Secondary Metabolites from *Marrubium velutinum*, Growing Wild in Greece

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From the aerial parts of *Marrubium velutinum*, two new phenylethanoid glycosides, velutinosides III-IV have been isolated together with four known phenylethanoid glycosides, as well as one new acylated flavone and five known flavonoids. The structures of the isolated compounds were established by means of NMR, MS, and UV spectral analyses.

Key words: Marrubium velutinum, Tetrasaccharidic Phenylethanoid Glycoside, Flavonoids

Introduction

Marrubium velutinum Sibth. & Sm. (Lamiaceae) is an endemic herb of central and southern Greece growing in dry rocky places in pastures [1]. In continuation of our research on this species [2], we report here on the isolation and identification of four phenylethanoid glycosides and six flavonoids.

Results and Discussion

From the methanolic extract of the aerial parts of *M. velutinum* five phenylethanoid glycosides (**2 – 7**) and six flavonoids (**8 – 13**) were isolated.

Compound **2** was obtained as an amorphous yellowish powder. Its MALDI-HRMS exhibited a pseudomolecular ion $[M+Na]^+$ at *m/z* 969.3201, compatible with the molecular formula $C_{42}H_{58}O_{24}$. The IR spectrum showed absorption bands typical of hydroxyl (3380 cm^{-1}), α,β-unsaturated ester (1685, 1620 cm^{-1}) and aromatic rings (1630, 1600, and 1520 cm^{-1}). The ^{1}H NMR spectrum of **2** exhibited proton signals characteristic of an *E*-feruloyl group (three aromatic protons resonating at $\delta = 7.21 - 6.85$ as an ABX system, two protons of a *trans* configured double bond at $\delta = 7.69$, 6.39 $J = 16.0$ Hz) as well as a methoxy group at $\delta = 3.89$ and a 3-hydroxy-4-methoxy-phenylethanol moiety (three aromatic protons resonating at $\delta = 6.86$, 6.78 and 6.74 as an ABX system, a double doublet at $\delta = 2.84$ due to a β-methylene and two non-equivalent protons at $\delta = 4.07$ and 3.77). Additionally, four signals assignable to anomeric protons indicated the presence of four sugar moieties in **2**: a doublet at $\delta = 4.45$ ($J = 7.8$ Hz, H-1' of inner β-glucose), a broad singlet at $\delta = 5.47$ (H-1" of α-rhamnose) and two doublets at $\delta = 4.34$ and 4.32 ($J =$ 6.8 Hz, H-1"" of α-arabinose and $J = 7.5$ Hz, H-1"' of outer β-glucose, respectively). These findings matched those in the HSQC/^{13}C NMR spectra, where four corresponding anomeric carbons resonated at $\delta = 104.2$, 102.4, 107.4, and 104.8, respectively. The downfield shift of C-6' indicated that this position is a glycosylation site. This finding was further confirmed by HMBC experiments, where a crosspeak between H-1"' and C-6' was observed. The carbon resonances assigned to the outer β-glucose unit showed no unusual chemical shifts, suggesting its terminal position. A further site of connectivity was proved to be C-2" of rhamnose, on the basis of ^{1}H and ^{13}C NMR spectra, as well as HMBC connectivities: H-1" and C-2" of rhamnose were highly deshielded as in the case of velutinosides I and II [2]. The site of attachment of arabinose was confirmed by crosspeaks between H-1""/C-2" and H-2"/C-1"" in the HMBC spectrum. Crosspeaks between H-1"/C-3' and H-3'/C-1" confirmed the usual linkage between glucose and rhamnose (Rha $^{1\rightarrow3}$ Glu), as can be observed for example in velutinoside I. The acylation site is on position C-4' of glucose and this was evident from the strong deshielding of H-4' ($\delta =$ 5.03). Detailed ^{1}H and ^{13}C NMR analysis showed a close relation to the stucture of velutinoside II, which

