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ANTHOCYANINS FROM FLOWERS OF *CROCUS* (IRIDACEAE)

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Key Word Index—*Crocus*; Iridaceae; flower pigments; malonated anthocyanins; HPLC.

Abstract—The perianth segments of three cultivars of *Crocus* were investigated by HPLC for their content of anthocyanins. The investigation revealed the presence of four known and two new anthocyanins. The novel anthocyanins were isolated from the blue flowers of *C. chrysanthus* 'Skyline', and identified as petunidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside) and malvidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside). The anthocyanins, isolated from the blue flowers of *C. sieberi* ssp. *sublimis* 'Tricolor', were identified as 3,5-β-D-diglucosides of delphinidin and petunidin, and from *C. chrysanthus* 'Eyecatcher' were as their 3-β-rutinosides. The complete structural determination of each compound was achieved by use of 1D and 2D NMR techniques and other spectral evidence. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The flowers of *Crocus* (Iridaceae) vary in colour from white, yellow, pale-brown, purple to lilac, mauve and blue. The genus differs from many other monocotyledonous genera in that no red flowers occur. Previously the cyanic colours (lilac, mauve and blue) were reported to be delphinidin-based; only delphinidin 3,5-diglucoside has been identified, and traces of petunidin have been found on the basis of simple colour reactions and chromatographic studies [1–3]. Traces of malvidin have also been detected [4] but no acylated anthocyanins have been reported earlier in *Crocus*, even though acylated anthocyanins seem to occur regularly in Iridaceae [5–9].

As a part of an ongoing chemotaxonomic work, structural determination of six anthocyanins is reported, including two novel compounds, that are present in the blue flowers of *Crocus* cultivars.

RESULTS AND DISCUSSION

Six anthocyanins (**1**–**6**) were detected and isolated by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. The UV-VIS and FAB mass spectra of all six compounds are shown in Table 1.

Compounds **1** and **2** were isolated from perianth segments of *C. chrysanthus* 'Skyline'. The FAB mass spectrum of **1** showed a $[M]^+$ at *m/z* 813, in good agreement with the mass calculated for $C_{34}O_{23}H_{37}^+$. Fragment peaks were observed at *m/z* 565 [M-248 (malonylhexose)]$^+$ and 317 [aglycone]$^+$, indicating **1** to be comprised of petunidin, and two malonylhexoses.

Analysis of the ^{1}H NMR spectrum of **1** revealed the presence of petunidin and two glucose residues, both acylated with malonic acid (Table 2). Although H-4 of the glucosides and the malonyl protons, observed at δ 3.44–3.37, were somewhat superimposed, the assignments of the two hexoses were carried out by 1D-HOHAHA spectra and ^{1}H-^{1}H-COSY. All vicinal coupling constants of both sugars were at 7.8–9.6 Hz including two anomeric protons at δ 5.37 (*d*, *J* = 7.8 Hz, glucoside A) and δ 5.23 (*d*, *J* = 7.8 Hz, glucoside B). Therefore, both sugar units must be β-D-glucopyranoside. The 6-methylene protons of glucose A and B are lowfield shifted by *ca* 0.6 ppm more than **3** and **4**. Thus, the malonyl groups were attached to the 6-OH of each glucoside [10]. The positions of the glucosidic linkages were determined by NOE difference spectra. A strong negative NOE was observed at H-4 (δ 8.92) of the petunidin nucleus by irradiation of the anomeric proton (δ 5.37) of glucoside A and strong

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Table 1. Analytical HPLC R_ts and spectral properties of anthocyanins found in *Crocus*

Anthocyanin	R_t* (min)	R_t† (min)	UV-VIS (0.1% HCl–MeOH) λ_{max} (nm)		FAB mass spectra $[M]^+$, fragment ions
1	16.1	11.9	269	536	813 [M], 565, 317
2	17.5	16.7	278	536	827 [M], 579, 331
3	9.2	4.5	269	538	627 [M], 465, 303
4	12.4	6.4	269	537	641 [M], 479, 317
5	13.5	7.5	276	543	611 [M], 303
6	16.6	13.2	275	542	625 [M], 317

* R_t on HPLC, linear gradient elution for 30 min using from 0 to 30% aq. CH_3CN containing 0.5% TFA.
† R_t on HPLC, isocratic solvent system with 14% aq. CH_3CN containing 0.5% TFA.

