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6-Hydroxypelargonidin glycosides in the orange–red flowers of *Alstroemeria*

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Abstract

Two 6-hydroxypelargonidin glycosides were isolated from the orange–red flowers of *Alstroemeria* cultivars, and determined to be 6-hydroxypelargonidin 3-*O*-(β-D-glucopyranoside) and 3-*O*-[6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside], respectively, by chemical and spectroscopic methods. In addition, five known anthocyanidin glycosides, 6-hydroxycyanidin 3-malonylglucoside, 6-hydroxycyanidin 3-rutinoside, cyanidin 3-malonylglucoside, cyanidin 3-rutinoside and pelargonidin 3-rutinoside were identified in the flowers.

Keywords: Alstroemeriaceae; *Alstroemeria* cultivars; Flower colour; Anthocyanin; 6-Hydroxypelargonidin (aurantinidin); 6-Hydroxypelargonidin 3-rutinoside; 6-Hydroxypelargonidin 3-glucoside

1. Introduction

Alstroemeria species are native to subtropical and temperate South America, and their hybrid cultivars are grown as popular ornamental plants with attractive flowers. 6-Hydroxycyanidin glycosides were isolated and identified from the red flowers of *Alstroemeria* cultivars (Saito et al., 1985), whereas 6-hydroxydelphinidin glycosides were found in purple–red flowers (Saito et al., 1988). Anthocyanins, namely 6-hydroxycyanidin 3-glucoside, four 3-rutinosides of cyanidin, delphinidin, 6-hydroxycyanidin and 6-hydroxydelphinidin, and a 3-malonylglucoside of 6-hydroxycyanidin, were also found in *Alstroemeria* cultivars (Saito et al., 1985, 1988; Tatsuzawa et al., 2001), in addition to 3-malonylglucosides of cyanidin and delphinidin (Nørbæk et al., 1996, 1998). In this paper, two anthocyanins, 6-hydroxypelargonidin 3-rutinoside **1** and 3-glucoside **2**, were isolated and identified from the orange-red cultivars of *Alstroemeria*. To the best of our knowledge, the occurrence of 6-hydroxypelargonidin was found only in *Impatiens aurantiaca* (Balsaminaceae; Clevenger, 1964) and no report on the occurrence of its glycoside has appeared to date. Therefore, this finding is the first report on the presence of 6-hydroxypelargonidin glycosides in plants. The occurrence of 6-hydroxypelargonidin in plants will provide important information for the biogenesis of anthocyanins, and also for the breeding of flowers with unprecedented colors.

2. Results and discussion

In a survey of three orange-red cultivars 'Oreiju', 'Mayprista' and 'Spotty-red' of *Alstroemeria* by HPLC analysis, seven anthocyanins, 6-hydroxycyanidin 3-rutinoside (11.3–13.5% determined by HPLC analysis), pigment **1** (32.7–64.0%), pigment **2** (0–0.3%), cyanidin 3-rutinoside (20.2–30.4%), 6-hydroxycyanidin 3-malonylglucoside (0–1.2%), pelargonidin 3-rutinoside (0–21.3%) and cyanidin 3-malonylglucoside (0–1.0%) were observed in the flower extracts as the main anthocyanins.

