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## Isolation and identification of a ferulic acid dehydrotrimer from saponified maize bran insoluble fiber

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**Abstract** Following saponification of maize bran insoluble fiber a ferulic acid dehydrotrimer was isolated using Sephadex LH-20 chromatography. Structural identification was carried out using UV-spectroscopy, mass spectrometry and 1D and 2D NMR experiments ( $^1\text{H}$ ,  $^{13}\text{C}$ , DEPT, COSY, TOCSY,  $^{13}\text{C}$ - $^1\text{H}$  HSQC, HMBC). UV-spectroscopy indicated characteristics of ferulate structures, mass spectrometry showed a trimeric ferulate structure, and the NMR spectra provided diagnostic evidence for its being a 5-5/8-O-4-coupled dehydrotrimer. Ferulic acid dehydrodimers are mainly derived from diferulates which cross-link polysaccharides. Because of the involvement of a 5-5-dehydrodiferulic acid unit in the identified trimer, this novel dehydrotriferulic acid from cereal grain fiber need not imply the cross-linking of three polysaccharide chains; molecular modeling of the ferulate dehydrodimerization in earlier studies showed that the 5-5-diferulate, uniquely, can form intramolecularly. This first identified ferulic acid dehydrotrimer nevertheless reveals that polysaccharide chains can be more extensively cross-linked than previously recognized.

**Keywords** *Zea mays* L. · Dietary fiber · Ferulic acid · Diferulic acid · Triferulic acid · Arabinoxylan · Cell wall cross-linking

### Introduction

Cross-linking of plant cell walls via ferulate dehydrodimerization reactions is well established [1, 2, 3, 4]. Ferulates acylate various polysaccharides, notably arabinoxylans in grasses [1] and in cereal grain dietary fiber, particularly in the insoluble fiber fraction [5, 6]. Ferulate dehydrodimerization is therefore a mechanism for linking two polysaccharide chains, providing structural integrity to the cell wall, but inhibiting fiber degradability [7]. Being phenolic entities capable of radical coupling reactions, ferulates and their dehydrodimers may also be incorporated into lignin polymers [2, 8], furthering the cross-linking of the wall, and further limiting the degradability of the polysaccharides [9].

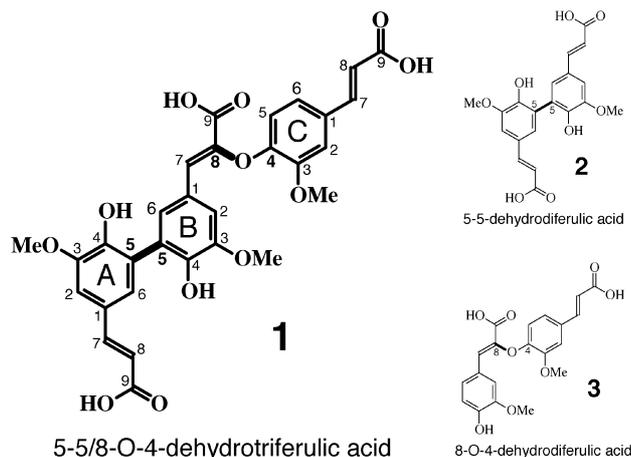
The dehydrodimerization reaction is via radical coupling [10]. As such, the reaction is somewhat combinatorial in nature, much like the radical coupling of monolignols to each other and to lignins [11]. A range of dehydrodiferulates can be found in a variety of materials [10, 12, 13, 14, 15, 16], as predicted and verified in 1994 [10]. The dehydrodiferulates are characterized by the new bond formed in the radical coupling reaction between the two ferulates (at their 4-O-, 5- or 8-carbons) as 5-5-, 8-8-, 8-5-, 8-O-4-, and 4-O-5-coupled dehydrodimers. Following saponification, or during the coupling process in the plant itself, several forms of the 8-8- and 8-5-coupled dehydrodimers are possible. Characterization of the cross-linking of walls via ferulate has therefore become considerably more interesting, but more complex than it was before the 1994 study, when only the 5-5-coupled dehydrodimer **2** (Fig. 1) was known.

