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Acylated apigenin glycosides from alfalfa (*Medicago sativa* L.) var. Artal

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Abstract

Three flavones, including 4′-*O*-[2′-*O*-*E*-feruloyl-*O*-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside]apigenin, 7-*O*-β-D-glucuronopyranosyl-4′-*O*-[2′-*O*-*E*-feruloyl-*O*-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside]apigenin and 7-*O*-β-D-glucuronopyranosyl-4′-*O*-[2′-*O*-*p*-*E*-coumaroyl-*O*-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside]apigenin have been identified in alfalfa var. Artal. The known flavone 7-*O*-{2-*O*-E-feruloyl-[β-D-glucuronopyranosyl(1→3)]-*O*-β-D-glucuronopyranosyl (1→2)-*O*-β-D-glucurono-pyranoside} apigenin was also isolated. The structures of these compounds were deduced on the basis of their spectral data.

Keywords: *Medicago sativa*; Aerial parts; Acylated apigenin glucuronides

1. Introduction

Alfalfa is a pasture crop rich in good quality protein. Its nutritional value has been, however, influenced by the presence of secondary metabolites including saponins and flavonoids. While saponins have been extensively studied (Oleszek, 2000), very little is known on alfalfa flavonoids (Bisby et al., 1994). In our preliminary HPLC screening of 50 alfalfa varieties obtained from USDA stocks it was clear that flavonoid profiles of all tested samples contained a number of glycosides and were, with some exceptions, very similar to each other (Stochmal et al., 1999). From the Polish variety Boja several novel, acylated with ferulic and coumaric acids apigenin and luteolin glycosides have been isolated and fully characterized (Stochmal et al., 2001). All these flavonoids had only glucuronic acid in the sugar moiety and the acylation occurred on the sugar attached at C-7 of the aglycone. The HPLC profile of the variety Artal showed some dissimilarities with other cultivars and thus, the aim of the present work was to isolate and chemically characterize the unknown compounds.

2. Results and discussion

Preliminary analyses with HPLC (diode array detection) of flavonoids in the aerial parts of alfalfa var. Artal indicated the presence of several flavones, out of which, the one dominant and two minor compounds had UV spectra identical to apigenin glycosides, but their retention times were different from the others so far characterized. The column chromatography of a purified flavonoid fraction afforded four compounds, which were fully characterized by spectral techniques. Compound **4** was identified as 7-*O*-{2-*O*-*E*-feruloyl-[-*O*-β-D-glucuronopyranosyl(1→3)]-*O*-β-D-glucuronopyranosyl (1→2)-*O*-β-D-glucuronopyranoside}apigenin by comparison of its NMR and FAB-MS spectra with previously reported literature data (Stochmal et al., 2001).

Compound **1** gave a molecular ion (ESI–HRMS, negative ion mode) at *m*/*z* 797.1575, which together with the ^{1}H and ^{13}C NMR spectral data (Tables 1 and 2) was consistent with the empirical formula $C_{37}H_{34}O_{20}$.

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Table 1
^{1}H NMR data of flavonoids **1–3** (CD_3OD)

H	**1**[a]	**2**	**3**
Apigenin			
3	6.87 *s*	6.61 *s*	6.61 *s*
6	6.19 *d J* = 1.9	6.48 *d J* = 2.0	6.46 *d J* = 2.0
8	6.51 *d J* = 1.9	6.79 *d J* = 2.0	6.78 *d J* = 2.0
2′ and 6′	7.98 *d J* = 8.7	7.84 *d J* = 9.0	7.83 *d J* = 9.0
3′ and 5′	7.10 *d J* = 8.7	7.15 *d J* = 9.0	7.14 *d J* = 9.0
Glucuronic acids			
1	5.32 *d J* = 7.4	5.37 *d J* = 7.1	5.35 *d J* = 6.8
2	3.56 *brdd*	3.74 *dd J* = 7.1, 5.1	3.73 *dd J* = 7.1, 6.8
3	3.40 (o)	3.62 (o)	3.63 (o)
4	3.40 (o)	3.62 (o)	3.63 (o)
5	3.97 *brd*	4.01 *d J* = 9.8	4.06 *d J* = 9.5
1′	4.93 *d J* = 8.2	5.09 *d J* = 7.8	5.07 *d J* = 8.1
2′	4.64 *dd J* = 8.7, 8.2	4.90 *dd J* = 9.0, 7.8	4.92 *dd J* = 8.8, 8.1
3′	3.40 (o)[b]	3.66 *dd J* = 9.0, 9.0	3.68 *dd J* = 9.0, 8.8
4′	3.40 (o)[b]	3.62 (o)	3.63 (o)
5′	3.71 *brd*	3.87 *d J* = 9.5	3.91 *d J* = 9.3
1″		5.18 *d J* = 7.6	5.19 *d J* = 7.6
2″		3.57 *brdd*	3.58 *brdd*
3″		3.62 (o)	3.63 (o)
4″		3.62 (o)	3.63 (o)
5″		4.07 *d J* = 9.0	4.12 *d J* = 9.5
Phenolic acid			
2	7.26 *d J* = 1.9	7.07 *d J* = 2.0	7.40 *d J* = 8.5
3	–	–	6.74 *d J* = 8.5
5	6.77 *d J* = 8.2	6.73 *d J* = 8.3	6.74 *d J* = 8.5
6	7.08 *dd J* = 8.2, 1.9	6.99 *dd J* = 8.3, 2.0	7.40 *d J* = 8.5
7	7.50 *d J* = 15.8	7.53 *d J* = 15.9	7.58 *d J* = 15.9
8	6.41 *d J* = 15.8	6.25 *d J* = 15.9	6.30 *d J* = 15.9
OCH_3	3.80 *s*	3.84 *s*	–

