



# 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

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## Abstract

Delphinidin 3-*O*- $\alpha$ -rhamnoside, malvidin 3-*O*-(6''-*O*- $\alpha$ -rhamnopyranosyl- $\beta$ -glucopyranoside) and the 3-*O*-(6''-*O*- $\alpha$ -rhamnopyranosyl-2''-*O*- $\beta$ -xylopyranosyl- $\beta$ -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*- $\alpha$ -rhamnopyranosyl-2''-*O*- $\beta$ -xylopyranosyl- $\beta$ -glucopyranosides) of petunidin and peonidin are novel compounds. © 2000 Elsevier Science Ltd. All rights reserved.

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## 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*- $\beta$ -glucopyranosides, 3-*O*- $\beta$ -galactopyranosides, 3-*O*- $\beta$ -arabinopyranosides and 3-*O*-sambubiosides (2''-*O*- $\beta$ -xylopyranosyl-*O*- $\beta$ -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  $-20^{\circ}\text{C}$ . The berries (100 g)

were extracted ten times with 100 ml MeOH containing 1% TFA at  $4^{\circ}\text{C}$ . 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 $\times$ 50 mm) using step elution with 20–60% MeOH–H<sub>2</sub>O (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 $\times$ 4.6 mm, 5  $\mu\text{m}$ ) and diode array detection. Two solvents were used for elution: A. HCO<sub>2</sub>H:H<sub>2</sub>O (1:9, v/v); B. MeOH:HCO<sub>2</sub>H:H<sub>2</sub>O (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<sup>-1</sup>. 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  $\mu\text{m}$  Millipore membrane filter. Peak percentages were estimated from several HPLC chromatograms obtained using gradients

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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<sub>2</sub>O (4:1:5, upper phase) and HCO<sub>2</sub>H:conc. HCl:H<sub>2</sub>O (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, <sup>1</sup>H-<sup>13</sup>C HSQC, <sup>1</sup>H-<sup>13</sup>C HMBC, <sup>13</sup>C SEFT) were obtained at 600.13 and 150.92 MHz for <sup>1</sup>H and <sup>13</sup>C, respectively, on a Bruker DRX-600 instrument at 25°C. The deuteriomethyl <sup>13</sup>C signal and the residual <sup>1</sup>H signal of the solvent (CF<sub>3</sub>CO<sub>2</sub>D:CD<sub>3</sub>OD; 1:19, v/v) were used as secondary references (49.0 and 3.4 ppm from TMS, respectively).

### 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 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 <sub>440</sub> /A <sub>max</sub> (%)	HPLC, t <sub>R</sub> (min)
<b>1</b>	531	27	18.2
<b>2</b>	540	30	19.3
<b>3</b>	540	20	20.1
<b>4</b>	531	31	24.8
<b>5</b>	540	22	26.8

Table 2

<sup>1</sup>H NMR chemical shifts and <sup>1</sup>H-<sup>1</sup>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**)

	<b>1</b> δ (ppm) J (Hz)	<b>4</b> δ (ppm) J (Hz)	<b>2</b> δ (ppm) J (Hz)	<b>5</b> δ (ppm) J (Hz)	<b>3</b> δ (ppm) J (Hz)
<i>Aglycone</i>					
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	
<i>3-O-glucoside</i>					
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, 9.2	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	
<i>2''-O-xylosyl</i>					
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		
<i>6''-O-rhamnosyl</i>					
1'v	4.73 d 1.5	4.73 d 1.5	4.73 d 1.5	4.73 d 1.5	3-O-rhamnoside 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  $^1\text{H}$  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  $^1\text{H}$  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 ratio between the peonidin aglycone and the three sugars. Starting from the doublet at 4.73 ppm (H-1<sup>IV</sup>) 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  $^1\text{H}$ - $^1\text{H}$  coupling constants were in agreement with an  $\alpha$ -linked rhamnopyranosyl. On the basis of a combination of COSY, TOCSY, HSQC, HMBC and SEFT NMR spectra it was possible to assign all the  $^1\text{H}$  (Table 2) and  $^{13}\text{C}$  (Table 3) resonances of the other sugar moieties, which were determined to be  $\beta$ -glucopyranosyl and  $\beta$ -

xylopyranosyl units. The HMBC spectrum of **4** revealed cross-peaks establishing that the glucosyl was attached to the aglycone 3-position H-1<sup>IV</sup>/C-3, 5.6/145.4 ppm), while the xylosyl and rhamnosyl residues were attached to the 2''- (H-1<sup>III</sup>/C-2'', 4.9/81.4 ppm) and the 6''-positions (H-1<sup>V</sup>/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- $\alpha$ -rhamnopyranosyl-2''-O- $\beta$ -xylopyranosyl- $\beta$ -glucopyranoside).

