Petunidin 3-*O*-α-Rhamnopyranoside-5-*O*-*β*-glucopyranoside and Other Anthocyanins from Flowers of *Vicia villosa*

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Three anthocyanins were isolated from the acidified methanolic extract of blue flowers of *Vicia villosa* Roth (hairy vetch) by a combination of ion-exchange resin and gel filtration. Mostly on the basis of homo- and heteronuclear two-dimensional nuclear magnetic resonance techniques, their structures were elucidated to be the 3-*O*-α-rhamnopyranoside-5-*O*-*β*-glucopyranosides of petunidin (71%), delphinidin (12%), and malvidin (9%), respectively.

Keywords: *Vicia villosa; Leguminosae; hairy vetch; anthocyanins; petunidin 3-O-α-rhamnopyranoside-5-O-β-glucopyranoside*

INTRODUCTION

Vicia villosa Roth (Leguminosae) has its native occurrence in Europe and Asia ranging from the boreal to the subtropical zones. It is used as forage and is harvested when it is flowering. In the United States and Canada the plant is also grown to increase the nitrogen content of the soil. Torck and Pinkas (1992) have identified the flavonoids of *V. villosa* to be the 3-rhamnosides of kaempferol and quercetin. Nozzolillo (1973) has identified the main anthocyanin in *V. villosa* Roth to be a delphinidin glycoside. Several other *Vicia* species have been analyzed with respect to their content of anthocyanins and flavonoids (Ishikura et al., 1978; Nozzolillo, 1973; Nozzolillo et al.,1989; Torck and Pinkas, 1992; Yoshitama et al., 1980). Here we report the complete structure of the three major anthocyanins isolated from flowers of *V. villosa*.

MATERIALS AND METHODS

Extraction and Separation. Flowers (~300 g) of *V. villosa* Roth (hairy vetch) were collected during March 1997 in Reggio Calabria prefecture (Italy). The identity was verified by Dr. G. Tripodi (University of Messina). The flowers were cut into pieces with a pair of scissors and extracted overnight with ~1 l MeOH/HOAC (19:1 v/v) at 4 °C. The filtered extract was concentrated under reduced pressure, purified by partition (three times) against 0.3 L of EtOAc, and applied to an Amberlite XAD-7 column. The pigments were partly purified by washing with ~3 L of water before being eluted from the XAD-7 column with ~1 L of MeOH/HOAC (19:1 v/v). The anthocyanins were separated on a Sephadex LH-20 column (100 × 1.0 cm, Pharmacia) using MeOH/H_2O/TFA (39.9:60.0:0.1 v/v) as eluent.

Analytical Chromatography. TLC was carried out on microcrystalline cellulose (F5565, Merck) with the solvents BAW (1-butanol/HOAc/H_2O, 4:1:5 v/v, upper phase) and FHW (HCO_2H-concentrated HCl−H_2O; 1:1:2 v/v). HPLC was performed with a ODS-Hypersil column (20 × 0.5 cm, 5 μm) and two solvents, HCO_2H/H_2O (1:9) (A) and HCO_2H/H_2O/MeOH (1:4:5) (B) using isocratic elution (90% A, 10% B) for 4 min followed by a linear gradient from 10% B to 100% B for 17 min and then isocratic elution (100% B) for 4 min followed by linear gradient from 100% B to 10% B for 1 min. The flow rate was 1.3 mL min^{-1}, and aliquots of 15 μL were injected. Prior to injection all samples were filtered through a 0.45 μm Millipore membrane filter.

Spectroscopy. UV−vis absorption spectra were recorded on-line during HPLC analysis using a photodiode array detector (HP 1050, Hewlett-Packard) and in the solvent 0.1% concentrated HCl in MeOH. Spectral measurements were made over the wavelength range 240−600 nm in steps of 2 nm. The relative quantitative data were based on the average values of the absorptions on every second nanometer between 500 and 540 nm, without the different molar absorption coefficients of the pigments taken into account. The NMR experiments were obtained at 600.13 and 150.90 MHz for ^1H and ^{13}C, respectively, on a Bruker DRX-600 instrument at 25 °C. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CD_3OD/CF_3CO_2D, 19:1 v/v) were used as secondary references (δ 49.0 and 3.4 from TMS, respectively). The 1D ^1H, 2D homonuclear correlation experiment (DQF−COSY), total correlation spectroscopy (TOCSY), and rotating frame Overhauser enhancement spectroscopy (ROESY) experiments were performed on a 5 mm multinuclear TXI-probe. The 1D ^{13}C one-pulse experiment, the heteronuclear APT experiments, and the 2D heteronuclear shift correlation (HSC) experiments were obtained on a 5 mm BBO probe.