[a] DMSO-d_6.
[b] (o) Overlapped with other signals.

Table 2
^{13}C NMR data of flavonoids **1–3** (CD_3OD)

	Apigenin				Glucuronic acids				Phenolic moiety		
C	**1**	**2**	**3**	**C**	**1**	**2**	**3**	**C**	**1**	**2**	**3**
2	166.1	165.9	166.0	1	99.7	99.1	99.4	1	127.8	127.8	127.3
3	104.8	104.9	105.0	2	82.9	82.6	82.9	2	111.7	111.6	131.2
4	183.9	184.0	184.0	3	76.5	76.4	76.5	3	149.3	149.2	116.7
5	163.2	162.9	162.8	4	72.8	72.9	72.9	4	150.5	150.6	161.2
6	100.2	101.3	101.2	5	76.1	76.2	76.1	5	116.4	116.4	116.7
7	165.5	164.5	164.5	6	172.5	172.6	172.6	6	124.2	124.2	131.2
8	95.1	96.0	95.9	1′	102.7	101.6	102.3	7	147.0	146.9	146.7
9	159.4	158.9	158.9	2′	75.2	75.2	75.2	8	115.7	115.5	115.4
10	105.4	107.3	107.3	3′	75.9	75.9	75.9	9	168.7	168.6	168.7
1′	126.1	125.8	125.8	4′	73.4	73.4	73.4	OCH_3	56.4	56.4	–
2′	129.1	129.2	129.3	5′	75.9	75.9	76.2				
3′	117.9	117.9	117.9	6′	172.5	172.6	172.6				
4′	161.3	161.4	161.4	1″		101.3	101.3				
5′	117.9	117.9	117.9	2″		74.4	74.4				
6′	129.1	129.2	129.3	3″		77.3	77.3				
				4″		73.1	73.0				
				5″		76.6	76.7				
				6″		173.0	172.8				

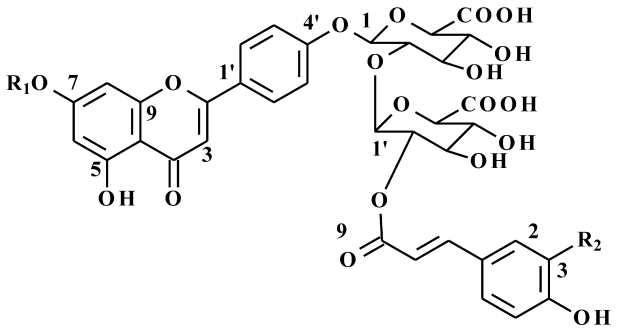
The downfield region of the ^{1}H NMR spectrum (Table 1) showed two spin systems correlated by ^{1}H–^{1}H shift correlation (COSY): an AA'BB' system at δ 7.98 (*d*, 8.7 Hz, H-2′ and H-6′) and δ 7.10 (*d*, 8.7 Hz, H-3′ and H-5′), and an AB system at δ 6.51 (*d*, 1.9 Hz, H-8) and δ 6.19 (*d*, 1.9 Hz, H-6). These two systems together with a singlet at δ 6.87 (H-3) indicated the presence of an apigenin aglycone in compound **1** (Stochmal et al., 2001). The UV and ^{13}C NMR (Table 2) spectra and the ROE correlations between the H-2′/H-6′ signal and the H-3 signal of the apigenin confirmed this structure.

