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Further anthocyanins from flowers of *Crocus antalyensis* (Iridaceae)

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Abstract

As a part of a continuing chemotaxonomic survey of pigments in *Crocus* two new and three known anthocyanins have been isolated from the blue perianth segments of *Crocus antalyensis* [Mathew, B., *The Crocus*. B.T. Batsford, London]. The novel anthocyanins were identified as delphinidin 3-*O*-(β-D-glucopyranoside)-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside and petudin 3,7-di-*O*-(β-D-glucopyranoside. Further 3,7-di-*O*-β-D-glucoside of delphinidin was isolated together with two minor components, 3,5-di-*O*-β-D-glucosides of delphinidin and petunidin. The complete structural determination of the compounds was achieved by use of 1D and 2D NMR techniques and other spectral evidence. © 1998 Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Crocus antalyensis*; Iridaceae; Flower pigments; Malonated anthocyanin; HPLC

1. Introduction

The flower pigments causing cyanic colors in *Crocus* (Iridaceae) have partly been identified by simple color reactions and chromatographic studies (Harborne & Williams, 1984; Garrido, Diez De Bethencourt, & Revilla, 1987; Hayashi, 1960; Lokar & Poldini, 1977). Recently we have isolated two new anthocyanins, 3,7-di-(6-malonyl)glucosides of petunidin and malvidin as well as 3-rutinosides and 3,5-diglucosides of delphinidin and petunidin from blue perianth segments of *Crocus* (Nørbæk & Kondo, 1998). Further studies on anthocyanin occurrence in *Crocus* proved another novel malonated anthocyanin along with the 3,7-diglucosides of petunidin and delphinidin. Delphinidin 3,7-diglucosides had already been isolated from maqui berries but was identified only by chromatographic methods (Diaz, Rosende, & Antunez, 1985). This is the first report on petunidin 3,7-diglucoside.

2. Results and discussion

Blue perianth segments of *Crocus antalyensis* were extracted with aqueous acetonitrile containing 0.5% TFA. HPLC chromatograms detected in the visible region of the crude anthocyanin extract showed the presence of three major anthocyanins as well as two minor pigments. The anthocyanins were isolated by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. The anthocyanins were identified as delphinidin 3-glucoside-5-(6-malonyl)glucoside (**1**), the 3,7-diglucosides of delphinidin (**2**) and petunidin (**3**) and as the 3,5-diglucosides of delphinidin (**4**) and petunidin (**5**). R_t's, UV-VIS and FAB mass spectra are shown in Table 1.

FAB-MS of **1** gave $[M]^+$ at m/z 713, in good agreement with the mass calculated for $C_{30}O_{20}H_{33}^{+}$. The fragment peaks were observed at m/z 551 $[M-162$ (hexose)$]^+$, 465 $[M-248$ (malonyl–hexose)$]^+$ and 303 $[\text{aglycone}]^+$, indicating **1** to be comprised of delphinidin, hexose and malonylhexoside. Acylation was also confirmed by the characteristically increase in retention time (R_t) of **1** compared with the original delphinidin 3,5-di-*O*-(β-D-glucopyranoside) (**4**) (Table 1).

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Table 1
Analytical HPLC retention times and spectral properties of anthocyanins found in blue perianth segments of *Crocus antalyensis*

Anthocyanin	R_t[a] (min)	R_t[b] (min)	UV-VIS (0.1% HCl–MeOH)		FAB-mass spectra
			λ_{max}	(nm)	$[M]^+$, fragment ions
1	14.3	17.4	271	538	713 [M], 551, 465, 303
2[c]	5.7	8.0	280	537	627 [M], 465, 303
3	8.2	9.4	279	537	641 [M], 479, 317
4[c]	9.2	10.8	269	538	627 [M], 465, 303
5	12.4	14.6	269	537	641 [M], 479, 317

[a] Retention time (R_t) on HPLC, linear gradient elution for 30 min using from 0–30% aq. CH_3CN containing 0.5% TFA.
[b] R_t on HPLC, elution profile see Section 3.
[c] FAB-mass spectrum obtained in a positive mode using NBA (1 drop of HCl aq. was added) as a matrix.

NMR data of compounds **1**–**3** are shown in Table 2, while data of the minor components have been described previously (Nørbæk & Kondo, 1998). The proton signals of delphinidin, the aglycone of both **1** and **2**, were assigned (Table 2) using information regarding coupling constants and chemical shifts. The ^1H NMR spectrum of **1** also revealed the presence of two glucose residues, one of them acylated with

Table 2
^1H-NMR spectral data of three new anthocyanins from *Crocus antalyensis* (in CD_3OD, containing 10% TFA-d)

