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FURTHER FLAVONOL GLYCOSIDES FROM *ANTHYLLIS ONOBRYCHIOIDES*

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Key Word Index—*Anthyllis onobrychioides*; Leguminosae; flavonol glycosides; rhamnazin 3-galactoside; rhamnazin 3-galactoside-4′-glucoside; rhamnetin 3-galactoside-3′,4′-diglucoside; ^{1}H NMR; ^{13}C NMR; LDMS; chemotaxonomy.

Abstract—The new triglycoside rhamnetin 3-*O*-β-D-galactopyranoside-3′,4′-di-*O*-β-D-glucopyranoside has been isolated from the aerial parts of *Anthyllis onobrychioides*. Two other new flavonol glycosides, rhamnazin 3-*O*-galactoside and rhamnazin 3-*O*-galactoside-4′-*O*-glucoside, were identified but not isolated as pure substances.

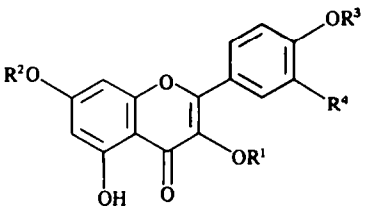
INTRODUCTION

Anthyllis onobrychioides Cav., a dwarf shrub very common in south-east Spain, belongs to a widely distributed but little studied genus of which some sixteen species are found in Spain [1]. With the exception of *A. vulneraria*, which has been extensively investigated by several groups [2–4] because of its wound-healing properties [5], few other species [6–10] have been studied from the chemical point of view. The chemotaxonomic knowledge of the genus *Anthyllis* is for these reasons very incomplete. Most papers on this genus are concerned with flavonoid content [2–4, 8–10] and since flavonoids are often excellent taxonomic markers [11, 12] an investigation of these constituents in *A. onobrychioides* should effectively contribute to a better chemotaxonomic description of this genus. We recently reported [13, 14] on the isolation of two new flavonol glycosides, rhamnocitrin 3-galactoside and rhamnocitrin 3-galactoside-4′-glucoside, and three known glycosides from aerial parts of *A. onobrychioides*. We now wish to report the complete results of our research on this species, which include the characterization of three additional new flavonol glycosides.

RESULTS AND DISCUSSION

The isolation and structural elucidation of compounds **1**–**5** have been described in our previous communications [13, 14]. Nevertheless, we include their NMR data in Tables 1 and 2 for comparison with the new compounds reported here. Compounds **6** and **7** were found by serendipity in mother liquors from crystallizations of **4** and **5**, respectively.

Compound **8** was isolated together with **5** [14] from an aqueous extract of the plant. The isolation was difficult because of its very low solubility in most solvents and the unusual mixture DMSO–$CHCl_3$ (1:10) had to be used for its crystallization. From the UV spectral analysis [15] only one free hydroxyl at C-5 could be ascertained (see Experimental). Complete hydrolysis of **8** gave rhamnetin (**14**), identified by mp and spectroscopic properties, and a 2:1 mixture of glucose and galactose (by GC of the TMSi ethers). The identification of the sugar residue at C-3 by H_2O_2 oxidation [16] was not very effective in this case since the sugar fraction was found to be (by GC of the TMSi ethers) a *ca* 1:1 mixture of glucose and galactose. More unequivocal results were obtained by controlled hydrolysis with aqueous TFA [17]. The sugar fraction



	R¹	R²	R³	R⁴
1	Gal	H	H	OH
2	Gal	H	H	OMe
3	Gal	H	H	H
4	Gal	Me	H	H
5	Gal	Me	Glc	H
6	Gal	Me	H	OMe
7	Gal	Me	Glc	OMe
8	Gal	Me	Glc	OGlc
9	H	H	H	OH
10	H	H	H	OMe
11	H	H	H	H
12	H	Me	H	H
13	H	Me	H	OMe
14	H	Me	H	OH

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Table 1. ^{1}H NMR spectra of compounds 1–14

