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FERULIC ACID ESTERS OF SUGAR CARBOXYLIC ACIDS FROM PRIMARY LEAVES OF RYE (*SECALE CEREALE*)

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Key Word Index—*Secale cereale*; Poaceae; rye; ^{1}H NMR; ^{13}C NMR; FABMS; ferulic acid; gluconic acid; sugar carboxylic acid; phenylpropane.

Abstract—New hydroxycinnamic acid esters have been isolated from primary leaves of rye and identified as positional isomers of (*E*)-*O*-feruloylgluconic acids on the basis of TLC, HPLC, FAB mass spectrometry, ^{1}H NMR and ^{13}C NMR spectroscopy. The 2-(*E*)-*O*-feruloylgluconic acid was the major isomer. A second ferulic acid ester, a very minor constituent in rye, was identified as 2(*E*)-*O*-feruloyl-4-methoxyaldaric acid, which is probably a glucaric acid derivative.

INTRODUCTION

In an earlier study on the phenylpropanoid metabolism in primary leaves of rye (*Secale cereale* L.) [1] we found, besides several flavonoid constituents (anthocyanins and *O*- and *C*-glycosylflavones), a pattern of simpler phenolic compounds, which were most likely composed of hydroxycinnamic acid conjugates. The flavones were recently identified as two isovitexin 2″-*O*-glycosides [2] and two luteolin *O*-glucuronides [3].

Two of the possible hydroxycinnamic acid conjugates have been investigated and in the present study we report on their structural elucidation.

RESULTS AND DISCUSSION

HPLC analyses of 80% aqueous methanolic extracts of fresh 7-day-old primary leaves of rye showed that compound **1a**, 2(*E*)-*O*-feruloylgluconic acid, constituted 3–4% of the UV (320 nm) absorbing components (*ca* 20 nmol/primary leaf) and compound **2a**, probably 2(*E*)-*O*-feruloyl-4-*O*-methoxyglucaric acid, was a very minor component 1%.

These two ferulic acid esters were isolated and their structures were assigned on the basis of the data presented below. The UV spectral data of **1** and **2** were identical (λ_{max}^{MeOH} nm: 217, 234, 296 shoulder, 324; $\lambda_{max}^{MeOH-NH_4OH}$ nm: 218, 248, 309, 376) and their colour reactions on TLC (under UV at 350 nm changing from blue to greenish-blue fluorescence when treated with NH_3 vapour) were consistent with those of ferulic acid esters. **1** and **2** exhibited the following R_f values on SS1: 0.51, 0.56; SS2: 0.44, 0.60 (severe diffusion); SS3: 0.71/0.79, 0.82/0.92 (see Experimental for compositions of solvent systems). Both compounds gave ferulic acid (co-chromatography with authentic ferulic acid on SS4) upon alkaline hydrolyses. Gluconic acid liberated from compound **1** was identified by co-chromatography with reference material on SS5 (R_f 0.39) and SS6 (R_f 0.44). The acid was visualized by spraying the dried chromatogram with bromocresol green in alkaline (sodium hydroxide) ethanol.

NMR spectroscopy identified components **1** and **2** as mixtures of closely related structures that could not be purified further by chromatographic methods (preparative scale). In both cases the negative ion FAB mass spectra showed a single molecular ion with a simple fragmentation pattern, clearly indicating that each component was a mixture of isomeric structures differing only in the position of attachment of the substituents. The identity and arrangement of substituents in the individual compounds were deduced from one-dimensional (1D) and two-dimensional (2D) ^{1}H NMR spectra.

The negative ion FAB mass spectrum of **1** gave a deprotonated molecular ion at *m/z* 371 and a sequence ion, characteristic of such compounds [4, 5], at *m/z* 195 corresponding to loss of a feruloyl moiety from an open-chain feruloyl monoester of gluconic acid. At the same time, these data excluded the possibility of the ester conjugate of the lactone of gluconic acid. In contrast to the mass spectra, the ^{1}H NMR spectra of **1** (Table 1) were complex and compatible with a mixture of isomeric compounds (Scheme 1). The proportions of each in the mixture was readily deduced from the characteristic feruloyl ester signals in the region 8.0–5.7 ppm and was compatible with three compounds in the ratio 3:1.8:1, each of which occurred as a 3:2 *E/Z* mixture. The assignment of the aromatic signals to each particular component in the mixture was taken from the cross peaks in the 2D COSY ^{1}H spectrum. The same spectrum allowed the assignment of the gluconic acid residue signals and consequently the position of attachment of the feruloyl moiety. The lowest signals, H-2, of the most abundant *E/Z* compound, **1a**, were doublets and could only arise from acylation at C-2 of gluconic acid. H-3 and H-4 were identified from cross peaks in the COSY spectrum, while the remaining protons, H-5, H-6A and H-6B, were in the multiplet of signals 3.9–3.6 ppm. Similarly the cross peaks in the COSY and relayed COSY ^{1}H

