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Isolation and structural characterisation of 8–O–4/8–O–4- and 8–8/8–O–4-coupled dehydrotriferulic acids from maize bran

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Abstract

Two new dehydrotriferulic acids were isolated from saponified maize bran insoluble fiber using Sephadex LH-20 chromatography followed by semi-preparative RP-HPLC. Based on UV-spectroscopy, mass spectroscopy and one- and two-dimensional NMR experiments, the structures were identified as 8–O–4,8–O–4-dehydrotriferulic acid and 8–8(cyclic),8–O–4-dehydrotriferulic acid. Which of the possible phenols in the initially formed 8–8-dehydrodiferulate was etherified by 4–O–8-coupling with ferulate has been unambiguously elucidated. The ferulate dehydrotrimers which give rise to these dehydrotriferulic acids following saponification are presumed, like the dehydrodiferulates, to cross-link polysaccharides. Neither dehydrotriferulic acid described here involves a 5–5-dehydrodiferulic acid unit; only the 5–5-dehydrodimer may be formed intramolecularly. However, whether dehydrotriferulates are capable of cross-linking more than two polysaccharide chains remains open. Although the levels of the isolated ferulate dehydrotrimers are lower than those of the ferulate dehydrodimers, the isolation now of three different dehydrotriferulates indicates that trimers contribute to a strong network cross-linking plant cell wall polysaccharides.

Keywords: *Zea mays* L.; Gramineae; Maize bran; Cell wall cross-linking; Triferulic acid; Triferulate; Ferulic acid; Ferulate; Arabinoxylans; Dietary fibre

1. Introduction

Hydroxycinnamic acids have a significant role in cross-linking plant cell wall polymers, especially polysaccharides and lignin (Lam et al., 1992; Ralph et al., 1998b, 2004a). Although sinapates (Bunzel et al., 2003b) and *p*-coumarates (Hartley et al., 1990a) also form dimers, ferulic acid is probably the most important hydroxycinnamic acid for cross-linking polysaccharides in the plant cell wall (Ishii, 1997; Ralph et al., 1998b, 2004a). In grasses, ferulic acid acylates the primary C5-OH of α-L-arabinosyl moieties of arabinoxylans (Kato and Nevins, 1985; Ishii, 1997). In some dicots, ferulic acid is linked to pectins (Ishii, 1997). Although ferulate dimerisation is possible by photochemical (Hartley et al., 1990b) and radical coupling (Ralph et al., 1994) mechanisms, radically coupled dehydrodiferulic acids (from dehydrodiferulates) usually predominate over photochemically coupled diferulate cyclobutane derivatives in alkaline hydrolysates of grasses. A whole range of dehydrodiferulates was synthesised (Ralph et al., 1994, 1998a) and identified in a variety of samples (Bunzel et al., 2001; Micard et al., 1997; Parker et al., 2003; Parr et al., 1997; Ralph et al., 1994; Waldron

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et al., 1997a). The dehydrodiferulates are characterised by the new bond formed between the two ferulates (by coupling at their 4–O-, 5- or 8-carbons) as 5–5-, 8–8-, 8–5-, 8–O–4-, and 4–O–5-coupled dehydrodimers.

Cross-linking of cell wall-polymers via diferulate bridges is of substantial interest in plant physiology, agricultural chemistry, food chemistry and food technology. Termination of the expansion of the cell growth in grasses (Kamisaka et al., 1990; Tan et al., 1992) and wall stiffening (Zarra et al., 1999), thermal stability of cell adhesion and maintenance of crispness of plant-based foods (e.g., water chestnuts) after cooking (Parker et al., 2003; Parr et al., 1996; Waldron et al., 1997b), gelling properties of sugar beet pectins and other food compounds (Neukom and Markwalder, 1978; Ng et al., 1997; Oosterveld et al., 1997), insolubility of cereal dietary fibres (Bunzel et al., 2001), and limited cell wall degradability by ruminants (Grabber et al., 1998; Hatfield et al., 1999) are related to the formation of diferulate cross-links.

Higher ferulic acid oligomers and polymers can be demonstrated in vitro (Ward et al., 2001) and have also been discussed for in vivo processes. Fry's group, investigating maize suspension cultures for these products (Fry et al., 2000), speculated that radical polymerization of ferulates does not stop at the dimeric stage in vivo, but forms higher oligomers. Recently, the first ferulic acid dehydrotrimer was isolated and structurally characterised (Bunzel et al., 2003a; Rouau et al., 2003). This dehydrotriferulic acid involves ferulates coupled by a 5–5- and an 8–O–4-linkage. From molecular modelling studies it is likely that the 5–5-diferulate is the only one that can form intramolecularly (Hatfield and Ralph, 1999). Owing to the involvement of a 5–5-linkage in the identified trimer, its isolation need not implicate the cross-linking of three polysaccharide chains. More indications for cross-linking three polysaccharide chains by ferulic acid dehydrotrimers would be attained by isolating trimers that contain only 8–8-, 8–5-, 8–O–4-, or 4–O–5-, but no 5–5-linkages.

In this paper, we describe the isolation and structural characterisation of an 8–O–4/8–O–4-coupled dehydrotrimer, and an 8–8/8–O–4-coupled dehydrotrimer, and discuss their significance for cell wall cross-linking.