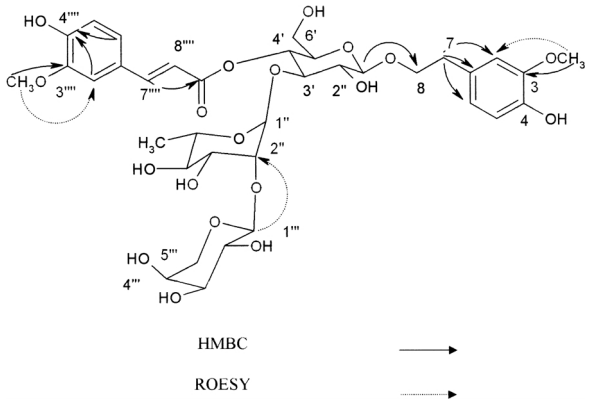


Fig. 1. Selected HMBC and rOe correlations for velutinoside IV.

contains the same sugar subunits (Ara $^{1\rightarrow2}$ Rha $^{1\rightarrow3}$ Glu $^{6\leftarrow1}$ Glu), except for the presence of one additional methoxy group belonging to the aglycon. The site of attachment of this methoxy group is established by HMBC spectrum (H-5, OCH_3/C-4). The presence of this methoxy group results in a deshielding of H-5 compared to velutinoside II (6.85 *vs* 6.71) partially overlapped by the signal of H-5””’. The site of attachment of arabinose was further confirmed by crosspeaks between H-1””/C-2” and H-2”/C-1”” in the HMBC spectrum. In the spectrum, same crosspeaks between H-1”/C-3’ and H-3’/C-1” confirmed the usual linkage between glucose and rhamnose (Rha $^{1\rightarrow3}$ Glu), as can be observed for example in Velutinoside I. The acylation site is on position C-4’ of glucose and this was evident from the strong deshielding of H-4’ ($\delta = 5.03$). The complete assignment of all proton and carbon resonances was achieved after careful analysis of ^{1}H-^{1}H COSY, HSQC, HMBC and ^{1}H-^{1}H TOCSY experiments.

The ^{1}H and ^{13}C NMR spectroscopic data of compound **3** showed that its structure is closely related to that stachysoside A formerly isolated from the same plant [2], with the exception of two singlets resonating at $\delta = 3.85$ (3H) and 3.89 (3H) due to methoxyl groups. ROESY crosspeaks between OCH_3/H-2”” and OCH_3/H-2 revealed that the site of attachment of these additional methoxyl groups are on C-3”” of the acyl chain and on C-3 of the aglycon, respectively. The complete assignment of all proton and carbon resonances was achieved for both compounds after careful analysis of ^{1}H-^{1}H COSY, HSQC, HMBC and ^{1}H-^{1}H TOCSY experiments. Some significant HMBC correlations are shown in Fig. 1.

Table 1. ^{1}H NMR (400 MHz, *J* in Hz) of **2** (CD_3OD + 2 drops D_2O) and **3** (CD_3OD).

Aglycone	**2**	**3**
H-2	6.78 br *s*	6.87 brs
H-5	6.86 *d* ($J = 7.8$)	6.70 *m*
H-6	6.74br *d* ($J = 7.8$)	6.70 *m*
H-7	2.84 *dd* ($J = 7.2$, 6.8)	2.86 *dd* ($J = 7.4$, 6.9)
H-8a	4.07 *m*	4.08 *m*
H-8b	3.77 *m*	3.75 *m*
OCH_3	3.82 *s*	3.85 *s*
Inner glucose		
H-1’	4.45 *d* ($J = 8.2$)	4.39 *d* ($J = 8.3$)
H-2’	3.41 *dd* ($J = 8.9$, 7.8)	3.39 *t* ($J = 8.3$)
H-3’	3.79*	3.77
H-4’	5.03*	4.92
H-5’	3.83*	3.54*
H-6a’	3.98*	3.63
H-6b’	3.66*	3.54*
Rhamnose		
H-1”	5.47 br *s*	5.50 brs
H-2”	3.99 br *s*	3.96 brs*
H-3”	3.68*	3.65 *dd*($J = 9.7$, 3.2)
H-4”	3.33*	3.28 *dd*($J = 9.7$, 10.6)
H-5”	3.52*	3.57*
H-6”	1.05 *d* ($J = 6.2$)	1.07 *d* ($J = 6.0$)
Outer glucose		
H-1”’	4.32 *d* ($J = 7.5$)	
H-2”’	3.24 *dd* ($J = 7.5$, 8.9)	
H-3”’	3.46*	
H-4”’	3.32*	
H-5”’	3.28*	
H-6a”’	3.87*	
H-6b”’	3.70*	
Arabinose		
H-1””	4.34 *d* ($J = 6.8$)	4.31 *d* ($J = 7.4$)
H-2””	3.60*	3.60*
H-3””	3.55*	3.49 *dd* ($J = 9.7$, 3.2)
H-4””	3.83*	3.77*
H-5a””	3.88*	3.85*
H-5b””	3.58*	3.53*
Feruloyl group		
H-2””’	7.21 br *s*	7.20 brs
H-5””’	6.85 *d* ($J = 7.9$)	6.81*d*($J = 8.3$)
H-6””’	7.12br *d* ($J = 8.5$)	7.09*dd*($J = 8.3$, 1.9)
H-7””’	7.69 *d* ($J = 16.0$)	7.66*d*($J = 16.2$)
H-8””’	6.39 *d* ($J = 16.0$)	6.38*d*($J = 15.7$)
OCH_3	3.89 *s*	3.89*s*

