**), we did not know which one of the six possible diastereomers of the suspected ferulate tetrahydrofuran "dimer" was isolated. The first synthetic path chosen produced the correct diastereomer. The stereochemistry might logically be the result of isomerization upon saponification; however, the stereochemistries of the ester and the acid appear to be the same.

The crucial step in the synthetic pathway chosen involves the coupling of cinnamate ester **9** with a β-ketoester **12** (Fig. 2) *via* $Mn(OAc)_3$.[36] The stereochemistry of the resulting dihydrofuran ring in **13** is assigned as *trans.* The coupling constant between the ring protons is 7 Hz which compares favorably with the coupling constant reported for the *trans*-isomer of diethyl 2-chloro-5-phenyl-4,5-dihydrofuran-3,4-dicarboxylate.[37] The coupling constant of the *cis*-isomer of the same compound is reported to be 11 Hz. The regio- and stereoselectivity for dihydrofuran **13** is consistent with that demonstrated previously for $Mn(OAc)_3$-mediated coupling of cinnamates and β-ketoesters.[36,38–41] Palladium catalyzed *cis*-hydrogenation and debenzylation of the dihydrotetrahydrofuran **13** resulted in the tetrahydrofuran product **4C2**, largely as the *cis–trans–trans*-isomer, with a small amount of the *cis–cis–trans*-isomer. Assignments are based on the assumption that *cis*-hydrogenation has occurred from the least hindered face of dihydrofuran **13**. Saponification of this product, or of the derived acetate **4C2-Ac**, gave the required di-acid **5C2**. After confirming this to be the same isomer released from the natural plant sources (see below), the isomeric form was elucidated by NMR and, on **4C2-Ac**, unambiguously validated by X-ray crystal structure, Fig. 4 (and ESI†).

### Identification and quantification of 8–8-THF–DFA in cereal grain fibers

In all extracts of investigated saponified insoluble cereal fibers, 8–8-THF–DFA (**5C2**, Fig. 1) was identified by comparison of its mass spectrum and its relative GLC retention time with that of the genuine compound. Identity was further confirmed by spiking some samples with the synthesized compound. Although the identification of 8–8-THF–DFA in the corresponding soluble fiber fraction was difficult due to the tiny amounts detected, it was found in all investigated cereals. Fig. 3 shows the MS-spectrum of trimethylsilylated 8–8-THF–DFA obtained *via* GC-MS; with originally two phenolic and two acid groups and, in comparison to the other DFAs, an additional water, its nominal molecular mass is 692. As HPLC-analysis with diode array detection of monomeric, dimeric[42] and trimeric hydroxycinnamic acids is becoming attractive, the UV-spectrum of 8–8-THF–DFA in MeOH–1 mM aqueous trifluoroacetic acid is provided in Fig. 3. Because both sidechains are saturated, there is no maximum at ~325 nm, typical of ferulic acid and the DFAs.

The amounts of 8–8-THF–DFA in insoluble cereal fibers were determined as follows [$\mu g\ g^{-1}$]: maize 692 ± 39; wheat 84 ± 8; spelt 109 ± 8; rye 132 ± 5; barley 133 ± 3; oats 149 ± 34; millet 242 ± 13; rice 109 ± 11; wild rice 115 ± 8. Regarding the DFA distribution,[13] 8–8-THF–DFA contributes between 2.5 and 4.9% to the absolute DFA content in cereal fibers. In all investigated insoluble cereal fibers 8–8-THF–DFA **5C2** was more prevalent than 4–O–5-DFA **5E**. Furthermore the amounts of DFA **5C2** ranged between 50% (in rice insoluble fiber) and 90% (in rye insoluble fiber) of the amounts of DFA **5C1**. In maize insoluble fiber DFA **5C2** was even more prevalent than DFA **5C1** (about 180%). Due to the tiny amounts in soluble cereal fibers, the amounts of DFA **5C2** in these samples were not unambiguously determined; the amounts often did not reach the determination limit. These very low amounts were surprising because the other 8–8-coupled DFAs (**5C1** and **5C3**) are often determined to be the major ones in soluble cereal fibers.