2. Results and discussion

2.1. Structural identification of compound 1 (8–O–4/8–O–4-dehydrotriferulic acid)

The recently developed method for the isolation of dehydrodiferulic acids and the 5–5/8–O–4-coupled dehydrotriferulic acid from saponified maize bran insoluble fiber on semi-preparative scale provided several fractions that contained multiple and/or unidentified phenolic compounds (Bunzel et al., 2004). Using Sephadex LH-20 chromatography, monomeric compounds (eluted with 0.5 mM TFA/MeOH 95/5, first eluent) were fully separated from dimeric/trimeric phenolic compounds. However, it was not possible to separate dimeric compounds from trimeric compounds. Compound **1** (Fig. 1) eluted with the third eluent (0.5 mM TFA/MeOH 40/60) in fraction **1** (Fig. 2). Analytical RP-HPLC and ^{1}H NMR-spectra showed that this fraction (7.7 mg) did not contain a pure compound. Further fractionation was achieved using semi-preparative RP-HPLC. The purity of compound **1** was estimated to be >97% (3.3 mg).

The UV-spectrum of compound **1** shows characteristics of ferulic acid structures (maxima at 294 and 319 nm) (Fig. 3, top). The molecular weight of this compound was determined by HPLC-ESI-MS. Negative-ion MS gave a high mass of *m/z* 577 $[M - H]^-$ (base peak) suggesting a molecular weight of 578. Positive-ion MS showed a base peak of *m/z* 617, deriving from the potassium adduct ion $[M + K]^+$. A sodium adduct ion was *m/z* 601 $[M + Na]^+$ (6% of base peak), also suggesting a molecular weight of 578 indicating a triferulic acid structure. Results from high-resolution mass spectrometry confirm the formula as $C_{30}H_{26}O_{12}$.

Using the usual array of 1D and 2D NMR experiments, compound **1** was unambiguously identified as the 8–O–4/8–O–4-coupled ferulic acid dehydrotrimer. ^{1}H and ^{13}C NMR (in acetone-d_6, Table 1) showed the signals of 22 protons and 30 carbons. The spectral data revealed three methoxyl groups, three carboxylic groups and three aromatic rings, supporting the MS data suggesting a dehydrotriferulic acid. Consideration of the coupling constants of the aromatic proton signals and the H,H-COSY-spectra indicates protons at the 2-, 5- and 6-carbons of each aromatic ring. This indicates that the ferulic acid units are linked via two 8–O–4-linkages. This contention is proven by presence of only two 16 Hz proton doublets (at 6.44 and 7.59 ppm), indicating just one *trans*-cinnamic acid side chain, but two singlet protons (at 7.38 and 7.42 ppm) characteristic of the unsaturated 7-protons in 8–O–4-coupled products. Based upon the 2D experiments, each of the three propenyl side chains was associated with its corresponding aromatic ring. Whereas the ferulic acid unit with both protons at the 7- and 8-carbons was clearly assigned as an terminal unit (C), the remaining ferulic acid units with only one proton in the sidechain (at carbon-7) could not definitively be assigned as terminal or interior units. The only difference between these two units is the free-phenolic hydroxyl group of the terminal ferulic acid unit (A). In order to observe a proton signal for the phenolic hydroxyl group and detect couplings to the aromatic protons, the standard 1D and 2D (for signal assignment) experiments were rerun in DMSO-d_6, a solvent which minimises proton exchange and therefore sharpens

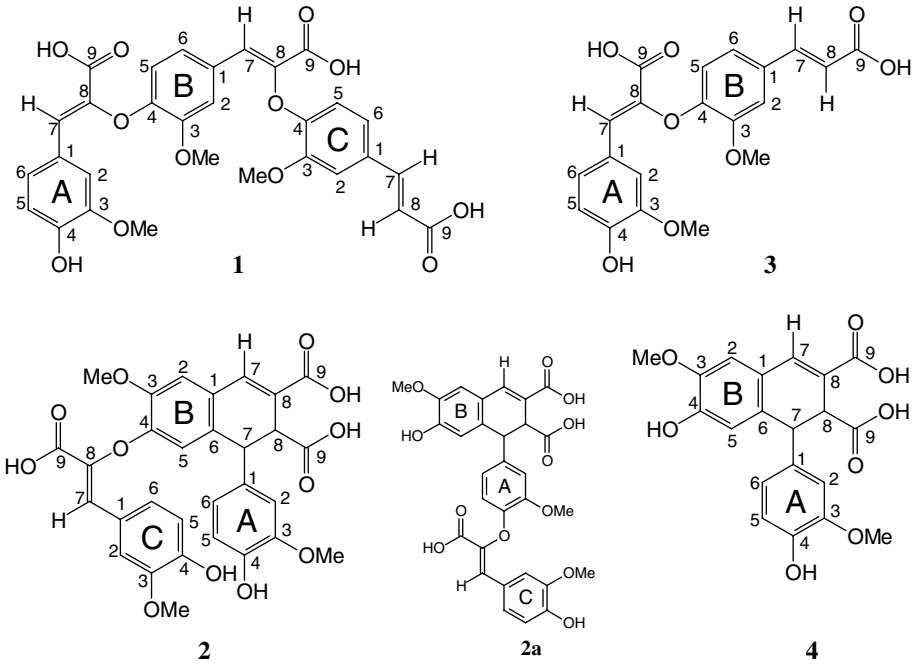

Fig. 1. Structures and numbering system of the new ferulic acid deydrotrimers **1** and **2** along with the dehydrodimers **3**, **4** and the structure of a hypothetical dehydrotrimer regioisomer **2a**.

