

## Structural elucidation of new ferulic acid-containing phenolic dimers and trimers isolated from maize bran

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**Abstract**—Four new phenolic dimers and trimers that contain ferulic acid moieties were isolated from the alkaline hydrolyzate of insoluble maize bran fiber and their structures were established by 1D/2D NMR and mass spectrometry. The biological role of one dimer remains unclear whereas the dimeric vanillin-ferulic acid-cross-product probably represents an oxidative degradation product from the corresponding diferulate. Both ferulic acid dehydrotrimers are able to cross-link polysaccharide chains. However, the 5-5/8-O-4(H<sub>2</sub>O)-triferulic acid may be a cross-link in its identified structure whereas we assume that the identified 8-O-4/8-5(non-cyclic)-triferulic acid arose from a natural 8-O-4/8-5(cyclic)-triferulate analog during the saponification process.

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Grasses and cereals contain substantial amounts of cell wall-bound hydroxycinnamate esters linked to polysaccharides.<sup>1</sup> Particularly, ferulates play an important role in cross-linking cell wall polysaccharides.<sup>2</sup> Ferulates dehydrodimerize via a radical, oxidative mechanism to form mainly 8-5-, 8-O-4-, 5-5-, 8-8-, and 4-O-5-coupled dehydrodiferulates (often simply referred to as diferulates), thus cross-linking two polysaccharide chains.<sup>3–8</sup> Polysaccharide cross-linking plays a significant role in the plant and plant derived products, for example, by effecting cell wall strength, fiber degradability, and food textural and processing properties.<sup>9–12</sup> Furthermore, ferulates and diferulates act as cross-links between polysaccharides and lignin.<sup>13,14</sup> Recently, three dehydrotriferulic acids have been isolated and identified for the first time,<sup>15–17</sup> indicating that also higher ferulate oligomers are involved in cross-linking cell wall polysaccharides as proposed by Fry et al.<sup>18</sup> However, to date it is not possible to decide whether triferulates cross-link three polysaccharide chains or whether their cross-link-

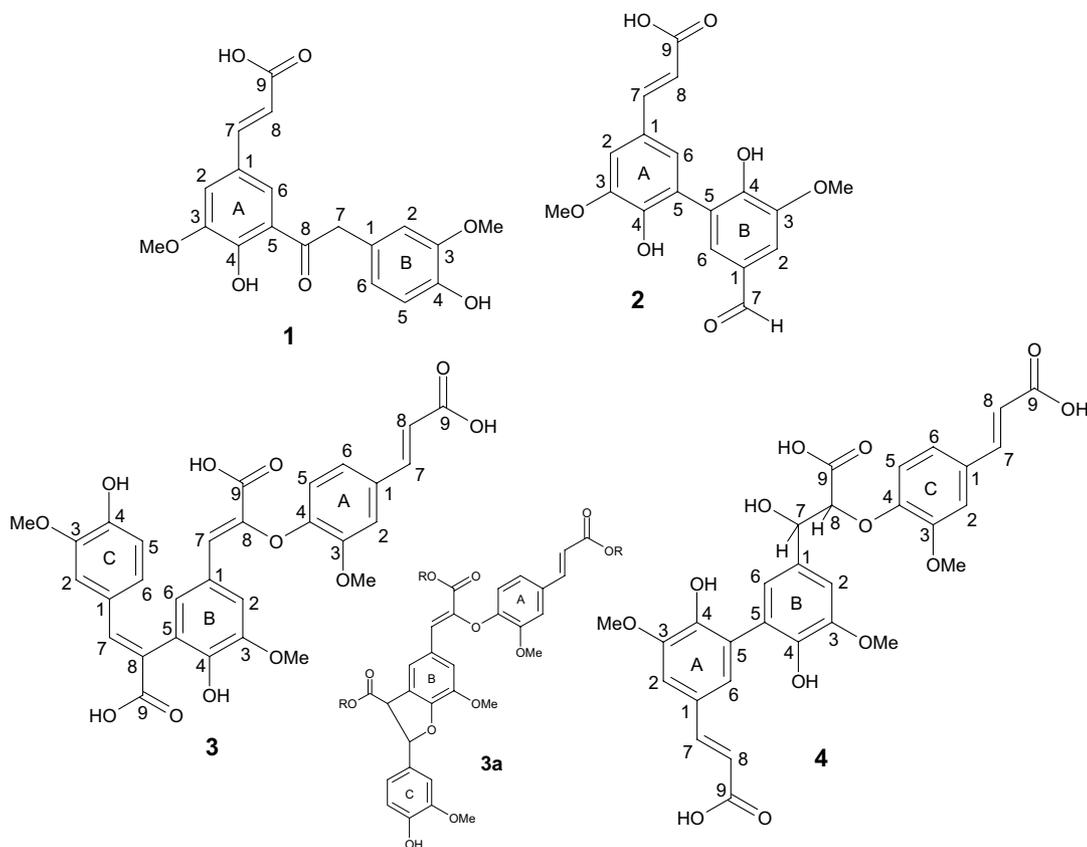
ing capacity is limited to two polysaccharide chains due to difficulties in the approach of three unwieldy polymer chains in a limited space.

