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Structure Determination of 6-Hydroxycyanidin- and 6-Hydroxydelphinidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) and Other Anthocyanins from *Alstroemeria* Cultivars

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The anthocyanin content of seven *Alstroemeria* cultivars was determined by combinations of chromatographic, spectroscopic and degradation techniques to be various amounts of the 3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) of cyanidin and delphinidin and the rare aglycones 6-hydroxycyanidin and 6-hydroxydelphinidin. While 6-hydroxycyanidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) was the major pigment in the cultivar Helios (49%) and 6-hydroxydelphinidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) in Cinderella (32%), cyanidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) was predominant in the other cultivars examined [King Cardinal (62%), Amanda (84%), Fiona (52%), White Libelle (75%), Madonna (34%)]. For complete structure determination of the 3-rutinosides of 6-hydroxycyanidin and 6-hydroxydelphinidin, in particular the unambiguous assignment of the aglycone hydroxy groups, the compounds were subjected to homo- and hetero-nuclear NMR techniques such as proton–proton shift correlation (COSY), heteronuclear *J*-modulated spin echo (SEFT), proton–carbon shift correlation in the direct mode (HSC) and inverse mode (HMBC).

Anthocyanins constitute the largest group of water-soluble pigments in the plant kingdom. Their colours vary from pink to violet and blue. Chemically, the anthocyanins are found in acidified extracts as glycosylated polyhydroxy- and/or polymethoxy-derivatives of 2-benzopyrylium salts. More than three hundred anthocyanins are known today. Nearly all of them are based on six anthocyanidins with their differences expressed in the B-ring [Fig. 1(a)]. Among the rarer anthocyanidins are those with *O*-methylation at the 5- or 7-hydroxy groups, those that lack a hydroxy residue at the 3-position (deoxyanthocyanidins) and those having three hydroxy groups on the A-ring. Among the latter is aurantinidin, which has been reported[1] to occur only in the petals of *Impatiens aurantiaca* (Balsaminaceae), and the 3-rutinoside of hydroxy-delphinidin and the 3-rutinoside and 3-glucoside of hydroxy-cyanidin, which have been found in *Alstroemeria* spp.[2,3]

Aurantinidin was first formulated[1] either as 6-hydroxypelargonidin or 8-hydroxypelargonidin [Fig. 1(b)]. Attempts to distinguish between these two possible structures by synthesis[4] failed, and it was suggested that unambiguous verification of aurantinidin-type structures is not possible by the direct use of known synthetic procedures, since the initial flavylium product may undergo reversible ring fission to yield the more thermodynamically stable isomer.[5] Instead, Jurd and Harborne[5] proposed 6-hydroxypelargonidin for aurantinidin on the basis of comparison of the λ_{max} of aurantinidin and some model flavylium salts. The ^{1}H NMR data of 3-rutinosides of hydroxy-delphinidin and hydroxy-cyanidin have been reported in addition to MS, UV–VIS and TLC information.[2,3] However, it is not possible to distinguish between 6- and 8-hydroxylation of the anthocyanidin A-ring from ^{1}H NMR data alone, and Saito *et al.*[2,3] based their structure assignments on a similar spectral comparison (λ_{max}) as Jurd and Harborne.[5]

The aim of this paper is to determine the qualitative and relative quantitative anthocyanin content of seven

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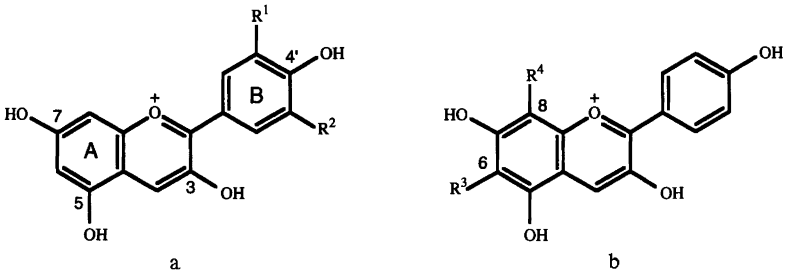


Fig. 1. (a) General structure of the six common anthocyanidins. R^1 and R^2 are H, OH or OCH^3. (b) R^3=OH, R^4= H:6-hydroxypelargonidin; R^3=H, R^4=OH:8-hydroxypelargonidin.

