Alfalfa (*Medicago sativa* L.) Flavonoids. 2. Tricin and Chrysoeriol Glycosides from Aerial Parts

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Ten flavone glycosides have been isolated and identified in aerial parts of alfalfa. These included six tricin, one 3′-*O*-methyltricetin, and three chrysoeriol glycosides. Most of these compounds were acylated with ferulic, coumaric, or sinapic acids, and acylation occurred on the terminal glucuronic acid. Eight of these compounds, including 7-*O*-*β*-D-glucuronopyranosyl-3′-*O*-methyltricetin, 7-*O*-*β*-D-glucuronopyranosyl-4′-*O*-*β*-D-glucuronopyranosidechrysoeriol, 7-*O*-[2′-*O*-feruloyl-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside]chrysoeriol, 7-*O*-{2′-*O*-feruloyl-[*β*-D-glucuronopyranosyl-(1→3)]-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}chrysoeriol, 7-*O*-[2′-*O*-sinapoyl-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside]tricin, 7-*O*-[2′-*O*- feruloyl-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside]tricin, 7-*O*-[2′-*O*-*p*-coumaroyl-*β*-D-glucuronopyranosyl-(1→2)-*O*-*β*-D-glucuronopyranoside]tricin, and 7-*O*-{2′-*O*-feruloyl-[*β*-D-glucuronopyranosyl(1→3)]-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}tricin, have not been reported previously in the plant kingdom. Two previously identified alfalfa flavones, 7-*O*-*β*-D-glucuronopyranosidetricin and 7-*O*-[*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside]tricin, were also isolated.

Keywords: *Medicago sativa; acylated flavones; tricin; tricetin; chrysoeriol*

INTRODUCTION

Alfalfa (*Medicago sativa* L.), a crop that has been used for centuries as an animal feedstuff, is gaining much attention as a human food component, consumed as a garnish, leaf protein concentrate, or food nutritional supplement. Thus, understanding of the alfalfa chemical composition remains one of the most important factors in designing new nutritional formulas. Although the primary composition (protein and carbohydrates) is well understood (*1*, *2*), secondary metabolites are not yet fully characterized. The best recognized secondary metabolite groups are carotenonoids (*3*) and saponins (*4*). The phenolic composition, including flavonoids, despite extensive work, is still poorly understood (*5*), and only a few glycosides have been fully characterized.

Our previous work on flavonoid composition indicated that flavonoid profiles of several alfalfa varieties are very similar and contain >20 individual compounds (*6*). A number of these compounds from the more polar fraction of the flavonoid mixture from the Boja cultivar were identifed (*7*). They were shown to be unique glucuronides of apigenin and luteolin, acylated at glucuronic acid residues with ferulic or coumaric acids. Three further acylated apigenin glucuronides, present only in trace amounts in the Boja cultivar grown in Poland, were characterized in the Artal cultivar collected in Portugal (*8*). In the present work we describe the remaining more hydrophobic glycosides, which form a quite uniform group in their flavonoid profile, characterized by a strong absorption in band I (337−355 nm) and two weak maxima in band II (247−270 nm), present in the Boja cultivar.

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MATERIALS AND METHODS

Spectral Analysis. Melting points are uncorrected. Optical rotations were obtained in a mixture of MeOH/H_2O/DMSO (45:45:10) at 24 °C on a Jasco P-1020 spectropolarimeter. ESI-HRMS were recorded on a Mariner Biospectrometry workstation (Per Septive Biosystems). ^1H, ^{13}C, and 2D NMR spectra were recorded in DMSO-d_6 or CD_3OD on a Varian UNITY-400 instrument operating at 400 MHz for ^1H NMR and at 100 MHz for ^{13}C NMR. Chemical shifts are reported in parts per million (δ), relative to TMS.

Plant Material. Alfalfa (*Medicago sativa* L. var. Boja) was grown at the Experimental Farm of the Institute of Soil Science and Plant Cultivation in Pulawy, Poland, in 1997. Plants were collected at the beginning of flowering, freeze-dried, and finely powdered.