In this paper, we describe the isolation and structural identification of four new ferulic acid-containing dimers and trimers from maize bran. These studies aim to characterize the range of such products to be found in cereal grains (and grass cell walls in general), and begin to understand their role and impact on grain properties.

Compounds **1–4** (Fig. 1) were isolated from maize bran according to a recently published method developed by our group to isolate dehydrodiferulic acids (DFA) on a semipreparative scale.<sup>19</sup> In brief, maize bran was defatted, milled, and destarched and partially deproteinated by treatment with  $\alpha$ -amylase, protease, and amyloglucosidase. Following centrifugation the residue was washed (hot water, ethanol, and acetone) and dried. Alkaline hydrolysis of insoluble maize fiber (40 g, compound **1**: 30 g) was carried out using 2 M NaOH under nitrogen and protected from light. After acidification (pH < 2), liberated phenolic acids were extracted into diethyl ether. The organic layer was purified by liquid–liquid extraction with NaHCO<sub>3</sub> solution and re-extracted into diethyl ether after acidification. The dried residue was redissolved in 10 mL MeOH/H<sub>2</sub>O 50/50 (v/v).

**Keywords:** *Zea mays* L.; Gramineae; Cell wall cross-linking; Triferulic acid; Triferulate; Ferulic acid; Ferulate; Phenolic dimers; Vanillin; Oxidative degradation; Arabinoxylans; Dietary fiber.

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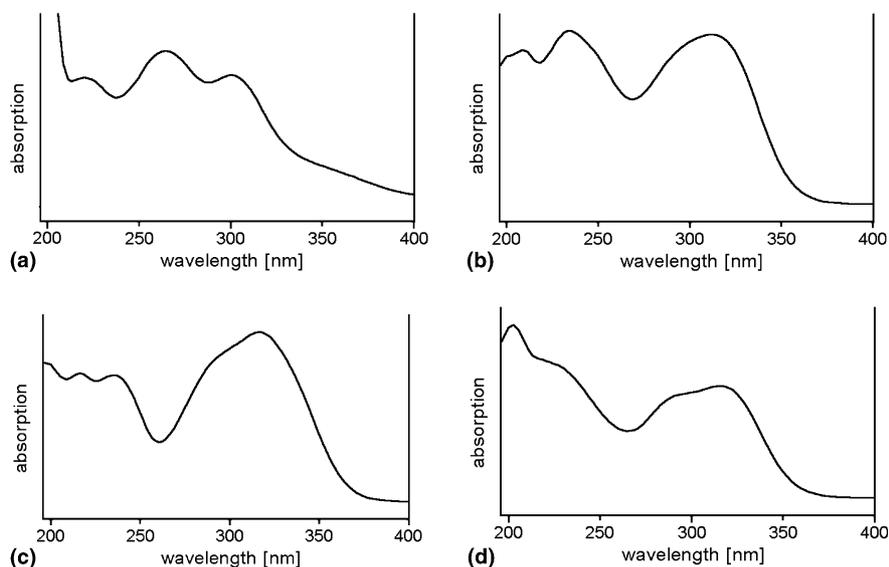
**Figure 1.** Structures and numbering systems used for the description of NMR data and trivial names of the new ferulic acid-containing phenolic dimers **1** and **2** (5-5-vanillin-ferulic acid cross-product) and trimers **3** (8-O-4/8-5(non-cyclic)-dehydrotriferulic acid), and **4** (5-5/8-O-4(H<sub>2</sub>O)-dehydrotriferulic acid) and of the hypothetical cell-wall-native component **3a** (8-O-4/8-5(cyclic)-dehydrotriferulate, R = arabinoxylan chain).