	1[a]	**2**[b]	**3**[b]
Aglycone			
4	9.07 *br s*	8.89 *br s*	8.97 *br s*
6	7.00 *d* (1.8)	7.17 *d* (1.8)	6.85 *d* (1.8)
8	7.06 *d* (1.8)	7.26 *d* (1.8)	7.28 *d* (1.8)
2′	7.83 *br s*	7.76 *br s*	8.00 *br s*
6′	7.83 *br s*	7.76 *br s*	7.86 *d* (2.4)
OMe			3.98 *br s*
Glucoside A			
1	5.31 *d* (7.8)	5.37 *d* (7.8)	5.40 *d* (7.8)
2	3.71 *t* (9.6)	3.76 *t* (9.0)	3.71 *t* (9.0)
3	3.54 *t* (9.6)	3.61 *t* (9.0)	3.59 *t* (9.0)
4	3.39 *t* (9.0)	3.50 *t* (9.0)	3.47 *t* (9.6)
5	3.64 *m*	3.60 *m*	3.60 *m*
6	3.95 *m*	3.95 *dd* (6.6; 12.3)	3.95 *m*
	3.70 *dd* (6.4; 12.7)	3.76 *dd* (6.6; 12.3)	3.72 *m*
Glucoside B			
1	5.16 *d* (7.8)	5.21 *d* (7.8)	5.20 *d* (7.8)
2	3.70 *t* (9.0)	3.58 *t* (9.0)	3.56 *t* (9.0)
3	3.57 *t* (9.6)	3.57 *t* (9.0)	3.54 *t* (9.0)
4	3.47 *t* (9.6)	3.44 *t* (9.0)	3.41 *t* (9.0)
5	3.80 *m*	3.66 *m*	3.63 *m*
6	4.55 *dd* (6.6; 12.6)	4.00 *dd* (6.0; 12.3)	3.98 *m*
	4.34 *dd* (6.6; 12.6)	3.75 *dd* (6.0; 12.3)	3.73 *m*
Malonic moiety	3.39–3.36		

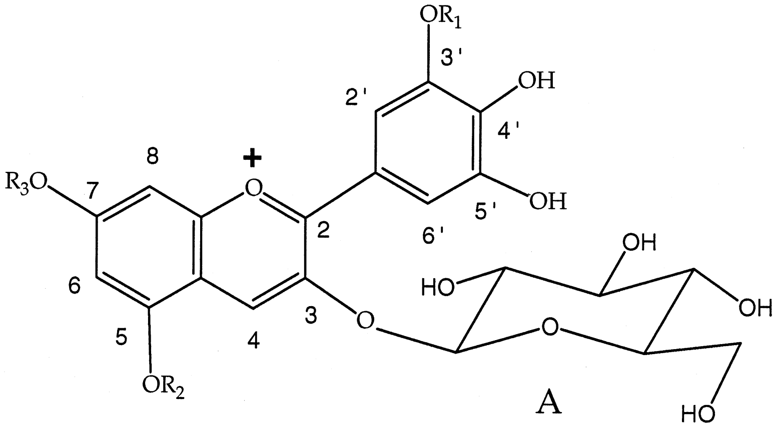
Coupling constants *J* (in Hz) in parentheses.
[a] By irradiation of H-1 of glucoside A in CD_3OD-d_6 containing 10% TFA-*d*, a strong negative NOE was observed at H-4 of the nucleus. By irradiation of H-1 of glucoside B, a strong negative NOE was observed at H-6 of the nucleus. A weak negative NOE was also observed between the same anomeric proton and H-4.
[b] By irradiation of H-1 of glucoside A in CD_3OD-d_6 containing 10% TFA-*d*, a strong negative NOE was observed at H-4 of the nucleus, and strong negative NOEs of H-6 and H-8 of the nucleus appeared by irradiation of H-1 of glucoside B. Concerning **3** a strong negative NOE was observed at H-2′ of the nucleus by irradiation of –OMe.

malonic acid, although H-4 of glucoside B and the malonyl protons, observed at δ3.39–3.36, were somewhat superimposed. The assignments of the two hexoses were perfectly carried out by 1D-HOHAHA spectra and ^{1}H–^{1}H-COSY. All vicinal coupling constants of both sugars were between 7.8–9.6 Hz including two anomeric protons at δ 5.31 (d, $J = 7.8$ Hz, glucoside A) and δ 5.16 (d, $J = 7.8$ Hz, glucoside B). Therefore, both sugar units must be β-D-glucopyranoside. The 6-methylene protons of glucoside B are lowfield shifted by ca 0.6 ppm compared to those in delphinidin 3,5-di-*O*-β-glucopyranoside (Nørbæk & Kondo, 1998) indicating acylation with the malonyl group at the 6-OH (Kondo, Ueda, & Goto, 1990). The positions of the glucosidic linkages were determined by NOE difference spectra (Goto & Kondo, 1991). A strong negative NOE was observed at H-4 (δ 9.07) of the delphinidin nucleus by irradiation of the anomeric proton (δ 5.31) of glucoside A. Glucoside B was deduced to be attached at the 5-OH of delphinidin through a glucosidic bond, because of the presence of a strong NOE between H-6 (δ 7.00) of delphinidin and the anomeric proton of glucoside B (δ 5.16) and a weak NOE between the same anomeric proton and H-4 (δ 9.07). Thus, **1** is delphinidin 3-*O*-(β-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (Scheme 1).