* Signal pattern unclear due to overlapping.

Compound **7** was identified as chrysoeriol 7-*O*-(6”-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside by 1D, 2D NMR and UV spectroscopic analyses and by MS spectrometry. ^{1}H and ^{13}C NMR spectra of **7** showed characteristic shift values and multiplicities of a 7-*O*-β-glucosylated chrysoeriol derivative [3]. Besides the 15 carbon signals of the flavonoid nucleus, the ^{13}C NMR spectrum of **7** exhibited six carbon resonances of a sugar moiety, and 9 carbon signals indicating the presence of one acyl group. An additional

Table 2. ^{13}C NMR (75.5 MHz, at 295 K) data of **1**, **2** (CD_3OD + 2 drops D_2O) and **3** (CD_3OD).

Aglycone	**1**	**2**	**3**
C-1	131.5	131.2	131.3
C-2	117.1	117.4	113.4
C-3	146.9	145.8	147.6
C-4	146.1	145.2	149.4
C-5	116.9	116.6	115.7
C-6	121.7	121.8	122.1
C-7	36.9	36.6	37.3
C-8	72.9	72.5	72.7
-OCH_3	−−	57.0	56.9
Inner glucose			
C-1'	104.2	104.2	104.7
C-2'	76.4	76.0	76.6
C-3'	82.9	82.9	82.8
C-4'	70.7*	70.6	71.0
C-5'	74.5	74.6	76.5
C-6'	69.6	69.5	62.9
Rhamnose			
C-1"	102.3	102.4	102.5
C-2"	82.8	82.8	83.3
C-3"	71.8	71.8	72.6
C-4"	74.0	73.8	74.7
C-5"	70.7*	70.8	70.9
C-6"	18.6	18.7	18.9
Outer glucose			
C-1"'	104.7	104.8	
C-2"'	75.0	75.2	
C-3"'	77.8	77.9	
C-4"'	71.5	71.6	
C-5"'	77.9	78.0	
C-6"'	62.6	62.6	
Arabinose			
C-1""	107.3	107.4	108.0
C-2""	72.9	73.2	72.3
C-3""	74.2	75.0	74.5
C-4""	70.0	70.1	70.3
C-5""	67.6	67.4	67.8
Feruloyl-group			
C-1""'	127.6	127.9	127.9
C-2""'	111.0	112.1	111.5
C-3""'	149.4	149.2	148.9
C-4""'	150.9	150.8	150.1
C-5""	116.8	116.6	116.1
C-6""'	124.8	124.3	124.2
C-7""'	148.7	148.6	147.8
C-8""'	115.0	115.0	115.0
C-9""'	167.8	167.8	167.8
OCH_3	56.9	57.1	56.3