Isolation of Flavonoids. Flavonoids were extracted with 70% aqueous MeOH from the Boja variety and first purified by solid phase extraction as previously described (*7*). The purified flavonoid fraction was loaded onto a preparative column (400 × 30 mm i.d., LiChroprep RP-18, 25−40 μm, Merck). The column was washed with water and then with increasing concentrations of MeOH in water (5% increments from 0 to 100% MeOH). Ten milliliter fractions were collected with a fraction collector. The first 400 fractions obtained by eluting the column with water and 5% MeOH contained the majority of apigenin and luteolin glycosides as previously described (*7*). Fractions 400−1250, obtained by washing the column with solvent containing >10% MeOH, showed the presence of compounds with characteristic strong absorption in band I. Fractions showing similar TLC profiles (DC-Alufolien Cellulose, Merck; solvent 15% HOAc) were combined. They were further separated/purified on a stainless Vertex column (250 × 8 mm i.d., Eurospher 100, RP18, 10 μm, Säulentechnik, Berlin, Germany) using an isocratic system (CH_3CN/1%H_3PO_4) optimized individually for each fraction

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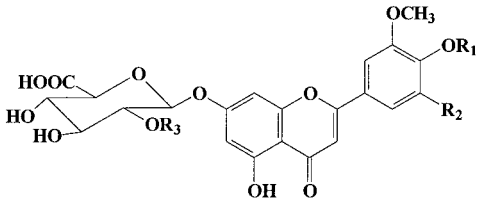

	R_1	R_2	R_3
1	-H	-OCH_3	-H
2	-H	-OH	-H
3	-GluA	-H	-H
4	-H	-OCH_3	-GluA
5	-H	-H	-GluA-2′-*O*-Feruloyl
6	-H	-OCH_3	-GluA-2′-*O*-Sinapoyl
7	-H	-OCH_3	-GluA-2′-*O*-Feruloyl
8	-H	-OCH_3	-GluA-2′-*O*-*p*-Coumaroyl
9	-H	-OCH_3	-GluA-[GluA(1→3)]-2′-*O*-Feruloyl
10	-H	-H	-GluA-[GluA(1→3)]-2′-*O*-Feruloyl

Figure 1. Isolated compounds from alfalfa (GluA = glucuronic acid).

with HPLC. Some fractions were separated on an analytical column (250 × 4.6 mm i.d., Eurospher 100, RP18, 5 μm, Säulentechnik) heated to 50 °C, using an isocratic system (CH_3CN/1%H_3PO_4). Separations were performed on HPLC (Waters with 996 PAD detector, 616 pump and Millenium software). These yielded several individual compounds as shown in Figure 1.

1: 163.1 mg, 0.408% yield; $C_{23}H_{21}O_{13}$; FABMS (negative ion mode), *m*/*z* 505 [M − H]$^-$, 329 [M − H − GluA]$^-$; UV, λ_{max}(nm) (MeOH) 247sh, 265sh, 352; for ^{1}H and ^{13}C NMR see Tables 1 and 2, respectively.

2: 4.6 mg, 0.012% yield; amorphous yellow powder; mp 219−220 °C; $[\alpha]_D^{24}$ −27.6 [MeOH − H_2O − DMSO (45:45:10), *c* 0.1]; HRMS, *m*/*z* 491.0832 [calcd for $C_{22}H_{19}O_{13}$ (M)$^-$, 491.0820]; FABMS (negative ion mode), *m*/*z* 491 [M − H]$^-$, 315 [M − H − GluA]$^-$; UV, λ_{max}(nm) (MeOH) 247sh, 265sh, 352; for ^{1}H and ^{13}C NMR see Tables 1 and 2, respectively.

3: 1.7 mg, 0.004% yield; amorphous yellow powder; mp 207−208 °C; $[\alpha]_D^{24}$ −47.4 [MeOH − H_2O − DMSO (45:45:10), *c* 0.1]; HRMS, *m*/*z* 651.1209 [calcd for $C_{28}H_{27}O_{18}$ (M)$^-$, 651.1192]; FABMS (negative ion mode), *m*/*z* 651 [M − H]$^-$, 475 [M − H − GluA]$^-$, 299 [M − H − 2GluA]$^-$; UV, λ_{max}(nm) (MeOH) 247sh, 271, 338; for ^{1}H NMR see Table 1.