Separation of the phenolic acids was carried out using Sephadex LH-20 chromatography with UV-detection at 325 nm. The sample was applied to the column (83 × 2 cm) beforehand conditioned with eluent 1 and chromatography was performed using the following eluents (given time periods may slightly differ from run to run; however, the elution profile given in Bunzel et al.<sup>19</sup> is very reproducible): (1) 0.5 mM TFA/MeOH 95/5 (v/v) for 73 h, flow rate: 1.5 mL min<sup>-1</sup>, (2) 0.5 mM TFA/MeOH 50/50 (v/v) for 53 h, flow rate: 1.0 mL min<sup>-1</sup>, (3) 0.5 mM TFA/MeOH 40/60 (v/v) for 64 h, flow rate: 1.0 mL min<sup>-1</sup>, and (4) rinsing step with 100% MeOH.

Compound **1** eluted in fraction 8<sup>19</sup> within the second elution step. Compounds **2**, **3**, and **4** co-eluted with 8-5-DFA (cyclic form) and 8-O-4-DFA (fraction 10).<sup>19</sup> Further fractionation was achieved by semipreparative RP-HPLC (Nucleosil 100-5 C18 HD; 250 × 10 mm) (flow rate: 2.7 mL min<sup>-1</sup>, column temperature: 45 °C, UV-detection at 325 nm). The gradient system to purify compound **1** consisted of A 1 mM TFA/MeOH 10/90 (v/v) and B 1 mM TFA/MeOH 90/10 (v/v): initially A 12%, B 88%, linear over 5 min to A 38%, B 62%, held isocratically for 5 min, linear over 5 min to A 50%, B 50%, held isocratically for 5 min, linear over 5 min to A 88%, B 12%, held isocratically for 5 min, following an equilibration step. A gradient system consisting of

A 1 mM TFA and B MeOH was used for the separation of compounds **2**, **3**, and **4**: initially A 90%, B 10%, linear over 10 min to A 60%, B 40%, held isocratically for 5 min, linear over 5 min to A 50%, B 50%, held isocratically for 5 min, linear over 5 min to A 20%, B 80%, held isocratically for 5 min, following an equilibration step. The structures of the purified compounds **1** (3.0 mg), **2** (1.7 mg), **3** (1.2 mg), and **4** (0.8 mg) were characterized as follows.

UV-spectra shown in Figure 2 were generally recorded in 1 mM TFA/MeOH 50/50 (v/v). Compound **1** shows absorption maxima at 221, 265, and 300 nm. HPLC-ESI-MS (generally performed in the negative and in the positive mode resulting in [M-H]<sup>-</sup> or in [M+H]<sup>+</sup>/[M+Na]<sup>+</sup>/[M+K]<sup>+</sup>/[M+NH<sub>4</sub>]<sup>+</sup>) identified the molecular weight of compound **1** to 358. Using the usual array of 1D and 2D NMR experiments (<sup>1</sup>H, <sup>13</sup>C, COSY, HSQC/HMQC, and HMBC) compound **1** was unambiguously identified as (2*E*)-3-{4-hydroxy-3-[(4-hydroxy-3-methoxyphenyl)acetyl]-5-methoxyphenyl}acrylic acid (Fig. 1). High resolution-mass spectrometry (HR-MS) confirmed the formula C<sub>19</sub>H<sub>18</sub>O<sub>7</sub> ([M+Na]<sup>+</sup> 381.09489; C<sub>19</sub>H<sub>18</sub>O<sub>7</sub>Na requires 381.09502). Proton NMR of compound **1** revealed two methoxy groups and five aromatic protons, suggesting two aromatic rings. However, two 16 Hz doublets indicate only one *trans*-cinnamic acid side chain, but an additional methylene group was