signal at $\delta = 56.0$ indicated a methoxylation. Accordingly, the ^{1}H NMR spectrum showed six protons resonating as doublets at $\delta = 7.48$ and 6.31 (each 1H, $J = 16.0$ Hz, H-7"', H-8"', respectively), as well as at $\delta = 7.35$ and 6.66 (each 2H, $J = 9.0$ Hz, H-2"'/H-6"' and $J = 8.5$ Hz, H-3"'/H-5"') indicating the presence of one *p*-coumaroyl moieties with *trans* configured double bond. This was confirmed by ^{13}C NMR spectral data (see Experimental Section) and all protons and carbons were assigned with the help of interpretation of ^{1}H-^{1}H COSY, HSQC and HMBC spectra. The linkage of the *trans* coumaroyl group to the sugar was deduced from the downfield shifted signals of H-6a", and H-6b" (at $\delta = 4.46$ and 4.14, respectively). Additionally, the attachment of the acyl group to glucose was confirmed by HMBC crosspeaks between C-9"'/H_2-6". It was also proved by the HMBC spectrum, that the β-glucopyranosyl moiety is attached to C-7, showing correlations between the anomeric proton H-1" ($\delta = 5.16$, $J = 7.5$ Hz, *d*) and C-7 ($\delta = 162.7$). Accordingly, the ROESY exhibited crosspeaks between H-1", H-8, and H-6. The position of the methoxy group was assigned to C-3' on the basis of HMBC correlations between C-3' and the protons of the methoxyl group. This was confirmed by ROE correlations between the methoxy group and H-2'. UV and MS data corroborated the results obtained from the NMR spectra (see Experimental Section).

As far as we know, the described phenylethanoid glycosides named velutinosides III (**2**), IV (**3**) and the flavonoid **8** are reported here for the first time. We also report in this paper, the ^{13}C NMR data of previously isolated velutinoside II (**1**) [2] (see Table 2).

On the basis of ^{1}H and ^{13}C NMR, UV and MS data compounds **4 – 7**, **9 – 13** were identified as lamiophlomiside (**4**) [4], cistanoside F (**5**) [5], stachysoside D (**6**) [6], martynoside (**7**) [7], apigenin (**9**) [8], luteolin 3', 4'-dimethyl ether (**10**) [9], rutin (**11**) [8], 6-hydroxykampferol rutinoside (**12**) [10], tiliroside (**13**) [11].

The results of this and the earlier investigation of *M. velutinum* [2] indicated the occurrence of a greater variety and abundance of the flavonoid content in this plant. Moreover, the isolation of rutin, 6-hydroxykaempferol rutinoside and tiliroside, in addition with the previously isolated flavonol derivatives from *M. velutinum* [2] is distinguishing this plant from the other taxa of subtribe Stachydeae, as the majority of their flavonoids are flavones [12, 13].

Experimental Section: Materials and Methods

General experimental procedures

NMR: 400 MHz (1D and 2D) 50.3 MHz (^{13}C). The NMR spectra were recorded using Bruker DRX 400 and Bruker AC 200 instruments at 295 K. Chemical shifts are given in δ (ppm) and the spectra were referenced against residual undeuterated solvent. UV: UV spectra were recorded

on a Shimadzu UV-160A spectrophotometer, according to Mabry *et al*. [14]. MS: ESI-MS were measured on a Waters Micromass ZQ, Cone voltage 25 V. The samples were solved in MeOH/H_2O. HR-MALDI mass spectra were measured on an Ionspec Ultima FTMS spectrometer using 2,5-dihydroxybenzoic acid (DHB) as matrix. FT-IR Spectrometer: Perkin-Elmer Paragon 500. The $[\alpha]_D$ values were obtained in MeOH at 20 °C on a Perkin-Elmer 341 Polarimeter.

Chromatography

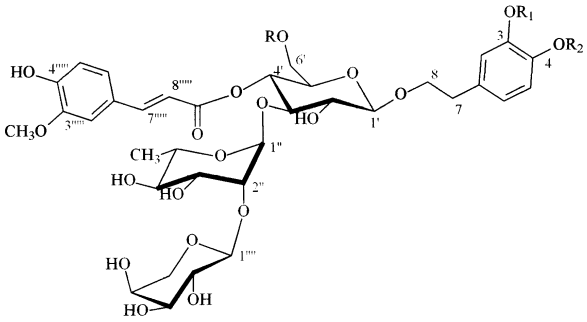
Vacuum liquid chromatography (VLC): silica gel 60H (Merck, Art. 7736); MPLC: Büchi 668, RP-silica gel 60 (Merck, Art. 10167), HPLC: Sykam S1021 solvent delivery system. UV/Vis detector S3200, Column: Kromasil C_{18}. Column chromatography (CC): silica gel 60 (Merck, Art. 9385), gradient elution with the solvents mixtures indicated in each case; Sephadex LH-20 (Pharmacia). TLC: Merck silica gel 60 F_{254} (Art. 5554); Merck cellulose (Art. 5552). Detection: UV-light, spray reagent [vanillin-H_2SO_4 on silica gel; Neu's reagent on cellulose [15].