FAB-MS of **2** and **4** both established [M$^+$] at *m/z* 627 supporting the molecular formula $C_{27}O_{17}H_{31}^{+}$ by two fragments corresponding to delphinidin 3-glucoside (*m/z* 465) and delphinidin (*m/z* 303). The diversity of **2** and **4** was found in the different chromatographic profiles, **2** having lower R_t values than **4** (Table 1).

The ^{1}H spectrum of the sugar moieties of **2** was almost identical to that of delphinidin 3,5-di-*O*-β-glucoside (Nørbæk & Kondo, 1998), but the glucosidic linkages determined by NOE difference spectra were 3,7-positions instead of 3,5-positions. A strong negative NOE was observed at H-4 (δ 8.89) of the delphinidin nucleus by irradiation of the anomeric proton (δ 5.37) of glucoside A and strong negative NOEs at H-6 (δ 7.17) and H-8 (δ 7.26) by irradiation of the anomeric proton of glucoside B (δ 5.21) (Table 2). Thus **2** is delphinidin 3,7-di-*O*-(β-D-glucopyranoside) (Fig. 1).

Same diversities and similarities were found between **3** and **5**. The retention times on anal. HPLC were different, **3** being eluted faster than **5**. The [M]$^+$ of **3** and **5** were 14 mass units larger than that of **2** and **4**



	R1	R2	R3
1	H	(6-*O*-malonyl)-β-D-glucopyranoside (B)	H
2	H	H	β-D-glucopyranoside (B)
3	CH_3	H	β-D-glucopyranoside (B)
4	H	β-D-glucopyranoside (B)	H
5	CH_3	β-D-glucopyranoside (B)	H

Scheme 1.

(Table 1) consistent with ^{1}H-NMR which revealed the presence of an aglycone containing –OMe (δ 3.98). By irradiation of the –OMe group a strong NOE was observed at H-2′ (δ 8.00) indicating petunidin. The ^{1}H spectrum of the sugar moieties of **3** was almost identical to that of petunidin 3,5-di-*O*-*β*-glucoside (Nørbæk & Kondo, 1998). However NOE spectra showed that glucoside B of **3** was attached at the 7-OH of petunidin, because of a strong NOE between H-6 (δ 6.85) and H-8 (δ 7.28) of petunidin and the anomeric proton of glucoside B (δ 5.20). The linkage position of glucoside A was not changed. Therefore, **3** is petunidin 3,7-di-*O*-(*β*-D-glucopyranoside) (Fig. 1).

3. Experimental

3.1. Plant material

Fieldgrown flowers of *Crocus antalyensis* were collected in Noordwijk, Holland, in March 1996.

3.2. Isolation of anthocyanins

Freeze-dried perianth segments of *C. antalyensis* (50 g) were extracted with 50% aq. CH_3CN containing 0.5% trifluoroacetic acid (TFA) at room temp. for 1 h. The conc. extract was adsorbed on an Amberlite XAD-7 column, washed with 0.5% TFA aq. soln. and then eluted stepwise from 4 to 16% aq. CH_3CN containing 0.5% TFA. For further purification, the crude anthocyanins were sepd. on prep. ODS-HPLC (20ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) using isocratic stepwise elution (5–16% aq. CH_3CN containing 0.5% TFA) at a flow rate of 7 ml min^{-1}. The pigment frs. were concd. to dryness in vacuo and stored at –80° as pure TFA salts; 10 mg (**1**), 20 mg (**2**), 10 mg (**3**), 5 mg (**4**) and 5 mg (**5**).

3.3. Analysis of anthocyanin

The relatively concentrations of delphinidin 3-glucoside-5-(6-malonyl)glucoside (**1**) (42%), the 3,7-diglucosides of delphinidin (**2**) (25%) and petunidin (**3**) (21%) and the 3,5-diglucosides of delphinidin (**4**) (7%) and petunidin (**5**) (5%), were determined by anal. HPLC. About 1 g of the freeze-dried perianth segments were extracted with 13 ml 50% aq. CH_3CN containing 3.0% TFA and after filtration the extract was analyzed by ODS-HPLC (4.6ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40°, monitoring on a 3D diode-array detector at 260–530 nm. Two gradient systems were used to verify the presence of the anthocyanins. One was, a linear gradient from 0–30% aq. CH_3CN containing 0.5% TFA during 30 min with a flowrate of 1 ml min^{-1}. The second elution profile was as follows: 0 min 16% B, 3 min 38% B, 10 min 44% B, 20 min 50% B, 25 min 67% B, 40–50 min 100% B using solvent A (H_2O–TFA, 99:1) and solvent B (CH_3CN–H_2O–TFA, 60:140:1), with a flowrate of 1.5 ml min^{-1}.

3.4. Spectroscopic analysis

UV-visible spectra were recorded in MeOH containing 0.1% HCl. FAB-MS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix. Exceptionally NBA was used as a matrix. ^{1}H NMR and other NMR-spectra were measured in CD_3OD containing 10% TFA*d* by 600 MHz (JNM alpha 600, JEOL) with internal standard CD_2HOD (3.326 ppm). 1D HOHAHA and 2D spectra were obtained using a pulse sequence supplied from JEOL.

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