Alstroemeria cultivars, and to make unambiguous assignments of the structures of the rare anthocyanins with three hydroxy-groups on the B-ring of the anthocyanidin. The flowers of the *Alstroemeria* species have considerable ornamental value.

Experimental

Plant material. Seven cultivars of *Alstroemeria* x *hybrida* L., King Cardinal, Cinderella, Helios, Amanda, Fiona, White Libelle and Madonna from van Staavern, The Netherlands, were grown at the Kvithamar Research Centre in mid-Norway. The plants were harvested in September 1994 and stored at −20 °C prior to analysis. The flowers were extracted twice with MeOH containing 0.1% conc. HCl. The filtered extracts were combined and concentrated under reduced pressure.

Chromatography. The anthocyanin extracts of the cultivars King Cardinal and Cinderella were purified by partition against EtOAc before application onto an Amberlite XAD-7 column.[6] The anthocyanins were further purified and separated by droplet counter-current chromatography (DCCC), with the lower layer of BAW (*n*-BuOH-AcOH-H_2O, 4:1:5) as the mobile phase. The chromatograph (Eyela Tokyo Rikakikai, Model A DCC) was fitted with 250 glass capillaries (40 cm × 2.0 mm i.d.). The final purification step was performed on a Sephadex LH-20 column (42 × 1.5 cm) using MeOH–HCl–H_2O (50, 40 or 30:0.1:50, 60 or 70, v/v) as eluents.

High performance liquid chromatography (HPLC) was performed on an HP-1050 module system (Hewlett Packard) using an ODS Hypersil column (20 × 0.5 cm, 5 μm). 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). The elution profile was 0–4 min, 10% B in A (isocratic); 4–21 min, 10–100% B in A (linear gradient). The flow rate was 1.2 ml min^{-1}. Prior to injection all samples were filtered through a 0.45 μm Millipore membrane filter.

The chromatograms were recorded as average values of absorptions on every second nm between 500 and 540 nm using a photodiode array detector (HP 1050). Relative amounts of each anthocyanin are reported as percentages of total peak area in each chromatogram without taking into account the different molar absorption coefficients. UV–VIS absorption spectra were recorded on-line during HPLC-analysis, and spectral measurements were made over the wavelength range 210–600 nm in steps of 2 nm.

Thin layer chromatography was carried out on cellulose sheets (0.1 mm, Schleicher & Schüll) using CH_3CO_2H–conc. HCl–H_2O (15:3:82, v/v) and HCO_2H–conc. HCl–H_2O (24:24:52, v/v) as eluents.

Hydrolysis. Acid hydrolysis and partial acid hydrolysis were carried out according to published procedures.[7]

NMR techniques. The ^1H and ^{13}C NMR spectra were obtained at 400.13 and 100.61 MHz, respectively, on a Bruker AM-400 instrument at 25 °C. The deuteriomethyl carbon signal and the residual proton signal of the solvent (CD_3OD containing one drop of DCl) were used as secondary references for the chemical shifts (δ 49.0 and 3.4 from Me_4Si, respectively). The ^1H–^1H homonuclear shift correlations were acquired using the standard COSY-90 sequence. The heteronuclear spin-echo experiment (SEFT) was performed with the gated decoupler method. The two-dimensional ^1H–^{13}C correlation experiments in the direct mode (HSC)[8] and inverse mode (HMBC)[9] were optimised for one-bond couplings of 160 Hz and long-range couplings of 7 Hz, respectively. The ^1H spectra, SEFT and HSC experiments were performed on a 5 mm ^1H–^{13}C dual probe, while the HMBC experiments were run on a 5 mm standard inverse or an inverse gradient probe.