4: 26.1 mg, 0.065% yield; $C_{29}H_{29}O_{19}$; FABMS (negative ion mode), *m*/*z* 681 [M − H]$^-$, 505 [M − H − GluA]$^-$, 329 [M − H − 2GluA]$^-$; UV, λ_{max}(nm) (MeOH) 247sh, 265sh, 352; for ^{1}H and ^{13}C NMR see Tables 1 and 2, respectively.

5: 13.1 mg, 0.033% yield; amorphous yellow powder; mp 235−236 °C; $[\alpha]_D^{24}$ −77.8 [MeOH − H_2O − DMSO (45:45:10), *c* 0.1]; HRMS, *m*/*z* 827.1667 [calcd for $C_{38}H_{35}O_{21}$ (M)$^-$, 827.1665]; FABMS (negative ion mode), *m*/*z* 827 [M − H]$^-$, 651 [M − H − ferulic acid]$^-$, 475 [M − H − ferulic acid − GluA]$^-$, 299 [M − H − ferulic acid − 2GluA]$^-$; UV, λ_{max}(nm) (MeOH) 248, 271sh, 338; for ^{1}H and ^{13}C NMR see Tables 1 and 2, respectively.

6: 2.9 mg, 0.007% yield; amorphous yellow powder; mp 227−228 °C; $[\alpha]_D^{24}$ −61.6 [MeOH − H_2O − DMSO (45:45:10), *c* 0.1]; HRMS, *m*/*z* 887.1889 [calcd for $C_{40}H_{39}O_{23}$ (M)$^-$, 887.1877]; FABMS (negative ion mode), *m*/*z* 887 [M − H]$^-$, 681 [M − H − sinapic acid]$^-$, 505 [M − H − sinapic acid − GluA]$^-$, 329 [M − H − sinapic acid − 2GluA]$^-$; UV, λ_{max}(nm) (MeOH) 223sh, 247sh, 269sh, 342; for ^{1}H and ^{13}C NMR see Tables 1 and 2, respectively.

7: 30.2 mg, 0.076% yield; amorphous yellow powder; mp 307−308 °C (with browning); $[\alpha]_D^{24}$ −70.2 [MeOH − H_2O − DMSO (45:45:10), *c* 0.1]; HRMS, *m*/*z* 857.1715 [calcd for $C_{39}H_{37}O_{22}$ (M)$^-$, 857.1771]; FABMS (negative ion mode), *m*/*z* 857 [M − H]$^-$, 681 [M − H − ferulic acid]$^-$, 505 [M − H − ferulic acid− GluA]$^-$, 329 [M − H − ferulic acid − 2GluA]$^-$; UV, λ_{max}(nm) (MeOH) 247sh, 269sh, 338; for ^{1}H NMR see Table 1.

8: 5.6 mg, 0.014% yield; amorphous yellow powder; mp 232−233 °C; $[\alpha]_D^{24}$ −14.7 [MeOH − H_2O − DMSO (45:45:10), *c* 0.1]; HRMS, *m*/*z* 827.1682 [calcd for $C_{38}H_{35}O_{21}$ (M)$^-$, 827.1665]; FABMS (negative ion mode), *m*/*z* 827 [M − H]$^-$, 681 [M − H − coumaric acid]$^-$, 505 [M − H − coumaric acid − GluA]$^-$, 329 [M − H − coumaric acid − 2GluA]$^-$; UV, λ_{max}(nm) (MeOH) 223sh, 271, 323; for ^{1}H NMR see Table 1.

9: 6.8 mg, 0.017% yield; amorphous yellow powder; mp 199−200 °C; $[\alpha]_D^{24}$ −35.3 [MeOH − H_2O − DMSO (45:45:10), *c* 0.1]; HRMS, *m*/*z* 1033.2104 [calcd for $C_{45}H_{45}O_{28}$ (M)$^-$, 1033.2092]; FABMS (negative ion mode), *m*/*z* 1033 [M − H]$^-$, 857 [M − H − ferulic acid]$^-$, 681 [M − H − ferulic acid − GluA]$^-$, 505 [M − H − ferulic acid − 2GluA]$^-$, 329 [M − H − ferulic acid − 3GluA]$^-$; UV, λ_{max}(nm) (MeOH) 247sh, 269sh, 338; for ^{1}H and ^{13}C NMR see Tables 1 and 2, respectively.

