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Acylated anthocyanins from leaves of *Oxalis triangularis*

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Abstract

The novel anthocyanins, malvidin 3-*O*-(6-*O*-(4-*O*-malonyl-α-rhamnopyranosyl)-β-glucopyranoside)-5-*O*-β-glucopyranoside (**2**), malvidin 3-*O*-(6-*O*-α-rhamnopyranosyl-β-glucopyranoside)-5-*O*-(6-*O*-malonyl-β-glucopyranoside) (**3**), malvidin 3-*O*-(6-*O*-(4-*O*-malonyl-α-rhamnopyranosyl)-β-glucopyranoside)-5-*O*-(6-*O*-malonyl-β-glucopyranoside) (**4**), malvidin 3-*O*-(6-*O*-(4-*O*-malonyl-α-rhamnopyranosyl)-β-glucopyranoside) (**5**) and malvidin 3-*O*-(6-*O*-(*Z*)-*p*-coumaroyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (**6**), in addition to the 3-*O*-(6-*O*-α-rhamnopyranosyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (**1**) and the 3-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (**7**) of malvidin have been isolated from purple leaves of *Oxalis triangularis* A. St.-Hil. In pigments **2**, **4** and **5** a malonyl unit is linked to the rhamnose 4-position, which has not been reported previously for any anthocyanin before. The identifications were mainly based on 2D NMR spectroscopy and electrospray MS.

Keywords: *Oxalis triangularis*; Purple shamrock; Leaves; Colour; Acylated anthocyanins; 4-Malonylated rhamnose; 2D NMR

1. Introduction

Oxalis triangularis A. St.-Hil. (purple shamrock or purple clover) in Oxalidaceae is an edible perennial plant, which is easily cultivated. The leaves are especially appreciated because of their sour and exotic taste. The plant has intensely purple leaves with a monomeric anthocyanin content of 195 mg/100 g on malvidin-3,5-diglucoside basis, which make them a potential source for natural colorants (Pazmiño-Durán et al., 2001). The anthocyanin content in the leaves of non-transformed plants has been shown to be about 40% higher than in transformed plants regenerated from hairy roots induced by A. rhizogenes LBA 9402 (Wielanek et al., 2004). Three major anthocyanins have previously been isolated from the leaves (Pazmiño-Durán et al., 2001). All of them shared the same basic structure, malvidin-3-rutinoside-5-glucoside. Two of these pigments were shown to be acylated with one and two molecules of malonic acid, respectively, however, the substitution positions of the acyl moieties were not determined (Pazmiño-Durán et al., 2001). From other species in Oxalidaceae, cyanidin 3-glucoside has been found as the main pigment in callus cultures of *O. reclinata* Jacq (Crouch et al., 1993), and in *Averrhoa bilimbi* and *A. carambola* (Gunasegaran, 1992). The latter species contained also cyanidin 3,5-diglucoside. More recently, malvidin 3-acetylglucoside-5-glucoside in addition to the 3,5-diglucosides of peonidin, petunidin and malvidin, and the 3-glucosides of peonidin, delphinidin, petunidin and malvidin have been found in colored tubers of Oca (*O. tuberosa* Mol.) var. Isla Oca (Alcalde-Eon et al., 2004). The objective of the present study was to isolate and determine the complete structures of the anthocyanins from leaves of *O. triangularis,* including five novel pigments (Fig. 1).

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2. Results and discussion

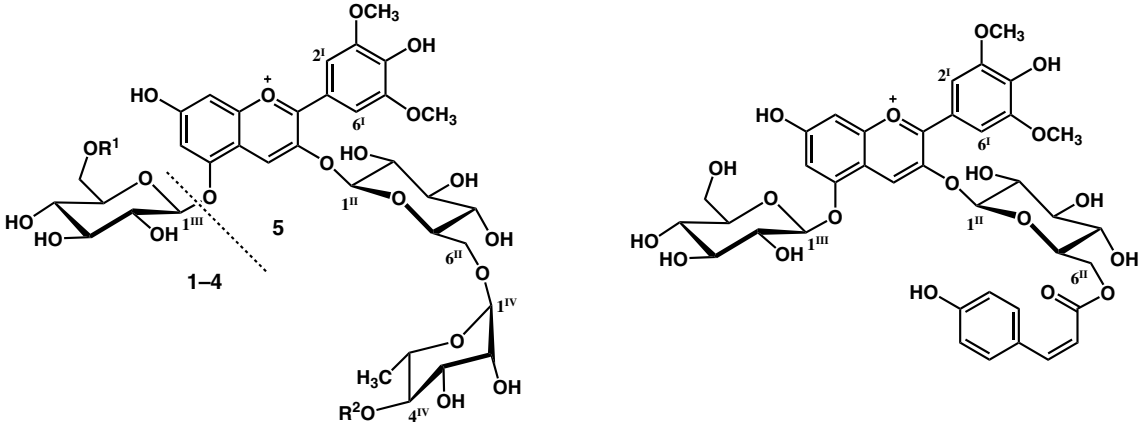
The aqueous concentrate of the acidified methanolic extract of leaves of *O. triangularis*, was purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography. The anthocyanins in the purified extract were fractionated by Sephadex LH-20 column chromatography. Individual pigments, **1**–**7**, were separated by preparative HPLC.