**Figure 2.** UV-spectra of (a) compound **1**, (b) compound **2**, (c) compound **3**, and (d) compound **4** recorded in 1 mM TFA/MeOH 50/50 (v/v).

proposed from the singlet at 4.41 ppm. Long-range  $^{13}\text{C}$ – $^1\text{H}$  correlations from the HMBC experiment showed the *trans*-cinnamic acid side chain attached to a 5-linked guaiacyl ring A (with aromatic protons at the 2- and 6-positions) and the methylene group attached to a guaiacyl ring B (with aromatic protons at the 2-, 5-, and 6-positions). Furthermore, the methylene group is linked via a keto group to the 5-carbon of ring A. The structure resulting from NMR interpretation is presented in Figure 1. The unambiguously assigned  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for compound **1** are shown in Table 1.

Compound **2** produces a UV-spectrum with absorption maxima at 235 and 313 nm. The molecular weight of 344 (from HPLC–ESI–MS) and the NMR data unambiguously identified compound **2** as (*E*)-3-(5'-formyl-2',6-dihydroxy-3',5-dimethoxybiphenyl-3-yl) acrylic acid. The results from HR–MS confirmed the formula  $\text{C}_{18}\text{H}_{16}\text{O}_7$  ( $[\text{M}+\text{Na}]^+$  367.07979;  $\text{C}_{18}\text{H}_{16}\text{O}_7\text{Na}$  requires 367.07937). Proton NMR showed signals for two methoxy groups, four aromatic protons, and only one *trans*-cinnamic acid side chain. A singlet at 9.86 ppm (Table 1) suggested an aldehyde group as confirmed by  $^{13}\text{C}$  NMR data (191.7 ppm, Table 1) and the molecular formula.

**Table 1.** NMR-data for compounds **1** and **2** in acetone- $d_6$ ,  $\delta$  in parts per million,  $J$  in hertz

Compound 1			Compound 2		
Dimer unit	H <sup>b</sup>	C <sup>c</sup>	Dimer unit	H <sup>a</sup>	C <sup>c</sup>
A1		126.21	A1		126.74
A2	7.58 (1H, d, $J = 1.9$ )	115.88!	A2	7.38 (1H, d, $J = 2.0$ )	110.16
A3		150.43	A3		148.86!!
A4		155.80	A4		147.41
A5		119.87	A5		124.93
A6	8.10 (1H, d, $J = 1.9$ )	124.85	A6	7.21 (1H, d, $J = 2.0$ )	125.99
A7	7.66 (1H, d, $J = 16.0$ )	144.83	A7	7.63 (1H, d, $J = 15.9$ )	145.84
A8	6.53 (1H, d, $J = 16.0$ )	117.69	A8	6.43 (1H, d, $J = 15.9$ )	116.35
A9		167.81	A9		168.01
OMe A3	3.94 (3H, s)	56.62	OMe A3	3.98 (3H, s)	56.58!!!
B1		126.50	B1		129.76
B2	7.02 (1H, d, $J = 1.9$ )	114.08	B2	7.45 (1H, d, $J = 1.9$ )	109.28
B3		148.37	B3		149.08!!
B4		146.50	B4		150.97
B5	6.79 (1H, d, $J = 8.1$ )	115.81!	B5		125.46
B6	6.83 (1H, dd, $J = 1.9, 8.1$ )	123.10	B6	7.53 (1H, d, $J = 1.9$ )	129.50
B7	4.41 (2H, s)	46.65	B7	9.86 (1H, s)	191.17
B8		206.25 <sup>d</sup>			
OMe B3	3.83 (3H, s)	56.28	OMe B3	3.98 (3H, s)	56.52!!!

!//!!!—assignments may be interchanged.

<sup>a</sup> Spectra were run at 500 MHz.

<sup>b</sup> Spectra were run at 360 MHz.

<sup>c</sup> Spectra were run at 90 MHz.

<sup>d</sup> From 2D HMBC experiment.