Plant material

Aerial parts of *Marrubium velutinum* Sibth. & Sm. were collected from Kellaria-Parnassos mountain (Sterea Hellas) in July 1998. Voucher specimen has been kept in the Herbarium of Patras University (UPA) under the number Skaltsa & Lazari 114.

Extraction and isolation

The air-dried powdered aerial parts of *M. velutinum* (0.63 kg) were successively extracted at room temperature with petroleum ether, ether, EtOAc and MeOH (2 l of each solvent, twice, 48 h). The dried MeOH extract (77.0 g) was subjected to VLC over silica gel (10×8 cm^2) with CH_2Cl_2-MeOH mixtures of increasing polarity to yield eight fractions (A-H) of 500 ml. Further purification of fraction D (2.1 g; eluted with CH_2Cl_2-MeOH 60:40) by CC over Sephadex LH-20 (MeOH) afforded ten fractions (D_1-D_{10}). Fraction D_{10} was further purified by CC over silica gel (CH_2Cl_2–MeOH–H_2O, mixtures of increasing polarity) and yielded compounds **8** (3.2 mg) and **13** (3.6 mg). Fraction D_1 was further purified by TLC and yielded compounds **9** (1.0 mg) and **10** (1.0 mg). Fraction E (13.4 g; eluted with CH_2Cl_2-MeOH 50:50 to 40:60) was applied to VLC over silica gel using mixtures of CH_2Cl_2–MeOH (9:1 – 3:7) and afforded four fractions (E_1-E_4). Fraction E_3 (6.5 g; eluted with CH_2Cl_2-MeOH 65:35 to 60:40) was applied to VLC over silica gel using mixtures of CH_2Cl_2–MeOH (9:1 – 1:9) yielded six fractions (E_{3a} – E_{3f}). Purification of fraction E_{3c} (334.5 mg; eluted with CH_2Cl_2-MeOH 75:25) by CC over silica gel followed by Sephadex LH-20 (MeOH) allowed the isolation of **5** (5.3 mg) and **7** (1.1 mg). Purification of fraction E_{3d} (2.31 g; eluted with CH_2Cl_2-MeOH 70:30) by repeated CC on silica gel with mixtures of CH_2Cl_2–MeOH–H_2O (97:3:0.3 – 30:7:0.7), on Sephadex LH-20 (MeOH) and on RP-18 HPLC (MeCN–H_2O 25:75) finally yielded compounds **3** (2.1 mg; *Rt* 18.0 min), **4** (4.8 mg; *Rt* 12.5 min) and **6** (1.3 mg; *Rt* 21.4 min). Fraction E_{3e} (2.63 g; eluted with CH_2Cl_2-MeOH 65:35 to MeOH 100%) was subjected to repeated CC and afforded **11** (4.8 mg) and **12** (3.6 mg). Fraction E_4 (3.3 g; eluted with CH_2Cl_2-MeOH 55:45 to MeOH 100%) was applied to MPLC (H_2O 100%-MeOH 50%, mixtures of decreasing polarity) to afford 20 fractions (E_{4a} – E_{4u}). Fraction E_{4u} (49.6 mg; eluted with H_2O-MeOH 70:30 to 65:35) was further subjected on RP-HPLC (MeOH–aq. AcOH 3.5% 35:65) to afford **2** (6.0 mg; *Rt* 11.7 min).



		R	R_1	R_2
1	Velutinoside **II**	glu	H	H
2	Velutinoside **III**	glu	H	CH_3
3	Velutinoside **IV**	H	CH_3	H

*Velutinoside III (***2***)*

Amorphous yellow powder (6.0 mg). $[\alpha]_d^{20}$ – 54.8° (MeOH, *c* 0.09). – UV/vis (MeOH:H_2O 1:1): λ_{max} (lgε) = 281.5 nm (27.70), 327.5 nm (26.35). – IR (film): $\tilde{\nu}$ = 3280 (OH), 1695 (C=O), 1638 (C=C), 1519 cm^{-1}. ESI-MS (pos) *m/z* 969 [M + Na]$^+$. MALDI-HRMS (pos.) *m/z*: 969.3201 [M + Na]$^+$, ([$C_{42}H_{58}O_{24}$ + Na]$^+$ requires for 969.3215). ^{1}H and ^{13}C NMR spectral data see Tables 1 and 2.