Results and discussion

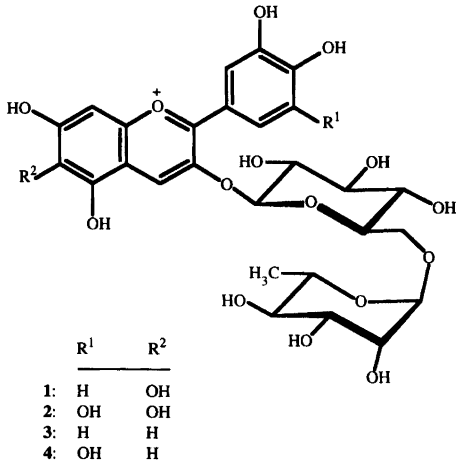
The anthocyanin extracts of the flowers of the *Alstroemeria* cultivars King Cardinal and Cinderella were partitioned against EtOAc and then chromatographed on Amberlite XAD-columns. The anthocyanins were separated by DCCC and finally purified with Sephadex LH-20 before they were examined by spectroscopy, and degradation techniques. The anthocyanins in the extracts of the other five cultivars were separated by HPLC, and their identifications were based on UV–VIS spectroscopic measurements and cochromatography (Table 1).

Table 1. Chromatographic and spectral data for the major anthocyanins in flowers of *Alstroemeria* cultivars.

Pigment[a]	t_R/min (HPLC)	λ_{max}/nm	A_{440}/A_{max} (%)	R_f (AcOH) (TLC)	R_f (FHW) (TLC)
1	14.78	502	36	0.22	0.41
2	13.19	509	29	0.18	0.23
3	16.94	518	33	0.32	0.53
4	15.10	525	29	0.22	0.41

[a] The 3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) of 6-hydroxycyanidin (**1**), 6-hydroxydelphinidin (**2**), cyanidin (**3**) and delphinidin (**4**).

Total acid hydrolysis of **1–4** gave in all instances rhamnose and glucose in addition to their aglycones.



	R^1	R^2
1:	H	OH
2:	OH	OH
3:	H	H
4:	OH	H

Partial acid hydrolyses gave rise to only the corresponding 3-glucoside intermediates of these pigments. The on-line UV–VIS spectrum of **1** and **2** showed visible maxima at 502 and 509 nm with A_{440}/A_{502} and A_{440}/A_{509} of 36 and 29%, respectively (Fig. 2). This is 16 nm less than the corresponding values of **3** and **4**, respectively (Table 1). The retention times of **1** and **2** were more than 2 min shorter than the corresponding values of **3** and **4**, respectively (Table 1). The pigments **3** and **4** cochromatographed (HPLC and TLC, Table 1) with the 3-rutinosides of cyanidin and delphinidin isolated from blackcurrant, *Ribes nigrum*. Their structures were confirmed by NMR spectroscopy[10] (Tables 2, 3) to be cyanidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) and delphinidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside), respectively.

In order to determine unambiguously the unusual

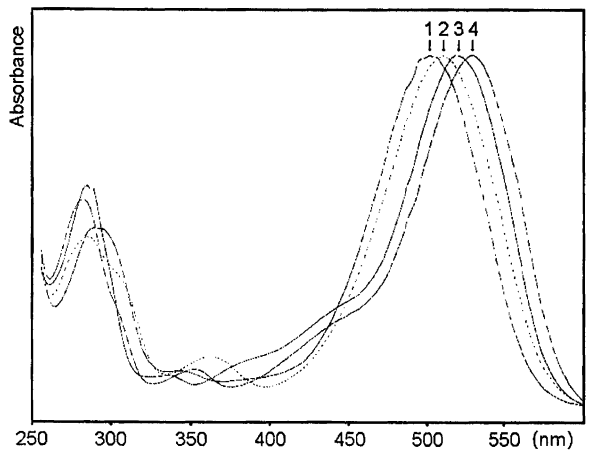


Fig. 2. Spectra taken on-line during HPLC analysis of the anthocyanins, **1–4**, isolated from flowers of *Alstroemeria* cultivars. The spectra of **1** and **2** show that the hydroxy group in the 6-position causes unusual hypsochromic shifts.

Table 2. ^{1}H NMR spectral data[a] for the 3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) of 6-hydroxycyanidin (**1**), 6-hydroxydelphinidin (**2**), cyanidin (**3**) and delphinidin (**4**) in CD_3OD (and one drop DCl) at 25 °C.