Long-range  $^{13}\text{C}$ - $^1\text{H}$  correlations revealed that the *trans*-cinnamic acid side chain and the aldehyde group are each attached to a 5-linked guaiacyl ring (A, B) (with aromatic protons only at the 2- and 6-positions), thus leading to the structure shown in Figure 1.

Compound **3** produces spectra with UV absorption maxima at 217, 235, and 317 nm and its molecular mass was determined to 578 (HPLC–ESI-MS). Data from 1D and 2D NMR experiments (Table 2) identified compound **3** as 8-O-4/8-5(non-cyclic)-dehydrotriferulic acid, (*2E*)-2-(5-((*Z*)-2-carboxy-2-[4-((*E*)-2-carboxyvinyl)-2-methoxyphenoxy]vinyl)-2-hydroxy-3-methoxyphenyl)-3-(4-hydroxy-3-methoxyphenyl)acrylic acid, consistent with results from HR-MS, suggesting  $\text{C}_{30}\text{H}_{26}\text{O}_{12}$  as formula ( $[\text{M}+\text{Na}]^+$  601.13188;  $\text{C}_{30}\text{H}_{26}\text{O}_{12}\text{Na}$  requires 601.13219). Three methoxy groups, eight aromatic protons, one *trans*-cinnamic acid side chain, and two further singlet vinylic protons were indicated by proton NMR, suggesting a ferulic acid dehydrotrimer structure. Long-range  $^{13}\text{C}$ - $^1\text{H}$  correlations from the HMBC experiment showed the attachment of the *trans*-cinnamic acid side chain to guaiacyl ring A with aromatic protons in 2-, 5-, and 6-position, suggesting a linkage via the

phenolic hydroxyl group. Two singlets at 7.42 and 7.75 ppm linked to carbons with chemical shifts at 128.43 and 141.75 ppm indicate that units B and C are linked via their 8-positions. The latter suggests the 8-O-4-coupling of units A and B. Unit B has two aromatic protons at its 2- and 6-positions indicating further linkage via its 5- position. The corresponding 8-5-coupling between units C and B was unambiguously shown in the HMBC experiment and all other correlation peaks from the HMBC experiment were fully consistent with the structure of compound **3** presented in Figure 1. The *E/Z*-configuration of the central vinyl groups is logically assumed from the appropriate dimers. The (*Z*)-configuration of the central vinyl group in the 8-O-4-coupled part (unit B, Fig. 1) has already been observed for the dimerization of ferulate esters<sup>6</sup> and also for (*E*)-coniferaldehyde dimerization and in the 8-O-4-cross-coupling between a ferulate ester and coniferyl alcohol oligomers.<sup>20</sup> Similarly, the configuration of the vinyl unit in the 8-5-coupled part (unit C) is logically assumed to be (*E*).

The UV-spectrum of compound **4** has an absorption maximum at 317 nm. The molecular weight of 596 (from