*Velutinoside IV (***3***)*

Amorphous yellow powder (2.1 mg). $[\alpha]_d^{20}$ – 24.6° (MeOH, *c* 0.19). – UV/vis (MeOH): λ_{max} (lgε) = 286.5 nm (7.89), 327.0 nm (10.44). – IR (film): $\tilde{\nu}$ = 3385 (OH), 1697 (C=O), 1630 (C=C), 1525 cm^{-1}. ESI-MS (pos) *m/z* 807 [M + Na]; MALDI-HRMS (pos.) *m/z*: 807.2695 ([$C_{36}H_{48}O_{19}$ + Na]$^+$ requires for 807.2688). ^{1}H and ^{13}C NMR spectral data see Tables 1 and 2.

*Chrysoeriol 7-O-(6"-O-E-p-coumaroyl)-β-D-glucopyranoside (***8***)*

Amorphous yellow powder (3.2 mg). $[\alpha]_d^{20}$ – 15° (MeOH, *c* 0.16). – UV/vis (MeOH): λ_{max} (lgε) = 271 nm (4.07),

318 nm (5.21), 348^{sh} nm (3.27). – IR (film): $\tilde{\nu}$ = 3330 (OH), 1710 (C=O, ester), 1655 (C=O, flavone), 1600, 1510, 1445 cm^{-1}. – ^{1}H NMR (400 MHz, CD_3OD): δ: aglycone: 7.58 (*d*, *J* = 9.0, 1H, H-6'), 7.56 (*d*, *J* = 2.0, 1H, H-2'), 6.94 (*s*, 1H, H-3), 6.92 (*d*, *J* = 8.5, 1H, H-5'), 6.84 (*d*, *J* = 2.5, 1H, H-8), 6.48 (*d*, *J* = 2.0, 1H, H-6), 3.88 (*s*, 3H,3'-OMe); glucose: 5.16 (*d*, *J* = 7.5, 1H, H-1"), 3.31 (m, 1H, H-2"), 3.36 (m, 1H, H-3"), 3.44 (*dd*, *J* = 10.0, 8.0, 1H, H-4"), 3.83 (*m*, 1H, H-5"), 4.46 (br *d*, *J* = 11.5, 1H, H-6a") and 4.14 (*dd*, *J* = 11.5, 6.5, 1H, H-6b"); *E*-p-coumaroyl moiety: 7.48 (*d*, *J* = 16.0, 1H, H-7"'), 7.35 (*d*, *J* = 9.0, 2H, H-2"'/H-6"'), 6.66 (*d*, *J* = 8.5, 2H, H-3"'/H-5"'), 6.31 (*d*, *J* = 16.0, 1H, H-8"'). – ^{13}C NMR: aglycone: 164.6 (C-2), 103.4 (C-3), 182.1 (C-4), 161.2 (C-5), 99.7 (C-6), 162.7 (C-7), 94.9 (C-8), 156.9 (C-9), 105.5 (C-10), 122.3 (C-1'), 110.2 (C-2'), 148.1 (C-3'), 151.5 (C-4'), 115.8 (C-5'), 120.6 (C-6'), 56.0 (OMe); glucose: 99.5 (C-1"), 73.0 (C-2"), 76.3 (C-3"), 70.1 (C-4"), 73.8 (C-5"), 63.5 (C-6"); *E*-p-coumaroyl moiety: 124.9 (C-1"'), 130.1 (C-2"', C-6"'), 115.7 (C-3"', C-5"'), 159.8 (C-4"'), 145.0 (C-7"'), 113.7 (C-8"'), 166.5 (C-9"'). – ESI-MS (pos) *m/z* 631 [M + Na]. MALDI-HRMS (pos.) *m/z*: 631.1432 ($[C_{31}H_{28}O_{13} + Na]^+$ requires for 631.1428).

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