The  $^1\text{H}$  and  $^{13}\text{C}$  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

$^{13}\text{C}$ NMR 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**)

	<b>1</b>	<b>4</b>	<b>2</b>	<b>5</b>	<b>3</b>
	$\delta$ (ppm)	$\delta$ (ppm)	$\delta$ (ppm)	$\delta$ (ppm)	$\delta$ (ppm)
<i>Aglycone</i>					
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	
<i>3-O-glucoside</i>					
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	
<i>2''-O-xylosyl</i>					
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		
<i>6''-O-rhamnosyl</i>					
1 <sup>IV</sup>	102.17	102.21	102.30	102.26	102.64
2 <sup>V</sup>	71.90	70.89	71.93	71.91	71.55
3 <sup>V</sup>	72.45	72.44	72.52	72.48	72.31
4 <sup>V</sup>	73.94	73.98	73.93	73.90	73.31
5 <sup>V</sup>	69.80	69.82	69.83	69.80	72.16
6 <sup>V</sup>	17.87	17.88	17.89	17.88	17.95

<sup>nd</sup> Not detected.

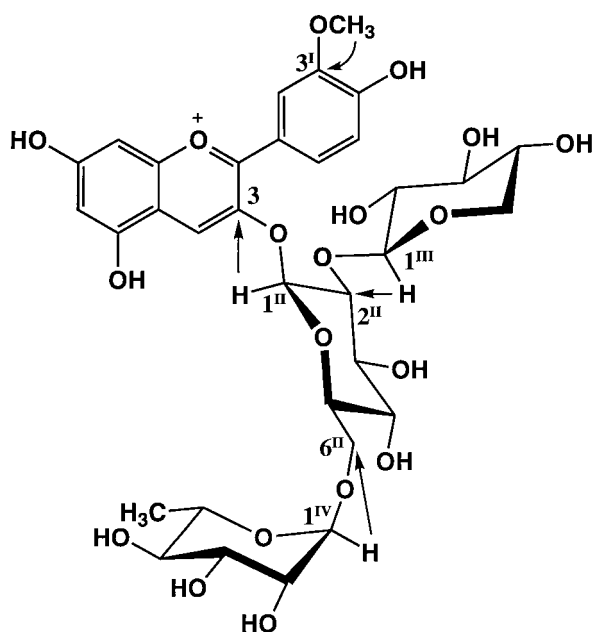


Fig. 1. The arrows refer to proton and carbon cross-peaks in the HMBC NMR spectrum of peonidin 3-O-(6''- $\alpha$ -rhamnopyranosyl-2''- $\beta$ -xylopyranosyl- $\beta$ -glucoside) showing linkage points between the different moieties.

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<sup>IV</sup>, 4.0/102.3 ppm). Thus, **2** was found to be petunidin 3-*O*-(6''-*O*- $\alpha$ -rhamnopyranosyl-2''-*O*- $\beta$ -xylopyranosyl- $\beta$ -glucopyranoside).

Based on similar <sup>1</sup>H and <sup>13</sup>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*- $\alpha$ -rhamnopyranosyl-2''- $\beta$ -xylopyranosyl- $\beta$ -glucopyranoside), delphinidin 3-rhamnopyranoside and malvidin 3-*O*-(6''-*O*- $\alpha$ -rhamnopyranosyl- $\beta$ -glucopyranoside), respectively.

#### 4. Conclusion

Pigments **2** and **4**, the 3-*O*-(6''-*O*- $\alpha$ -rhamnopyranosyl-2''-*O*- $\beta$ -xylopyranosyl- $\beta$ -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*- $\alpha$ -rhamnopyranosyl- $\beta$ -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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