We have recently provided the first detailed characterization of releasable diferulates from a range of cereal grains [16]. Cereal grains are one of the most important food groups. Their fiber properties and even some of their health benefits can likely be attributed to the nature of their cell wall polymers and their chemical architecture. We have also recently discovered disinapates and sinapate-ferulate cross-products in wild rice, rice, wheat and

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**Fig. 1** Structures and numbering system used for the new dehydrotriferulic acid (**1**) along with dehydrodimers **2** (a 5-5-dehydrodiferulic acid) and **3** (an 8-O-4-dehydrodiferulic acid). The actual bonds formed in the radical coupling steps are in bold

other grains, implicating sinapates in similar polysaccharide cross-linking reactions in the wall [17].

The idea that ferulates might continue their radical polymerization reactions and form higher oligomers in the wall has long been discussed, but rarely in the literature. Certainly, dehydrogenation oligomers [18] and polymers can be made *in vitro*. However, ferulates encumbered by their direct attachment to an extensive polysaccharide chain were thought unlikely to be able to approach two other such ferulates. A possible exception is at the ends of chains, or if there is rather extreme clustering of ferulates on polysaccharide chains such that dimerization (cross-linking two polysaccharide chains) might create a dehydrodiferulate in sufficient proximity to another ferulate on one of the chains that a further coupling reaction could occur. This dehydrotrimer formation would still cross-link only two polysaccharide chains, not three. In view of the finding reported in this paper, such possibilities should be examined through the use of molecular modeling.

Molecular modeling of the ferulate dehydrodimerization process led Hatfield et al. to conclude that one dimer was unique in that it could be formed intramolecularly [19]. If ferulates on arabinosyl residues were attached to xylose units in a chain with two intervening xylose units (i.e. located on one xylose unit and on the fourth xylose unit from it), and with no other arrangement, it appears to be possible to form intramolecular 5-5-dehydrodiferulate, and only that dehydrodimer. Thus the 5-5-diferulate is likely the only one that can form intramolecularly and this may explain the prevalence of this dimer in various plant cell walls when its formation from low molecular-weight ferulate ester models *in vivo* is rather minor; ferulates may simply be positioned on wall polymers such that this coupling is relatively favored.

More recently, Fry et al. have provided evidence for higher ferulate oligomers in maize suspension cultures [20]. No actual trimers or higher oligomers were structurally characterized, but they were strongly implied. In

this paper, we report on the isolation and structural identification of a ferulate dehydrotrimer from maize bran insoluble fiber, and discuss its implications for cell wall cross-linking in cereal grains.

## Materials and Methods

**General.** Sephadex LH-20 was from Amersham Pharmacia Biotech (Freiburg, Germany). Heat-stable  $\alpha$ -amylase Termamyl 120 L (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. Methanol (HPLC grade) was from Baker (Deventer, Holland), methanol- $d_4$  from Deutero (Kastellaun, Germany) and acetone- $d_6$  and  $D_2O$  from Cambridge Isotope Laboratories (Andover, Mass., USA). All other chemicals were from Merck (Darmstadt, Germany).

**Plant material.** Maize bran (*Zea mays* L.) was kindly provided by Hammermühle Maismühle GmbH, Kirrweiler, Germany.

**Preparation of insoluble maize fiber.** Maize bran was defatted and extracted with acetone for 4 h using Soxhlet equipment. The following procedure was performed four times: defatted maize bran (20 g, milled to a particle size <0.5 mm) was suspended in phosphate buffer (pH 6.0, 0.08 M, 300 ml), and  $\alpha$ -amylase (1.5 ml) was added. Beakers were heated in a boiling water bath for 30 min with occasional shaking. After cooling down to room temperature, the pH was adjusted to 7.5 with 0.275 M NaOH (~60 ml). Samples were incubated with protease (600  $\mu$ l) at 60 °C for 30 min with continuous agitation. After the pH had been adjusted to 4.5 with 0.325 M HCl (~60 ml), amyloglucosidase (700  $\mu$ l) was added and the mixture was incubated at 60 °C for 30 min (continuous agitation). Following centrifugation, the residue was washed twice each with hot water (70 °C), 95% (v/v) ethanol, and acetone, and was finally dried at 40 °C overnight in a vacuum oven.