Compound	Aromatic protons						Anomeric protons†			OMe
	H-6	H-8	H-2′	H-3′	H-5′	H-6′	H-1″	H-1‴	H-1⁗	
1	6.21 *d* (2.0)	6.41 *d* (2.0)	7.55 *d* (2.2)		6.83 *d* (8.5)	7.66 *dd* (8.5; 2.2)	5.36 *d* (7.6)			
2	6.22 *d* (2.1)	6.45 *d* (2.1)	8.02 *d* (2.1)		6.92 *d* (8.5)	7.53 *dd* (8.5; 2.1)	5.50 *d* (7.6)			3.87 *s*
3	6.21 *d* (2.0)	6.44 *d* (2.0)	8.07 *d* (9.0)	6.87 *d* (9.0)	6.87 *d* (9.0)	8.07 *d* (9.0)	5.39 *d* (7.6)			
4	6.37 *d* (2.1)	6.74 *d* (2.1)	8.09 *d* (8.9)	6.88 *d* (8.9)	6.88 *d* (8.9)	8.09 *d* (8.9)	5.40 *d* (7.5)			3.86 *s*
5	6.38 *d* (2.2)	6.73 *d* (2.2)	8.17 *d* (9.0)	7.15 *d* (9.0)	7.15 *d* (9.0)	8.17 *d* (9.0)	5.38 *d* (7.5)	4.99 *d* (7.3)		3.86 *s*
6	6.37 *d* (2.1)	6.75 *d* (2.1)	8.03 *d* (2.0)		6.94 *d* (8.5)	7.55 *dd* (8.5; 2.0)	5.52*d* (7.5)			3.85 *s*
7	6.39 *d* (2.2)	6.73 *d* (2.2)	8.03 *d* (2.0)		7.23 *d* (8.6)	7.64 *dd* (8.6; 2.0)	5.47 *d* (7.5)	5.04 *d* (7.8)		3.87 *s* 3.86 *s*
8	6.39 *d* (2.2)	6.82 *d* (2.2)	7.89 *d* (2.0)		7.22 *d* (8.8)	7.98 *dd* (8.8; 2.0)	5.42 *d* (7.6)	4.99 *d* (7.0)	4.92 *d* (7.3)	3.86 *s*
9	6.20 *d* (2.0)	6.40 *d* (2.0)	7.67 *d* (2.2)		6.90 *d* (8.5)	7.54 *dd* (8.5; 2.2)				
10	6.21 *d* (2.1)	6.47 *d* (2.1)	7.77 *d* (2.1)		6.96 *d* (8.5)	7.69 *dd* (8.5; 2.1)				3.87 *s*
11	6.21 *d* (2.0)	6.44 *d* (2.0)	8.03 *d* (8.9)	6.94 *d* (8.9)	6.94 *d* (8.9)	8.03 *d* (8.9)				
12	6.34 *d* (2.2)	6.73 *d* (2.2)	8.07 *d* (8.8)	6.93 *d* (8.8)	6.93 *d* (8.8)	8.07 *d* (8.8)				3.85 *s*
13	6.35 *d* (2.2)	6.78 *d* (2.2)	7.70–7.80 *m*		6.94 *d* (8.4)	7.70–7.80 *m*				3.86 *s* 3.84 *s*
14	6.34 *d* (2.0)	6.70 *d* (2.0)	7.71 *d* (2.0)		6.89 *d* (8.5)	7.56 *dd* (8.5; 2.0)				3.85 *s*

*At 200.13 MHz in DMSO-d_6 (room temp.); δ values are followed by multiplicity and below, in parentheses, coupling constants in Hz. Only aromatic, anomeric and methoxyl signals are given, sugar non anomeric protons showing consistently a broad absorption in the range δ3.20–3.80. The 5-OH originates a broad singlet at δ12.5–12.7 in all compounds.

†″indicates the sugar bound to 3-OH (galactose), ‴and⁗ indicate the sugars bound to 4′-and 3′-OH (glucose), respectively.

was a 1:4 mixture of glucose and galactose, which suggested that there was one galactose molecule bound to C-3 and that the two glucose molecules were attached to C-3′ and C-4′. This conclusion was confirmed by isolation of a flavonol derivative from the hydrolysis mixture which showed a bright yellow colour under UV light and gave typical flavonol UV absorptions (see Experimental). By complete acid hydrolysis, this derivative gave only rhamnetin and glucose. Compound **8** is thus rhamnetin 3-galactoside-3′,4′-diglucoside.