2.1. Identification

The HPLC profile of the acidified methanolic crude extract of leaves of *O. triangularis* showed two major and several minor anthocyanins (Fig. 2). Pigment **1** was identified as the known pigment malvidin 3-*O*-(6^{II}-*O*-α-rhamnopyranosyl-β-glucopyranoside)-5-*O*-β-glucopyranoside by NMR, UV–Vis spectroscopy and electrospray MS (Tables 1–3). **1** has previously been identified in *O. triangularis* (Pazmiño-Durán et al., 2001).

The aromatic region of the 1D ^{1}H NMR spectrum of **2** showed a 1H singlet at δ 9.05 (H-4), a 2H singlet at δ 8.07 (H-2^{I}/6^{I}) and an AX system at δ 7.21 (*d*, 1.8 Hz; H-8) and δ 7.11 (*d*, 1.8 Hz; H-6), revealing an anthocyanin having a symmetrically substituted B-ring. The 6H singlet at δ 4.09 (OCH_3) confirmed the identity of the aglycone of **2** to be malvidin. The 14 ^{13}C resonances belonging to the aglycone in the 1D ^{13}C CAPT spectrum of **2** were assigned by the observed crosspeaks in the HMBC spectrum (Fig. 3). The sugar regions of the 1D ^{1}H and 1D ^{13}C CAPT spectra of **2** showed the presence of two glucose units and one rhamnose unit (Tables 2 and 3). All the ^{1}H sugar resonances were assigned by the DQF-COSY and the TOCSY experiments, and the corresponding ^{13}C resonances were then assigned by the ^{1}H–^{13}C HSQC experiment. The anomeric coupling constants (7.9, 7.9 and 1.5 Hz, respectively) and the 18 ^{13}C resonances in the sugar region of the ^{13}C CAPT spectrum of **2** were in accordance with two β-glucopyranose units and one rhamnose unit (Fossen and Andersen, 2000). The downfield shift of H-4^{IV} (δ 4.93) belonging to the rhamnose unit indicated the presence of acyl substitution. The acyl moiety was identified as malonic acid by the 2H singlet at δ 3.44 (H-2 malonyl) in the 1D ^{1}H spectrum and the three signals at δ 168.36 (C-1 malonyl), δ 170.29 (C-3 malonyl) and δ 41.97 (C-2 malonyl) in the 1D ^{13}C CAPT spectrum. The linkages between the aglycone, sugar units and the malonyl were determined by the long-range correlations in the 2D HMBC spectrum (Fig. 3). A molecular ion at *m*/*z* 887 in the ESI-MS spectrum of **2** corresponded to malvidin malonyl-rhamnosyl-glucosyl-glucoside and the fragment ions at *m*/*z* 655, *m*/*z* 493 and *m*/*z* 331 corresponded to malvidin diglucoside (loss of terminal malonylated rhamnosyl), malvidin glucoside and malvidin aglycone, respectively. A molecular ion at *m*/*z* 887.249 in the high resolution ESI-MS spectrum, which was in accordance with the molecular formula $C_{38}H_{47}O_{24}$, confirmed the identity of **2** to be the novel anthocyanin malvidin 3-*O*-(6^{II}-*O*-(4^{IV}-*O*-malonyl-α-rhamnopyranosyl)-β-glucopyranoside)-5-*O*-β-glucopyranoside (Fig. 1).