**Alkaline hydrolysis and extraction.** Insoluble maize fiber (4x5 g) was weighed into four screw-cap tubes (200 ml). Saponification with NaOH (2 M, 100 ml, degassed with  $N_2$ ) was carried out under nitrogen and protected from light. The mixture was acidified (pH < 2) with concentrated HCl (~19 ml). Liberated phenolic acids were extracted into diethyl ether (40 ml, three times). The volume of the combined ether extracts was reduced to 50 ml (rotary evaporation) and extracted with  $NaHCO_3$  solution (5%, 25 ml, three times). The combined aqueous layers were acidified (pH < 2) with concentrated HCl and phenolic acids were extracted into diethyl ether (30 ml, three times). The combined ether extracts were dried over  $Na_2SO_4$  and evaporated to dryness. The residue was further dried under a stream of nitrogen and finally redissolved in 10 ml MeOH/ $H_2O$  (50/50, v/v).

**Separation of phenolic acids using Sephadex LH-20 chromatography.** Following conditioning of the Sephadex LH-20 column (83x2 cm) with 0.5 mM aqueous trifluoroacetic acid (TFA)/MeOH (95/5, v/v) the sample was applied. A four-step elution was performed:

1. Elution with 0.5 mM TFA/MeOH (95/5, v/v) for 73 h, with a flow rate of 1.5 ml  $min^{-1}$  (L-6000 pump, Merck/Hitachi, Darmstadt, Germany)
2. Elution with 0.5 mM TFA/MeOH (50/50, v/v) for 53 h, with a flow rate of 1.0 ml  $min^{-1}$
3. Elution with 0.5 mM TFA/MeOH (40/60, v/v) for 64 h, with a flow rate of 1.0 ml  $min^{-1}$
4. Rinsing step with 100% MeOH

**Table 1** <sup>1</sup>H NMR data for ferulic acid dehydrotrimer **1** along with ferulic acid dehydromers (DFA) **2** and **3** for comparison

		H-2	H-5	H-6	H-7	H-8	OMe
In acetone-d <sub>6</sub> /D <sub>2</sub> O 3:1 <sup>a</sup>	<b>1</b> , trimer unit-A	7.207 (d, 2.0)	–	6.90 (d, 2.0)	7.51 (d, 15.9)	6.29 (d, 15.9)	3.87 (s)
	<b>1</b> , trimer unit-B	7.37 (d, 2.0)	–	7.209 (d, 2.0)	7.36 (s)	–	3.72 (s)
	<b>1</b> , trimer unit-C	7.31 (d, 2.0)	6.75 (d, 8.3)	7.06 (dd, 8.3, 2.0)	7.51 (d, 15.9)	6.34 (d, 15.9)	3.84 (s)
In acetone-d <sub>6</sub> <sup>a</sup>	<b>2</b> , 5-5-DFA (A) <sup>c</sup>	7.35 (d, 2.0)	–	7.21 (d, 2.0)	7.64 (d, 15.9)	6.42 (d, 15.9)	3.97 (s)
	<b>3</b> , 8-O-4-DFA (B) <sup>d</sup>	7.52 (d, 2.0)	6.82 (d, 8.2)	7.23 (dd, 8.2, 2.0)	7.42 (s)	–	3.73 (s)
	<b>3</b> , 8-O-4-DFA (C) <sup>d</sup>	7.44 (d, 2.0)	7.13 (d, 8.3)	7.59 (dd, 8.3, 2.0)	6.43 (d, 15.9)	4.00 (d, 15.9)	– (s)
In methanol-d <sub>4</sub> <sup>b</sup>	<b>1</b> , trimer unit-A	7.19	–	6.94	7.59	6.31	3.94
	<b>1</b> , trimer unit-B	7.42	–	7.21	7.44	–	3.77
	<b>1</b> , trimer unit-C	7.30	6.80	7.08	7.61	6.39	3.89

