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A dehydrotrimer of ferulic acid from maize bran

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Abstract

A new phenolic acid trimer was detected by coupled liquid chromatography/mass spectroscopy in alkali extracts of maize bran. The trimer was purified by preparative silica gel chromatography. The structure of the new compound was elucidated on the basis of 1D and 2D NMR and corresponded to a 4-*O*-8′, 5′-5″ dehydrotriferulic acid.

Keywords: *Zea mays* L.; Maize; Bran; Phenolic acid; Ferulic acid trimer

1. Introduction

Ferulic acid (3-methoxy-4-hydroxy-cinnamic acid) is the most common phenolic acid found in the Gramineae family. It is particularly abundant in cereal grains. Although present in grains as free acid and low molecular weight conjugates, ferulic acid occurs mostly as an ester-linked substituent of cell-wall heteroxylans, especially in the brans. Several dehydrodimers of ferulic acids have been isolated from the cell walls. They result from oxidative coupling of ferulate esters and represent mainly products of 8-5, 8-*O*-4, and 5-5 radical coupling (Ralph et al., 1994). Ferulate dehydrodimers are particularly abundant in maize bran (Lapierre et al., 2001) comprising mostly aleurone layer and pericarp. No definitive evidence has been given that all ferulic acid dehydrodimers cross-link cell wall polysaccharides. An alternative is that they form intra-polymeric loops rather than inter-polymeric bridges. They play, however, a determinant role in the mechanical properties of the tissues (Kamisaka et al., 1990; Sanchez et al., 1996; Wakabayashi et al., 1997; Peyron et al. 2002) and their enzymatic degradability (Grabber et al., 1998). It has been shown recently by ^{14}C experiments that in 2- and 4-day old maize cell cultures, trimers and larger oligomers represented the major products of ferulate coupling (Fry et al., 2000). However, the structure of such natural products has not been determined so far, although trimers of specified structure have recently been generated enzymically in vitro (Ward et al., 2001). In analyzing the phenolic compounds of maize bran, besides *p*-coumaric, ferulic and dehydro-diferulic acids, we have detected in appreciable amounts, based on HPLC peak area, an unknown compound with a typical hydroxycinnamic acid UV spectrum. In the present paper, we report on the isolation and the structural characterization of this new compound.

2. Results and discussion

The phenolic acid content of maize bran was determined after saponification. Commercial destarched and deproteinized maize bran was treated with diluted alkali at 35 °C. The saponified extract was extracted with Et_2O, vacuum dried then redissolved in 50% MeOH. The extract was analyzed by HPLC on a C18 reverse phase column with a diode array detection. From this separation (Fig. 1), the major peaks were identified and quantified using standards, as **1** *p*-coumaric acid, **2** *E*-ferulic acid, **3** 8-5′-dehydrodiferulic acid, **4** 8-*O*-4′-dehydrodiferulic acid, **5** 8-5′-benzofuran-dehydrodiferulic

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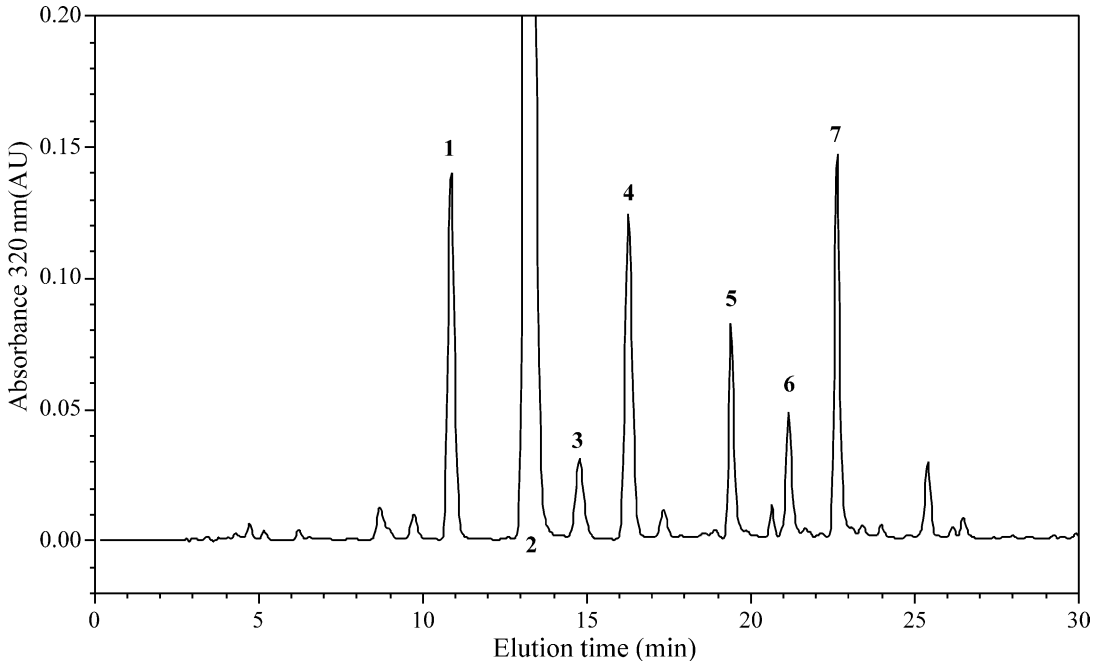

