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Food Chemistry 69 (2000) 33–36

Food Chemistry

www.elsevier.com/locate/foodchem

Anthocyanin trisaccharides in blue berries of *Vaccinium padifolium*

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Received 19 April 1999; received in revised form 16 September 1999; accepted 16 September 1999

Abstract

Delphinidin 3-*O*-α-rhamnoside, malvidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside) and the 3-*O*-(6″-*O*-α-rhamnopyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranosides) of cyanidin, petunidin and peonidin were isolated by various chromatographic techniques from the edible berries of *Vaccinium padifolium*. Their complete structures were elucidated mainly by one- and two-dimensional nuclear magnetic resonance spectroscopy. Together they account for 7% of the anthocyanin content in this species. No anthocyanidin 3-triglycoside, 3-rutinoside or 3-rhamnoside had previously been found in the genus *Vaccinium*. The 3-*O*-(6″-*O*-α-rhamnopyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranosides) of petunidin and peonidin are novel compounds.

1. Introduction

Vaccinium padifolium is a deciduous shrub endemic to Madeira island (Portugal). Its berries have been used as food preserves and in local ethnopharmacology (cough, colds, bronchitis, dysentery) (Rivera & Obón, 1995), and exported for commercial production of ophthalmic specialities (Vieira, 1992). We have recently reported twenty different anthocyanins, including the 3-*O*-β-glucopyranosides, 3-*O*-β-galactopyranosides, 3-*O*-β-arabinopyranosides and 3-*O*-sambubiosides (2″-*O*-β-xylopyranosyl-*O*-β-glucopyranosides) of delphinidin, cyanidin, petunidin, peonidin and malvidin to occur in its berries (Cabrita & Andersen, in press).

The aim of this study is to present the isolation and structure elucidation of five additional anthocyanins in *V. padifolium*, which have not been reported in this genus before, including two novel anthocyanidin 3-triglycosides.

2. Materials and methods

2.1. Extraction and separation

Ripened berries of Uveira, (*Vaccinium padifolium*) (Ericaceae) were collected in Madeira island (Portugal) in October 1995 and stored at −20°C. The berries (100 g) were extracted ten times with 100 ml MeOH containing 1% TFA at 4°C. The combined extract was filtered, concentrated under reduced pressure and partitioned against ethyl acetate. The purified extract was then adsorbed on a column packed with Amberlite XAD-7, washed with water, eluted with MeOH containing 0.1% TFA and dried. The sample was fractionated on a Sephadex LH-20 column (1000×50 mm) using step elution with 20–60% MeOH–H_2O (containing 0.1% TFA) and the collected fractions were further purified by semi-preparative HPLC.

2.2. HPLC and TLC

Analytical and semi-preparative high performance liquid chromatography (HPLC) separations were performed on a HP-1050 module system (Hewlett–Packard) using an ODS Hypersil column (200×4.6 mm, 5 μm) and diode array detection. Two solvents were used for elution: A. HCO_2H:H_2O (1:9, v/v); B. MeOH:HCO_2H:H_2O (5:1:4, v/v). Analytical HPLC: the elution profile was 0–4 min, 10% B in A (isocratic); 4–21 min, 10–100% B in A (linear gradient), 21–25 min, 100% B (isocratic). The flow rate was 1.2 ml min^{-1}. Semi-preparative HPLC: 0–4 min, 10% B in A (isocratic); 4–30 min, 10–100% B, 30–35 min, 100% B. Prior to injection all samples were filtered through a 0.45 μm Millipore membrane filter. Peak percentages were estimated from several HPLC chromatograms obtained using gradients

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0308-8146/00/$ - see front matter
PII: S0308-8146(99)00230-7

with different steepness, since no single elution program could resolve all peaks.

Thin-layer chromatography (TLC) was carried out on microcrystalline cellulose F (Merck) with the solvents 1-BuOH:HOAc:H_2O (4:1:5, upper phase) and HCO_2H:-conc. HCl:H_2O (1:1:2).