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These pigments were isolated from the flowers of the three cultivars with MAW (MeOH–HOAc–H_2O, 4:1:5) solvent, and purified using Diaion HP-20 column chromatography (CC), prep. HPLC and TLC. The structures of the known anthocyanins were identified on the basis of TLC, HPLC and spectral analysis with authentic anthocyanins as shown in Sections 3.4. and 3.5. (Harborne, 1967; Saito et al., 1988; Torskangerpoll et al., 1999; Tatsuzawa et al., 2001). However, the structures of pigments **1** and **2** could not be determined at this stage, since they exhibited unusual short λ_{max} (493 and 493 nm) values in the visible region (Harborne, 1967). Upon acid hydrolysis of pigments **1** and **2,** only one anthocyanidin, 6-hydroxypelargonidin was obtained, and glucose and rhamnose were detected for pigment **1**, and only glucose for pigment **2** as their sugar components. On addition of $AlCl_3$ this anthocyanidin did not exhibit a bathochromic shift, indicating that it lacks a vicinal hydroxyl group in its B-ring (see Section 3.4.). Moreover, the aglycone showed an λ_{max} of 500 nm in 0.1% HCl–MeOH in the visible region, at shorter wavelength than that (520 nm) of pelargonidin (see Section 3.4). On TLC analysis of Forestal (HOAc–HCl–H_2O, 30:3:10) the aglycone showed a smaller R_f value (0.46) than that (0.70) of pelargonidin, but closely related to the value (0.41) of cyanidin (see Section 3.4.). The FAB mass measurement of the aglycone gave a molecular ion $[M]^+$ at m/z 287, in agreement with the mass calculated for 6-hydroxypelargonidin ($C_{15}H_{11}O_6$). The elemental component was unambiguously confirmed by HR-FABMS (see Section 3.4.1.). Based on these spectral data, the anthocyanidin was presumed to be 6-hydroxypelargonidin. To confirm the structure of the anthocyanidin, its 500 MHz ^1H NMR spectra were obtained for CF_3CO_2D–DMSO-d_6 (1:9) solution, and analyzed. The chemical shifts of four aromatic protons in the B-ring were readily assigned by 2D COSY spectral analysis (see Section 3.4.). Signals at 8.88 (1H, *s*) and 7.27 (1H, *s*) ppm were assigned H-4 and H-8, respectively, based on previous work, where those signals of pelargonidin 3,5-diglucoside were observed at 8.83 and 7.21 ppm (Toki et al., 1995). However, the signal of H-6 (6.97 ppm of pelargonidin 3,5-diglucoside) was not observed in the spectrum suggesting the presence of a hydroxyl group at the 6-position. Therefore, this anthocyanidin was determined to be 6-hydroxypelargonidin.

2.1. Pigment 1

The FAB mass spectrum of pigment **1** showed a molecular ion $[M]^+$ at m/z 595 ($C_{27}H_{31}O_{15}$). The elemental components of anthocyanin **1** were confirmed by HR-FABMS (see in Section 3.4.2.). The results indicated that this pigment is composed of 6-hydroxypelargonidin with one molecule each of glucose and rhamnose. The detailed structure of anthocyanin **1** was elucidated based on measurements of its ^1H NMR spectra including 2D COSY and negative difference NOE (DIFNOE) spectral techniques. Six aromatic proton signals of 6-hydroxypelargonidin were assigned based on an analysis of its ^1H–^1H COSY spectrum, as shown in Table 1. The signals of two anomeric protons of the sugars appeared at 5.39 ppm (*d*, $J = 7.7$Hz, H-1 of glucose) and 4.56 ppm (*s*, H-1 of rhamnose). Based on the observed coupling constants, the glucose had a β-D-pyranose form and the rhamnose had a α-L-pyranose form. The linkage between 3-OH of 6-hydroxypelargonidin and C-1′ of glucose was confirmed by analysis of its DIFNOE spectra (Fig. 1). The methylene protons in the glucose moiety were shifted to rather low field (3.94 and 3.44 ppm), indicating that the 6-OH of glucose is linked to the rhamnose unit. This linkage was

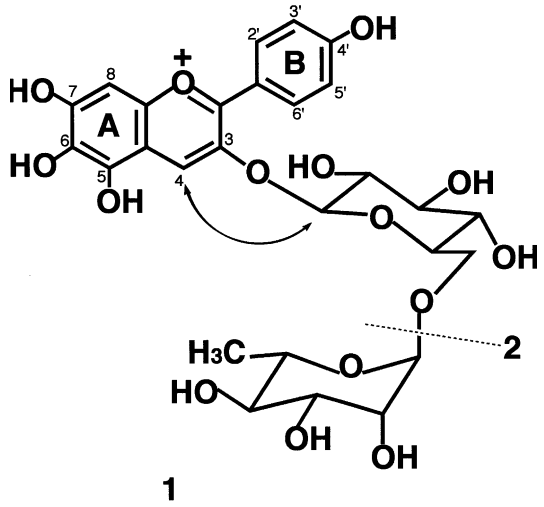
Table 1
NMR spectral data for 6-hydroxypelagonidin glycosides from the flowers of *Alstroemeria* (500 and 125.78 MHz, in CF_3CO_2D–DMSO-d_6 (1:9), TMS as internal standard, *J* Hz in parentheses)