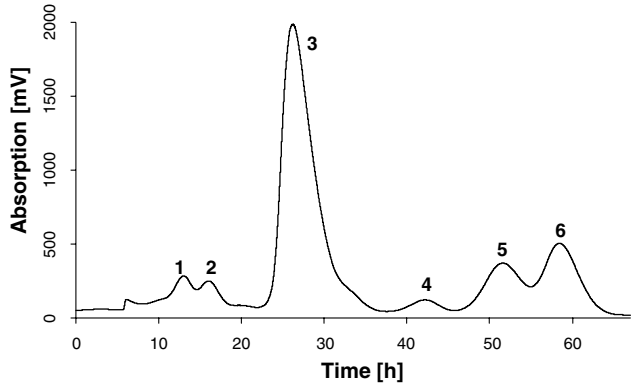

Fig. 2. Sephadex LH-20 chromatography to isolate compound **1**. Compound **1** eluted in fraction 1 using the third eluent (0.5 mM TFA/MeOH 40/60). Fraction 3 contains 5–5-dehydrodiferulic acid, fraction 5 5–5/8–O–4-dehydrotriferulic acid, fraction 6 the decarboxylated form of 8–5-dehydrodiferulic acid and fractions 2 and 4 contain unknown compounds.

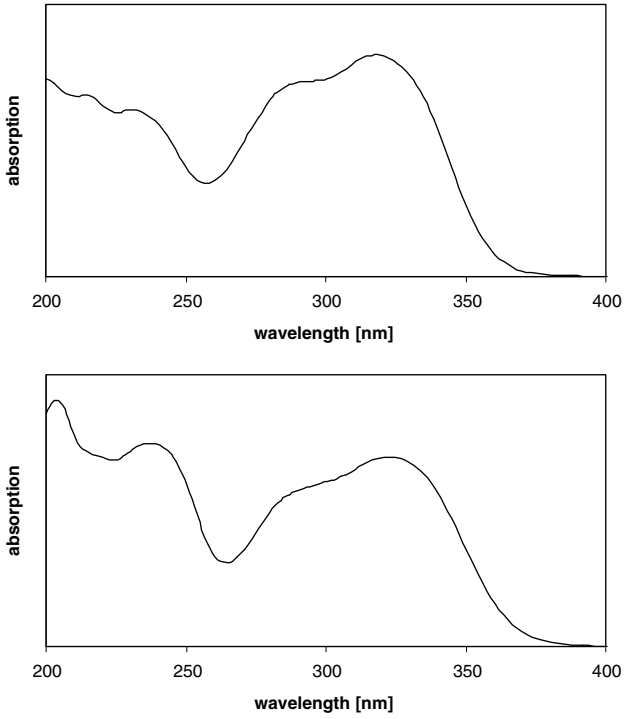

Fig. 3. UV-spectra of compounds **1** (8–O–4/8–O–4-dehydrotriferulic acid) (top) and **2** (8–8(cyclic)/8–O–4-dehydrotriferulic acid) (bottom).

OH-resonances (including those from phenols). The proton spectra showed a singlet at 9.53 ppm, caused by the proton of the phenolic hydroxyl group. The ROESY-spectra showed a coupling between this proton and the proton H5 of one ferulic acid unit, which therefore was assigned as belonging to unit A. The unambiguously assigned proton NMR data for compound **1** in DMSO-d_6 is summarized in Table 1. The ^{13}C NMR data are not listed because the small sample amount impeded definitive assignments for all signals within the time constraints for acquiring spectra; full ^{13}C NMR data are provided for the compound in acetone-d_6. Table 1 also shows the comparison of the individual trimer units A, B and C with units A and B of the dimer 8–O–4-dehydrodiferulic acid (**3**, Fig. 1). The trimer units A and C are well modelled by this compound, whereas it is not possible to model trimer unit B satisfactorily with this

Table 1
NMR-data for compound **1**, 8–O–4/8–O–4-dehydrotriferulic acid, δ in ppm, J in Hz