2.3. Spectroscopy

UV-vis absorption spectra (240–600 nm, 2 nm steps) were recorded in 0.01% conc. HCl in MeOH. On-line absorbance signals were recorded for every second nm between 500 and 540 nm.

The NMR experiments (DQF-COSY, 1D TOCSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC, ^{13}C SEFT) were obtained at 600.13 and 150.92 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DRX-600 instrument at 25°C. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (CF_3CO_2D:CD_3OD; 1:19, v/v) were used as secondary references (49.0 and 3.4 ppm from TMS, respectively).

Table 1
UV-visible data and retention times (HPLC) for the 3-(6″-rhamnosyl-2″-xylosylglucosides) of cyanidin (**1**), petunidin (**2**) and peonidin (**4**), delphinidin 3-rhamnoside (**3**) and malvidin 3-rutinoside (**5**)

Anthocyanin	Vis max (nm)	A_{440}/A_{max} (%)	HPLC, t_R (min)
1	531	27	18.2
2	540	30	19.3
3	540	20	20.1
4	531	31	24.8
5	540	22	26.8

3. Results and discussion

The berries were extracted with an acidified methanolic solution. The extract was purified by liquid–liquid partition and washed on XAD-7. The eluate was concentrated under vacuum to dryness (1.2 g), and separation of the sample (1.0 g) on Sephadex LH-20 yielded 44 fractions that were analysed by TLC and analytical HPLC. After further purification by semi-preparative reversed-phase HPLC, fractions 4–7 yielded pigments **1** (1.1 mg), **2** (3.3 mg) and **4** (2.5 mg); fractions 6–9 afforded

Table 2
^{1}H NMR chemical shifts and ^{1}H-^{1}H coupling constants for the 3-(6″-rhamnosyl-2″-xylosylglucosides) of cyanidin (**1**), peonidin (**4**) and petunidin (**2**), malvidin 3-rutinoside (**5**) and delphinidin 3-rhamnoside (**3**)