The NMR resonances of pigment **3** shared many similarities with the corresponding resonances of **2** (Tables 2 and 3), in accordance with malvidin 3-rutinoside-5-



R1	R2
1: H	H
2: H	malonyl
3: malonyl	H
4: malonyl	malonyl
5: ----	malonyl

Fig. 1. Structures of the novel anthocyanins **2**, **3**, **4**, **5** and **6** and the known pigment **1** isolated from purple leaves of *Oxalis triangularis* A. St.-Hil. Compound **7** is similar to compound **6**, but the acyl moiety has an *E*-configuration.

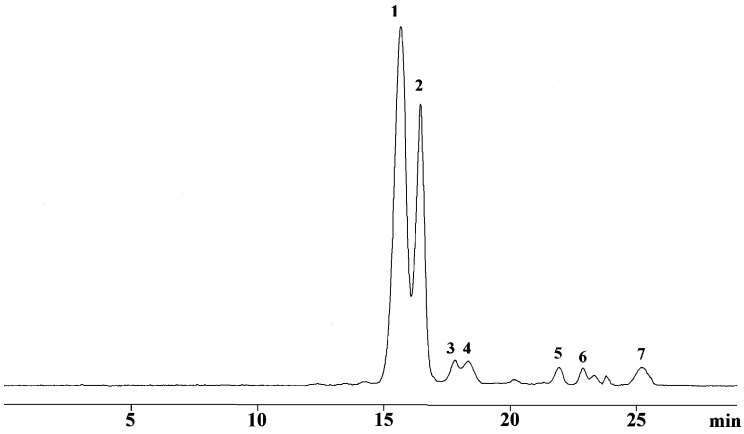


Fig. 2. HPLC chromatogram of anthocyanins from purple leaves of *Oxalis triangularis* A. St.-Hil. detected at 520 ± 20 nm. See Fig. 1 for structures.

Table 1
Chromatographic (HPLC) and spectral (UV–Vis and MS) data recorded for **1**–**7**

Compound	On-line HPLC							ESI-MS	
	Relative proportions (%)[*]	Vis_{max} (nm)	Local UV_{max} (nm)	$A_{440}/A_{vis\text{-}max}$ (%)	$A_{320}/A_{vis\text{-}max}$ (%)	$A_{UV\text{-}max}/A_{vis\text{-}max}$ (%)	t_R (min)	M^+ *m/z*	M^+ *m/z* calculated
1	54	530	278	14	13	104	15.63	801.245	801.224
2	31	533	278	11	10	87	16.42	887.249	887.246
3	3	532	278	13	14	110	17.81	887.249	887.246
4	3	536	278	11	11	94	18.34	973.247	973.246
5	–[**]	534	280	24	6	62	21.9	725[a]	725[a]
6	2	538	280	14	51	97	22.88	801.225	801.224
7	3	535	284	13	172	220	25.19	801[a]	801[a]

M^+ = molecular ion.
[a] Low-resolution MS.
[*] Unknown pigments: 2%.
[**] <2%.

glucoside acylated with malonic acid. However, the chemical shift values of H-6A^{III} (δ 4.66), H-6B^{III} (δ 4.51) and C-6A^{III} (δ 65.44) of **3** occurred significantly downfield, and the chemical shift value of H-4^{IV} (δ 3.36) occurred significantly upfield, respectively, compared to the analogous chemical shift values of **2**, indicating that **3** is acylated with malonic acid at the 6-hydroxyl of the 5-glucosyl unit. The crosspeaks at δ 4.66/168.7 (H-6A^{III}/C-1 malonyl) and δ 4.51/168.7 (H-6B^{III}/C-1 malonyl) in the HMBC-spectrum confirmed the linkage between the malonyl and the 5-glucose to be at the 6^{III}-hydroxyl. A molecular ion at *m/z* 887 in the ESI-MS spectrum of **3** corresponded to malvidin malonyl-rhamnosyl-glucosyl-glucoside and the fragment ions at *m/z* 639, *m/z* 579 and *m/z* 331 corresponded to malvidin rhamnosyl-glucoside (loss of malonylated glucosyl), malvidin malonyl-glucoside and malvidin aglycone, respectively. A molecular ion at *m/z* 887.249 in the high resolution ESI-MS spectrum, which was in accordance with the molecular formula $C_{38}H_{47}O_{24}$, confirmed the identity of **3** to be the novel anthocyanin malvidin 3-*O*-(6^{II}-*O*-α-rhamnopyranosyl-β-glucopyranoside)-5-*O*-(6^{III}-*O*-malonyl-β-glucopyranoside) (Fig. 1).