Trimer unit	H			C	
	In acetone-d_6	Ref. in acetone-d_6	In DMSO-d_6	In acetone-d_6	Ref. in acetone-d_6
A1				125.1	125.3[a]
A2	7.45 (1H, *d*, 1.9)	7.52 (1H, *d*, 2.0)[a]	7.28 (1H, *d*, 1.7)	113.8	113.8[a]
A3				148.3	148.3[a]
A4				149.4	149.5[a]
A5	6.79 (1H, *d*, 8.2)	6.82 (1H, *d*, 8.2)[a]	6.71 (1H, *d*, 8.2)	115.9	116.0[a]
A6	7.19 (1H, *dd*, 8.2, 1.9)	7.23 (1H, *dd*, 8.2, 2.0)[a]	7.07 (1H, *dd*, 8.2, 1.7)	125.9	126.1[a]
A7	7.38 (1H, *s*)	7.42 (1H, s)[a]	7.29 (1H, *s*)	128.2	128.5[a]
A8				138.3	138.3[a]
A9				164.7	164.5[a]
A3-OMe	3.65 (3H, *s*)	3.73 (3H, *s*)[a]	3.53 (3H, *s*)	55.8	55.9[a]
A4-OH			9.53 (1H, s)		
B1				127.9	130.1[b]
B2	7.63 (1H, *d*, 1.9)	7.44 (1H, *d*, 2.0)[b]	7.50 (1H, *d*, 1.6)	114.6	112.4[b]
B3				149.7	150.2[b]
B4				148.3	148.9[b]
B5	6.78 (1H, *d*, 8.5)	6.83 (1H, *d*, 8.3)[b]	6.64 (1H, *d*, 8.2)	114.4	114.4[b]
B6	7.23 (1H, *dd*, 8.5, 1.9)	7.13 (1H, *dd*, 8.3, 2.0)[b]	7.14 (1H, *dd*, 8.2, 1.6)	125.0	122.9[b]
B7	7.42 (1H, *s*)	7.59 (1H, *s*)[b]	7.33 (1H, *s*)	127.4	145.3[b]
B8				139.6	117.5[b]
B9				164.5	167.9[b]
B3-OMe	3.82 (3H, *s*)	4.00 (3H, *s*)[b]	3.72 (3H, *s*)	56.0	56.5[b]
C1				130.2	130.1[b]
C2	7.44 (1H, *d*, 1.9)	7.44 (1H, *d*, 2.0)[b]	7.43 (1H, *d*, 1.7)	112.4	112.4[b]
C3				150.1	150.2[b]
C4				148.7	148.9[b]
C5	6.82 (1H, *d*, 8.2)	6.83 (1H, *d*, 8.3)[b]	6.67 (1H, *d*, 8.2)	114.2	114.4[b]
C6	7.12 (1H, *dd*, 8.2, 1.9)	7.13 (1H, *dd*, 8.3, 2.0)[b]	7.10 (1H, *dd*, 8.2, 1.7)	122.8	122.9[b]
C7	7.59 (1H, *d*, 16.0)	7.59 (1H, *s*)[b]	7.50 (1H, *d*, 16.0)	145.1	145.3[b]
C8	6.44 (1H, *d*, 16.0)	6.43 (1H, *d*, 15.9)[b]	6.45 (1H, *d*, 16.0)	117.7	117.5[b]
C9				168.2	167.9[b]
C3-OMe	3.99 (3H, *s*)	4.00 (3H, *s*)[b]	3.89 (3H, *s*)	56.5	56.5[b]

[a/b]From a previous study (Ralph et al., 1994), and in NMR database (Ralph et al., 2004b), compound number 2040, 8–O–4-dehydrodiferulic acid (Fig. 1, **3**).
[(a)]Ring A, [(b)]ring B.
Abbreviations used: *s* – singlet, *d* – doublet, *dd* – doublet of doublets.
Note that the modelling of the aromatic ring in trimer unit B by this compound will not be particularly good since it is additionally 8-linked. The C-ring of the trimer is well modelled by ring B of the dimer.

8–O–4-dehydrodiferulic acid due to the additional 8-coupling in comparison to unit B of the dimer.

2.2. Structural identification of compound **2** (8–8(cyclic)/8–O–4-dehydrotriferulic acid)

Compound **2** eluted as a fraction (50.2 mg) together with 8–5-coupled dehdrodiferulic acid (non-cyclic form) and some unidentified compounds with the second eluent (0.5 mM TFA/MeOH 50/50). Further semi-preparative RP-HPLC separated compound **2** from the major compound in this fraction, 8–5(non-cyclic)-dehydrodiferulic acid, and some other minor compounds to yield 3.0 mg of fairly clean compound **2** (> 90%).

The UV-spectrum shows a maximum at 323 nm characteristic of a ferulic acid derivative (Fig. 3, bottom). HPLC-ESI-MS was used to determine the molecular weight of this compound. Again, the negative-ion MS gave a high mass of *m/z* 577 $[M - H]^-$ (base peak) suggesting a weight of 578. Using positive-ion MS the potassium adduct ion *m/z* 617 $[M + K]^+$ formed the base peak. Together with the sodium adduct ion *m/z* 601 $[M + Na]^+$ (6% of base peak) it also suggested a molecular weight of 578 indicating a triferulic acid structure. High-resolution mass spectrometry confirmed the formula as $C_{30}H_{26}O_{12}$.