	1 δ (ppm) *J* (Hz)	**4** δ (ppm) *J* (Hz)	**2** δ (ppm) *J* (Hz)	**5** δ (ppm) *J* (Hz)	**3** δ (ppm) *J* (Hz)
Aglycone					
4	8.97 s	9.04 s	8.99 s	9.10 s	9.04 s
6	6.76 d 1.8	6.77 d 1.8	6.76 d 1.8	6.82 d 1.8	6.74 *d* 2.0
8	6.98 dd 2.0, 0.7	7.03 d 0.9	7.00 d 1.1	78.09 d 1.5	6.94 dd 1.8, 0.6
2′	8.13 d 2.2	8.14 d 2.2	7.86 d 2.2	8.13 s	7.65 s
5′	7.1 d 8.8	7.14 d 8.8			
6′	8.37 dd 8.8, 2.2	8.49 dd 8.7,2.2	7.99 d 2.2	8.13 s	7.65 s
OMe		4.12 s	4.12 s	4.10 s	
3-O-*glucoside*					
1″	5.53 d 7.7	5.53 d 7.7	5.54 d 7.7	5.41 d 7.7	
2″	4.06 dd 7.7, 9.2	4.02 dd 7.7,91.2	4.03 dd 7.7, 9.2	3.73 dd 7.7,9.2	
3″	9.86 t 9.2	3.86 t 9.2	3.85 t 9.2	3.63 t 9.2	
4″	3.56 t 9.2	3.54 t 9.2	3.55 t 9.4	3.48 t 9.2	
5″	3.82 ddd 9.2, 5.5, 1.8	3.82 ddd 8.8, 6.6, 1.8	3.82 ddd 9.2, 6.6, 1.8	3.81 ddd 9.5, 6.6, 1.8	
6″ A	4.13 dd 11.4, 1.8	4.13 dd 11.4, 1.8	4.12 dd 11.4, 1.8	4.14 dd 11.4, 1.8	
6″ B	3.70 dd 11.4, 5.5	3.69 dd 11.4, 6.6	3.70 dd 11.4, 6.6	3.68 dd 11.4, 6.6	
2″-O-*xylosyl*					
1‴	4.85 d 7.7	4.85 d 7.7	4.77 d 7.7		
2‴	3.25 dd 7.7, 9.2	3.23 dd 7.7, 9.0	3.23 dd 7.7, 9.2		
3‴	3.40 t 9.2	3.39 t 9.2	3.36 t 9.2		
4‴	3.49 ddd 10.3, 9.2, 5.5	3.48 ddd 10.2, 9.2, 5.5	3.42 ddd 10.3, 9.2, 5.5		
5‴ A	3.78 dd 11.4, 5.5	3.77 dd 11.4, 5.5	3.67 dd 12.1, 5.5		
5‴ B	3.14 dd 11.4, 10.3	3.14 dd 11.4, 10.3	3.05 dd 12.1, 10.3		
6″-O-*rhamnosyl*					3-*O*-rhamnoside
1′ᵛ	4.73 d 1.5	4.73 d 1.5	4.73 d 1.5	4.73 d 1.5	5.82 d 1.5
2′ᵛ	3.86 dd 1.5, 3.3	3.85 dd 1.5, 3.3	3.85 dd 1.5, 3.3	3.87 dd 1.5, 3.3	4.35 dd 1.5, 3.3
3′ᵛ	3.71 dd 3.3, 9.5	6.39 dd 3.3, 9.2	3.69 dd 3.3, 9.5	3.69 dd 3.3, 9.2	4.02 dd 3.3, 9.3
4′ᵛ	3.41 t 9.3	3.40 t 9.5	3.40 t 9.4	3.40 t 9.3	3.66 t 9.4
5′ᵛ	3.65 dd 9.2, 6.2	3.69 dd 9.5, 6.2	3.64 dd 9.2, 6.2	3.31 dd 9.5, 6.2	3.73 dd 9.5, 6.2
6′ᵛ	1.23 d 6.2	1.23 d 6.2	1.22 d 6.2	1.23 d 6.2	1.37 d 6.2

pigment **5** (4.9 mg), and pigment **3** (7.5 mg) was recovered from fractions 35–37 (Table 1).

The low-field part of the ^{1}H NMR spectrum of **4** showed 6 resonances (see Table 2). On the basis of chemical shifts and coupling-patterns, the signals of the AMX system at 8.14, 7.14 and 8.49 ppm were assigned to H-2′, H-5′ and H-6′, respectively, the 2H AX system at 7.03 and 6.77 ppm to H-8 and H-6, and the singlet at 9.04 ppm to H-4. Together with the 3H singlet at 4.12 ppm (OMe), which in the HMBC spectrum correlated with C-3′ (4.1/149.8 ppm) (Fig. 1), these assignments confirmed the identity of the aglycone to be peonidin.

The anomeric proton signals in the ^{1}H NMR spectrum of **4** appear considerably downfield for the other sugar resonances, and thus the three doublets at 5.53, 4.86 and 4.73 ppm, together with the integration data, defined a 1:1:1:1 ratio between the peonidin aglycone and the three sugars. Starting from the doublet at 4.73 ppm (H-1^{IV}) the observed crosspeak with the signal at 3.85 ppm in the DQF-COSY permitted assignment of H-2. The chain of coupled protons H-2, H-3, H-4, H-5 and H-6, was, thereafter, assigned using the same spectrum (Table 2). Subsequently the chemical shifts of the corresponding carbon atoms (Table 3) were assigned from the HSQC experiment, which together with ^{1}H-^{1}H coupling constants were in agreement with an α-linked rhamnopyranosyl. On the basis of a combination of COSY, TOCSY, HSQC, HMBC and SEFT NMR spectra it was possible to assign all the ^{1}H (Table 2) and ^{13}C (Table 3) resonances of the other sugar moieties, which were determined to be β-glucopyranosyl and β-xylopyranosyl units. The HMBC spectrum of **4** revealed cross-peaks establishing that the glucosyl was attached to the aglycone 3-position H-1″/C-3, 5.6/145.4 ppm), while the xylosyl and rhamnosyl residues were attached to the 2″- (H-1‴/C-2″, 4.9/81.4 ppm) and the 6″-positions (H-1^{IV}/C-6″, 4.8/67.7 ppm) of the glucosyl moiety, respectively (Fig. 1). Thus, the identity of **4** was found to be peonidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranoside).