RESULTS AND DISCUSSION

The HPLC chromatogram of the crude extract of the flowers of *V. villosa* Roth detected in the visible region showed one major and two minor anthocyanins, in addition to several pigments occurring in trace amounts (Figure 1). The UV−vis spectra of the dominant anthocyanins (**1**−**3**) showed λ_{max} around 536 nm (Table 1), indicating anthocyanins with a B-ring substituted with three oxygen functions. The relatively short retention time on RP18-HPLC for **1**−**3** indicated lack of acylation; however, all three pigments showed very high mobility both in aqueous and in alcoholic TLC solvents (Table 1).

The downfield part of the ^1H NMR spectrum of **2** showed a 1H singlet at δ 9.05 (H-4), a 2H AB system at δ 7.67 (*d*, J = 1.9 Hz, H-2′) and 7.60 (H-6′), and a 2H

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10.1021/jf980558e CCC: $15.00
Published on Web 10/21/1998

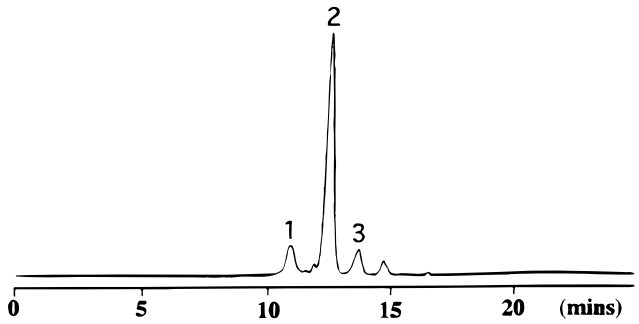


Figure 1. HPLC chromatogram of the extract of *V. villosa* detected at 520 ± 20 nm. The identities of the chromatographic peaks are explained in Figure 3.

Table 1. Chromatographic and Spectral Data of the 3-*O*-α-Rhamnopyranoside-5-*O*-β-glucopyranosides of Delphinidin (1), Petunidin (2), and Malvidin (3) Found in *V. villosa*

	TLC (R_f)		UV−vis spectroscopy		on-line HPLC		
compd	FHW	BAW	λ_{max} (nm)	$A_{440}/A_{vis-max}$ (%)	λ_{max} (nm)	$A_{440}/A_{vis-max}$ (%)	t_R (min)
1	0.86	0.39	537	14	524	17	10.86
2	0.93	0.56	536	14	526	18	12.43
3	0.96	0.63	536	14	528	17	13.66
Dp 3-glc[a]	0.62	0.18	537	22	526	27	12.20

[a] Isolated from black currant.

Table 2. ^{1}H NMR Spectral Data for 1−3 in CD_3OD/CF_3COOD (19:1) at 25 °C

	1 δ_H	**2** δ_H	**3** δ_H
aglycon			
4	9.11	9.05	9.22
6	7.12	7.07	7.14
8	7.13	7.08	7.25
2′	7.70	7.67	7.93
6′	7.70	7.60	7.93
OMe		4.02	4.10
3-*O*-α-rhamnopyranoside			
1″	6.00	5.98	6.04
2″	4.37	4.32	4.36
3″	4.02	4.02	4.01
4″	3.65	3.59	3.65
5″	3.65	3.60	3.65
6″	1.34	1.37	1.35
5-*O*-β-glucopyranoside			
1‴	5.28	5.28	5.30
2‴	3.74	3.69	3.75
3‴	3.64	3.59	3.64
4‴	3.54	3.49	3.54
5‴	3.67	3.61	3.68
6A‴	4.03	3.99	4.06
6B‴	3.83	3.79	3.85

AB system at δ 7.08 (*d*, J = 1.9 Hz, H-8) and 7.07 (H-6) (Andersen et al., 1991). A singlet at δ 4.02 integrating for 3H was in agreement with one methoxyl group (Table 2). The corresponding aglycon carbons were assigned by the heteronuclear shift correlation (HSC) experiment, and the signals of the quarternary carbon atoms (Table 3) were found by an APT experiment to be in accordance with the aglycon petunidin (Andersen et al., 1991). The two anomeric carbon signals appear considerably downfield from the other sugar resonances, and thus the cross-peaks at δ 6.0/102.1 and 5.3/102.5 in the HSC spectrum of **2** together with the nine carbon resonances between 80 and 60 ppm, in addition to the methyl carbon at δ 18.05, indicated two hexoses with pyranose form (Nygård et al., 1996). The two sugars were identified as β-glucopyranosyl and α-rhamno-