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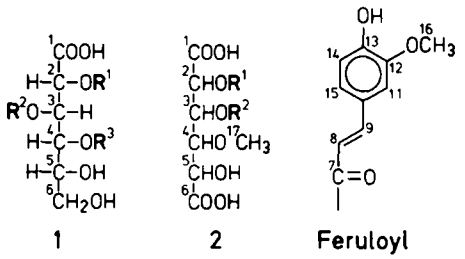
Table 1. ^{1}H NMR spectral data of compound **1** in CD_3OD

	1a		**1b**		**1c**	
	E	*Z*	*E*	*Z*	*E*	*Z*
Shifts (ppm)						
H-2	5.177	5.138	4.102	4.097	4.31	4.29
H-3	4.258	4.229	4.317	4.290	5.530	5.505
H-4	3.777	3.752	5.276	5.260	4.00	3.98
H-5	3.86–3.63		4.03		3.86–3.63	
H-6A			3.69			
H-6B			3.58			
H-8	6.518	5.994	6.449	5.894	6.455	5.919
H-9	7.732	6.89	7.678	6.89	7.718	6.89
H-11	7.252	7.879	7.257	7.819	7.234	7.837
H-14	6.855	6.792	6.848	6.789	6.855	6.789
H-15	7.18–7.10		7.18–7.10		7.18–7.10	
H-16	3.940	3.899	3.940	3.903	3.932	3.909
Couplings (Hz)						
(2–3)	6.5	6.6	3.2	3.0	4.4	4.4
(3–4)	1.9	1.9	4.0	4.3	3.2	3.3
(4–5)			6.7	6.7		
(8–9)	15.9	12.9	15.9	12.9	15.9	12.8
(11–15)	2.0	2.0	2.0	2.0	2.0	2.0
(14–15)	8.2	8.2	8.2	8.2	8.2	8.2



Scheme 1. Structures of rye constituents **1** (**a**, **b**, **c**) and **2** (**a**, **b**). **1a**: R^1 = feruloyl, $R^2 = R^3$ = H; **1b**: R^3 = feruloyl, $R^1 = R^2$ = H; **1c**: R^2 = feruloyl, $R^1 = R^3$ = H; **2a**: R^1 = feruloyl, R^2 = H; **2b**: R^2 = feruloyl, R^1 = H.

spectra indicated that the lowest field signal, H-4, of the second most abundant compound, **1b**, had two separate vicinal protons, both of which possessed further separate vicinal protons indicating substitution at C-4. For the third compound, **1c**, the lowest field signal was similar to that of **1b** in being coupled to only two vicinal protons which were not from a CH_2OH group. Hence **1c** was a gluconic acid conjugate substituted at C-3. The complexity of **1** and the amount of material available precluded the measurement of a ^{13}C NMR spectrum.

The negative ion FAB mass spectrum of component **2** gave a deprotonated molecular ion at *m/z* 399 and fragmentation ions of *m/z* 223 $[M - C_{10}H_9O_3]^-$, *m/z* 205 $[M - C_{10}H_9O_3 - H_2O]^-$ and *m/z* 193 $[C_{10}H_9O_4]^-$ corresponding to cleavage of the feruloyl moiety. The ^{1}H NMR spectrum (Table 2) was again complex and indicated the presence of two compounds in the ratio 6:1, each of which occurred as a 3:2 *E*/*Z* mixture. In addition to the feruloyl moiety, the ^{1}H NMR and