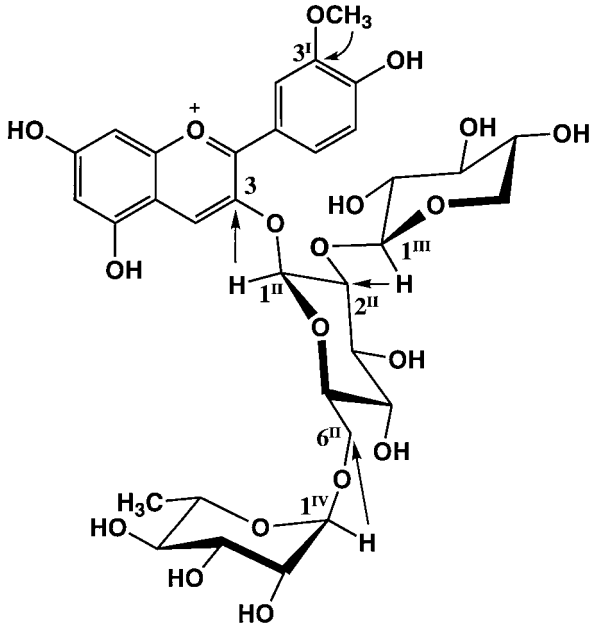

Fig. 1. The arrows refer to proton and carbon cross-peaks in the HMBC NMR spectrum of peonidin 3-*O*-(6″-α-rhamnosyl-2″-β-xylosyl-β-glucoside) showing linkage points between the different moieties.

The ^{1}H and ^{13}C shifts of **2** (Tables 2 and 3) were assigned by the same one- and two-dimensional homo- and heteronuclear NMR techniques as used for **4**, showing the same sugar moiety. The aglycone B-ring signals of **2** at 7.86 and 7.99 ppm were assigned to H-2′ and H-6′, respectively, and the cross-peak between the

Table 3
^{13}CNMR chemical shifts for the 3-(6″-rhamnosyl-2″-xylosylglucosides) of cyanidin (**1**), peonidin (**4**) and petunidin (**2**), malvidin 3-rutinoside (**5**) and delphinidin 3-rhamnoside (**3**)

	1 δ (ppm)	**4** δ (ppm)	**2** δ (ppm)	**5** δ (ppm)	**3** δ (ppm)
Aglycone					
2	163.64	164.29	164.38	163.98	164.49
3	145.31	145.32	145.34	145.74	144.51
4	136.43	136.39	136.51	136.68	135.51
5	159.01	159.08	159.36	159.16	159.11
6	103.50	103.58	103.43	102.39	103.38
7	170.38	170.64	169.52	170.76	170.34
8	94.63	94.60	94.80	nd	95.08
9	157.97	157.80	157.77	157.91	157.71
10	113.78	113.39	113.09	113.59	113.36
1′	121.26	121.10	122.30	119.87	120.00
2′	118.60	115.19	108.83	110.83	112.11
3′	147.53	149.76	149.58	149.85	147.73
4′	154.93	156.77	147.43	146.39	144.89
5′	117.44	116.05	144.48	149.85	147.73
6′	128.33	130.04	115.05	110.83	112.11
OMe		56.87	57.29	57.34	
3-O-glucoside					
1″	101.33	101.82	101.77	103.77	
2″	81.66	81.37	82.54	74.98	
3″	78.11	78.15	77.94	77.61	
4″	70.98	70.98	70.87	71.37	
5″	77.37	77.45	77.53	78.18	
6″	67.62	67.71	67.73	67.85	
2″-O-xylosyl					
1‴	105.75	105.43	105.20		
2‴	75.75	75.60	75.68		
3‴	71.63	71.63	71.74		
4‴	70.99	70.99	70.92		
5‴	67.23	67.14	67.01		
6″-O-rhamnosyl					3-*O*-rhamnoside
1′^{v}	102.17	102.21	102.30	102.26	102.64
2′^{vv}	71.90	70.89	71.93	71.91	71.55
3′^{v}	72.45	72.44	72.52	72.48	72.31
4′^{v}	73.94	73.98	73.93	73.90	73.31
5′^{v}	69.80	69.82	69.83	69.80	72.16
6′^{v}	17.87	17.88	17.89	17.88	17.95