Definitive support for the proposed structure as well as the sugar ring size and stereochemistry of the three anomeric centres were deduced from both the ^{1}H and ^{13}C NMR spectra. The 3′,4′-dioxygenated B-ring appeared as an AMX system with signals at δ7.22 (*d*, J = 8.8 Hz), 7.89 (*d*, J = 2 Hz) and 7.98 (*dd*, J = 8.8, 2.0 Hz), which were assigned to the protons H-5′, H-2′ and H-6′, respectively. The shifts produced in the δ values of rhamnetin by the glycosylation were more or less as expected [18, 19]. The downfield shift of H-5′ (*ca* 0.3 ppm) shows a normal value as also does that of H-2′ (*ca* 0.2 ppm), whereas the downfield shift of H-6′ is perhaps somewhat higher (*ca* 0.4 ppm) than expected (Table 1). The two doublets at δ6.39 and 6.82 (J = 2.2 Hz) were attributed to H-6 and H-8, respectively. The sharp singlet at δ3.86 was originated by the methoxyl group at C-7 and the three anomeric protons gave rise to three doublets at δ5.42 (J = 7.6 Hz, galactose at C-3), 4.99 (J = 7.0 Hz) and 4.92 (J = 7.3 Hz, glucose at C-4′ and C-3′), which pointed to the presence of three pyranose rings with β-stereochemistry [20]. The ^{13}C NMR spectrum led to the same conclusion (see Table 2). Three characteristic signals at δ101.14, 101.67 and 102.01 were assigned to the three anomeric carbons, the rest of the spectrum showing all the expected signals [21, 22].

Glycosylation at C-4′ has been reported to produce a

Table 2. ^{13}C NMR spectra of compounds 1–5, 8 and 12*

Carbon number	Aromatic region 1	2	3	4	5	8	12	Carbon number	Sugar region† 1	2	3	4	5	8
2	156.18[a]	156.09[a]	156.11[a]	156.31[a]	156.30[a]	156.19[a]	147.23	1″	101.93	101.65	101.68	101.78	101.93	102.01[e]
3	133.49	133.14	133.18	133.59	134.19	134.35	135.95	2″	71.15	71.21	71.18	71.20	71.27	71.16
4	177.37	177.32	177.35	177.67	177.64	177.55	176.00	3″	73.17	73.09	73.06	73.13	73.21	73.44[f]
5	161.10	161.12	161.11	160.95	160.85	160.72	160.34	4″	67.84	67.86	67.82	67.82	67.89	67.79
6	98.56	98.61	98.58	97.77	97.79	97.75	97.42	5″	75.69	75.81	75.78	75.70	75.70	75.74
7	164.05	164.10	164.04	165.15	165.20	165.13	164.87	6″	60.04	60.21	60.18	60.09	60.16	59.99
8	93.38	93.57	93.52	92.22	92.26	92.31	92.06	1‴					100.29	101.67[e]
9	156.20[a]	156.28[a]	156.32[a]	156.72[a]	156.04[a]	155.27[a]	156.08	2‴					73.21	73.27[f]
10	103.82	103.92	103.88	104.95	105.04	104.95	104.01	3‴					76.52	76.16
1′	121.05	121.02	120.96	120.72	123.65	124.76[b]	121.55	4‴					69.84	69.86[g]
2′	115.08	113.57	130.81	130.90	130.50	118.96[c]	129.43	5‴					76.96	76.83
3′	144.66	149.35	114.95	115.03	115.92	149.68[d]	115.41	6‴					60.80	60.87[h]
4′	148.31	146.93	159.82	160.11	159.25	146.60[d]	159.30	1⁗						101.14[e]
5′	115.94	115.08	114.95	115.03	115.92	116.87[c]	115.41	2⁗						73.08[f]
6′	121.79	121.84	130.81	130.90	130.50	124.29[b]	129.43	3⁗						76.16
3′-OMe		55.92						4⁗						69.65[g]
7-OMe				55.94	55.92	55.87	55.99	5⁗						76.83
								6⁗						60.64[h]

*At 50.32 MHz in DMSO-d_6 (75°). The signals with the same superscript ([a, b ...]) may be interchanged within the corresponding spectrum.