Table 2. ^1H NMR spectral data of anthocyanins from 3 *Crocus* cultivars (in CD_3OD, containing 10% TFA-*d*)

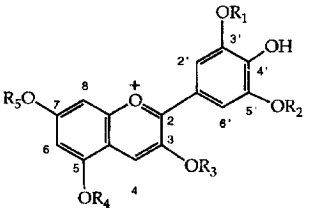
	1*	**2***	**3†**	**4†**	**5‡**	**6‡**
Aglycone						
4	8.92 *br s*	8.98 *br s*	9.04 *br s*	9.09 *br s*	8.86 *br s*	8.88 *br s*
6	6.85 *d* (1.8)	6.86 *br s*	7.04 *d* (2.4)	7.05 *d* (2.4)	6.66 *d* (2.0)	6.66 *d* (2.2)
8	7.26 *d* (1.8)	7.35 *br s*	7.02 *d* (1.8)	7.06 *d* (2.4)	6.85 *d* (2.0)	6.87 *d* (2.2)
2′	8.04 *d* (2.4)	8.09 *br s*	7.76 *br s*	7.98 *d* (1.8)	7.75 *br s*	7.91 *d* (2.2)
6′	7.87 *d* (1.8)	8.09 *br s*	7.76 *br s*	7.80 *d* (1.8)	7.75 *br s*	7.75 *d* (2.2)
OMe	4.00 *br s*	4.01 *br s*		3.98 *br s*		3.97 *br s*
Glucoside A						
1	5.37 *d* (7.8)	5.38 *d* (7.2)	5.31 *d* (7.2)	5.32 *d* (7.2)	5.29 *d* (7.8)	5.31 *d* (7.8)
2	3.71 *t* (9.6)	3.68 *t* (9.0)	3.74 *t* (8.4)	3.69 *t* (9.6)	3.68 *t* (9.5)	3.66 *t* (9.5)
3	3.58 *t* (9.6)	3.57 *t* (9.0)	3.58 *t* (9.6)	3.57 *t* (9.6)	3.56 *t* (9.5)	3.55 *t* (9.5)
4	3.43 *t* (9.6)	3.42 *t* (9.0)	3.44 *t* (9.6)	3.42 *t* (9.6)	3.43 *t* (9.5)	3.41 *t* (9.5)
5	3.87 *m*	3.87 *m*	3.64 *m*	3.63 *m*	3.72 *m*	3.73 *m*
6	4.54 *dd* (6.6; 12.3)	4.52 *dd* (6.6; 12.3)	3.97 *m*	3.97 *m*	4.06 *dd* (1.5; 11.3)	4.07 *m*
	4.31 *dd* (6.6; 12.3)	4.31 *dd* (6.6; 12.3)	3.73 *dd* (3.0; 12.3)	3.72 *dd* (4.8; 12.0)	3.59 *dd* (1.5; 11.5)	3.57 *m*
Glucoside B						
1	5.23 *d* (7.8)	5.26 *d* (7.2)	5.15 *d* (7.2)	5.14 *d* (7.2)		
2	3.56 *t* (9.0)	3.54 *t* (9.0)	3.68 *t* (8.4)	3.66 *t* (9.6)		
3	3.54 *t* (9.0)	3.53 *t* (9.0)	3.56 *t* (9.6)	3.56 *t* (9.6)		
4	3.44 *t* (9.0)	3.44 *t* (9.0)	3.47 *t* (9.0)	3.47 *t* (9.6)		
5	3.85 *m*	3.85 *m*	3.57 *m*	3.54 *m*		
6	4.51 *dd* (6.6; 12.3)	4.49 *dd* (6.0; 12.3)	3.93 *m*	3.93 *m*		
	4.36 *dd* (6.6; 12.3)	4.35 *dd* (6.0; 12.3)	3.75 *dd* (4.8; 11.7)	3.76 *dd* (4.8; 12.0)		
Rhamnoside						
1					4.65 *br s*	4.65 *br s*
2					3.80 *m*	3.79 *m*
3					3.64 *dd* (3.3; 9.5)	3.61 *dd* (3.6; 9.5)
4					3.32 *t* (9.5)	3.17 *t* (9.5)
5					3.54 *m*	3.54 *m*
6					1.15 *d* (6.0)	1.15 *d* (6.6)
malonic moiety						
	3.42–3.37	3.42–3.37				