[nd] Not detected.

methoxyl group and C-3′ (4.1/149.6 ppm) in the HMBC spectrum confirmed the identity of the aglycone to be petunidin. The HMBC spectrum further revealed the connectivities between the aglycone and the 3-glucosyl (H-1″/C-3, 5.6/145.3 ppm), between the 3-glucosyl and the 2″-xylosyl (H2″/C1‴, 4.0/105.2 ppm) and the 6″-rhamnosyl (H6″A/C1^{IV}, 4.0/102.3 ppm). Thus, **2** was found to be petunidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranoside).

Based on similar ^{1}H and ^{13}C NMR assignments as used for **2** and **4** (Tables 2 and 3), the identities of **1**, **3** and **5** were found to be cyanidin 3-*O*-(6″-*O*-α-rhamnopyranosyl- 2″–β-xylopyranosyl-β-glucopyranoside), delphinidin 3-rhamnopyranoside and malvidin 3-*O*-(6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), respectively.

4. Conclusion

Pigments **2** and **4**, the 3-*O*-(6″-*O*-α-rhamnpyranosyl-2″-*O*-β-xylopyranosyl-β-glucopyranosides) of petunidin and peonidin, respectively, are novel compounds. Other anthocyanidin 3-*O*-(6″-rhamnosyl-2″-xylosylglucosides) seem to be relatively rare in nature. The corresponding derivative of delphinidin has been found in flowers of *Linum grandiflorum* cv Scarlet Flax (Toki, Saito, Harada, Shigihara & Honda, 1995), while the cyanidin derivative has a more widespread distribution, including the genera *Ribes* and *Rubus* (Mazza & Miniati, 1993). Also malvidin 3-*O*-(6″-*O*-α-rhamnpyranosyl-β-glucopyranosides), **3**, and delphinidin 3-rhamnopyranoside, **5**, seem to have a rather limited occurrence in plants (Harborne & Grayer, 1988; Strack & Wray, 1994).

The anthocyanidin 3-triglycosides constitute less than 2% of the total anthocyanin content, while the anthocyanidin 3-diglycosides account for 14%, where 1% is malvidin 3-rutinoside and the rest are the 3-sambubiosides (Cabrita & Andersen, in press). The monoglycosides altogether contribute with 86%, where 3.9% is delphinidin 3-rhamnoside. The pigments described in this paper account for approximately 7% of the anthocyanin content in *Vaccinium padifolium*.

From a chemotaxonomic point of view it is interesting to note the presence of anthocyanidin 3-triglycosides and 3-disaccharides together with delphinidin 3-rhamnoside in *V. padifolium*, since none of these anthocyanin classes has been identified in any other *Vaccinium* species before. The berries of these latter species seem to be characterized only by anthocyanidin 3-monoglucosides, 3-monogalactosides and 3-monoarabinosides (Mazza & Miniati, 1993). However, most *Vaccinium* species contain a complex anthocyanin mixture, which is difficult to separate in a scale necessary for proper pigment elucidation, at least for pigments occurring in relatively low amounts.

Acknowledgements

LC is grateful to Mr Victor Freitas (Univ. Madeira) for collecting berries of *Vaccinium padifolium* and to FCT (Fundação para a Ciência e Tecnologia, Portugal) for a PRAXIS XXI BD scholarship.

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