Similarly, the NMR spectra of **4** resembled those of **2** and **3** (Tables 2 and 3), in accordance with malvidin 3-rutinoside-5-glucoside acylated with two units of malonic acid. The downfield chemical shift values of H-6A^{III} (δ 4.65), H-6B^{III} (δ 4.47), C-6A^{III} (δ 65.34) and H-4^{IV} (δ 4.94) indicated the substitution positions of the malonyl units to be at the 5-glucosyl 6^{III}-hydroxyl and the rhamnosyl 4^{IV}-hydroxyl, respectively. The crosspeaks at δ 4.65/168.5 (H-6A^{III}/C-1 malonyl), δ 4.47/168.5 (H-6B^{III}/C-1 malonyl) and δ 4.94/168.0 (H-4^{IV}/C-1 malonyl) in the HMBC-spectrum confirmed this substitution pattern. A molecular ion at *m/z* 973 in the ESI-MS spectrum of **4** corresponded to malvidin dimalonyl-rhamnosyl-glucosyl-glucoside, and the fragment ions at *m/z* 887, *m/z* 579 and *m/z* 331 corresponded to malvidin malonyl-rhamnosyl-glucosyl-glucoside, malvidin malonyl-glucoside and malvidin aglycone, respectively. A molecular ion at *m/z* 973.247 in the high resolution ESI-MS spectrum, which was in accordance with the molecular

Table 2
^{1}H NMR spectral data for **1**–**7**, in CF_3COOD–CD_3OD (5:95; v/v) at 25 °C