Table 3. ^{13}C NMR Spectral Data for 1−3 in CD_3OD/CF_3COOD (19:1) at 25 °C

	1 δ_C	**2** δ_C	**3** δ_C	SEFT[a]
aglycon				
2	165.12	164.14	164.51	↑
3	146.09[b]	145.49[b]	145.69[b]	↑
4	133.80	133.90	134.25	↓
5	157.08[c]	156.97[c]	157.30[c]	↑
6	105.39	105.43	105.77	↓
7	169.27	169.48	169.59	↑
8	97.84	97.33	97.45	↓
9	156.75[c]	156.69[c]	156.73[c]	↑
10	*d*	113.31	113.55	↑
1′	119.83	119.68	119.72	↑
2′	112.49	108.56	110.51	↓
3′	145.45	149.84	149.96	↑
4′	146.24[b]	147.61[b]	147.00[b]	↑
5′	145.45[b]	145.76[b]	149.96	↑
6′	112.49	113.90	110.51	↓
OMe		57.16	57.28	↓
3-*O*-α-rhamnopyranoside				
1″	101.99	102.14	102.14	↓
2″	71.40	71.50	71.57	↓
3″	72.30	72.36	72.39	↓
4″	73.28	73.29	73.29	↓
5″	72.21	72.25	72.32	↓
6″	18.04	18.05	18.04	↓
5-*O*-β-glucopyranoside				
1‴	102.58	102.50	102.58	↓
2‴	74.58	74.59	74.57	↓
3‴	77.82	77.82	77.85	↓
4‴	71.09	71.04	71.08	↓
5‴	78.69	78.63	78.71	↓
6‴	62.37	62.32	62.37	↑

[a] SEFT, coupling modulated spin−echo NMR experiment: Cq and CH_2, ↑; CH and CH_3, ↓. [b,c] Assignments with the same superscript may be reversed. [d] Hidden under solvent peak.

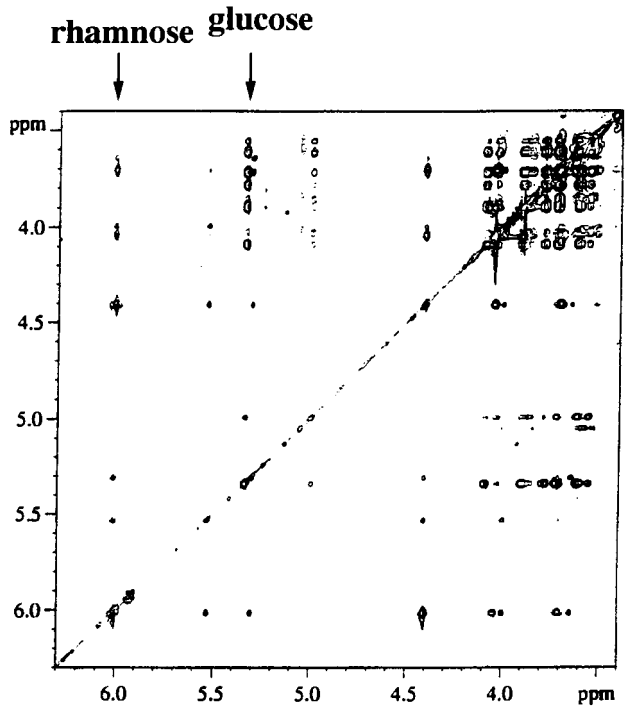
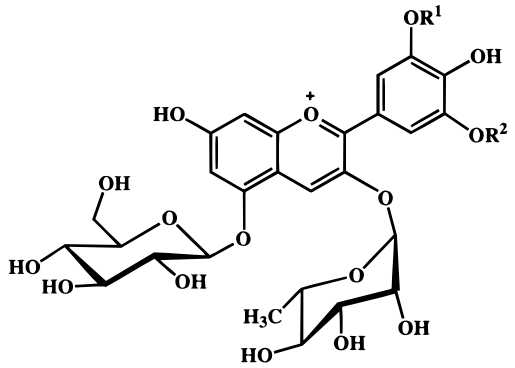


Figure 2. Main sugar region of the TOCSY NMR spectrum of petunidin 3-rhamnopyranoside-5-glucopyranoside (**2**) isolated from *V. villosa*. The arrows are pointing at the columns of correlations between the glucose and rhamnose anomeric protons, respectively, and the rest of the sugar protons belonging to each of these two sugar units. Be aware that the correlations between the methyl group and the rest of the rhamnosyl protons are not shown in this part of the spectrum.