Table 2. ^{1}H NMR spectral data of compound **2** in CD_3OD

	2a		**2b***	
	E	*Z*	*E*	*Z*
Shifts (ppm)				
H-2	5.283	5.239	4.51	4.49
H-3	4.378	4.350	5.75	5.74
H-4	3.713	3.669	3.96	3.93
H-5	4.321	4.302	4.44	4.42
H-8	6.511	5.976	6.40	5.86
H-9	7.768	6.944	7.69	6.88
H-11	7.270	7.882	7.22	7.80
H-14	6.863	6.798	6.83	6.78
H-15	7.149	7.172	7.10	7.14
H-16	3.946	3.899	3.93	3.91
H-17	3.574	3.550	3.52	3.51
Couplings (Hz)				
(2–3)	4.0	4.0		
(3–4)	5.2	5.3		
(4–5)	3.7	3.9		
(8–9)	15.9	13.0		
(11–15)	2.0	2.3		
(14–15)	8.2	8.2		

*The shifts of **2b** were determined from the 2D COSY ^{1}H spectrum.

^{13}C NMR spectra showed the presence of an additional methoxyl group per molecule and a hydroxydicarboxylic acid (aldaric acid). The nature of the main compound, **2a**, was readily deduced from cross peaks in the 2D COSY ^{1}H spectrum, which showed that the acyl group was at C-2.

The presence of two carboxylic acid carbons was confirmed in the ^{13}C NMR spectrum (Table 3), where the difference in chemical shifts of these was indicative of β-acylation. Comparison of the ^{13}C shifts in the region 85–70 ppm with mucinic acid indicated that the signals at *ca* 84 ppm corresponded to the carbon carrying the methoxyl group and that those at 75.5 ppm to C-2. Correlation of the ^{1}H NMR spectrum with the ^{13}C NMR spectrum by selective ^{1}H decoupling showed unambiguously that the methoxyl group was at C-4.

The identity of the minor component **2b** followed directly from the cross peaks in the 2D COSY ^{1}H spectrum.

The relative geometries of the various asymmetric centres in molecules **2a** and **2b** could not be deduced from these spectra as reference materials were not available.

The observation of a series of positional isomers is a well-known phenomenon which occurs with esters of phenolic acids with polyhydroxy compounds (cf. ref. [6]). Isomer conversion was probably enhanced due to the slightly alkaline solvent used in polyamide chromatography (see below). As expected for component **2**, only two such isomers were observed as the methoxyl group effectively blocks further isomerization. It is difficult to say at the moment whether all the observed compounds are actually present in the plant. However, HPLC analysis of freshly prepared extracts showed the major compound in both cases, the *E*-isomer of **1a** and **2a**, to be the predominant structures and only a few per cent of structures **1b**, **1c** and **2b** were present. The latter rapidly increased in quantity during our isolation procedure.

To the best of our knowledge, feruloylgluconic acid (**1**) is a new naturally occurring hydroxycinnamic acid ester. Compound **2** might be related to the glucaric acid conjugates which were found to be present as caffeic acid esters in *Lycopersicon esculentum* [6] and *Cestrum euanthes* [7]. Whether biosyntheses of these conjugates proceed through acyl-coenzyme-A or acyl-glucose mediated carboxyl-group activation [8] is presently being studied in our laboratory.

It is interesting to note that in addition to the hydroxycinnamic acid sugar acid conjugates described herein, two major glucuronic acid derivatives of the flavonoid metabolism were found to be present in developing rye primary leaves: luteolin-7-*O*-(β-D-glucuronosyl-1,2-β-D-glucuronide) and its 4′-*O*-β-D-glucuronide derivative [3].

Table 3. ^{13}C chemical shifts (ppm) of compound **2a** in CD_3OD

Carbon	*E*	*Z*
C-1	173.02	173.02
C-2	75.48	75.48
C-3	72.36	72.25
C-4	84.33	84.25
C-5	72.85	72.85
C-6	176.52	176.52
C-7	168.45	168.45
C-8	115.13	*
C-9	147.41	146.22
C-10	128.03	127.88
C-11	111.87	*
C-12	†	†
C-13	†	†
C-14	116.50	*
C-15	124.19	126.90
C-16	56.50	56.50
C-17	60.19	60.19

*116.12; 115.39; 115.30.
†151.08; 150.82; 150.67; 149.39.

EXPERIMENTAL

Plant material. Young plants (7 days old) of rye (*Secale cereale* L. var. Kustro) (F. von Lochow-Petkus, Bergen, West Germany) were grown as described in ref. [1].