†″ indicates the sugar residue bound to 3-OH (galactose), ‴ and ⁗ indicate the sugar residues bound to 4′- and 3′-OH (glucose), respectively.

distinctive downfield shift of 2–4 ppm in the signal of C-1′ [21, 23]. This is indeed the case for **8**, as can be seen by comparing the chemical shift of C-1′ with that of its aglycone **14** [22]. Lastly, another independent confirmation was sought via mass spectrometry. The compound failed to give a molecular peak either by the FD or the FAB mode [24, 25]. Nevertheless, we were successful with the laser desorption (LD) mode [26]. Even without the addition of cationizing agents, traces of salts in the surface of the probe tip were sufficient to promote the ionization of the sample. Thus, peaks at *m/z* 841 $[M+K]^+$, 825 $[M+Na]^+$, 679 $[M+K-C_6H_{10}O_5]^+$, 663 $[M+Na-C_6H_{10}O_5]^+$, 641 $[M+H-C_6H_{10}O_5]^+$, 501 $[M+Na-2C_6H_{10}O_5]^+$, 479 $[M+H-2C_6H_{10}O_5]^+$ and 317 $[M+H-3C_6H_{10}O_5]^+$ supplied the final evidence for the structure of **8**.

As already stated, the discovery of both **6** and **7** was rather fortuitous. On running a ^{1}H NMR spectrum of the residual mother liquor from two crystallizations of **4**, several sharp signals, in addition to those of **4**, were noticeable, even though only one spot was visible on TLC plates. The ^{1}H NMR spectrum (Table 1) suggested the presence of a 3′,4′-dioxygenated B-ring. Total hydrolysis of the mother liquor gave an aglycone fraction, which gave two constituents on polyamide TLC-plates. While one corresponded as expected to rhamnocitrin, the other was shown to be rhamnazin (**13**) by its mp and spectral behaviour. The sugar fraction consisted exclusively of galactose (GC). Since the UV spectrum of the product mixture was indistinguishable from that of authentic rhamnocitrin 3-galactoside, we assume this minor component is rhamnazin 3-galactoside. In the FD mass spectrum [24] of the product mixture, peaks at *m/z* 515 $[M+Na]^+$, 492 $[M]^+$, 353 $[M+Na-C_6H_{10}O_5]^+$ and 330 $[M-C_6H_{10}O_5]^+$ tended to support this structural assignment. Although a melting point cannot be given for **6**, its R_f values (absolute and relative to cacticin, **2**) were identical with those of **4** [13]. According to the ^{1}H NMR spectrum, there was *ca* 20% of **6** in the mother liquors of **4**.

The identification of product **7** was carried out in a similar manner. Mother liquors from crystallizations of **5** were found to be enriched with a new component (*ca* 20%), which was detected by NMR examination. The aglycone of **7** was identified as rhamnazin, which was isolated together with rhamnocitrin from the hydrolysis mixture. The sugar fraction was shown by GC to be a 1:1 mixture of glucose and galactose. Controlled hydrolysis (aqueous TFA) [17] gave only galactose (GC), which together with the NMR data (Table 1) suggested that the minor component was rhamnazin 3-galactoside-4′-glucoside. A mass spectrum (FD) of the product mixture showed peaks at *m/z* 678 $[M+H+Na]^+$, 677 $[M+Na]^+$, 515 $[M+Na-C_6H_{10}O_5]^+$, 353 $[M+Na-2C_6H_{10}O_5]^+$ and 330 $[M-2C_6H_{10}O_5]^+$, in addition to the peaks from **5**. The R_f values (absolute and relative to **2**) of **7** are the same as those of **5**, which are given in Table 3.