	R^1	R^2
1:	H	H
2:	Me	H
3:	Me	Me

Figure 3. Structures of the anthocyanins identified in *V. villosa*.

pyranosyl on the basis of coupling constants, ^{1}H NMR integration data, and the chemical shifts of the sugars (Tables 2 and 3), which were assigned by homo- and heteronuclear shift correlations. The A_{440}/A_{536} of 14% was in accordance with an anthocyanidin 3,5-diglycoside (Harborne, 1958). The TOCSY experiment gave the correlations from the two anomeric protons to the rest of the protons of each sugar unit (Figure 2). The cross-peak at δ 9.0/6.0 in the ROESY spectrum between the rhamnose anomer and H-4 confirmed that the rhamnosyl and glucosyl units were connected to the aglycon 3- and 5-positions, respectively. Thus, the identity of **2** was found to be petunidin 3-*O*-α-rhamnopyranoside-5-*O*-*β*-glucopyranoside.

The UV−vis spectra (Table 1) of **1** and **3** were nearly identical to and the proton and carbon NMR spectra (Tables 2 and 3) showed many similarities with the corresponding spectra of **2**. Assignments of the protons and carbon resonances by homo- and heteronuclear NMR techniques revealed more symmetry on the aglycon B-rings of **1** and **3** than of **2**. The singlets at δ 7.70 and 7.93 in the ^{1}H NMR spectra of **1** and **3**, respectively, were integrating for two protons each. The downfield regions of the ^{13}C NMR spectra of the two pigments showed each two signals less than the corresponding region of **2**. Each of these regions also contained two signals with twice the intensity of the other resonances in the same regions. The NMR spectra of **1** showed no signals corresponding to methoxy signals; however, the ^{1}H NMR spectrum of **3** contained a singlet at δ 4.10 integrating for six protons. Thus, the identities of **1** and **3** were confirmed to be the 3-*O*-α-rhamnopyranoside-5-*O*-*β*-glucopyranosides of delphinidin and malvidin, respectively.

In our survey of the anthocyanin content of flowers with blue colors, we found no evidence for a delphinidin glycoside as the major pigment in flowers of *V. villosa* (Nozzolillo, 1973). The anthocyanin content of the flower of this plant was determined to be the 3-*O*-α-rhamnopyranoside-5-*O*-*β*-glucopyranosides of petunidin (71%), delphinidin (12%), and malvidin (9%), respectively. These pigments seem to occur in several species in the families Leguminosae and Vitaceae (Ishikura et al., 1978; Yoshitama, 1992) and may have some chemotaxonomic importance.

LITERATURE CITED

Andersen, Ø. M.; Opheim, S.; Aksnes, D. W.; Frøystein, N. Å. Structure of petanin, an acylated anthocyanin isolated from *Solanum tuberosum*, using homo- and hetero-nuclear two-dimensional nuclear magnetic resonance techniques. *Phytochem. Anal.* **1991**, *2*, 230−236.

Harborne, J. B. Spectral methods of characterizing anthocyanins. *Biochem. J.* **1958**, 22−28.

Ishikura, N.; Ito, S.; Shibata, M. Paper chromatographic survey of anthocyanins in Leguminosae. III. Identification and distribution pattern of anthocyanins in twenty-two legumes. *Bot. Mag.* **1978**, *91*, 25−30.

Nozzolillo, G. A survey of anthocyanin pigments in seedling legumes. *Can. J. Bot.* **1973**, *51*, 911−915.

Nozzolillo, C.; Ricciardi, L.; Lattanzio, V. Flavonoid constituents of seed coats of *Vicia faba* (Fabaceae) in relation to genetic control of their color. *Can. J. Bot.* **1989**, *67*, 1600−1604.

Nygård, A. M.; Aksnes, D. W.; Andersen, Ø. M.; Bakken, A. K. Structure determination of 6-hydroxycyanidin-, 6-hydroxydelphinidin-3-(6″-*O*-α-L-rhamno-pyranosyl-*β*-D-glucopyranosides) and other anthocyanins from *Alstroemeria* cultivars. *Acta Chem. Scand.* **1996**, *51*, 108−112.

Torck, M.; Pinkas, H. Les flavonoides du genre *Vicia*. *Biochem. Syst. Ecol.* **1992**, *20*, 453−457.

Yoshitama, K.; Ishii, K.; Yasuda, H. A chromatographic survey of anthocyanins in the flora of Japan. *J. Fac. Sci.* **1980**, *15*, 19−26.

Yoshitama, K.; Ishikura, N.; Fuleki, T.; Nakamura, S. Effect of anthocyanin, flavonol co-pigmentation and pH on the color of the berries of *Ampelopsis brevipedunculata*. *J. Plant Physiol.* **1992**, *139*, 513−518.

Received for review May 26, 1998. Revised manuscript received September 1, 1998. Accepted September 9, 1998. This research is supported by grants from The Norwegian Research Council (T.F.) and The University of Messina (G.C.).

JF980558E