	1	**2**	**3**	**4**	**5**	**6**	**7**
	ppm Hz	ppm Hz	ppm Hz	ppm Hz	ppm Hz	ppm Hz	ppm Hz
Aglycone							
4	8.96 *s*	9.05 *s*	9.11 *d* 0.8	9.13 *d* 0.8	9.08 *d* 0.8	8,81 *d* 0.7	9.07 *d* 0.8
6	7.08 *d* 1.9	7.11 *d* 1.8	7.11 *d* 2.0	7.11 *d* 1.9	6.78 *d* 2.1	7.07 *d* 2.0	7.10 *d* 1.9
8	7.13 *d* 1.9	7.21 *d* 1.8	7.25 *dd* 2.0, 0.8	7.26 *dd* 1.9, 0.8	7.07 *dd* 2.1, 0.8	7.00 *dd* 2.0, 0.7	7.07 *dd* 1.9, 0.8
$2^I/6^I$	7.96 *s*	8.07 *s*	8.13 *s*	8.14 *s*	8.11 *s*	8.03 *s*	8.03 *s*
OMe	4.05 *s*	4.09 *s*	4.11 *s*	4.11 *s*	4.11 *s*	4.12 *s*	4.08 *s*
3-*O*-β-Glucopyranoside							
1^{II}	5.60 *d* 7.7	5.60 *d* 7.9	5.52 *d* 7.7	5.53 *d* 7.7	5.45 *d* 7.7	5.54 *d* 7.8	5.51 *d* 7.7
2^{II}	3.77 *m*	3.78 *dd* 7.9, 9.2	3.76 *dd* 7.7, 9.2	3.77 *m*	3.74 *dd* 7.7, 9.1	3.82 *dd* 7.8, 9.1	3.80 *m*
3^{II}	3.69 *m*	3.68 *t* 9.2	3.66 *t* 9.2	3.65 *t* 9.1	3.64 *m*	3.67 *t* 9.1	3.68 *m*
4^{II}	3.52 *t* 9.4	3.53 *dd* 9.2, 8.8	3.53 *dd* 9.2, 9.8	3.55 *dd* 9.5, 9.1	3.52 *m*	3.53 *dd* 9.1, 9.7	3.59 *m*
5^{II}	3.90 *ddd* 2.1, 6.5, 9.0	3.90 *ddd* 2.2, 6.6, 8.8	3.85 *ddd* 1.8, 5.7, 9.8	3.84 *m*	3.81 *m*	4.03 *m*	3.96 *m*
$6A^{II}$	4.12 *dd* 12.0, 2.1	4.10 *dd* 11.9, 2.2	4.09 *dd* 11.4, 1.8	4.06 *dd* 11.6, 1.9	4.11 *dd* 11.1, 1.9	4.71 *dd* 12.0, 9.0	4.56 *m*
$6B^{II}$	3.77 *m*	3.76 *m*	3.76 *m*	3.79 *m*	3.72 *m*	4.46 *dd* 12.0, 2.6	4.56 *m*
5-*O*-β-Glucopyranoside							
1^{III}	5.30 *d* 7.7	5.29 *d* 7.9	5.31 *d* 7.7	5.31 *d* 7.7		5.28 *d* 7.7	5.25 *d* 7.9
2^{III}	3.79 *m*	3.77 *m*	3.79 *dd* 7.7, 9.0	3.77 *m*		3.86 *dd* 7.7, 9.1	3.83 *m*
3^{III}	3.68 *m*	3.66 *m*	3.68 *t* 9.0	3.67 *t* 9.1		3.67 *t* 9.1	3.65 *m*
4^{III}	3.68 *m*	3.65 *m*	3.63 *dd* 9.0, 9.5	3.58 *m*		3.57 *dd* 9.1, 9.6	3.52 *m*
5^{III}	3.71 *m*	3.70 *ddd* 2.0, 5.3,*	3.93 *ddd* 2.1, 7.0, 9.5	3.91 *ddd* 2.2, 7.0, 9.7		3.71 *ddd* 2.1, 6.0, 9.6	3.69 *m*
$6A^{III}$	4.06 *dd** 2.1,	4.05 *dd* 2.0, 12.1	4.66 *dd* 2.1, 12.2	4.65 *dd* 2.2, 12.0		4.14 *dd* 12.0, 2.1	4.06 *m*
$6B^{III}$	3.95 *dd* 12.3, 5.0	3.92 *dd* 12.1, 5.3	4.51 *dd* 7.0, 12.2	4.47 *dd* 7.0, 12.0		3.91 *dd* 12.0, 6.0	3.82 *m*
6^{II}-*O*-α-Rhamnopyranosyl[a]							
1^{IV}	4.74 *d* 1.5	4.76 *d* 1.5	4.74 *d* 1.5	4.78 *d* 1.6	4.77 *d* 1.7		
2^{IV}	3.79 *m*	3.84 *dd* 3.5, 1.5	3.81 *dd* 3.4, 1.5	3.88 *dd* 3.4, 1.6	3.92 *dd* 3.4, 1.7		
3^{IV}	3.67 *dd* 3.5, 9.5	3.86 *dd* 3.5, 9.7	3.67 *dd* 3.4, 9.6	3.84 *dd* 3.4, 9.7	3.86 *dd** 3.4		
4^{IV}	3.36 *t* 9.5	4.93 *t* 9.7	3.36 *t* 9.6	4.94 *t* 9.7	4.97 *t* 9.8		
5^{IV}	3.60 *dd* 9.5, 6.3	3.76 *m*	3.60 *dd* 9.6, 6.4	3.73 *dd* 9.7, 6.3	3.76 *dd* 9.8, 6.3		
6^{IV}	1.22 *d* 6.3	1.12 *d* 6.4	1.19 *d* 6.4	1.08 *d* 6.3	1.12 *d* 6.3		
6^{III}-*O*-Malonyl							
2			3.44 *s*	3.47 *s*			
4^{IV}-*O*-Malonyl[a]							
2		3.44 *s*		3.47 *s*	3.44 *s*		
6^{II}-*p*-Coumaroyl						Z-config.	E-config.
α						5.83 *d* 12.7	6.28 *d* 15.9
β						6.62 *d* 12.7	7.45 *d* 15.9
2/6						7.30 *′d′* 8.7	7.36 *′d′* 8.7
3/5						6.47 *′d′* 8.7	6.84 *′d′* 8.7

m = multiplet or overlap with solvent peak.
[a] ^{1}H resonances belonging to the rhamnose unit of pigment **5** are annotated as H-1^{III}–H-6^{III}.
* Overlap.