Signals of 21 protons and 30 carbon signals were observed in the 1D NMR spectra in acetone-d_6. Carbon signals at 173.2, 168.2 and 164.4 ppm (Table 2) indicated three carboxylic groups. Due to their chemical shifts (3.61, 3.69, and 3.97 ppm), three proton signals (in relation with their carbon shifts) each comprising three protons were logically assigned to be methoxyl groups. Two doublets of doublets at 6.36 and 7.18 ppm with the coupling constants J = 8.2/2.1 and J = 8.2/1.9 Hz indicated two aromatic systems with protons in *ortho* and *meta* positions (protons at the 2-, 5-, and 6-carbons in ferulic acid units) showing that these units are not coupled at

Table 2
NMR-data for compound **2**, 8–8(cyclic)/8–O–4-dehydrotriferulic acid, δ in ppm, J in Hz

Trimer unit	H			C		
	In acetone-d_6	Ref. in acetone-d_6	In DMSO-d_6	In acetone-d_6	Ref. in acetone-d_6	In DMSO-d_6
A1				135.5	136.1[c]	134.1[*]
A2	6.59 (1H, *d*, 2.1)	6.79 (1H, *d*, 2.0)[c]	6.46 (1H, *bs*)	111.9	112.0[c]	111.3
A3				148.0	148.1[c]	147.1
A4				146.0	146.1[c]	144.9
A5	6.57 (1H, *d*, 8.2)	6.64 (1H, *d*, 8.2)[c]	6.48 (1H, *d*, 8.2)	115.4	115.5[c]	115.0
A6	6.36 (1H, *dd*, 8.2, 2.1)	6.42 (1H, *dd*, 8.2, 2.0)[c]	6.15 (1H, *dd*, 8.2, 1.3)	120.4	120.7[c]	118.9
A7	4.57 (1H, *d*, 1.9)	4.61 (1H, *br d*, 1.8)[c]	4.40 (1H, *s*)	45.7	46.0 [c]	43.9
A8	3.97 (1H, *d*, 1.9)	3.88 (1H, *d*, 1.8)	n.d.	47.5	48.1[c]	n.d.
A9				173.2	173.6[c]	172.9
A3-OMe	3.61 (3H, *s*)	3.74 (3H, *s*)[c]	3.51 (3H, *s*)	55.9!!!	56.2[c]	55.2
A4-OH			8.71 (1H, s)			
B1				127.2	124.6[d]	125.8
B2	7.23 (1H, *s*)	7.04 (1H, *s*)[d]	7.24 (1H, *bs*)	114.2	113.1[d]	113.1
B3				149.1!!	147.5[d]	147.3
B4				148.4!	149.3[d]	n.d.
B5	6.72 (1H, *s*)	6.71 (1H, *s*)[d]	6.53 (1H, *s*)	115.4	116.9[d]	113.6
B6				131.4	132.4[d]	130.0
B7	7.66 (1H, *s*)	7.60 (1H, *s*)[d]	n.d.	137.4	137.6[d]	n.d.
B8				125.2	124.3[d]	n.d.
B9				168.2	169.3[d]	n.d.
B3-OMe	3.97 (3H, *s*)	3.86 (3H, *s*)[d]	3.87 (3H, *s*)	56.6	56.4[d]	55.9
C1				125.3	125.3[a]	123.4
C2	7.46 (1H, *d*, 1.9)	7.52 (1H, *d*, 2.0)[a]	7.29 (1H, *d*, 1.4)	113.8	113.8[a]	113.2
C3				148.2!	148.3[a]	147.4
C4				149.3!!	149.5[a]	148.5
C5	6.79 (1H, *d*, 8.2)	6.82 (1H, *d*, 8.2)[a]	6.72 (1H, *d*, 8.3)	115.9	116.0[a]	115.5
C6	7.18 (1H, *dd*, 8.2, 1.9)	7.23 (1H, *dd*, 8.2, 2.0)[a]	7.08 (1H, *dd*, 8.3, 1.4)	126.0	126.1[a]	124.6
C7	7.36 (1H, *s*)	7.42 (1H, *s*)[a]	7.26 (1H, *s*)	128.3	128.5[a]	127.0
C8				138.4	138.3[a]	137.1
C9				164.4	164.5[a]	164.0
C3-OMe	3.69 (3H, *s*)	3.73 (3H, *s*)[a]	3.57 (3H, *s*)	56.0!!!	55.9[a]	55.0
C4-OH			9.55 (1H, *s*)			

[c/d]From a previous study (Ralph et al., 1994), and in NMR database (Ralph et al., 2004b), compound number 2036, 8–8(cyclic)-dehydrodiferulic acid (Fig. 1, 4). [(c)]ring A, [(d)]ring B.
!/!!/!!! – assignments may be interchanged.
Abbreviations used: *s* – singlet, *d* – doublet, *dd* –doublet of doublets, *bs* – broad singlet, *br d* – broad doublet, n.d. – not unambiguously detected.
Note that the modelling of the aromatic ring in trimer unit B by this compound will show some deviations since it is additionally 4–O-linked.
[a] From a previous study (Ralph et al., 1994), and in NMR database (Ralph et al., 2004b), compound number 2040, 8–O–4-dehydrodiferulic acid (Fig. 1, 3). [(a)]Ring A.
[*] From 2D-HMBC experiment.