**Table 2.** NMR-data for compounds **3** and **4** in acetone- $d_6$ ,  $\delta$  in parts per million,  $J$  in hertz

Compound 3			Compound 4		
Trimer unit	H <sup>a</sup>	C <sup>b</sup>	Trimer unit	H <sup>a</sup>	C <sup>b</sup>
A1		130.12 <sup>d</sup>	A1		126.68 <sup>d</sup>
A2	7.35 (1H, d, $J = 1.8$ )	112.20 <sup>c</sup>	A2	7.33 (1H, d, $J = 2.1$ )	109.77 <sup>c</sup>
A3		150.15 <sup>d</sup>	A3		148.97 <sup>d</sup>
A4		148.84 <sup>d</sup>	A4		147.28 <sup>d</sup>
A5	6.78 (1H, d, $J = 8.2$ )	114.17 <sup>c</sup>	A5		126.61 <sup>d</sup>
A6	7.09 (1H, dd, $J = 1.8, 8.2$ )	122.94 <sup>c</sup>	A6	7.12 (1H, d, $J = 2.1$ )	126.29 <sup>c</sup>
A7	7.57 (1H, d, $J = 15.9$ )	145.31 <sup>c</sup>	A7	7.61 (1H, d, $J = 15.8$ )	145.99 <sup>c</sup>
A8	6.41 (1H, d, $J = 15.9$ )	117.42 <sup>c</sup>	A8	6.39 (1H, d, $J = 15.8$ )	116.11 <sup>c</sup>
A9		167.88 <sup>d</sup>	A9		168.00 <sup>d</sup>
OMe A3	3.94 (3H, s)	56.30 <sup>c</sup>	OMe A3	3.96 (3H, s)	56.50 <sup>c</sup>
B1		126.31 <sup>d</sup>	B1		132.31 <sup>d</sup>
B2	7.57 (1H, d, $J = 1.9$ )	112.83 <sup>c</sup>	B2	7.29 (1H, d, $J = 1.9$ )	110.70 <sup>c</sup>
B3		148.67 <sup>d</sup>	B3		148.19 <sup>d</sup>
B4		147.44 <sup>d</sup>	B4		144.29 <sup>d</sup>
B5		125.44 <sup>d</sup>	B5		124.63 <sup>d</sup>
B6	7.19 (1H, d, $J = 1.9$ )	127.97 <sup>c</sup>	B6	7.07 (1H, d, $J = 1.9$ )	122.88 <sup>c</sup>
B7	7.42 (1H, s)	128.43 <sup>c</sup>	B7	5.24 (1H, d, $J = 4.1$ )	74.97 <sup>c</sup>
B8		138.57 <sup>d</sup>	B8	4.90 (1H, d, $J = 4.1$ )	83.55 <sup>c</sup>
B9		164.50 <sup>d</sup>	B9		170.41 <sup>d</sup>
OMe B3	3.78 (3H, s)	55.36 <sup>c</sup>	OMe B3	3.89 (3H, s)	56.40 <sup>!!c</sup>
C1		127.69 <sup>d</sup>	C1		129.69 <sup>d</sup>
C2	6.57 (1H, d, $J = 1.9$ )	112.84 <sup>c</sup>	C2	7.32 (1H, d, $J = 2.1$ )	112.24 <sup>c</sup>
C3		147.76 <sup>d</sup>	C3		151.13 <sup>d</sup>
C4		148.90 <sup>d</sup>	C4		150.75 <sup>d</sup>
C5	6.68 (1H, d, $J = 8.2$ )	115.50 <sup>c</sup>	C5	6.81 (1H, d, $J = 8.3$ )	116.16 <sup>c</sup>
C6	6.81 (1H, dd, $J = 1.9, 8.2$ )	126.64 <sup>c</sup>	C6	7.11 (1H, dd, $J = 2.1, 8.3$ )	123.06 <sup>c</sup>
C7	7.75 (1H, s)	141.75 <sup>c</sup>	C7	7.56 (1H, d, $J = 15.9$ )	145.27 <sup>c</sup>
C8		126.45 <sup>d</sup>	C8	6.40 (1H, d, $J = 15.9$ )	117.32 <sup>c</sup>
C9		168.52 <sup>d</sup>	C9		167.83 <sup>d</sup>
OMe C3	3.29 (3H, s)	56.15 <sup>c</sup>	OMe C3	3.88 (3H, s)	56.40 <sup>!!c</sup>

!—Assignments may be interchanged; !!—one unresolved contour in HSQC.

<sup>a</sup> Spectra were run at 600 MHz.

<sup>b</sup> Spectra were run at 150 MHz (F1).

<sup>c</sup> Signal assignments were from HSQC.

<sup>d</sup> Signal assignments were from HMBC.