	δ_H			
	1	**2**	**3**	**4**
Aglycone				
4	8.99	8.92	8.97	8.90
6	—	—	6.80	6.80
8	7.20	7.16	7.02	6.95
2′	8.07	7.76	8.12	7.81
5′	7.09	—	7.12	—
6′	8.27	7.76	8.34	7.81
3-*O*-β-D-glucopyranoside				
1″	5.42	5.45	5.40	5.44
2″	3.76	3.81	3.82	
3″	3.66	3.67	3.67	
4″	3.50	3.52	3.51	
5″	3.83	3.86	3.81	
6A″	4.14	4.16	4.14	
6B″	3.68	3.86	3.86	
6″-*O*-α-L-rhamnopyranosyl				
1‴	4.77	4.75	4.75	4.75
2‴	3.92	3.92	3.91	
3‴	3.78	3.80	3.73	
4‴	3.41	3.42	3.42	
5‴	3.65	3.67	3.65	
6‴	1.28	1.24	1.23	1.25

[a] Coupling constants (J_{HH}) in Hz: 6-hydroxycyanidin, H4 (s), H8 (s), H2′ (d, 2.2), H5′ (d, 8.7), H6′ (8.7, 2.2); 6-hydroxydelphinidin, H4 (s), H8 (s), H2′/6′ (s); cyanidin, H4 (s), H6 (d, 2.1), H8 (dd, 2.1, 0.8), H2′ (d, 2.3), H5′ (d, 8.7), H6′ (dd, 8.7, 2.3); delphinidin, H4 (s), H6 (d, 2.0), H8 (dd, 0.8, 2.2), H2′/6′ (s); 3-glucopyranose, H1″ (d, 7.6–7.7); 6″-rhamnopyranosyl, H1‴ (d, 1.2–1.6), H6‴ (d, 6.1–6.4).

Table 3. ^{13}C NMR spectral data of the 3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) of 6-hydroxycyanidin (**1**), 6-hydroxydelphinidin (**2**), cyanidin (**3**) and delphinidin (**4**) in CD_3OD (and one drop DCl) at 25 °C.

	δ_C			
	1	**2**	**3**	**4**
Aglycone				
2	162.28	161.62	164.05	163.77
3	145.85	145.94	145.54	145.94
4	134.39	133.47	136.19	135.93
5	141.95	141.64	158.96	158.81
6	135.62	135.53	101.08	103.50
7	159.37	159.78	170.46	170.40
8	95.24	95.07	95.33	95.22
9	151.56	151.17	157.55	157.32
10	114.04	113.87	113.19	113.01
1′	121.38	120.10	121.17	119.85
2′	118.11	112.06	119.01	112.53
3′	147.19	147.25	147.32	147.27
4′	154.97	143.69	155.75	—
5′	117.51	147.25	117.49	147.27
6′	127.68	143.69	128.43	112.53
3-*O*-β-D-glucopyranoside				
1″	103.39	103.00	103.43	102.99
2″	74.68	74.58	74.66	74.63
3″	77.98	77.86	77.94	77.91
4″	71.27	71.20	71.21	71.18
5″	77.42	77.34	77.35	77.32
6″	67.87	67.73	67.76	67.73
6″-*O*-α-L-rhamnopyranosyl				
1‴	102.10	102.00	102.56	102.07
2‴	71.85	71.61	71.81	71.78
3‴	72.32	72.25	72.33	72.92
4‴	73.84	73.79	73.58	73.64
5‴	69.80	69.73	69.70	69.78
6‴	17.90	17.87	17.93	18.03

structure of the aglycones of **1** and **2**, it was necessary to apply homo- and hetero-nuclear NMR techniques in tandem. The low-field part of the ^1H NMR spectrum of **1** showed five resonances (see Table 2). On the basis of the chemical shifts and coupling-patterns the signals at 8.27, 8.07 and 7.09 ppm were assigned to H6′, H2′ and H5′ of the B-ring, respectively. The remaining singlets of the aglycone at 8.99 and 7.20 ppm were attributed to the H4 and H8 protons, respectively, in analogy with earlier results on **3**.