Fig. 1. RP-HPLC profile of 50% aq. MeOH extracts from saponified maize bran. **1** *p*-coumaric acid, **2** *E*-ferulic acid, **3** 8-5abm5-dehydrodiferulic acid, **4** 8-*O*-4′-dehydrodiferulic acid, **5** 8-5′-benzofuran dehydrodiferulic acid, **6** trimer, **7** 5-5′-dehydrodiferulic acid.

acid, **7** 5-5′-dehydrodiferulic acid. An unknown compound (**6**) was eluted in between the dehydrodimers, with a UV absorption spectrum in HPLC solvent (MeCN/aq. CH_3COONa buffer pH 4.6 30:70) typical to hydroxycinnamic acids (max. 316 nm, 295 nm; min. 262 nm). On-line detection with a mass spectrometer equipped with an electrospray ionisation source and an ion trap mass analyser revealed that compound **6** was detected as the deprotonated $[M–H]^-$ form at $m/z = 577$ in the negative ion mode, meaning that it exhibited a 578 molecular weight, compatible with the structure of a dehydro-triferulic acid.

In order to characterize the structure of compound **6**, milligram amounts were prepared from maize bran. The saponified extract from maize bran was repeatedly chromatographed on columns of silica gel to afford compound **6**. The purification was followed at each step by TLC and reverse-phase HPLC. Twenty-one milligrams of compound **6** were purified from 100 g of maize bran. The molecular weight of purified compound **6** was confirmed by FAB mass spectrometry in negative mode at 577.14 ($M–H^-$). 1D and 2D ^{13}C and ^{1}H NMR experiments were performed on pure compound **6**. The NMR spectral data revealed the presence of three aromatic rings, three methoxyl groups and three carboxylic groups. The broad band decoupled ^{13}C NMR spectrum showed 30 carbon signals. The ^{1}H and HSQC NMR data revealed the presence of 12 protons in addition to three methyl signals (three protons each) confirming the trimeric structure and indicating that the two linkages between the three ferulic acid moieties were respectively one C–C bond and one ether bond. Examination of the coupling constants showed the presence of two H7–H8 signals in the *trans* position (at 6.31 and 7.49 ppm and at 6.28 and 7.47 ppm), of one phenolic ring with the same substitution pattern as ferulic acid (ABC spin system corresponding to H2, H5, H6 at 7.31 ppm, 6.73 ppm and 7.02 ppm, respectively) and of two aromatic rings bearing only two protons in *meta* position (H2, H6, at 6.93 and 7.22 ppm for one ring and at 7.22 and 7.43 ppm for the other), meaning that one C7 or C8 and two C5 carbons were substituted in the trimeric structure. Protonated carbons were assigned with HSQC spectrum and quaternary carbons by HSQC long range correlations. Thus long range correlations between the H7 of a C7–C8 unsubstituted double bond (7.49 ppm) and the carbons of the unsubstituted ferulic ring (111.73, C2; 122.45, C6; 129.56, C1) showed that one of the ferulic units was involved only in an ether linkage, and was thus in terminal position. Short and long range correlations allowed us to distinguish the C1, C2, C4, C5 and C6 signals of the residual phenolic rings and to attribute H2 (at 7.43 and 7.22 ppm) and H6 (at 6.96 and 7.22 ppm) in each ring. Long range correlations of both H6 protons with both substituted C5 carbons indicated that one of the linkages was a 5–5 bond. Finally, long range correlations of the carbon signals of the phenolic rings respectively with the H7 of the remaining unsubstituted double bond (at 7.47 ppm) and with the residual proton of the substituted double bond (at 7.35 ppm) allowed us to distinguish between the C moiety, involved only in the 5–5 linkage, and the B moiety, substituted both at C5 and at C8 and thus involved in both 5–5 and 8–*O*–4 bonds (Table 1).