Coupling constants *J* (in Hz) in parentheses.

* By irradiation of H-1 of glucoside A in DMSO-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.

† On ^1H-^1H NOESY a NOE in DMSO-d_6 containing 10% TFA-*d* was observed between H-4 of the nucleus and H-1 of glucoside A and a NOE between H-6 of the nucleus and H-1 of glucoside B. A weak negative NOE was also observed between H-4 of the nucleus and H-1 of glucoside B.

‡ On ^1H-^1H-NOESY a NOE in CD_3OD, containing 10% TFA-*d* was observed between H-4 of the nucleus and H-1 of glucoside. A weak negative NOE was also observed between H-6 of glucoside and H-1 of rhamnoside.



	R_1	R_2	R_3	R_4	R_5
1	CH_3	H	β-(6-*O*-malonyl-glucoside) (A)	H	β-(6-*O*-malonyl-glucoside) (B)
2	CH_3	CH_3	β-(6-*O*-malonyl-glucoside) (A)	H	β-(6-*O*-malonyl-glucoside) (B)
3	H	H	β-glucoside (A)	β-glucoside (B)	H
4	CH_3	H	β-glucoside (A)	β-glucoside (B)	H
5	H	H	β-rutinoside	H	H
6	CH_3	H	β-rutinoside	H	H

Fig. 1.

negative NOEs at H-6 (δ 6.85) and H-8 (δ 7.26) by irradiation of that of glucoside B (δ 5.23). Thus, **1** is petunidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside) (Fig. 1).

The $[M]^+$ of **2** was 14 mass units larger than that of **1** (Table 1). The ^1H NMR spectrum of **2** showed two equivalent aromatic protons on the B-ring and two CH_3- (δ 4.01), thus the aglycone of **2** is malvidin. ^1H spectrum of the sugar moieties were almost identical to that of **1** and also the assignment was confirmed by 1D HOHAHA, ^1H-^1H-COSY and NOE difference spectrum (Table 2). Thus **2** is malvidin 3-*O*-(6-*O*-malonyl-β-D-glucoside)-7-*O*-(6-*O*-malonyl-β-D-glucoside) (Fig. 1).

Compounds **3** and **4** were found in flowers of *C. sieberi* ssp. *sublimis* 'Tricolor' while **5** and **6** were isolated from *C. chrysanthus* 'Eyecatcher' (Fig. 1). Also in flowers of *C. chrysanthus* 'Skyline' **5** and **6** were detected. These compounds are very common in plant species of Iridaceae [5–8, 11–13]. The structures of **3**–**6** were determined by FAB mass spectrometry (Table 1) and finally elucidated by the complete assignments of the ^1H NMR signals deduced with 1D and 2D techniques (Table 2).

By inspection of ^1H-^1H-NOESY the linkage position of glucoside B in **3** and **4** was found to be different from the position in **1** and **2**. Glucoside B was deduced to be attached at the OH-5 of delphinidin of **3** through a glucosidic bond, because of the presence of a strong NOE between H-6 (δ 7.04) of delphinidin and H-1 of glucoside B (δ 5.15) and a weak NOE between same anomeric proton and H-4 (δ 9.04) of delphinidin. This linkage position of glucose B was also obtained for **4**.

