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STRUCTURES OF TWO DIACYLATED ANTHOCYANINS FROM *PETUNIA HYBRIDA* cv. SURFINIA VIOLET MINI

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Key Word Index—*Petunia hybrida*; Solanaceae; acylated anthocyanin; ^{1}H and ^{13}C-NMR; vacuolar pH.

Abstract—Two novel diacylated anthocyanins and two known monoacylated anthocyanins were isolated from violet petals of *Petunia hybrida* cv. Surfinia Violet Mini. Their structures were elucidated by spectral methods. The major novel pigment was identified as malvidin 3-*O*-(6-*O*-(4-*O*-(4-*O*-(6-*O*-caffeoyl-β-D-glucopyranosyl)-*p*-coumaroyl)-α-L-rhamnosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside and the minor novel pigment was determined as malvidin 3-*O*-(6-*O*-(4-*O*-(4-*O*-(6-*O*-caffeoyl-β-D-glucopyranosyl)-caffeoyl)-α-L-rhamnosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside.

INTRODUCTION

Flower colours are mainly determined by anthocyanin structures, co-pigments and vacuolar pH [1]. The anthocyanin biosynthetic pathway of *Petunia hybrida* has been extensively studied in terms of genetics and molecular biology, and has been well established [2]. According to the pathway, petunia anthocyanins contain one acyl group. Some flowers with cyanic colour, for example *Gentiana makinoi* [3], *Platycodon grandiflorum* [4] and *Ipomoea tricolor* [5] contain diacylated or polyacylated anthocyanins as major pigments. However, no diacylated or polyacylated anthocyanins have hitherto been isolated from *P. hybrida*. This report describes the isolation and the structural elucidation of diacylated anthocyanins from a mutant of *P. hybrida*, *P. hybrida* cv. Surfinia Violet Mini.

RESULTS AND DISCUSSIONS

Petunia hybrida cv. Surfinia Purple Mini (PM) (Fig. 1) developed by Suntory Ltd and Keisei Rose Ltd has a reddish purple flower and contains monoacylated anthocyanins as major pigments. The violet sport from PM is named *Petunia hybrida* cv. Surfinia Violet Mini (VM) (Fig. 1). The change of flower colour from reddish purple to violet in the sport is thought to be due to difference in their anthocyanin compositions.

In order to elucidate the difference in pigment compositions between both petunias, we analyzed flower petals of both varieties by HPLC equipped with a photodiode-array detector. Two peaks (**1** and **2**) were observed in the extract of VM (Fig. 2A), but not in PM (Fig. 2B).

Violet flower petals of VM were extracted with aqueous acetonitrile containing TFA. The extract was roughly fractionated by chromatography on a HP-20 column. The crude pigment fraction was purified repeatedly by prep. reverse phase HPLC. Four pigments were finally isolated from violet flower petals of VM. **4** and **5** were identified as malvidin 3-*O*-(6-*O*-(4-*O*-caffeoyl-α-L-rhamnosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside and malvidin 3-*O*-(6-*O*-(4-*O*-*p*-coumaroyl)-α-L-rhamnosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside [6], respectively, by comparison of the spectral data (UV and MS) and the retention times of HPLC with authentic samples. Two new pigments (**1** and **2**) were isolated from VM, and their structures were elucidated as follows.

After 6N-HCl hydrolysis of **2**, the reaction mixture was extracted with organic solvent. From the organic solvent layer, malvidin as aglycon, and *p*-coumaric acid and caffeic acid as acyl groups were identified by reverse phase HPLC analyses. The HPLC analysis using NH_2 column of the water layer showed that glucose and rhamnose were in the ratio 3:1. The

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hydrolytic result demonstrated that **2** contained two acyl groups. Two peaks at around 290 nm (E_{UV}) and at around 540 nm (E_{VIS}) in electronic spectra of **2** are 1:1 in intensity, as shown in Fig. 2C. This finding also provided evidence that **2** has two acyl groups per molecule, because the approximate number of aromatic acyl groups could be estimated from the intensities of E_{UV}/E_{VIS}, using Harbone's rule [7].