their 5-positions. Due to the absence of proton signals showing coupling constants of around 16 Hz it was clear that compound **2** did not include any intact *trans*-cinnamic acid side-chains. However, the singlets at 7.36 and 7.66 ppm, in conjunction with their corresponding carbon shifts, are prime indicators that at least two units are 8-linked. Long-range correlations showed that one 8-coupled side chain is coupled to an aromatic ring with protons at carbons 2, 5 and 6 (C-unit, **2**, Fig. 1). The second 8-linked side-chain is coupled to an aromatic system with two protons *para* to each other showing singlets (H2 and H5, ring B, **2**, Fig. 1), indicating an additional linkage of this unit via its 6-position. Therefore, it was logical that units A and B form an 8–8(cyclic)-coupled unit, comparable to that in 8–8(cyclic)-dehydrodiferulic acid (**4**, Fig. 1). This assumption is supported by two proton doublets at 4.57 ppm (carbon shift 45.7 ppm) and 3.97 ppm (carbon shift 47.5 ppm) showing the saturated side-chain of unit A. Accordingly, in the HMBC experiment proton A7 (4.57 ppm) shows correlations to the aromatic systems of unit A and B as well to the side chain (carbon-8) of unit B. Full analysis of the 1D and 2D spectra provided the assignment given in Table 2 and proved the 8–8(cyclic)/8–O–4-triferulic acid structure. From the NMR-data in acetone-d_6 it is not possible to decide unambiguously if the trimer has the structure **2** or **2a** (Fig. 1). Although a comparison of the NMR-data with those data of 8–8(cyclic)-dehydrodiferulic acid and 8–O–4-dehydrodiferulic acids as models (see Table 2) slightly favours structure **2**, structure **2a** cannot excluded from these data. Thus the regiochemistry of the coupling reaction of ferulate with the preformed 8–8(cyclic)-dehydrodimer remained ambiguous. Again, NMR-experiments in DMSO-d_6 sharpened the

phenolic hydroxyl resonances and allowed observation of their correlated nuclei in 2D experiments. Phenolic hydroxyls had chemical shifts at 8.71 and 9.55 ppm. Long-range correlations showed that the phenolic proton at 8.71 ppm coupled to carbons of the aromatic ring A, whereas the phenolic proton at 9.55 ppm coupled to carbons being part of the aromatic system C. This indicates free phenolic hydroxyl-groups on units A and C whereas the phenolic group of unit B is etherified. Using this information structure **2a** was excluded and structure **2** was unambiguously established.

2.3. Ferulate dehydrotrimers as polysaccharide cross-links in plant cell walls

The isolation of 8–O–4/8–O–4- and 8–8(cyclic)/8–O–4-coupled dehydrotriferulic acids simplistically suggests that radical-coupled ferulate dehydrotrimers may be capable of cross-linking three polysaccharide chains in the plant cell wall. The former isolation of a 5–5/8–O–4-coupled dehydrotrimer (Bunzel et al., 2003a; Rouau et al., 2003) supported the results of Fry's group (Fry et al., 2000), suggesting higher ferulic acid oligomers, and was the first structurally characterised ferulic acid trimer. However, due to the involvement of a 5–5-coupled diferulic acid moiety in this trimer, we reported that the detection of this compound need not implicate the cross-linking of three polysaccharide chains (Bunzel et al., 2003a). Hatfield and Ralph (1999) used molecular modelling to evaluate the feasibility of intramolecular diferulate formation. These studies showed that it is rather unlikely that diferulates couple arabinoxylans in grass cell walls intramolecularly, with one exception: the 5–5-coupled diferulate is the only diferulate that can be formed without bond strain if the ferulates are positioned strategically on arabinose units attached to xylose units with two intervening xylose units on the same xylan backbone. The results from the molecular modelling study were consistent with the observations that low molecular weight ferulate esters do not favour 5–5-coupling, but the 5–5-diferulate is found in considerable amounts in plant cell walls, e.g. in plant cell walls of the maize grain, where it is the second most abundant after the 8–5-coupled diferulate (Bunzel et al., 2001). Therefore, the detection of the 8–O–4/8–O–4- and 8–O–4/8–8-coupled trimers is the first real hint that ferulates may couple three polysaccharide chains. However, coupling of three polysaccharide chains via a ferulic acid dehydrotrimer appears problematic as polysaccharide chains cross-linked through diferulates are rather rigid entities and it seems challenging to arrange another ferulate in sufficient proximity to produce radical-coupled triferulates. Although the possibility of cross-linking three polysaccharide chains by the identified compounds should not be excluded another possibility should be considered. The mentioned modelling studies did not include the possibility of "back-crossing", as detailed recently (Ralph et al., 2004a). Back-crossing means that two ferulates on two different polysaccharide chains may couple, followed by coupling of another ferulate on these chains with either of the two ferulate moieties already involved in the diferulate. The possibility remains, therefore, that even these newly isolated trimers may still only cross-link two polysaccharide chains. Determining whether two or three polysaccharide chains are involved in dehydrotriferulate cross-linking remains a formidable challenge.

The amounts of the isolated 8–O–4/8–O–4-triferulic acid (3.3 mg out of 30 g maize fibre) and 8–8(cyclic)/8–O–4-triferulic acid (3.0 mg from 40 g maize fibre) are rather low in comparison to the amount of isolated 5–5/8–O–4-triferulic acid (10 mg from 20 g maize fibre) and of diferulic acids (12.6 mg total dehydrodiferulic acids per g insoluble whole grain maize fibre (Bunzel et al., 2001)). However, it is difficult to draw unambiguous quantitative conclusions from preparative results. To assess the significance of dehydrotriferulic acids for cross-linking plant cell wall polymers it will be necessary to develop a method for the quantitative determination of these compounds and to screen these compounds in different plant cell walls.