<sup>a</sup> Spectra in acetone-d<sub>6</sub>:D<sub>2</sub>O 3:1 and acetone-d<sub>6</sub> were run at 360 MHz. Assignments were fully authenticated by the usual array of 1D and 2D methods. The full data will be placed in our NMR database as compound Number 3063 [24]

<sup>b</sup> Spectra in methanol-d<sub>4</sub> were run at 500 MHz. Coupling constants were essentially the same as the above (to within the data-point resolution). Note that protons A7 and C7, and protons A2 and B6, fully resolve in methanol-d<sub>4</sub>

<sup>c</sup> From a previous study [10], and in NMR Database [24], compound number 2056

<sup>d</sup> From a previous study [10], and in NMR Database [24], compound number 2040. Note that the modeling of the aromatic ring in trimer unit B by this compound will not be particularly good since it does not have its 5-position substituted; the C-ring is well modeled

**Table 2** <sup>13</sup>C NMR data for ferulic acid dehydrotrimer **1** along with ferulic acid dehydromers (DFAs) **2** and **3** for comparison. Data are for triferulate **1** in acetone-d<sub>6</sub>:D<sub>2</sub>O 3:1 and dimers **2** and **3** in acetone-d<sub>6</sub>, and were acquired at 90 MHz. Assignments were fully

authenticated by the usual array of 1D and 2D methods. The full data will be placed in our NMR database as compound Number 3063 [24]

	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	OMe
<b>1</b> , trimer unit-A	126.2	109.8	148.8	147.1	125.6	125.5	145.9	115.9	169.52	56.4
<b>1</b> , trimer unit-B	124.3	112.5	148.3	146.3	125.5	127.3	127.8	139.1	165.9	56.0
<b>1</b> , trimer unit-C	129.5	112.0	149.6	148.5	113.9	122.7	145.2	117.3	169.46	56.2
<b>2</b> , 5-5-DFA (A) <sup>a</sup>	126.6	110.0	148.9	147.4	125.6	126.1	145.9	116.3	168.4	56.5
<b>3</b> , 8-O-4-DFA (B) <sup>b</sup>	125.3	113.8	148.3	149.5	116.0	126.1	128.5	138.3	164.5	55.9
<b>3</b> , 8-O-4-DFA (C) <sup>b</sup>	130.1	112.4	150.2	148.9	114.4	122.9	145.2	117.5	167.9	56.5

<sup>a</sup> From a previous study [10], and in NMR Database [24], compound number 2056

<sup>b</sup> From a previous study [10], and in NMR Database [24], compound number 2040. Note that the modeling of the aromatic ring in trimer unit B by this compound will not be particularly good since it does not have its 5-position substituted; the C-ring is well modeled

An UV-detector equipped with a preparative flow cell (L-7400, Merck/Hitachi, Darmstadt, Germany) was used for the detection at 325 nm. Fractions were collected every 18 min. The ferulic acid trimer **1** (Fig. 1) eluted in the third elution step (0.5 mM TFA/MeOH, 40/60) between 45 h and 53.5 h.

**Analytical HPLC.** An analytical HPLC method usually applied to the separation of diferulic acids was used to confirm the purity of the isolated ferulic acid trimer. Separation was performed by RP-HPLC (L-6200 Intelligent pump, T-6300 Column thermostat (Merck/Hitachi, Darmstadt, Germany) using a Nucleosil 100-5 C 18 HD column (250×4 mm, i.d. 5 μm, Macherey-Nagel, Düren, Germany). Elution was carried out using a binary gradient system consisting of aqueous 1 mM TFA and MeOH: initially MeOH 10% and 1 mM TFA 90%, linear over 10 min to MeOH 40% and 1 mM TFA 60%, held isocratically for 5 min, linear over 5 min to MeOH 50% and 1 mM TFA 50%, held isocratically for 5 min, linear over 5 min to MeOH 80% and 1 mM TFA 20%, held isocratically for 5 min, following an equilibration step. The injection volume was 20 μl, the column temperature 45 °C, and the flow rate was maintained at 1 ml min<sup>-1</sup>. Detection was carried out using a Waters

994 programmable photodiode array detector (Waters, Eschborn, Germany). Detection wavelengths were 325 nm and 280 nm or 210 nm. Using the HPLC-method described, trimer **1** eluted after 23.5 min.