	Pigment **1**		Pigment **2**
	δ_C	δ_H	δ_H
6-Hydroxypelargonidin			
2	164.5		
3	144.6		
4	133.9	8.93 *s*	9.02 *s*
4a	113.4		
5	141.1		
6	134.6		
7	172.5		
8	94.7	7.17 *s*	7.28 *s*
9	150.2		
1′	120.0		
2′	134.5	8.57 *d* (8.9)	8.49 *d* (9.2)
3′	117.1	7.08 *d* (8.9)	7.07 *d* (9.2)
4′	160.7		
5′	117.1	7.08 *d* (8.9)	7.07 *d* (9.2)
6′	134.5	8.57 *d* (8.9)	8.49 *d* (9.2)
Glucose			
1	102.6	5.39 *d* (7.7)	5.35 *d* (ca.7)
2	73.5	3.49 *m*	3.20–3.75
3	76.7	3.43 *m*	3.20–3.75
4	70.1	3.26 *t* (8.9)	3.20–3.75
5	76.4	3.70 *m*	3.20–3.75
6	66.8	3.49 *m*, 3.94 *brd* (10.7)	3.20–3.75
Rhamnose			
1	101.1	4.56 *s*	
2	70.7	3.66 *brs*	
3	71.1	3.49 *m*	
4	72.3	3.19 *t* (9.2)	
5	68.9	3.43 *m*	
CH_3	18.3	1.09 *d* (6.1)	

further confirmed by detection of free rutinose in the H_2O_2 degradation product of pigment **1** (Harborne, 1984). Thus, the structure of pigment **1** was determined to be 6-hydroxypelargonidin 3-*O*-[6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranoside], which is a new anthocyanin. This structure was also confirmed by analyses of its ^{13}C NMR and ^{1}H–^{13}C COSY spectra (Table 1). In the ^{13}C NMR spectra, the chemical shift (134.6 ppm) of C-6 was apparently shifted to lower field than that (104.1 ppm) of pelargonidin 3,5-diglucoside (Toki et al., 1995), indicating oxygenation at C-6.

2.2. Pigment 2

The chromatographic and spectral data of pigment **2** are shown in Section 3.4. The FAB mass spectrum of pigment **2** showed a molecular ion $[M]^+$ at *m/z* 449 corresponding to the presumed molecular formula $C_{21}H_{21}O_{11}$ (449.1083). Unfortunately, the HR-FABMS data for pigment **2** was not measured because of the small amount of the pigment **2** obtained. The structure of pigment **2** was elucidated in a manner similar to that used for pigment **1**. The six aromatic protons in the aglycone were assigned as shown in Table 1, and these values were identical with those of pigment **1**. The proton signals of the glucose moiety appeared in the region of 5.35–3.20 ppm, and an anomeric proton signal was observed at 5.35 ppm (*d*, *J* = ca. 7 Hz). By HPLC analysis, the R_t (min) of pigment **2** is identical with that of 6-hydroxypelargonidin 3-glucoside, obtained by partial acid hydrolysis of pigment **1**. Therefore, pigment **2** is determined to be 6-hydroxypelargonidin 3-*O*-(β-D-glucoside), which is a new naturally occurring anthocyanin.



1

Fig. 1. 6-Hydroxypelargonidin glycosides from the orange-red flowers of *Alstroemeria*. 1: pigment **1**, 2: pigment **2**. Observed NOE correlations are indicated by arrows.

3. Experimental

3.1. General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using seven mobile phases: BAW (*n*-BuOH–HOAc–H_2O, 4:1:2), BuH (*n*-BuOH–2N HCl, 1:1), 1% HCl and AHW (HOAc–HCl–H_2O, 15:3:82) for anthocyanins, and BAW, EAA (EtOAc–HOAc–H_2O, 3:1:1), ETN (EtOH–NH_4OH–H_2O, 16:1:3) and EFW (EtOAc–HCOOH–H_2O, 5:2:1) for organic acids and sugars. Analytical HPLC was performed on a LC-10A system (Shimadzu), using a Waters C18 (4.6∅×250 mm) column at 40 °C with a flow rate of 1 ml/min and monitoring at 530 nm. The eluant was applied as a linear gradient from 20 to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O). UV–vis spectra were recorded on a MPS-2400 (Shimadzu) in 0.1% HCl–MeOH (from 200 to 700 nm), whereas FAB mass spectra were obtained in the positive ion mode using the magic bullet. NMR spectra were acquired at 500 MHz for ^{1}H spectra and at 125.78 MHz for ^{13}C spectra in DMSO-d_6–CF_3COOD (9:1). Chemical shifts are reported relative to a TMS int. standard (δ) and coupling constants are in Hz.

3.2. Plant materials

The orange-red flowers of *Alstroemeria* cultivars 'Oreiju', 'Mayprista' and 'Spotty-red' were purchased from the Takii nursery (Japan). The flowers exhibited a red color (Red 43A by R.H.S. color chart and its chromaticity values, $b^*/a^* = 0.75$–0.97) and the fresh flowers were dried overnight at 37 °C and stored at 10 °C until needed.