Table 3
^{13}C NMR spectral data for **1**–**4** and **7** in CF_3COOD–CD_3OD (5:95; v/v) at 25 °C

	1	**2**	**3**	**4**	**7**
Aglycone					
2	163.98	164.31	164.9	164.92	164.89
3	146.05	146.19	146.3	146.40	146.00
4	134.56	134.80	135.3	135.46	136.38
5	156.65	156.75	156.5	156.58	157.34
6	105.55	105.64	106.0	106.03	106.11
7	169.88	169.93	170.25	169.8	170.01
8	97.68	97.71	97.6	97.65	97.70
9	157.14	157.29	157.3	157.47	156.87
10	113.14	113.28	113.4	113.47	113.47
1^{I}	119.50	119.58	119.69	119.65	119.58
$2^{I}/6^{I}$	110.87	110.92	111.14	111.07	110.95
$3^{I}/5^{I}$	149.82	149.87	149.95	149.93	149.84
4^{I}	147.27	147.28	147.3	147.37	147.21
OMe	57.31	57.29	57.32	57.31	57.26
3-*O*-β-Glucopyranoside					
1^{II}	102.59	102.78	103.4	103.50	103.46
2^{II}	74.74	74.75[a]	74.70[a]	74.70[a]	74.77
3^{II}	78.34	78.28	78.24	78.14	78.15
4^{II}	71.47	71.46	71.27	71.25	71.89
5^{II}	77.74	77.59	77.83	77.47	75.88
6^{II}	67.51	67.50	67.30	66.95	64.11
5-*O*-β-Glucopyranoside					
1^{III}	102.75	102.85	102.50	102.40	103.18
2^{III}	74.74	74.81[a]	74.95[a]	74.93[a]	74.83
3^{III}	77.89	77.91	78.24	77.75	77.91
4^{III}	70.75	70.82	71.24	71.25	71.28
5^{III}	78.67	78.66	76.10	76.02	78.92
6^{III}	62.08	62.10	65.44	65.34	62.59
6^{II}-*O*-α-Rhamnopyranosyl					
1^{IV}	102.21	102.11	102.02	101.67	
2^{IV}	71.93	71.94	72.05	72.07	
3^{IV}	72.28	70.14	72.30	70.14	
4^{IV}	73.86	76.43	73.85	76.51	
5^{IV}	69.77	67.50	69.82	67.55	
6^{IV}	17.94	17.62	17.94	17.60	
6^{III}-*O*-Malonyl					
1			168.79	168.48	
2			nd	nd	
3			169.65	168.92	
6^{II}-*O*-*p*-Coumaroyl					
1					126.81
2/6					131.39
3/5					116.84
4					161.36
α					114.58
β					146.98
C=O					169.02
4^{IV}-*O*-Malonyl					
1		168.36		167.95	
2		41.9		nd	
3		170.29		nd	

nd = not detected.
[a] Assignments may be reversed.

formula $C_{41}H_{49}O_{27}$, confirmed the identity of **4** to be the novel anthocyanin malvidin 3-*O*-(6^{II}-*O*-(4^{IV}-*O*-malonyl-α-rhamnopyranosyl)-β-glucopyranoside)-5-*O*-(6^{III}-*O*-malonyl-β-glucopyranoside) (Fig. 1).

The NMR resonances of pigment **5** shared many similarities with the corresponding resonances of **2** (Tables 2 and 3), in accordance with malvidin 3-rutinoside acylated with one unit malonic acid. The downfield shift of

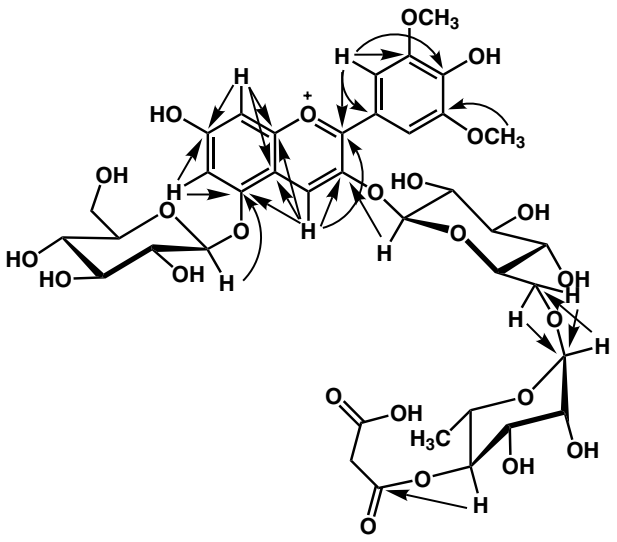


Fig. 3. Long-range ^{1}H–^{13}C correlations in the HMBC spectrum of pigment **2**.

H-4^{III} (δ 4.97) showed the linkage between the malonyl and the terminal rhamnose unit at the 4^{III}-hydroxyl. Thus, **5** was identified as the novel pigment malvidin 3-*O*-(6^{II}-*O*-(4^{III}-*O*-malonyl-α-rhamnopyranosyl)-β-glucopyranoside) (Fig. 1). A molecular ion at *m/z* 725 corresponding to malvidin malonyl-rhamnosyl-glucoside confirmed this identification.