3. Conclusion

The first ferulic acid dehydrotrimers devoid of 5–5-coupled moieties were identified from maize bran fiber. Such trimers are derived from initial ferulate dehydrodimers that cannot be formed intramolecularly. Unfortunately, the logical conclusion that cross-linking of three polysaccharide chains is involved may still not be warranted; the possibility that preformed dehydrodiferulates can "back-cross" to another ferulate monomer on one of the two chains cannot be discounted. Such possibilities now need to be considered more seriously, although unequivocal proof that dehydrotriferulates cross-link three polysaccharide chains will be a formidable challenge. With three dehydrotriferulic acids now identified, it is becoming clear that higher oligomers of ferulate, like the dehydrodimers from which they logically derive, play a role in extensively cross-linking cell wall polysaccharides.

4. Experimental

4.1. General experimental procedures

Heat-stable α-amylase Termamyl 120 L (E.C. 3.2.1.1, from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase 2.4 L (E.C. 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g) and the amyloglucosidase AMG 300 L

(E.C. 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were kindly donated by Novo Nordisk, Bagsvaerd, Denmark. Sephadex LH-20 was from Amersham Pharmacia Biotech (Freiburg, Germany). Equipment for Sephadex LH-20 chromatography (L-6000 pump, L-7400 UV-detector) was from Merck/Hitachi (Darmstadt, Germany). HPLC-columns were purchased from Macherey-Nagel (Düren, Germany). Semi-preparative and analytical RP-HPLC were carried out using the following instrumentation: L-6200 intelligent pump, T-6300 column thermostat, L-7400 preparative UV-detector (Merck/Hitachi, Darmstadt, Germany), analytical photodiode array detector (Model 994, Waters, Eschborn, Germany). HPLC-MS instrumentation was from Hewlett–Packard (Waldbronn, Germany): HP Series 1100: autosampler G 1313, pump G 1312A, mass spectrometer G 1946A. High-resolution ESI-mass spectrometry was performed on a Thermo-Finnigan (Austin, USA) instrument (MAT 95 XL). NMR experiments were performed on Bruker instruments (Rheinstetten, Germany): DMX 600i (cryogenic probe), DRX-500 and DRX-360.

4.2. Material

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

4.3. Preparation of insoluble maize fibre

Maize bran was pre-treated by extraction with acetone for 4 h using Soxhlet equipment. Pre-treated maize bran (20 g) was used to prepare insoluble maize fibre by an enzymatic procedure as described by Bunzel's group (Bunzel et al., 2001). Deviating from the referenced procedure 1.5 ml Termamyl, 600 µl protease and 700 µl amyloglucosidase were used and the heating time during the α-amylase treatment was prolonged to 30 min owing to doubled amount of starting material. The isolation procedure was performed several times.

4.4. Alkaline hydrolysis and extraction

The described dehydrotriferulic acids were isolated in two similar isolation processes, process **a** and process **b**, that differ in their details. The following procedure was performed: (**a**) six times, (**b**) eight times: Insoluble maize fiber (5 g) was saponified (2 M NaOH, 100 ml, degassed with N_2) in screw-cap tubes (200 ml) under nitrogen and protected from light for 18 h. Following acidification of the mixture (pH < 2, ~19 ml of 37% HCl) liberated phenolic acids were extracted into Et_2O (40 ml, three times). The combined Et_2O extracts of all (**a**) six preparations, (**b**) eight preparations were reduced to (**a**) 50 ml, (**b**) 60 ml (rotary evaporation) and extracted with $NaHCO_3$ solution (5%) ((**a**) 25 ml, three times; (**b**) 30 ml, four times). The aqeous layers were combined and acidified (pH < 2) with HCl (37%) and phenolic acids were re-extracted into Et_2O ((**a**) 30 ml, three times; (**b**) 35 ml, four times). The combined ether extracts were dried over Na_2SO_4 and evaporated to dryness. The residue was further dried under a stream of N_2 and finally redissolved in (**a**) 14.5 ml, (**b**) 22.5 ml MeOH/H_2O 50/50 (v/v).

4.5. Sephadex LH-20 chromatography

Phenolic acids were separated using Sephadex LH-20 chromatography. Application of Sephadex LH-20 chromatography to the separation of monomeric from dimeric/trimeric acids was recently detailed (Bunzel et al., 2004).