3.3. Isolation of anthocyanins

Dried flowers (50 g) of *Alstroemeria* cultivars were immersed overnight in 10% HOAc–MeOH (1:l) at room temp. The extract was concd (100 ml) and purified by TLC (BAW, 4:1:2) and prep. HPLC by previous procedures (Tatsuzawa et al., 2001). Prep. HPLC utilised a Waters C_{18} (19∅×150 mm) column at 40 °C with a flow rate of 4 ml/min monitoring at 485 nm for anthocyanins. The solvent systems used were as follows: linear gradient elution for 15 min from 40 to 60% solvent B in solvent A. Fractions (about 50 ml) were transferred to a DIAION HP-20 column. Anthocyanins were eluted with 5% HOAc–MeOH, evapn residues were dissolved in a small volume of 5% HOAc–EtOH followed by addition of excess Et_2O, and then dried to give pigment powders, 6-hydroxypelargonidin 3-rutinoside **1** (5 mg) and 6-hydroxypelargonidin 3-glucoside **2** (2 mg). In addition, other five known pigments (ca. 1–3 mg) were also obtained.

3.4. Analysis of anthocyanins

Characterization of pigments were carried out by using TLC and HPLC, and UV–vis, FABMS and NMR spectral data are shown in Table 1. Furthermore, products of the pigments **1** and **2** were analyzed by TLC after acid hydrolysis, partial acid hydrolysis (2% HCl–MeOH) and H_2O_2 degradation (Harborne, 1984). 6-Hydroxypelargonidin 3-glucoside and 6-hydroxypelargonidin were detected and confirmed in the partial hydrolysis-products of pigment **1**, and obtained as purified pigment powders. The UV–vis and ^{1}H NMR spectra of both pigment products were measured, which confirmed their structures as shown in Table 1 and this section. The sugars involved in pigments **1** and **2** were confirmed to be rutinose in pigment **1** (BAW = 0.24, EAA = 0.20, ETN = 0.59, EFW = 0.12), and glucose in pigment **2** (BAW = 0.29, EAA = 0.26, ETN = 0.65, EFW = 20), respectively, by TLC analysis after the H_2O_2 degradation of both pigments.

3.4.1. 6-Hydroxypelargonidin

UV: λ_{max} 500, 272 nm, $E440/E_{max}$ (%) = 37, $AlCl_3$ shift 0, ^{1}H NMR; δ 8.88(1H, *s*, H-4), 8.50 (2H, *d*, J = 8.9 Hz, H-2′ and 6′), 7.27 (1H, *s*, H-8), 7.12 (2H, *d*, J = 8.9 Hz, H-3′ and 5′), TLC: R_f-values BAW 0.66, BuHCl 0.69, 1% HCl 0.01, AHW 0.07, Forestal 0.46, HPLC: R_t (min) 23.58. HR-FAB mass calc. of $C_{15}H_{11}O_6$: 287.0556. Found: 287.0563.

3.4.2. Pigment 1 (6-hydroxypelargonidin 3-rutinoside)

UV: λ_{max} 493, 271 nm, $E440/E_{max}$ (%) = 38, $AlCl_3$ shift 0, TLC: R_f-values BAW 0.34, BuHCl 0.18, 1% HCl 0.16, AHW 0.35, HPLC: R_t (min) 16.97. HR-FAB mass calc. for $C_{27}H_{31}O_{15}$: 595.1663. Found: 595.1635.

3.4.3. Pigment 2 (6-hydroxypelargonidin 3-glucoside)

UV: λ_{max} 493, 270 nm, $E440/E_{max}$ (%) = 41, $AlCl_3$ shift 0, TLC: R_f-values BAW 0.28, BuHCl 0.16, 1% HCl 0.05, AHW 0.17, HPLC: R_t (min) 15.10.

3.5. Reference of anthocyanins

Cyanidin, cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-malonylglucoside, 6-hydroxycyanidin, 6-hydroxycyanidin 3-glucoside, 6-hydroxycyanidin 3-rutinoside and 6-hydroxycyanidin 3-malonylglucoside were isolated from the red flowers of *Alstroemeria* cultivars (Saito et al., 1985, 1988; Tatsuzawa et al., 2001). Pelargonidin, pelargonidin 3-glucoside and pelargonidin 3-rutinoside were isolated from the orange-red flowers of *Turipa* cultivars (Harborne, 1967; Torskangerpoll et al., 1999). These pigments were fully identified by spectroscopic and chemical methods.

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