Therefore, the structure of **6** was assigned as (*Z*)-3-{5′-[(*E*)-2-carboxyvinyl]-6,2′-dihydroxy-5,3′-dimethoxybiphenyl-3-yl}-2-{4-[(*E*)-2-carboxyvinyl]-2-methoxyphenoxy}-acrylic acid, named 4-*O*-8′, 5′-5″ dehydrotriferulic acid (Fig. 2) in agreement with usual nomenclature for hydroxycinnamic acids (Ralph et al., 1994).

A response factor of 6 relative to the HPLC internal standard (TMCA) was determined at 320 nm. It allowed us to determine the ferulate trimer content of maize bran: 1.8 mg/g. This value represents approx. 12% (w/w) of the ferulic acid oligomers (dehydrodimers + trimer) detected. It appears therefore that this compound cannot be neglected as a structural compound of the grain cell-wall. It is possible moreover that maize bran contains other trimers and higher ferulate oligomers that may have escaped the extraction and separation by procedures described here.

From the experiments reported in this paper, it is not known whether this trimer, recovered after saponification, constitutes a covalent bridge between cell-wall polymers. It is, however, theoretically possible that the trimer cross-links up to three polymer chains. Further experiments will be necessary to determine the structural role of this compound.

3. Experimental

3.1. General

1D and 2D homo and heteronuclear NMR spectra of the trimer were recorded at 300 K on a Bruker Avance DRX 400 MHz spectrometer in acetone-d_6. *J* values are given in Hz. The central solvent signals were used as internal reference (^{1}H, δ_H 2.04; ^{13}C, δ_c 29.8). 1D and 2D NMR spectra were obtained using standard Bruker pulse programs. ^{1}H–^{1}H correlation information was obtained with a delayed COSY experiment, whereas ^{1}H–^{13}C correlation information was obtained with the usual combination of the inverse detected one-bond and long-range ^{1}H–^{13}C correlation experiments, HMQC and HMBC. The mass experiment on purified compound 6 was performed on a Jeol JMS-DX300 mass spectrometer by the FAB ionisation method in positive and negative mode. Analytical RP-HPLC with UV absorbance detection was carried out using an Alltima (Alltech, Deerfield, USA) C_{18} column 5 μm (250×4.6 mm i.d.). Linear gradient elution was performed by MeCN and aq. CH_3COONa buffer 0.05 M, pH 4.6, at a flow rate of 1 ml min^{-1} at 35 °C, from 15:85 to 35:65 in 24 min, from 35:65 to 60:40 in 0.5 min, from 60:40 to 15:85 in 4.5 min and maintained at 15:85 for 5 min. Detection used a 996 Waters photodiode array detector (Waters, Milford, MA). Response factors of ferulic acid dehydrodimers determined by Saulnier et al. (1999) were used. Products were identified using their UV absorption spectra (Waldron et al., 1996). Chromatographic separation with on-line mass detection used a Lichrospher 100-RP18 5 μm (250×2 mm i.d) column (Merck, Darmstadt, Germany). Gradient elution was at a flow rate of 0.25 ml min^{-1} with MeCN and aq. CH_3COONH_4 buffer pH 4.6, maintained at 10:90 up to 7 min then modified to 30:70 in 28 min. Mass detection was achieved with a ThermoFinnigan LCQ Advantage (San Jose, California) mass spectrometer equipped with an ESI source and an ion trap mass analyser which was controlled by the LCQ navigator software. The mass spectrometer was operated in the negative ion mode

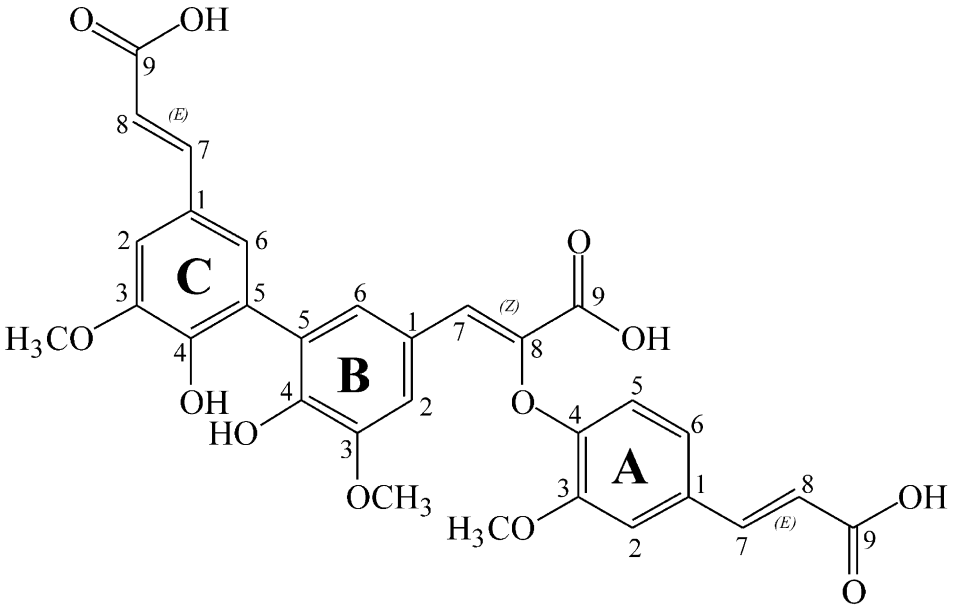

Fig. 2. Structure of compound **6**.