The UV–Vis spectrum of pigment **7** showed an increased absorption around 320 nm indicating the presence of aromatic acylation (Table 1). The 1D ^{1}H and 1D ^{13}C NMR spectra of pigment **7** showed malvidin aglycone, two glucose units and one *p*-coumaroyl unit (Tables 2 and 3). The coupling constants of H-α and H-β (15.9 Hz) of the *p*-coumaroyl revealed *E*-configuration. All the ^{1}H sugar resonances were assigned by the DQF-COSY and the TOCSY experiments, and the corresponding ^{13}C resonances were then assigned by the ^{1}H–^{13}C HSQC experiment. The crosspeaks at δ 5.51/146.1 (H-1^{II}/C-3) and δ 5.25/157.1 (H-1^{III}/C-5) showed that the glucose units were attached to the aglycone 3- and 5-hydroxyls, respectively. The downfield chemical shift values of H-6A^{II} (δ 4.56), H-6B^{II} (δ 4.56) and C-6A^{II} (δ 64.11), respectively, indicated that the *p*-coumaroyl was attached to the 6^{II}-hydroxyl. The crosspeaks at δ 4.56/169.2 (H-6A^{II}, H-6B^{II}/C=O *p*-coumaroyl) confirmed the linkage between the 3-glucose and the *p*-coumaroyl moiety to be at the 6^{II}-hydroxyl. A molecular ion at *m/z* 801 corresponding to malvidin coumaroyl-diglucoside and fragment ions at *m/z* 639, *m/z* 493 and *m/z* 331 corresponding to malvidin coumaroylglucoside, malvidin glucoside and malvidin aglycone, respectively, in the ESI-MS spectrum confirmed the identity of **7** to be malvidin 3-*O*-(6^{II}-*O*-(*E*)-*p*-coumaroyl-β-glucopyranoside)-5-*O*-β-glucopyranoside. Hrazdina and Franzese (1974) reported malvidin 3-*O*-(6^{II}-*O*-*p*-coumaroyl-β-glucopyranoside)-5-*O*-β-glucopyranoside in Ives grapes (Vitaceae), however, without determining the configuration of the double bond of the acyl moiety. Saito and Harborne (1992) reported malvidin 3-*O*-(6^{II}-*O*-*E*-*p*-coumaroyl-β-glucopyranoside)-5-*O*-β-glucopyranoside in several Labiatae spp., and Tamura et al. (1994) have previously given proton NMR data for this pigment. This anthocyanin has not previously been identified in Oxalidaceae.

The UV–Vis spectrum of pigment **6** showed an increased absorption around 320 nm indicating the presence of aromatic acylation. The 1D ^{1}H spectrum and the 2D DQF-COSY and the TOCSY spectra of pigment **6** shared many similarities with that of **7** showing malvidin aglycone, two glucose units and one unit *p*-coumaric acid (Table 2). However, contrary to that of **7**, the coupling constants of H-α and H-β (12.9 Hz) of the *p*-coumaroyl moiety of **6** revealed *Z*-configuration. A molecular ion at *m/z* 801.225 in the high resolution ESI-MS spectrum of **6** was in accordance with the molecular formula $C_{38}H_{41}O_{19}$ corresponding to malvidin coumaroyl-diglucoside. Thus, pigment **6** was identified as the novel pigment malvidin 3-*O*-(6^{II}-*O*-(*Z*)-*p*-coumaroyl-β-glucopyranoside)-5-*O*-β-glucopyranoside (Fig. 1).

In the pigments **2**, **4** and **5** a malonyl unit is linked to the rhamnose 4-position, which has not been reported for any anthocyanin before. Among the thirty-seven different anthocyanins, which previously have been reported to contain an acylated rutinosyl group, thirty-two are acylated with one or more cinnamoyl unit (Andersen and Jordheim, 2005), and none with a malonyl unit as found in pigment **2**, **4** and **5**. Anthocyanins acylated with aromatic acids (**6** and **7**) have previously not been reported to occur in the family Oxalidaceae.