Briefly, the column (83 × 2 cm) was conditioned with 0.5 mM aq. TFA/MeOH 95/5 (v/v). Owing to the temporarily precipitation of extracted material at the beginning of the separation, an interspace of about 0.5 cm between floating punch and gel bed was filled with solvent (Bunzel et al., 2004). Following the sample application using a six-way valve, the sample was flushed onto the column eluting with 0.5 mM TFA/MeOH 80/20 (v/v) for 20 min, flow rate: 1.0 ml min^{-1}. Subsequent elution was carried out as follows (four step elution): (1) elution with 0.5 mM TFA/MeOH 95/5 (v/v) for (**a**) 90 h, (**b**) 145 h, flow rate: 1.5 ml min^{-1}; (2) elution with 0.5 mM TFA/MeOH 50/50 (v/v) for (**a**) 71 h, (**b**) 67 h flow rate: 1.0 ml min^{-1}; (3) elution with 0.5 mM TFA/MeOH 40/60 (v/v) for (**a**) 67 h, (**b**) 80 h, flow rate: 1.0 ml min^{-1}; (4) rinsing step with 100% MeOH. A preparative UV-detector was used for the detection at 325 nm. Fractions were collected every 18 min. In the isolation process **a**, the fraction containing compound **1** (2-(4-{2-carboxy-2-[4-(2-carboxy-vinyl)-2-methoxy-phenoxy]-vinyl}-2-methoxy-phenoxy)-3-(4-hydroxy-3-methoxy-phenyl)-acrylic acid) eluted in the third elution step (0.5 mM TFA/MeOH 40/60 (v/v)) between 670 and 850 min (equal to fraction F12 (Bunzel et al., 2004) and Fig. 2). Using isolation process **b**, compound **2** (7- [1-carboxy-2-(4-hydroxy-3-methoxy-phenyl)-vinyloxy]-1-(4-hydroxy-3-methoxy-phenyl)-6-methoxy-1,2-dihydro-naphthalene-2,3-dicarboxylic acid) eluted in the second elution step (0.5 mM TFA/MeOH 50/50 (v/v)) together with 8–5-diferulic acid (non-cyclic form) between 1944 and 2448 min (equal to fraction F9 (Bunzel et al., 2004)).

4.6. Semi-preparative RP-HPLC

Further separation of the GPC-fractions was achieved by means of semi-preparative RP-HPLC using a Nucleosil 100 C 18 HD column (250 × 10 mm i.d., 5 µm). The detection wavelength was 325 nm, the injection volume 70 µl (100 µl sample loop). The flow rate

was maintained at 2.7 ml min^{-1}. A binary elution system consisting of MeOH/1 mM aq. TFA 90/10 (v/v) (a) and MeOH/1 mM aq. TFA 10/90 (v/v) (b) was used to purify compound **1**: 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 (column temperature 45 °C). Compound **2** was separated from 8–5-diferulic acid (non-cyclic) and unidentified compounds using the following system: initially (a) 25%, (b) 75%, linear over 22 min to (a) 60%, (b) 40%, following a rinsing and equilibration step (column temperature 35 °C). Fractions were collected according to the chromatograms.

4.7. Analytical RP-HPLC

The purity of compounds **1** and **2** was checked by analytical RP-HPLC on a Nucleosil 100 C 18 HD column (250 × 4 mm i.d., 5 μm). The detection wavelengths were 325 and 280 nm, using an analytical photodiode array detector. The injection volume was 20 μl and the flow rate was maintained at 1 ml min^{-1}. Elution system: eluent a: MeOH; eluent b: 1 mM aq. TFA. Initially (a) 10%, (b) 90%, linear over 10 min to (a) 40%, (b) 60%, 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) 80%, (b) 20%, held isocratically for 5 min, following an equilibration step (column temperature 45 °C).

4.8. HPLC-MS

Molecular mass determination was achieved by HPLC-MS using atmospheric pressure-electrospray ionisation (AP-ESI) in the positive and negative mode (fragmentor voltage: 60 V (negative mode), 40 V (positive mode); scan range: *m/z* 100–750). Fast elution of the isolated trimer was realized by the following HPLC conditions: eluent a: acetonitrile; eluent b: 1 mM ammonium formate in doubly-distilled H_2O (pH 3.0). Initially (a) 40%, (b) 60%, held isocratically for 2 min, linear over 5 min to (a) 60%, (b) 40%, following an equilibration step (column temperature 40 °C). The injection volume was 20 μl and the flow rate was maintained at 1 ml min^{-1}.

AP-ESI MS, peaks >5% of base peak. Positive-ion mode, mass (% of base peak), compound **1**: 617 $[M + K]^+$ (100), 601 $[M + Na]^+$ (6), 561 (21), 543 (48). Compound **2**: 617 $[M + K]^+$ (100), 601 $[M + Na]^+$ (6), 596 (45), 579 (6), 561 (10), 533 (20), 425 (11). Negative-ion mode, compound **1**: 577 $[M - H]^-$ (100). Compound **2**: 577 $[M - H]^-$ (100).

High-resolution ESI-MS was performed by direct inlet using a syringe pump. Compound **1**: found $[M + Na]^+$ 601.13280, $C_{30}H_{26}O_{12}Na$ requires 601.13219; compound **2**: found $[M + Na]^+$ 601.13267, $C_{30}H_{26}O_{12}Na$ requires 601.13219.

4.9. Structural identification by NMR

Structural identification was performed using the usual array of one- and two-dimensional NMR experiments (^1H, ^{13}C, H,H-COSY, HSQC/HMQC, HMBC). A complete one-dimensional ^{13}C spectra of compound **1** in DMSO-d_6 was not acquired due to sensitivity limitations. Compound **1** in DMSO-d_6 was also analysed using a ROESY experiment using correlations from the phenolic-OH to distinguish A and B moieties. Chemical shifts (δ) were referenced to the central solvent signals (acetone-d_6: δ_H 2.04 ppm; δ_C 29.8 ppm; DMSO-d_6: δ_H 2.49 ppm; δ_C 39.5 ppm). *J*-values are given in Hz. NMR-assignments follow the numbering shown in Fig. 1.

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