The other group of signals in the downfield region of the ^{1}H NMR spectrum corresponded to a phenolic acyl moiety. An ABX system at δ; 7.26 (*d*, 1.9 Hz, H-2); δ 7.08 (*dd*, 8.2 Hz, 1.9 Hz, H-6) and δ; 6.77 (*d*, 8.2 Hz, H-5), and two doublets at δ 7.50 (H-7) and δ 6.41 (H-8) with a coupling constant of 15.8 Hz typical of an *E*-double bound were observed. The ROE correlation between a methoxyl group signal at δ 3.80 (*s*) with H-2 and the correlation between the double bound signals and the aromatic protons H-2 and H-6 (Fig. 1) suggested the presence of a ferulic acyl moiety, confirmed with the presence of nine signals in the ^{13}C NMR spectrum (Ibrahim and Barron, 1989) (Table 2).

The sugar region of the ^{1}H NMR spectrum showed the presence of two sugar units, with anomeric proton signals at δ 5.32 (*d*, 7.4 Hz, H-1) and δ 4.93 (*d*, 8.2 Hz, H-1′). The ^{13}C NMR spectrum showed 12 signals for two hexose units. Two typical C-6 signals of carboxyl groups at δ 172.5 suggested two glucuronic acid moieties. Their β-configuration was evident from the coupling constant of the anomeric protons.

The connection between the glucuronyl moieties and apigenin was established as 4′-[glucuronopyranosyl (1→2)glucuronopyranosyl] apigenin, from the observed ROE correlation between H-3′ and H-5′ of apigenin and H-1 of the first glucuronic acid and H-2 and H-1′ of both glucuronic acids. The pronounced downfield shift of H-2′ of the second glucuronic acid (δ 4.64) indicated the linkage between the sugar and the acyl moiety at C-2′, and thus **1** is 4′-*O*-[2′-*O*-*E*-feruloyl-*O*-β-D-glucurono pyranosyl(1→2)-*O*-β-D-glucuronopyranoside]apigenin.

Compound **2** showed a molecular ion (ESI–HRMS, negative ion mode) at *m*/*z* 973.1903 according with a molecular formula $C_{43}H_{41}O_{26}$ and peak at 269.0452 corresponding to the aglycone. The ^{1}H and ^{13}C NMR spectra of **2** (Tables 1 and 2) were very similar to those of compound **1**. The additional signals that appeared in its spectra corresponded to a third glucuronic acid moiety. The observed ROE correlation between H-6 and H-8 of the apigenin moiety and H-1″ of the third glucuronic acid determined the structure of **2** as 7-*O*-β-D-glucuronopyranosyl-4′-*O*-[2′-*O*-*E*-feruloyl-*O*-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside]apigenin.



1: R_1=H R_2=OCH_3
2: R_1=glucuronic acid R_2=OCH_3
3: R_1=glucuronic acid R_2=H

Fig. 1. Chemical formula of compounds **1**–**3**.

Compound **3** gave a molecular ion (ESI–HRMS, negative ion mode) at *m*/*z* 943.1765 with a molecular formula $C_{42}H_{40}O_{26}$ and peak at 269.0470 corresponding to the aglycone. The only spectroscopic difference between the spectra of compounds **2** and **3** was in the phenolic moiety signals, the ^{1}H NMR spectrum (Table 1) of **3** had two doublets δ 7.40, *d*, 8.5 Hz, H-2 and H-6 and δ 6.74, *d*, 8.5 Hz, H-3 and H-5) typical of a *p*-coumaroyl moiety. The ^{13}C NMR spectrum (Table 2) was in agreement with the structure of the acyl moiety and the ROE effects observed confirmed an identical connection of apigenin, glucuronic acids and coumaroyl moiety as compound **2**. The structure of **3** was stablished as 7-*O*-β-D-glucuronopyranosyl-4′-*O*-[2′-*p*-*E*-coumaroyl-*O*-β-D-glucuronopyranosyl (1→2)-*O*-β-D-glucuronopyranoside]apigenin.

As far as we know, the three flavone glycosides (**1**, **2** and **3**) are reported here for the first time as natural compounds. They, similarly to previous identified apigenin glycosides in alfalfa (Stochmal et al., 2001), possess glucuronic acid as a sole sugar subunit in the sugar chain, and acylation with ferulic and coumaric acids occurred on the glucuronic acid moieties. However, the difference with previously identified compounds is in the acylation position. The three identified glycosides are acylated on sugars attached at C-4′, while all previously reported compounds were acylated on sugars at C-7. These differences may be specific for this particular variety or they may reflect the influence of environmental conditions. Plant material from which present flavonoids were separated was collected from plants grown in Portugal. The four isolated compounds were tested for their antioxidant activity in β-carotene/linoleic acid heterogenous system (Pratt, 1992), and for radical scavenging activity in methanolic solution of DPPH (Lee et al., 1998). They showed no activity in both tests performed. More research is needed, in order to shed some light on the function of acylated glucuronides in the host plant on their adaptation to environmental conditions.