The NMR tables deserve some comment. In contrast to many recommendations in the literature [20, 27] about the use of TMSi ethers in ^{1}H NMR spectroscopy, we prefer to utilize DMSO-d_6 for both ^{1}H and ^{13}C NMR spectra because of its general solubilizing ability for all kinds of flavonoid compounds and easy elimination [28]. Unfortunately, no other publication has appeared in the literature, since the papers of Batterham and Highet [18] and Hillis and Horn [19], reviewing general ^{1}H NMR data of flavonoids in this solvent. We have thus included in Table 1 NMR data of the known aglycones **9–14**, which slightly differ from published ones [18, 19], to allow an

Table 3. R_f values for compounds 5* and 8

Solid phase	Solvent system	$R_f \times 100$ Absolute 5	Absolute 8	Relative to cacticin (2) 5	Relative to cacticin (2) 8
Silica gel	EtOAc–MeCOEt–HCOOH–H_2O (5:3:3:1)	18	3	28	5
(Merck)	$CHCl_3$–MeOH–H_2O (7:3:0.5)	27	11	53	22
Polyamide	$CHCl_3$–MeOH–MeCOEt–2,4-pentandione	65	7	94	10
(Macherey & Nagel TLC-11)	(20:10:5:1)				
Cellulose	H_2O	43	56	226	295
(Merck)	Phenol–H_2O (4:1)	82	52	94	60
Paper	15% HOAc	64	55	160	138
(Macherey & Nagel 218)	*t*-BuOH–HOAc–H_2O (3:1:1)	60	50	80	67

*R_f values for compound 7 are identical to those of 5.

easy comparison with the corresponding glycosides. As can be expected, 3-*O*-glycosylation produces noticeable shifts only at H-2′ and H-6′, and negligible ones (less than 0.1 ppm) at the other protons. The effects on H-2′ and H-6′ are, however, erratic and do not show any particular highfield or downfield trend. Glycosylation of the hydroxyl at C-4′ appears to give more predictable effects and originates a distinct downfield shift (*ca* 0.3 ppm) at H-3′ and H-5′. As commented above, glycosylation of the hydroxyl groups at C-3, C-3′ and C-4′ (compound **8**) gives rise to expected shifts in the signals of H-2′, H-5′ and H-6′, although lack of structurally similar glycosides in the literature precludes a comparison.

The ^{13}C δ values behave, however, in a quite predictable manner. The ^{13}C NMR data of aglycones **9**–**11**, **13** and **14** have been reported in the bibliography [22, 29] and are not given here, but the ^{13}C NMR spectrum of rhamnocitrin **12**, to the best of our knowledge, has not been described as yet. In general, the methylation and glycosylation induced shifts correspond acceptably to predictions [21, 22]. Assignment of individual signals has been made according to the literature.

From the chemotaxonomic point of view, *A. onobrychioides* displays clear similarities with the well studied *A. vulneraria*. In fact, five of the six flavonol aglycones isolated from the former species had also been identified in the latter [2, 30]. Remarkably, neither flavones, isoflavonoids nor 5-deoxyflavonols have been detected in *A. onobrychioides* although they are very common constituents in eight of the nine tribes of Leguminosae [31]. However, isoflavonoids are rather unusual in the tribe Loteae, to which the genus *Anthyllis* belongs. On the other hand, 5-deoxyflavonols occur throughout the Leguminosae [31] and have indeed been found in *A. vulneraria* [30].

It should also be mentioned that three of the above mentioned aglycones, rhamnocitrin (**12**), rhamnazin (**13**) and rhamnetin (**14**) are common constituents of the Rhamnaceae but are not frequently found in other plant families [31]. Until recently [32], only ten glycosides of rhamnetin, five glycosides of rhamnocitrin and three glycosides of rhamnazin had been described, almost all of which having one sugar residue bound to the hydroxyl group at C-3. As far as we know, compound **8** is the first 3,3′,4′-triglycosylated flavonol to be found in nature.

EXPERIMENTAL

^1H and ^{13}C NMR spectra were run at 200.13 and 50.32 MHz on a Bruker AC-200 NMR spectrometer. The solvent signals at δ2.49 (^1H) and δ39.5 (^{13}C) were used as reference. ^1H NMR spectra were measured at room temp., no significant differences being observed with those registered at higher temp. [13, 14]. ^{13}C NMR spectra were run at high temp. (75°) as recommended [21], although it did not appear to give differences in some cases. FDMS were measured on a Varian MAT-731 mass spectrometer and required the addition of NaI [24, 25]. The LD mass spectrum was measured with a Nicolet 2000 FT mass spectrometer.