10: 2.8 mg, 0.007% yield; amorphous yellow powder; mp 254−255 °C; $[\alpha]_D^{24}$ −37.6 [MeOH − H_2O − DMSO (45:45:10), *c* 0.1]; HRMS, *m*/*z* 1003.2009 [calcd for $C_{44}H_{43}O_{27}$ (M)$^-$, 1003.1986]; FABMS (negative ion mode), *m*/*z* 1003 [M − H]$^-$, 827 [M − H − ferulic acid]$^-$, 651 [M − H − ferulic acid − GluA]$^-$, 475 [M − H − ferulic acid − 2GluA]$^-$, 299 [M − H − ferulic acid − 3GluA]$^-$; UV, λ_{max}(nm) (MeOH) 248, 269sh, 338; for ^{1}H NMR see Table 1.

RESULTS AND DISCUSSION

Extraction and preliminary purification of flavonoids from alfalfa (*M. sativa*) cultivar Boja afforded a mixture containing over 20 individual glycosides as shown by analytical liquid chromatography. The flavonoid profile contained two basic groups of flavonoids, one with apigenin and luteolin aglycons, having spectra with two distinct absorption bands, and the other one characterized by strong absorption in band I (337−355 nm) and only weak shoulders in the range of 247−270 nm. The first fraction, containing luteolin and apigenin glycosides, was more polar and easily separated into individual compounds on a C18 preparative column, eluted with water and 5% aqueous MeOH. These flavonoids were described previously (*7*). The second fraction, which consisted of 10 compounds of similar UV characteristics, was much more hydrophobic than the first fraction and more difficult to separate into individual compounds. In most cases separation on a semipreparative column failed (compounds **2**, **3**, **5**, **6**, **9**, and **10**), and an analytical HPLC system with a column heated to 50 °C was required. This allowed successful separation of all compounds and their full characterization with spectral techniques.

All isolated compounds (**1**−**10**) had ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) consistent with a structure of a glycosylated flavonoid (*9*). The downfield region of the ^{1}H NMR spectrum (Table 1) of these compounds showed a 2H AX system, correlated by 2D-COSY with typical shifts of an A ring of flavonoid with oxygenation at C-5 and C-7.

The B-ring signals in seven compounds (**1**, **2**, **4**, and **6**−**9**) consisted of a two-proton singlet resonance arising from a pair of degenerated protons (H-2′, H-6′), indicating oxygenation at C-3′, C-4′, and C-5′. The oxygenation of the B-ring of the other three compounds (**3**, **5**, and **10**) had to be at C-3′ and C-4′, which was shown by a three-proton ABX system, with coupling constants (2 and 8 Hz) characteristic for meta and ortho coupling, in their ^{1}H NMR spectra. The rotating frame effect (ROE) correlations (Figure 2) between the H-2′ and H-6′ signals and the H-3 singlet signal in each compound confirmed the substitution pattern of the aglycones. Together with the substitution patterns, the ^{1}H NMR

Table 1. ^{1}H NMR Data of Compounds 1−10 in DMSO[a,b]