**Structural identification (NMR and MS).** Trimer **1** (Fig. 1) was firmly identified by its mass spectra under negative and positive ion conditions, and by the usual diagnostic range of NMR experiments. A correct IUPAC name for trimer **1** is: 3-[5'-(E)-(2-carboxyvinyl)-6,2'-dihydroxy-5,3'-dimethoxy-biphenyl-3-yl]-2-(E)-[4-(E)-(2-carboxyvinyl)-2-methoxyphenoxy]-acrylic acid; we prefer to name it where possible based on the cinnamic acid residue: (Z)-8-[4-[(E)-2-carboxyvinyl]-2-methoxyphenoxy]-(E, E)-4,4'-dihydroxy-5,5'-dimethoxy-3,3'-bicycinnamic acid. Note that the stereochemistry of all three cinnamic acid residues are *trans* (E), but unit B must be designated as (Z) in the latter name since the aryl-substituent linked via O, OAr, has higher priority than the carboxyl.

NMR spectra at 360 MHz in acetone-d<sub>6</sub>/D<sub>2</sub>O (3/1) were acquired on a Bruker DRX-360 instrument (Bruker, Rheinstetten, Germany) fitted with a 5-mm <sup>1</sup>H/broadband gradient probe with inverse geometry (proton coils closest to the sample). The central

acetone solvent peak was used as internal reference ( $\delta_C$  29.8,  $\delta_H$  2.04 ppm). We used the standard Bruker implementation (inviet-gssi) of the gradient-selected sensitivity-improved inverse ( $^1H$ -detected) HSQC [21], the standard (inv4gslplrnd) gradient-selected inverse-detected HMBC [22] with a 100 ms long-range coupling delay, and gradient COSY experiments for the fully authenticated structural assignments in Table 1 and Table 2. Spectra at 500 MHz in methanol- $d_4$  were acquired on a 500 MHz Bruker AMX-500 instrument. Chemical shifts were referenced to tetramethylsilane as internal standard. These experiments and their applications to lignins are detailed in [23]. The NMR data are presented in Table 1 and Table 2, and will also be displayed in the next release of our NMR Database of Lignin and Cell Wall Model Compounds (as entry number 3063) [24].

Molecular weight was determined using flow injection analysis-mass spectrometry (HP Series 1100: autosampler G1313, pump G 1312A, mass spectrometer G 1946A), Hewlett-Packard, Waldbronn, Germany). MS parameters: atmospheric pressure-electrospray ionisation (AP-ESI) positive and negative mode; drying gas flow 10 l  $min^{-1}$ , drying gas temperature 350 °C, nebulizer pressure 50 psi, capillary voltage 4,000 V, fragmentor voltage 100 V. A mixture of acetonitrile/5 mM ammonium formate in doubly distilled water (50/50, v/v) was used as solvent.

AP-ESI MS, positive-ion mode, mass (% of base peak): 617 ( $M+39$ ) $^+$  (6), 601 ( $M+23$ ) $^+$  (18), 543 (15), 384 (9), 339 (100), 284 (8), 283 (18), 219 (5), 129 (9); negative-ion mode: 577 ( $M-H$ ) $^-$  (100), 371 (10), 353 (9), 339 (96).