3. Experimental

3.1. General

Flavonoids were chromatographed on Cellulose (Merck) ready to use plates, developed with 15% AcOH and after drying plates were observed under a UV lamp. High-performance liquid chromatography was performed on an HPLC system (Waters) consisting of 616 pump, a 600 s controller, and a 996 photodiode array detector. The Millenium Chromatography Manager was used to monitor chromatographic parameters and to process the data.

^{1}H, ^{13}C and 2D NMR spectra were recorded in CD_3OD and DMSO-d_6 on a Varian UNITY-400 instrument operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Chemical shifts are reported in ppm (δ). ESI–HRMS were recorded in the negative ion mode on a Mariner Biospectrometry Workstation (Per Septive Biosystems). UV spectra were recorded on a HP 8453 UV/vis spectrophotometer. The α values were obtained in MeOH at 20°C on a Jasco P-1020 spectropolarimeter. Melting points were uncorrected.

3.2. Plant material and isolation

The lucerne var. Artal (American variety) was seeded in March 1999 in the north of Portugal, at Vairco (near Porto), and the samples were collected on the 23 August 1999, and they were from a 2nd harvest. The material was freeze dried, powdered and used for extraction.

Powdered plant material was extracted in a Soxhlet with chloroform to remove chlorophyl and fats. After drying it was extracted with 70% MeOH at room temperature. After filtration the extract was evaporated in vacuo (40°C) yielding a brown solid. The solid was suspended in water and loaded onto a C18 filled column (30×70 mm, 60 μm, Baker) equilibrated with water. The column was washed with water to remove carbohydrates, and flavonoids were washed out with 40% MeOH. The 40% MeOH fraction was evaporated to dryness, redissolved in 20% MeOH and loaded onto a C18 column (40 ×300 mm, 25–40 μm, Merck). The column was washed with MeOH-H_2O (linear gradient 20–70% MeOH) and 10 ml fractions were collected with a fraction collector. Fractions showing identical chromatographic characteristics (TLC, HPLC) were combined. Four single flavonoids were obtained.

3.2.1. 4′-*O*-[2′-*O*-*E*-Feruloyl-β-D-glucuronopyranosyl (1→2)-*O*-β-D-glucuronopyranoside]apigenin (**1**)

Amorphous yellow powder (15 mg); mp. 197–198°C; UV λ_{max}^{MeOH} nm: 271 *sh*, 325; $[\alpha]_D^{20}$ −74.2°(MeOH, *c* 0.1); HRMS *m*/*z*: 797.1575 [calc. for $C_{37}H_{33}O_{20}$ $[M\text{-}1]^-$, 797.1560], 269.0457 [M–H–ferulic acid–2GluA]$^-$. For ^{1}H and ^{13}C NMR see Tables 1 and 2, respectively.

*3.2.2. 7-O-β-D-Glucuronopyranosyl-4′-O-[2′-O-E-feruloyl -O-β-D-glucuronopyranosyl (1→2)-O-β-D-glucurono pyranoside]apigenin (**2**)*

Amorphous yellow powder (3 mg); mp. 197–198°C; UV λ_{max}^{MeOH} nm: 270 *sh*, 319; $[\alpha]_D^{20}$ −10.23° MeOH, *c* 0.1); HRMS *m/z*: 973.1903 [calc. for $C_{43}H_{41}O_{26}$ $[M\text{-}1]^-$, 973.1881], 269.0452 [M–H–ferulic acid–3GluA]$^-$. For ^1H and ^{13}C NMR see Tables 1 and 2, respectively.

*3.2.3. 7-O-β-D-Glucuronopyranosyl-4′-O-[2′-p-E-coumaroyl-O-β-D-glucuronopyranosyl (1→2)-O-β-D-glucuronopyranoside]apigenin (**3**)*

Amorphous powder (3 mg); mp. 197–198°C; UV λ_{max}^{MeOH} nm: 272 sh, 314; α_D^{20} −52.45° λ_{max}MeOH *c* 0.1); HRMS *m/z*: 943.1575 [calc. for $C_{42}H_{39}O_{25}$ $[M\text{-}1]^-$, 943.1775], 269.0470 [M–H–coumaric acic–3GluA]$^-$. For ^1H and ^{13}C NMR see Tables 1 and 2, respectively.

*3.2.4. 7-O-{2-E-Feruloyl-[β-D-glucuronopyranosyl(1→3)] -O-β-D-glucuronopyranosyl(1→2)-O-β-D-glucurono pyranoside}apigenin (**4**) (10 mg)*

UV, HRMS and NMR data were in accordance with literature data (Stochmal et al., 2001).

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