	δ									
H	**1**	**2**	**3**[c]	**4**	**5**	**6**	**7**	**8**	**9**	**10**
aglycons										
3	7.06 *s*	6.90	6.74	7.07	6.95	6.99	6.96	7.01	7.03	6.92
6	6.47 *d*	6.45	6.49	6.44	6.42 *brs*	6.42 *d*	6.40 *brd*	6.41 *d*	6.42	6.41
8	6.91 *d*	6.83	6.84	6.81	6.78 *brs*	6.82 *d*	6.80 *brd*	6.82 *d*	6.83	6.76
2′	7.35 *s*	7.18	7.55 *d*	7.36 *s*	7.58 (o)	7.33 *s*	7.32	7.33	7.35	7.57 (o)
5′			7.27 *d*		6.94 (o)					6.95 *d*
6′	7.35 *s*	7.18	7.60 *dd*	7.36 *s*	7.57 (o)	7.33 *s*	7.32	7.33	7.35	7.56 (o)
OCH_3	2 × 3.88 *s*	3.87	3.96	2 × 3.88	3.89	2 × 3.88	2 × 3.88	2 × 3.87	2 × 3.88	3.89
sugars										
1	5.24 *d*	5.27	5.17	5.41	5.38	5.38	5.36	5.35	5.36	5.36
2	3.28 *dd*	3.28	3.55	3.54	3.55	3.56	3.53	3.53 *brdd*	3.53 (o)	3.54 (o)
3	3.33 *dd*	3.33	3.60 (o)	3.46 *dd*	3.40 (o)	3.41 (o)	3.42 (o)	3.41 (o)	3.41 (o)	3.38 (o)
4	3.38 *dd*	3.39	3.62 (o)	3.49 *dd*	3.40 (o)	3.41 (o)	3.42 (o)	3.41 (o)	3.41 (o)	3.38 (o)
5	3.98 *d*	4.03	4.07 *d*	4.10	4.02	3.99	3.92	3.97	4.01 *brd*	3.97 *d*
1′			5.12 *d*	4.55	4.91	4.92	4.93	4.92	4.98	4.98
2′			3.60 (o)	3.01 *dd*	4.64	4.64	4.63	4.62	4.80	4.79
3′			3.60 (o)	3.22 *dd*	3.40 (o)	3.41 (o)	3.42 (o)	3.41 (o)	3.86 (o)	3.79 (o)
4′			3.62 (o)	3.26 *dd*	3.40 (o)	3.41 (o)	3.42 (o)	3.41 (o)	3.53 (o)	3.49 (o)
5′			3.99 *d*	3.66	3.72	3.69	3.57 *brd*	3.69 *d*	3.82	3.79 (o)
1″									4.37 *d*	4.32
2″									2.96 *dd*	2.95
3″									3.07 *dd*	3.06
4″									3.25 *dd*	3.20
5″									3.62 *d*	3.49 (o)
phenolic acids										
2					7.25 *brs*	6.92 *s*	7.17 *brs*	7.47 *d*	7.22 *brs*	7.19 *brs*
3								6.74 *d*		
5					6.76 *d*		6.70 *brd*	6.74 *d*	6.77	6.74
6					7.07 *brd*	6.92 *s*	7.00 *brdd*	7.47 *d*	7.06 *brd*	7.03
7					7.50 *d*	7.48	7.44	7.48	7.46	7.44
8					6.41 *d*	6.42	6.32	6.31	6.37	6.33
OCH_3					3.80 *s*	2 × 3.76	3.76		3.80	3.79

[a] Multiplicities are not repeated if identical to those of previous column. [b] Coupling constant values (Hz) for aglycons: **1**−**4**, **6**, **8**−**10**, $J_{6-8} = 2$; **3**, $J_{2'-6'} = 2$; **3**, **10**, $J_{5'-6'} = 9$. Coupling constant values (Hz) for sugars: **1**−**10**, $J_{1-2} = 7$; **1**−**4**, **6**, **7**, $J_{2,3} = 8$; **1**, **2**, **4**, $J_{3,4} = 9$; **1**−**4**, **6**, **8**, **9**, $J_{4,5} = 9$; **3**−**10**, $J_{1'-2'} = 8$; **4**−**10**, $J_{2',3'} = 9$; **4**, $J_{3',4'} = 9$; **3**−**6**, **8**, **9**, $J_{4',5'} = 9$; **9**, **10**, $J_{1''-2''} = 7$; **9**, **10**, $J_{2'',3''} = 8$; **9**, **10**, $J_{3'',4''} = 9$; **9**, **10**, $J_{4'',5''} = 10$. Coupling constant values (Hz) for phenolic acids: **5**, **7**−**10**, $J_{5,6} = 8$; **5**−**10**, $J_{7-8} = 16$. [c] Spectrum obtained in CD_3OD.