On ^1H-^1H-NOESY a NOE was observed between H-4 (δ 8.88) of petunidin and H-1 of glucoside (δ 5.31) of **6**. A weak negative NOE in DMSO-d_6 containing 10% TFA-*d* was also observed between H-6 of glucoside (δ 3.57; 4.07) and H-1 of rhamnoside (δ 4.65). Therefore, **6** is petunidin 3-β-rutinoside. The linkage of rutinose was further confirmed by H_2O_2 oxidation of **6**, followed by positive FAB mass spectrometry (327 $[M+1]^+$.

EXPERIMENTAL

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

Isolation of anthocyanins. Freeze-dried perianth segments of *C. chrysanthus* 'Skyline' (50 g), *C. sieberi* ssp. *sublimis* 'Tricolor' (50 g) and *C. chrysanthus* 'Eyecatcher' (40 g) were extracted with 50% aq. CH_3CN containing 0.5% TFA at room temp. for 1 hr. The concd extracts were adsorbed on an Amberlite XAD-7 column, washed with 0.5% TFA aq. soln and then eluted stepwise from 4 to 20% aq. CH_3CN containing 0.5% TFA. For further purification, the crude anthocyanins were applied to prep. ODS-HPLC (20 ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) by monitoring at 280 nm. The elution was carried out stepwise with 5 to 16% aq. CH_3CN containing 0.5% TFA (at a flow rate of 7 ml min^{-1}, at 40°, eluent

solvents were distilled just before use). The pigment frs were concd to dryness *in vacuo* and stored at −80°. From *C. chrysanthus* 'Skyline' **1** (5 mg) and **2** (4.5 mg), from *C. sieberi* ssp. *sublimis* 'Tricolor' **3** (80 mg) and **4** (18 mg) and from *C. chrysanthus* 'Eyecatcher' **5** (19 mg) and **6** (15 mg) were isolated as pure TFA salts.

Analysis of anthocyanin. Identification of the anthocyanins in *Crocus* cultivars was performed by analytical HPLC using two solvent systems (Table 1). About 1 g of the freeze-dried perianth segments was extracted with 13 ml 50% aq. CH_3CN containing 3.0% TFA and after filtration the extract was analysed by ODS-HPLC (4.6 ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at 40°, at a flow rate of 1 ml min^{-1}, monitoring on a 3D diode-array detector at 260–530 nm. In the first, a linear gradient elution for 30 min using from 0 to 30% aq. CH_3CN containing 0.5% TFA was carried out, and in the second, an isocratic solvent system with 14% aq. CH_3CN containing 0.5% TFA for 30 min.

Spectroscopic analysis. UV-visible spectra of the isolated anthocyanins were recorded in MeOH containing 0.1% HCl. FABMS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix. ^1H 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). Exceptionally DMSO (TMS as internal standard) containing 10% TFA-*d* was used as solvent. 1D HOHAHA and 2D spectra were obtained using a pulse sequence supplied from JEOL.

H_2O_2 degradation. The H_2O_2 degradation of **6** was carried out according to [10, 14]. To a soln of petunidin 3-β-rutinoside (7 mg) in H_2O (0.5 ml) and CH_3CN (0.2 ml) was added 30% aq. H_2O_2 (0.6 ml). After 4 hr 10% palladium on charcoal (10 mg) was added to the colourless solr. and the mixt. was allowed to stand for 3 hr. The catalyst was removed by filtration and the filtrate was evapd to dryness. The residue was treated with 28% aq. NH_3 (4 ml) and EtOH (9 ml) at room temp. for 14 hr. The product was partitioned between H_2O and Et_2O and the aq. layer was dried to give rutinose, detected by FABMS using NBA as the matrix (*m/z* 327 $[M+1]^+$ corresponding to rutinose and *m/z* 479 $[M+NBA]^+$.

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