### Nature of dehydrodiferulates in the plant

We have already established that saponification of esters **4C1** and **4C3** yield only their corresponding acids **5C1** and **5C3** respectively; for example, the ester **4C1** does not produce acid **5C3**. 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 **5C1-3** result from a single ester precursor in the wall such as the tetrahydrofuran **4C2**. To test the plausibility of a common precursor, the formerly synthesized methyl ester of **5C2** (**4C2**, X = Me, Fig. 1, or the ethyl analog) was saponified in a cell-wall-like matrix consisting of cellulose–xylan 2 : 3 or in pure cellulose. These experiments yielded predominantly the tetrahydrofuran **5C2**. The non-cyclic 8–8-DFA **5C1** was not detected in any saponification experiment, thus excluding **4C2** as precursor of the non-cyclic 8–8-DFA **5C1**. The situation was different with the cyclic 8–8-DFA **5C3**. Although in all saponification experiments the 8–8-THF **5C2** clearly dominated the HPLC-UV and GC-FID-chromatograms, in the HPLC runs two small peaks were detected showing UV-spectra and mass peaks consistent with cyclic 8–8-DFAs. Based on peak areas at 280 nm the single peaks were typically 0.4 to 5.8% of the peak area of **5C2**. However, some unwelcome variability is noted—in a single saponification experiment the peak areas were 9 and 19% of the peak area of **5C2**. In a further saponification experiment the alkaline hydrolysate was investigated using GC-MS. Chromatograms showed two additional peaks, one of them having the same retention time and mass spectrum as the cyclic 8–8-DFA **5C3**. The second, slightly bigger peak with the same mass spectrum elutes very close to **5C3**. It is assumed that this compound is an isomer of **5C3** (Fig. 1), presumably having alternate ring-B C7 and C8 stereochemistry. Re-evaluation of the GC-MS chromatograms that were used for the identification of ferulate dimers in cereal grains[13] revealed that this assumed isomer of **5C3** was not produced during the saponification of plant cell walls. This indicates that saponification produces no detectable conversion from arabinoxylan esters of **5C2** to the cyclic 8–8-DFA products. Furthermore, the saponification experiments using the methyl ester of **5C2** showed that **5C2** is always the predominant product. The small amounts of cyclic 8–8-DFA **5C3** formed upon saponification cannot explain the determined amounts of **5C3** from the wall.

It therefore now appears that 8–8-coupling in the plant cell wall leads to three distinct 8–8-diferulates (**4C1**, **4C2**, **4C3**, X = arabinoxylan, Fig. 1) that are the precursors for the detected acids **5C1**, **5C2** and **5C3**. There is an urgent need to investigate the

nature of the dehydrodiferulates *in vivo* and not only in alkaline hydrolysates.

As with any chemical reaction that produces multiple products, the ratio of products is dependent upon 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 (2005, 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 more readily add water nucleophilically at low pH resulting in tetrahydrofuran **5C2**; such addition is protonation-dependent. At high pH, the elimination reactions producing **5C1** are more likely.

Cursory mass spec analysis indicates no other isomers of **5C2** at a significant level (trace amounts of one more isomer are possible from the selected ion mode in the GC-MS) although the synthesis and coupling reactions with methyl ferulate appear to produce other isomers. Determining whether other isomers of the ester **4C2** produce the same acid isomer **5C2** following saponification is beyond the current study, which sought to identify the product liberated from natural sources.

## Conclusions

Alkaline hydrolysates of cereal grain and grass walls contain a previously unauthenticated component derived from ferulate. Strictly DFA **5C2** is not a true dehydrodimer since an additional oxygen (from water) is incorporated. Its higher molecular mass is responsible for its being missed as a dimeric ferulate product previously. It is still formed *via* radical coupling, but the post-coupling steps are different from those in the previously identified DFAs.[2] DFA **5C2** is a substantial component that should also be quantified as resulting from 8–8-dehydrodimerization. Only one of the six potential diastereomers of **5C2**, the *cis–trans–trans*-isomer, was detected among the products from saponified plant cell walls. The finding of this 8–8-tetrahydrofuran DFA **5C2** along with the previously identified **5C1** and **5C3** implicates at least three 8–8-coupling products of ferulate in the cell walls of these plants and suggests that cell wall cross-linking may occur under acidic conditions.


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