Table 2. ^{13}C NMR Data of Compounds 1,2, 4−6, and 9 in DMSO-d_6

	δ											
C	**1**	**2**	**4**	**5**	**6**	**9**	C	**1**	**2**	**4**	**5**	**6**	**9**
aglycons							sugars						
2	164.2	164.5	164.2	164.2	164.1	164.2	1′			104.5	100.8	100.4	100.7
3	103.9	103.6	103.8	103.5	103.8	103.8	2′			74.2	75.7	75.4	75.8
4	182.1	182.0	182.2	182.1	182.0	182.1	3′			75.9	73.8	73.9	81.3
5	161.2	161.2	161.2	161.2	161.2	161.2	4′			70.8	71.4	71.3	71.4
6	99.3	99.2	99.2	99.2	99.2	99.2	5′			74.9	74.9	74.8	74.8
7	162.6	162.5	162.3	162.2	162.2	162.2	6′			169.9	169.8	169.9	169.8
8	95.1	94.7	95.3	94.9	95.2	95.2	1″						102.7
9	156.9	157.0	156.8	156.9	156.8	156.8	2″						71.4
10	105.5	105.5	105.5	105.5	105.5	105.5	3″						75.2
1′	120.2	121.3	120.3	121.4	120.3	120.3	4″						70.3
2′	104.6	102.6	104.5	110.4	104.5	104.6	5″						75.0
3′	148.2	148.6	148.2	148.0	148.2	148.2	6″						169.7
4′	140.1	138.9	140.0	150.9	140.0	140.0	phenolic acid						
5′	148.2	146.1	148.2	115.8	148.2	148.2	1				125.8	124.5	125.9
6′	104.6	107.8	104.5	120.4	104.5	104.6	2				111.2	105.9	111.3
OCH_3	2 × 56.4	56.3	2 × 56.4	56.0	2 × 56.4	2 × 56.4	3				149.1	148.0	149.0
sugars							4				147.9	138.1	147.8
1	99.3	99.2	97.8	97.3	97.2	97.4	5				115.5	148.0	115.5
2	72.8	72.8	82.6	79.9	79.8	80.2	6				122.8	105.9	122.7
3	75.7	75.6	75.6	73.4	73.4	72.8	7				144.6	144.9	144.4
4	71.3	71.3	71.6	71.9	71.9	71.9	8				115.0	115.3	114.9
5	75.2	75.6	75.0	75.0	74.9	75.0	9				165.8	165.7	165.5
6	170.3	170.2	170.2	170.0	170.1	170.2	OCH_3				55.7	2 × 56.0	55.7

spectra indicated the presence of methoxyl groups in each aglycon. Six compounds (**1**, **4**, and **6**−**9**) showed a six-proton singlet at δ 3.88, which had an ROE correlation with the singlet signal of H-2′ and H-6′. This is characteristic of two methoxyl groups at C-3′ and C-5′, so the aglycon part for these compounds was determined to be tricin.

Compound **2** had the same oxygenation pattern as compound **1**, but its ^{1}H NMR spectrum showed a three-proton singlet at δ 3.87, corresponding to a methoxyl group in the molecule. The ^{13}C NMR spectrum of **2** showed a signal at δ 56.3 for this methoxyl group, characteristic of either one or no *ortho* substituents (*10*), eliminating the possibility that the methoxyl group was

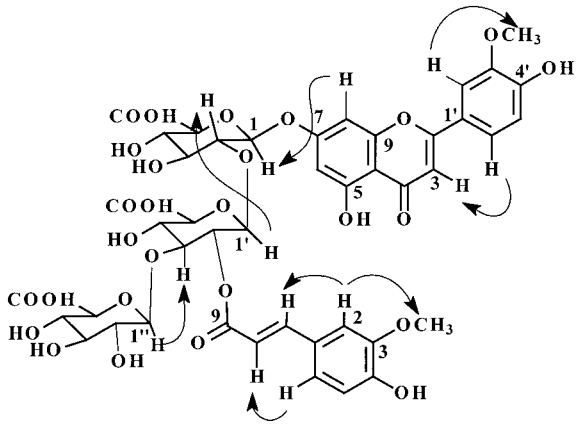

Figure 2. Example of ROE correlations, compound **10**.

located at C-4′. On the other hand a ROE correlation between the H-2′ and H-6′ signals and the methoxyl group signal was observed. This confirmed the localization of the methoxyl group at the C-3′ in the aglycon of compound **2**. Thus, the aglycon of **2** was established as 3′-*O*-methyltricetin.