The SEFT spectrum of **1** showed 27 resonances, i.e., 11 positive resonances, originating from 10 quaternary carbons and 1 methylene carbon, and 16 negative resonances attributed to 15 methine carbons and 1 methyl carbon. Once the ^1H spectrum had been completely assigned, the 17 resonances from the protonated carbons were determined using the heteronuclear shift correlation (HSC) experiment (see Table 3). According to published chemical shift data on related compounds the resonances of the protonated C6 and C8 carbons should appear around 101 and 95 ppm, respectively.[10] The negative SEFT signal at 95.24 ppm is therefore assigned to a protonated C8 carbon, whereas the downfield-shifted positive signal at 135.62 ppm is attributed to the hydroxylated quaternary carbon at C6. This shows that the extra hydroxy substituent in **1** compared with **3**, is situated in the 6 position of the A-ring and not in the 8-position. The assignment of the 10 quaternary carbon resonances of the aglycone was accomplished by comparison with cyanidin (**3** and Ref. 10) and by use of the heteronuclear multiple bond correlation (HMBC) experiment. For example, the resonance at 162.28 ppm was assigned to C2 from its three-bond couplings to H4, H2′ and H6′. Since C3 and C5 showed correlations only with H4, their resonances could not be unambiguously assigned from the HMBC spectrum. However, by comparison with cyanidin the signals at 145.85 and 141.95 ppm were attributed to C3 and C5, respectively.

In the ^1H spectrum of **1** the doublets at 5.42 and 4.77 ppm split by 7.6 and 1.6 Hz, respectively, were readily assigned to the anomeric protons of β-D-glucose and α-L-rhamnose, respectively. The remaining ^1H resonances of the sugar rings were assigned by analogy with **3** since the aglycone has a negligible effect on the sugar resonances. Finally, the points of attachment between the three units constituting **1** were determined from the downfield chemical shift of C6″ and the three-bond correlation between C3/H1″ in the HMBC experiment.

The ^1H spectrum of **2** showed three resonances in the aromatic region as expected for a 6- or 8-hydroxylated delphinidin derivative (Table 2). The strong singlet at 7.76 ppm was readily assigned to the equivalent H2′,6′ protons, whereas the weaker singlet at 8.92 ppm was attributed to H4. The singlet at 7.16 ppm assigned to H8, in accordance with **1**, implies that the A-ring is hydroxylated at the 6-position. The ^1H resonances of the sugar region of **2** were assigned by analogy with cyanidin 3-rutinoside and **1**. The very similar chemical shift values observed for **1** and **2** show, in agreement with other evidence, that the two compounds contain the same sugar units.

The SEFT spectrum of **2** showed 25 resonances as expected since the B-ring is symmetrically substituted (Table 3). The 11 positive signals were attributed to the 10 different quaternary carbons of the aglycone and one methylene carbon of the β-D-glucopyranose. The 14 negative resonances were identified as three different methine carbons of the aglycone and 10 methine carbons and one methyl carbon of the sugar units. The ^{13}C spectrum of **2** was completely assigned using the HSC and HMBC experiments (Table 3). Thus **1** and **2** were determined to be 6-hydroxycyanidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) and

Table 4. Distribution of anthocyanin in seven *Alstroemeria* cultivars.

Cultivar	Pigment[a]	Distribution (%)[b]
King Cardinal	**1**	33.9
	3	61.5
Cinderella	**1**	13.6
	2	32.0
	3	27.4
	4	14.4
Helios	**1**	49.4
	3	42.1
Amanda	**1**	5.5
	2	10.4
	3	84.2
Fiona	**1**	10.7
	2	11.1
	3	51.8
	4	17.4
White Libelle	**1**	12.9
	2	5.1
	3	75.3
	4	6.8
Madonna	**1**	5.6
	2	4.3
	3	33.6

[a] The 3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) of 6-hydroxycyanidin (**1**), 6-hydroxydelphinidin (**2**), cyanidin (**3**) and delphinidin (**4**). [b] Where the anthocyanin content does not equal 100%, the remaining percentages refer to unidentified components.

6-hydroxydelphinidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside), respectively, and not their 8-hydroxy-analogues.

The relative anthocyanin content of the cultivars examined are given in Table 4. All the cultivars contained the 3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides) of cyanidin and 6-hydroxycyanidin. While 6-hydroxycyanidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) was the major pigment in the cultivar Helios (49%) and 6-hydroxydelphinidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) in Cinderella (32%), cyanidin-3-(6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) was the major component in the other cultivars examined [King Cardinal (62%), Amanda (84%), Fiona (52%), White Libelle (75%), Madonna (34%)].

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