The relative proportions of the anthocyanins **1**–**7** in the acidified methanolic extract of leaves of *O. triangularis* are presented in Table 1. The two major anthocyanins malvidin 3-rutinoside-5-glucoside (**1**) and its 4^{IV}-malonyl derivative (**2**) constitute 85% of the total anthocyanin content. Anthocyanins acylated with malonic acid and *p*-coumaric acid constitute above 37% and 5%, respectively, of the total anthocyanin content in leaves of *O. triangularis*.

3. Experimental

3.1. Isolation of pigments

Purple shamrock was cultivated in Bergen. A voucher specimen has been deposited in Bergen Herbarium, University of Bergen (accession number H/505).

Leaves (100 g) were cut into pieces and extracted (2 times) with 0.5% TFA in MeOH at 4 °C. The filtered extract was concentrated under reduced pressure, purified

by partition (three times) against EtOAc (equal volume), and then subjected to Amberlite XAD-7 column chromatography (Goto et al., 1982). The anthocyanins were further purified on a Sephadex LH-20 column (100 × 5 cm) using MeOH–H_2O–TFA (29.7:70:0.3; v/v) to MeOH-H_2O-TFA (69.7:30:0.3; v/v) (stepwise gradient elution). The flow rate was 2.5 ml min^{-1}. Pure anthocyanins were then isolated by preparative HPLC.

Preparative HPLC (Gilson 305/306 pump equipped with an HP-1040A detector) was performed with an ODS-Hypersil column (25 × 2.2 cm, 5 μm) using the solvents HCOOH–H_2O (1:19, v/v) (A) and HCOOH–H_2O–MeOH (1:4:5, v/v) (B). The elution profile consisted of a linear gradient from 10% B to 100% B for 45 min, isocratic elution (100% B) for the next 13 min, followed by linear gradient from 100% B to 10% B for 1 min. The flow rate was 14 ml min^{-1}, and aliquots of 300 μl were injected. Altogether 80 mg of pigment **1**, 20 mg of pigment **2**, 5 mg of pigment **3**, 5 mg of pigment **4**, 2 mg of pigment **6**, 7 mg of pigment **7**, in addition to 2 mg of a mixture of pigment **5** and malvidin 3-rutinoside, respectively, were isolated.

3.2. Analytical HPLC

Analytical HPLC was performed with an ODS-Hypersil column (25 × 0.3 cm, 5 μm) using the solvents HCOOH–H_2O (1:19) (A) and HCOOH–H_2O–MeOH (1:4:5) (B). The gradient consisted of a linear gradient from 10% B to 100% B for 23 min, 100% B for the next 5 min, followed by linear gradient from 100% B to 10% B for 1 min. The flow rate was 0.75 ml min^{-1}, and aliquots of 15 μl were injected.

3.3. Spectroscopy

UV–Vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240–600 nm in steps of 2 nm.

The NMR experiments were obtained at 600.13 and 150.90 MHz for ^1H and ^{13}C, respectively, on a Bruker DRX-600 instrument equipped with a multinuclear inverse probe for the 1D ^1H and the 2D Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Bond Correlations (HMBC) and Double Quantum Filtered Correlation Spectroscopy (DQF-COSY) experiments. The ^{13}C 1D experiment was performed on a $^1H/^{13}C$ BBO probe. Sample temperatures were stabilised at 25 °C. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CF_3CO_2D–CD_3OD; 5:95, v/v) were used as secondary references (δ 49.0 and δ 3.40 from TMS, respectively). See Fossen et al. (2003) for more experimental details.

Low-resolution mass spectral data were achieved by a LCMS system (Waters 2690 HPLC-system connected to Micromass LCZ mass spectrometer) with electrospray ionisation in positive mode (ESP+). See Fossen et al. (2003) for more experimental details. High resolution mass spectra were recorded at the Gesellschaft für Biotechnologische Forschung (GBF), Abteilung Strukturbiologie (Braunschweig, Germany). The pigments were dissolved in a 1:1 (vol:vol) methanol/1% formic acid solution. Approximately 3 μl of this solution (final concentration ca. 20 pmol/l) were filled into a gold-coated nanospray glass capillary (Protana, Odense, Denmark). The tip of the capillary was placed orthogonally in front of the entrance hole of a quadrupole time-of-flight (QTOF 2) mass spectrometer (Micromass, Manchester, Great Britain) equipped with a nanospray ion source, and a voltage of approximately 1000 V was applied. The isotopic composition of the sample was determined in the accurate mass mode using stachyose and cyclodextrane (Glc7) ($[M + H]^+$ = 667.0994; 1135,3776 Da) as internal reference compounds.

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