Compounds **3**, **5**, and **10** showed in their ^{1}H NMR spectra a three-proton singlet at δ 3.89 in DMSO-d_6, assigned to a methyl group at C-3′ as indicated by the correlation between the H-2′ signal and the methoxyl signal observed in the ROE spectra of the compounds (Figure 2). This substitution determined the nature of their aglycon as chrysoeriol.

The UV (*11*) and ^{13}C NMR (*12*) (Table 2) spectra of each compound confirmed their aglycon structures deduced above (Figure 1). Their molecular formulas were further supported by FAB mass spectrometry, which in negative ion mode gave clear aglycon peaks, which for compounds **3**, **5**, and **10** were found at *m*/*z* 299 (chrysoeriol), for compound **2** at *m*/*z* 315 (methyltricetin), and for compounds **1**, **4**, and **6**−**9** at *m*/*z* 329 (tricin).

The nature of the sugars was similar to the apigenin and luteolin glycosides as previously reported (*7*). The only sugar unit comprising the glycosidic moieties was glucuronic acid. This was evident from FAB mass spectrometry, showing for each compound losses of 176 amu, which was characteristic for a uronic acid. The signals of ^{1}H and ^{13}C NMR spectra corresponding to the sugar moieties were in agreement with previous values for glucuronic acid (*7*). Their β-configuration was deduced from the coupling constant of the anomeric protons.

The connection between glucuronyl moieties and aglycons were established in each compound to be at the hydroxyl group of C-7. The ROE correlation between H-6 and H-8 of the A-ring and H-1 of the first glucuronic acid was observed (Figure 2). Thus, compounds **1** and **2** had one glucuronic acid attached to C-7. This was supported by the FAB mass spectra, which showed in negative ion mode molecular ions at *m*/*z* 505 and 491, respectively. Thus, the structure of **1** was elucidated as 7-*O*-β-D-glucuronopyranosidetricin, a compound previously identified in alfalfa (*13*), and that of **2** as 7-*O*-β-D-glucuronopyranoside-3′-*O*-methyltricetin, which is a novel structure.

Compound **3** had one additional glucuronic acid in comparison to **2**, as indicated by the FAB mass spectrum showing a molecular ion at *m*/*z* 651 and additional peaks at 475 and 299 corresponding to the loss of one and two uronic acids, respectively. The attachment of the additional glucuronic acid was established to be at C-4′ of the B-ring of chrysoeriol, which was evident from ROE correlations between H-5′ (δ 7.27 *d*, 9 Hz) and H-1′ of the second glucuronic acid (δ 5.12 *d*, 8 Hz). Thus, **3** was identified as 7-*O*-β-D-glucuronopyranosyl-4′-*O*-β-D-glucuronopyranosidechrysoeriol.

The difference between compounds **4** and **1** was in the presence of an additional glucuronic acid unit. The FAB mass spectrum of the former showed a molecular ion at *m*/*z* 681 and two other ions at *m*/*z* 505 and 329 arising from the loss of one and two uronic acids, respectively. The ^{13}C NMR values (Table 2) were consistent with a 1→2 linkage between two glucuronic acids, which was confirmed with the ROE correlation between H-2 and H-1′ of the first and second sugars (Figure 2). Thus, **4** was identified as 7-*O*-[β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside]-tricin. The presence of this compound in alfalfa was previously reported (*13*).

The other five compounds (**5**−**10**) had additional signals in the downfield region of their ^{1}H NMR spectrum, corresponding to a phenolic acyl moiety (*14*). Two coupled doublets (J = 16 Hz) in the region of 7.50 and 6.31 ppm indicated the presence of a trans double bound of the phenolic acyl moiety, and three signals in their ^{13}C NMR at δ 165.8, 144.6, and 115.0 (values for **5**) corresponded to C-9, C-7, and C-8, respectively (Table 2). The other signals belonged to the aromatic ring of the phenolic acyl moiety.