Table 1
NMR spectral data of compound **6** (400 MHz, acetone-d_6)

No.	C	H
A1	129.56	
A2	111.73	7.31 (1H, *d*, *J* = 1.86 Hz)
A3	148.33	
A4	149.67	
A5	113.81	6.73 (1H, *d*, *J* = 8.19 Hz)
A6	122.45	7.02 (1H, *dd*, *J* = 1.86, 8.34 Hz)
A7	144.94	7.49 (1H, *d*, *J* = 15.92 Hz)
A8	115.79	6.31 (1H, *d*, *J* = 15.98 Hz)
A9	164.09	
B1	129.18	
B2	111.99	7.43 (1H, *d*, *J* = 1.93 Hz)
B3	149.28	
B4	147.01	
B5	125.65	
B6	127.86	7.22 (2H, *d*, *J* = 1.9 Hz)
B7	128.18	7.35 (1H, *s*)
B8	138.00	
B9	167.45	
C1	124.14	
C2	109.39	7.22 (2H, *d*, *J* = 1.9 Hz)
C3	147.38	
C4	147.01	
C5	125.65	
C6	127.86	6.96 (1H, *d*, *J* = 1.9 Hz)
C7	147.80	7.47 (1H, *d*, *J* = 15.97 Hz)
C8	114.51	6.28 (1H, *d*, *J* = 15.77 Hz)
C9	167.66	
OMe A3, B3 or C3	56.03	3.67 (3H, *s*)
OMe A3, B3 or C3	55.86	3.78 (3H, *s*)
OMe A3, B3 or C3	55.65	3.83 (3H, *s*)

Chemical shifts are δ values; assignments were confirmed by decoupling and 2D NMR experiments (COSY 1H–1H, HMQC and HMBC).

under the following conditions : source voltage, 4.5 kV; capillary voltage, −4 V; capillary temperature, 200 °C; tube lens voltage, −35 V. During the chromatographic run, mass spectra of the eluate were recorded from *m*/*z* 100 to *m*/*z* 800. Silica gel 60 F 254 plates (Merck) were used for TLC in $CHCl_3$/MeOH/HOAc 90:10:1) or (hexane/EtOAc/HOAc 25:75:1). For R_f values see experimental section below.

3.2. Material

Maize bran provided by ULICE (Riom, France) was destarched and deproteinized as previously reported (Saulnier et al., 1995).

3.3. Analytical extraction of phenolic acids

Ground samples (20 mg) were saponified for 2 h in the dark with 2.0 M NaOH (10 ml) at 35 °C under slow agitation. An internal standard, 3,4,5-tri-methoxy-*(E)*-cinnamic Acid (TMCA, Sigma, St Louis, Ms USA) was added and the solution was adjusted to pH 2.0 with 4 M HCl. Phenolic acids were extracted twice with Et_2O (5 ml). Ether phases were evaporated in the presence of argon. The dry extract was dissolved in 50:50 MeOH/H_2O (v/v), filtered (0.45 μm) and injected (20 μl) onto RP-HPLC column.

3.4. Preparative extraction and purification

A 1 l alkali solution (NaOH 2M) was degassed for 30 min in the dark and under Ar atmosphere at 35 °C, and 100 g of maize bran were added. After the mixture had been stirred for 1.5 h at 35 °C under Ar, the solution mixture was adjusted to pH 4.0 with 500 ml of 4 M HCl and saturated with NaCl. The solution was extracted 4 times with Et_2O (400 ml). The organic layer was dried over Na_2SO_4 and evaporated to give a crude product (5.371 g), which was submitted to several silica gel chromatography (35–70 μm, 60A Merck), first with $CHCl_3$/MeOH/HOAc and after with hexane/EtOAc/HOAc to afford compound **6** after evaporation as a pale yellow powder (21 mg). R_f values ($CHCl_3$/MeOH/HOAc 90:10:1) : **6** (0.11); ferulic acid (0.44); 5-5′ dehydrodiferulic acid (0.24). R_f values (hexane/EtOAc/HOAc 25:75:1) : **6** (0.05); ferulic acid (0.46); 5-5′ dehydrodiferulic acid (0.17).

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