Isolation and purification of ferulic acid esters. Primary leaves (1 kg fr. wt) were cut into small pieces and extracted with 80% aq. MeOH with an Ultra-Turrax homogenizer. After filtration, the extract was concentrated under vacuum to a vol. of *ca* 10 ml and was fractionated on a polyamide column (CC-6, 4.5 × 34 cm; Macherey, Nagel, Düren, West Germany) using H_2O (3 l.), MeOH (3 l.) and 0.03% NH_4OH in MeOH (2 l.). The last fraction was evaporated to dryness and the resulting residue redissolved in *ca* 3 ml 50% aq. MeOH. This was chromatographed on a Sephadex LH-20 column (3 × 68 cm; Pharmacia, Uppsala, Sweden) using H_2O as solvent. Elution was monitored continuously by UV absorption at 254 nm and fractions of the eluant showing high UV absorption were collected and examined by TLC. Two bright-blue fluorescent compounds (**1** and **2**) were separated from other phenolics by TLC in SS1. Purifications were achieved initially with polyamide CC as described above and finally with Sephadex LH-20 CC (twice with H_2O as solvent).

Hydrolysis of **1**. Compound **1** was dissolved in 1 N aq. NaOH and kept for 30 min at room temp. The hydrolysate was neutralized by means of cation exchange CC (Dowex 50W X8, Serva, H^+ form).

TLC. On microcrystalline cellulose ('Avicel', Macherey, Nagel): SS1, $CHCl_3$–HOAc–H_2O (3:2, H_2O saturated); SS2, *n*-BuOH–HOAc–H_2O (6:1:2); SS3, 10% aq. HOAc; SS4, toluene–HOAc–H_2O (2:1, H_2O saturated); SS5, *n*-BuOH–HOAc–H_2O (2:1:1); SS6, Me_2CO–MeOH–HOAc–H_2O (4:2:1:1).

HPLC. The HPLC apparatus (LKB) and the data processor (Shimadzu) are described in ref. [9]. The chromatographic column (250 mm long, 4 mm inner diameter) was prepacked with MN-Nucleosil C_{18} (5 μm) (Macherey, Nagel); detection at 320 nm; elution system: linear gradient elution within 25 min from solvent A (1.5% H_3PO_4 in H_2O) to 50% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A; flow rate = 1 ml/min. **1a** and **2a** gave retention times of 17.7 and 20 min, respectively.

NMR and MS. ^{1}H NMR (400 MHz) and ^{13}C NMR (75 MHz) spectra were recorded at ambient temp. on Bruker WM 400 and Bruker AM 300 NMR spectrometers locked to the deuterium resonance of the solvent, CD_3OD. Two-dimensional COSY ^{1}H spectra were recorded with a 90°-t_1–90°-FID(t_2) pulse sequence. The spectral width was F2 2000 Hz and F1 ± 1000 Hz with 1 K data points in t_2 and 512 data points in t_1. 104 Pulses were taken for each t_1 increment with a relaxation delay of 0.5 s between pulse sequences to give a total accumulation time of 13.3 hr. The data were multiplied by sine-bell functions, and one level of zero-filling was used for both t_1 and t_2. A 2D COSY with relayed coherence transfer ^{1}H spectrum was recorded with a 90°-t_1–90°-D2–180°-D2–90°-FID(t_2) pulse sequence with a constant delay,

D2, of 20 ms. The other parameters were those of the normal COSY spectrum. All 1D and 2D spectra were recorded using the standard Bruker software package. Chemical shifts are recorded in ppm relative to TMS and coupling constants are in Hz.

Negative-ion fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS50 mass spectrometer equipped with a Kratos FAB source. Glycerol was used as matrix.

Note added in proof. HPLC under stringent conditions showed more than 20 different hydroxycinnamic acid conjugates. Our current enzymatic studies proved that synthesis of feruloylgluconic acid proceeds via feruloyl-CoA. In studies on enzyme acceptor specificity, glucaric and galactaric acids turned out to be efficient acceptor molecules for the CoA-thioester dependent acyltransferase(s) and by this we tentatively identified *p*-coumaroyl- and feruloylglucaric acids as major constituents with 10–15% of the hydroxycinnamic acid conjugates in the rye primary leaves and feruloylgalactaric acid as a minor component.

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