In the ^{1}H NMR spectrum of **8** a pair of two-proton, ortho-coupled doublets (J = 8 Hz) arising from two pairs of degenerate protons [(H-2, H-6, δ 7.47) and (H-3, H-5, δ 6.74)] were observed, which is typical for a coumaroyl group. The FAB mass spectrum showed a molecular ion at *m*/*z* 827 and additional peaks at *m*/*z* 505 and 329 corresponding to the loss of coumaric acid and glucuronic acid, respectively. The pronounced downfield shift of H-2′ of the second glucuronic acid (δ 4.64), suggested the linkage between the sugar and the acyl moiety at C-2′ as previously reported (*7*). Thus, flavonoid **8** was the acylated form of compound **4**, and its structure was established as 7-*O*-[2′-*O*-*p*-coumaroyl-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside]tricin.

In the ^{1}H NMR spectra of **5**, **7**, **9**, and **10** a three-proton ABX system characteristic of ortho and meta couplings typical for a feruloyl moiety was observed. The position of the methoxyl group at C-3 of the feruloyl residue was confirmed by ROE correlation between the signal at δ 3.80 (*s*) and H-2. Compounds **5**, **7**, **9**, and **10** showed molecular ions at *m*/*z* 827, 857, 1033, and 1003, respectively. The pronounced downfield shift of H-2′ of a second glucuronic acid (δ 4.64 and 4.63) in **5** and **7** suggested the linkage between the sugar and the acyl moiety at C-2′ as previously reported (*7*). Thus, flavonoids **5** and **7** were identified as 7-*O*-[2′-*O*-feruloyl-β-D-glucuronopyranosyl(1→2)-*O*-β-D-glucuronopyranoside]-chrysoeriol and 7-*O*-[2′-*O*-feruloyl-β-D-glucuronopyranosyl-(1→2)-*O*-β-D-glucuronopyranoside]tricin. Flavonoids **9** and **10** were analogues of **7** and **5**, respectively, each possessing one more glucuronic acid molecule. The ROE correlation between this third sugar and the rest of the molecule indicated its attachment at C-3″ of the second glucuronic acid (Figure 2). On the basis of these findings structures **9** and **10** were established as 7-*O*-{2′-*O*-feruloyl-[β-D-glucuronopyranosyl(1→3)]-*O*-β-D-glu-

curonopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}tricin and 7-*O*-{2′-*O*-feruloyl-[*β*-D-glucuronopyranosyl(1→3)]-*O*-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside}-chrysoeriol.

In the ^{1}H NMR spectrum of **6** a two-proton singlet (*δ* 6.92 *s*) was observed, indicating three oxygenated positions in the phenolic moiety, together with a six-proton signal corresponding to two methoxyl groups (*δ* 3.76 *s*). The ROE correlation between the double-bond signals and the aromatic protons singlet (Figure 2) suggested that the oxygenated positions were at C-3, C-4, and C-5, and the correlation between methoxyl and aromatic singlets confirmed the attachment of the methoxyls at C-3 and C-5, which is characteristic for a sinapoyl residue. The presence of this acyl group was further confirmed by the FAB mass spectrum, showing a molecular ion at *m*/*z* 857, which was 30 mu different ($-OCH_2-$) from that found for flavonoid **7**. Thus, the structure of **6** was established as 7-*O*-[2′-*O*-sinapoyl-*β*-D-glucuronopyranosyl(1→2)-*O*-*β*-D-glucuronopyranoside]-tricin. The nature of the coumaroyl, feruloyl, and sinapoyl groups was confirmed also by their ^{13}C NMR spectra (Table 2), which were consistent with previously published data (*14*).

To the best of our knowledge flavones **2**, **3**, and **5**−**10** are novel structures, not identified previously in the plant kingdom. Isolated novel flavonoids confirm our previous findings that alfalfa flavonoids are unique compounds as they all possess only glucuronic acid as the sole sugar in the sugar chains (*7*). Most of these glycosides are acylated with ferulic, coumaric, or synapic acid, and acylation takes place on a terminal glucuronic acid molecule. It was also established that acylation takes place on the sugar chain attached at the C-7 position. These were generally found in the Polish cultivar Boja, but our studies on the cultivar Artal grown in Portugal showed that the dominant flavonoid was acylated at the sugar chain attached at the C-4′ position. These differences may reflect variety specificity or may be generated by the environment. The function of acylated flavone glucuronides in the host plant remains unclear. The nutritional implications of such structures are also uncertain. More research is required to establish the role and bioactivity of these flavonoids.

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