Plant material. The plant material and its treatment have been already described [13, 14]. An improvement in the isolation of **5** and **8** was obtained in the following way: the aq. extract remaining after continuous extraction with Et_2O and EtOAc was re-extracted with *n*-BuOH leaving sugars in the aq. layer. The *n*-BuOH extract was concd *in vacuo* and eluted from Sephadex LH-20 with 70% aq. MeOH to give **8** (*ca* 10 mg) and **5**+**7** (*ca* 50 mg).

Rhamnetin 3-galactoside-3′,4′-diglucoside (**8**). Compound **8** cryst. from DMSO–$CHCl_3$ (1:10) as pale yellow needles, mp 223–225°. For R_f values (absolute and relative to **2**) see Table 3. UV λ_{max}^{MeOH} nm: 273, 282 sh, 338, 390 sh; $\lambda_{max}^{MeOH+NaOMe}$ nm: 286, 316 sh, 386; $\lambda_{max}^{AlCl_3}$ nm: 258, 280, 294 sh, 342, 390 sh; $\lambda_{max}^{AlCl_3+HCl}$ nm: 256, 280, 294 sh, 342, 388 sh; λ_{max}^{NaOAc} nm: 275, 285, 342 sh; $\lambda_{max}^{NaOAc+H_3BO_3}$ nm: 270, 336. For NMR data see Tables 1 and 2. LDMS (probe) *m/z* (rel. int.): 841 $[M+K]^+$ (35), 825 $[M+Na]^+$ (40), 679 $[M+K-C_6H_{10}O_5]^+$ (37), 663 $[M+Na-C_6H_{10}O_5]^+$ (100), 641 $[M+H-C_6H_{10}O_5]^+$ (35), 501 $[M+Na-2C_6H_{10}O_5]^+$ (56), 479 $[M+H-2C_6H_{10}O_5]^+$ (28), 317 $[M+H-3C_6H_{10}O_5]^+$ (73).

Total hydrolysis was performed under the usual conditions (2N HCl, 100°, 30 min). Partial hydrolysis was carried out according to Markham [17] with 1 N TFA (80°, 10 min). The hydrolysis mixture was extracted with EtOAc giving a small monoglycoside and aglycone fraction, and then with *n*-BuOH, which gave the main diglycoside fraction, with sugars remaining in the aq. layer. The *n*-BuOH extract was concd *in vacuo* and the residue percolated through a short Sephadex LH-20 column using MeOH as eluant. The diglycoside displayed a typical flavonol UV spectrum: λ_{max}^{MeOH} nm: 253, 269, 290 sh, 328 sh, 366, 425 sh; $\lambda_{max}^{MeOH+NaOMe}$ nm: 262, 265 sh, 285 sh, 351, 437; $\lambda_{max}^{AlCl_3}$ nm: 259, 265 sh, 300 sh, 357, 422; $\lambda_{max}^{AlCl_3+HCl}$ nm: 244, 259, 265 sh, 300 sh, 357, 422; λ_{max}^{NaOAc} nm: 262, 335 sh, 418:

$\lambda_{max}^{NaOAc+H_3BO_3}$ nm: 270, 329 sh, 368. The above data are consistent with a 3,5-dihydroxyflavone carrying substituents (other than OH) at C-3′ and C-4′ [15]. Total hydrolysis of this glycoside yielded a sugar fraction which consisted only of glucose. The aq. layers of the total and partial hydrolyses of **8** were conc. to dryness and studied by GC (see Results and Discussion). Oxidative degradation of **8** was performed according to ref. [16].

Compounds **6** and **7** were found in mother liquors from crystallizations of **4** and **5**, respectively but no chromatographic system was found to separate these pairs of products. For R_f values (absolute and relative to **2**) see Table 3 and ref. [13]. For ^{1}H NMR data, see Table 1. For FDMS data, see Results and Discussion. Partial hydrolysis of the mixture **5** + **7** was performed at 70° (10 min) [17]. Extraction with EtOAc gave a monoglycoside fraction which showed two spots on TLC (silica gel) with almost identical R_f values. The sugar fraction was analysed as above by GC.

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