HPLC–ESI–MS) exceeds the molecular weight of a tri-ferulic acid by 18. The assumption that this compound contains an additional water was confirmed by the formula  $C_{30}H_{28}O_{13}$  as determined by HR–MS ( $[M+Na]^+$  619.14268  $C_{30}H_{28}O_{13}Na$  requires 619.14275). Compound **4** was unambiguously identified as 5-5/8-O-4( $H_2O$ )-dehydrotriferulic acid, (2*E*)-3-(4-{1-carboxy-2-[5'-((*E*)-2-carboxyvinyl)-2',6-dihydroxy-3',5-dimethoxybiphenyl-3-yl]-2-hydroxyethoxy}-3-methoxyphenyl)acrylic acid, using 1D and 2D NMR experiments (data in Table 2). Proton NMR revealed three methoxy groups, seven aromatic protons, two *trans*-cinnamic acid side chains, and two characteristic protons showing doublet signals at 5.24 and 4.90 ppm ( $J = 4.1$  Hz). Long-range  $^{13}C$ – $^1H$  correlations showed one *trans*-cinnamic acid side chain attached to an unsubstituted guaiacyl ring C (with aromatic protons at the 2-, 5-, and 6-positions), suggesting the linkage of unit C via its phenolic hydroxyl group. The second *trans*-cinnamic acid side chain was attached to a 5-linked guaiacyl ring A (aromatic protons only at its 2- and 6-positions). Guaiacyl ring B with only two protons, at its 2- and 6-positions, was shown to be 5-5-linked with unit A. The side chain of unit B is hydrated explaining the two characteristic doublet signals at 5.24 and 4.90 ppm. Next to the coupling to unit A via a 5-5-linkage, unit B is linked by an 8-O-4-linkage to unit C as unambiguously shown by long-range correlations. The resulting structure of compound **4** is presented in Figure 1.

The biological significance of compound **1** in the plant remains unclear. This compound is obviously not involved in polysaccharide cross-linking since it has only one carboxylic acid group. To date, compounds related to **1** are not known in grasses.

Although we cannot exclude the possibility that compound **2** is a natural product, we rather assume that compound **2** derived from the corresponding 5-5-coupled diferulate by oxidative degradation. Oxidative degradation from ferulic acid to vanillin is a known reaction and we assumed a similar process for the 5-5-DFA although we tried to exclude oxygen during the saponification process. To confirm this theory we simulated worst case conditions and stirred 5-5-DFA in 2 M NaOH in an open pyrex tube for 138 h. The reaction mixture was analyzed by HPLC–DAD. Under these conditions several reaction products were found, one of them being compound **2**. The peak area at 280 nm of compound **2** represented 17% of the peak area of the parent 5-5-DFA.

Both newly identified triferulates **3** and **4** represent possible polysaccharide cross-links. Although these compounds are minor compounds in maize bran fiber, they indicate that cross-linking of polysaccharides via triferulates is an important mechanism within the plant cell wall, strengthening the polymer network. However, we suppose that compound **3** does not naturally occur in the plant but derives from an 8-O-4/8-5(cyclic)-coupled triferulate (**3a**, Fig. 1). This assumption is based on studies of 8-5-coupled diferulates.<sup>6</sup> The cyclic form of the 8-5-dehydrodiferulate is the only one formed under biometric conditions using  $H_2O_2$ /peroxidase. Saponification

of the ester (e.g., the ethyl ester) gives rise to three compounds, the cyclic form, the non-cyclic (open), and the decarboxylated form of the 8-5-coupled DFA. We reason by analogy that not compound **3** but the related cyclic form **3a** should be the natural product in the plant.

Regarding the structure of triferulate **4**, the addition of water to the side chain of unit B is surprising. In the formation of 8-O-4-diferulates, the elimination of the acidic 8-proton of the quinone methide directly resulting from the coupling reaction proved to be faster than the addition of water.<sup>6</sup> Consequently, no analogous 8-O-4-diferulate with additional water was found from plant extracts so far. Although such a dimer cannot be excluded it is also possible that addition of water to the 7-position of the quinone intermediate during the radical coupling process is some peculiar feature of the trimerization process.

In conclusion, the four new products identified here from maize bran expand the number of products potentially involved in cell wall cross-linking. Compound **2** is the least novel, likely arising via degradation of the known 5-5-diferulate. Trimer **3** indicates the presence of an 8-O-4/8-5-triferulate (never identified previously), and trimer **4** is a novel variation on the 5-5/8-O-4-trimer observed previously.<sup>15</sup> The derivation and likely role of compound **1** remains uncertain. In total, however, these findings imply that cross-coupling reactions in the cell wall are more extensive and more varied than previously recognized.

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