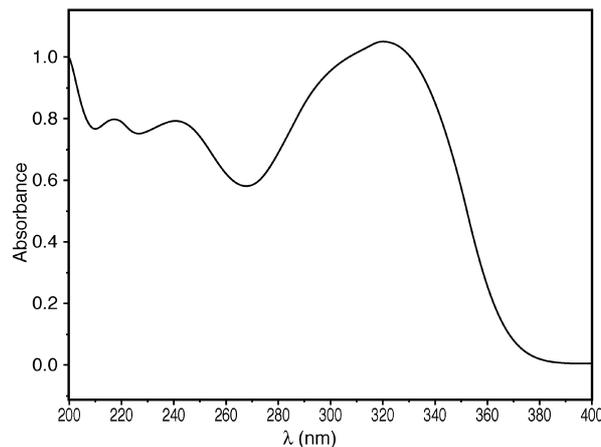
## Results and discussion

### Dehydrotriferulate isolation

Our group in Hamburg has recently developed a method for isolating milligram quantities of diferulates from maize bran as an alternative to laborious chemical synthesis (as will be fully described elsewhere). The method involves the alkaline hydrolysis of ester-bound compounds, a clean-up using liquid-liquid partitioning and separation of the liberated phenolic acids (monomeric, dimeric and trimeric ferulic acids and other phenolic acids). A key step in the separation procedure is Sephadex LH-20 chromatography using organic solvents. Using Sephadex LH-20 as stationary phase, fractionation is based not only on molecular weight differences but also on secondary interactions between the stationary phase and the phenolic compounds. Trimer **1** (Fig. 1) elutes within the third elution step using methanol (60%) and 0.5 mM TFA (40%) as eluent. Analytical HPLC shows that the trimer is quite pure (>95%, based on peak areas at 325 nm and 210 nm, respectively) following the Sephadex LH-20 chromatography. Using the method described, 10 mg of trimer **1** were isolated.

### Structural identification

The UV-spectrum acquired via analytical HPLC with photodiode-array-detection has characteristics of ferulate structures (Fig. 2). The mass-spectrum indicated trimeric ferulate structures. Negative-ion MS produced an ( $M-H$ ) $^-$  of  $m/z$  577 suggesting a molecular weight of 578. Positive-ion MS gave a high-mass  $M+23$  of  $m/z$  601 ( $M+Na$ ) $^+$  and  $M+39$  of  $m/z$  617 ( $M+K$ ) $^+$ , suggest-



**Fig. 2** UV spectrum of dehydrotriferulic acid (**1**) recorded under conditions pertaining to the analytical HPLC procedure described in the Materials and methods section

ing the same molecular weight. Several potential ferulate dehydrotrimers have that mass. Interpretation of the MS proved to be consistent with the final structure more unambiguously elucidated from the NMR data below.

The NMR spectra (Fig. 3) provided diagnostic evidence for the 5-5/8-O-4-dehydrotrimer **1**. Proton NMR revealed three methoxyls and enough aromatic protons to suggest three aromatic rings, confirming the product as a trimer. There were two *trans*-cinnamic acid side chains. Some of these protons were better separated in methanol- $d_4$  than in acetone- $d_6$  (Table 1). Long-range  $^{13}C$ - $^1H$  correlations via the HMBC experiment showed that one (A) was attached to a 5-linked guaiacyl ring (with aromatic protons only on the 2- and 6-carbons), whereas one (C) was linked to a normal (4-O-linked) guaiacyl unit (with protons on 2-, 5- and 6-carbons). A singlet proton at 7.36 ppm appeared to indicate an 8-O-4-linked structure for the third unit (B); the HMBC spectrum revealed that this side chain was on a 5-linked guaiacyl ring. Full analysis of the usual array of 1D and 2D NMR experiments ( $^1H$ ,  $^{13}C$ , DEPT, COSY, TOCSY,  $^{13}C$ - $^1H$  HSQC, HMBC) provided the assignments in Table 1 and Table 2, all of which are fully consistent with structure **1**. The structural assignment is unambiguous since no other isomers of this compound match the data.

### Implications for cell wall cross-linking

The isolation of this trimer provides concrete evidence for at least one ferulate dehydrotrimer, confirming the evidence from Fry et al. [20] for ferulate oligomers. Unfortunately, with this structure it is not possible to tell the order in which the coupling reactions occurred, i.e. was the compound formed by 8-O-4-coupling of two ferulates followed by coupling at the product's 5-position with the third ferulate at its 5-position, or did the 5-5-



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