Full assignments of the ^{1}H-NMR and the ^{13}C-NMR signals of **2** using H,H-COSY, C,H-COSY, 2D-HMBC and 1D-HOHAHA have been carried out (Table 1). In the ^{1}H NMR spectrum the lowest field signal (8.92 ppm, 1H, s) was assigned at C-4 proton, and additional signals at 3.93 ppm (6H, s) were attributed to C-3′ and C-5′ methoxyl protons in flavylium form of the anthocyanidin nucleus. Therefore, the anthocyanidin nucleus was ascertained to be malvidin. The observation of the two sets of olefinic proton signals (5.88 and 7.33 ppm, each *d*, $J = 16$, and 6.12 and 7.40 ppm, each *d*, $J = 16$) indicated the presence of two molecules of cinnamic acid derivative in **2**. Furthermore, detailed analysis of the remaining signals in the aromatic proton's region were assigned to one molecule of *p*-coumaric acid and one molecule of caffeic acid. By 1D-HOHAHA spectra with irradiation at four anomeric protons (5.52, 5.20, 4.91 and 4.78 ppm), four sugars were elucidated to be three glucopyranosides and one rhamnopyranoside (Table 1).

Fast-atom bombardment mass spectrum (FAB-MS) of **2** showed prominent product ions at *m/z* 1271, 1109 and 947, which were assigned to molecular ion and the series of ions formed by glucose or caffeic acid losses from *m/z* 1271, respectively, indicating that **2** is an anthocyanin with glucose or caffeic acid at the molecular terminus.

By NMR, the methylene proton signals at C-6 of Glc-1 moiety and the methine proton signal at C-4 of rhamnosyl moiety were observed at a lower field than those of glucose and rhamnose. The 6 position of Glc-1 and the 4 position of rhamnosyl moiety were esterified.

In order to distinguish the linkages of caffeic acid and *p*-coumaric acid, 2D-HMBC spectra were measured. The signal of C-4 proton of rhamnosyl moiety was correlated to the ^{13}C signal of the carbonyl carbon of the *p*-coumaric acid moiety and the signals of the methylene protons at C-6 of Glc-3 moiety were also correlated to the ^{13}C signal of carbonyl carbon of the caffeic acid moiety. Additionally, based on the NOESY and ROESY spectra, the positions of three glucosidic linkages were determined to be C-3 and C-5 in the malvidin moiety, and C-4 in the *p*-coumaric acid moiety, respectively. Further, the NOE was observed between the anomeric proton of the rhamnose moiety and the methylene protons at C-6 of the Glc-1 moiety.

The structure of **2** was therefore determined as malvidin 3-*O*-(6-*O*-(4-*O*-(4-*O*-(6-*O*-caffeoyl-β-D-glucopyranosyl)-*p*-coumaroyl)-α-L-rhamnosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside (Fig. 3).

The FAB-MS spectrum of **1** showed prominent product ions at *m/z* 1287, 1125 and 963, which were assigned to molecular ion and the series of ions formed by glucose or caffeic acid losses from *m/z* 1287, respectively, and these peaks were 16 mass units higher than those of **2**. Absorption maxima in electronic spectra of **1** were observed at 290 and 540 nm, and appeared to be very similar to that of **2**. The intensity of E_{UV}/E_{VIS} was also 1:1 as shown in Fig. 2C. The ^{1}H NMR spectra data of **1** were similar to those of **2**, except for the proton signals of C-1 in the Glc-3 moiety and C-2–C-6, C_α and C_β in the *p*-coumaric acid moiety (Table 1). The aromatic proton signal at 7.00 ppm due to C-3 in the *p*-coumaric acid moiety in **2** is absent in **1**. The ROESY was observed between the anomeric proton of the Glc-3 moiety and the C-5 proton (7.04 ppm, *d*, $J = 9$) of the caffeic acid moiety (Caf-1) in **1**. These findings indicated that the *p*-coumaric acid moiety in **2** was replaced by the caffeic acid moiety in **1**.

The structure of **1** was therefore deduced as malvidin 3-*O*-(6-*O*-(4-*O*-(4-*O*-(6-*O*-caffeoyl-β-D-glucopyranosyl)-caffeoyl)-α-L-rhamnosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside (Fig. 4).

Two new diacylated anthocyanins together with two known monoacylated anthocyanins were isolated from petals of the violet flower of VM. The diacylated anthocyanins comprised about 40% of the total pigments in the petals of VM (Fig. 2A). Although some cultivars (*P. hybrida* cv. Titan Mid Blue or Falcon Blue (Sakata Seeds, Co. Ltd, Japan)) with petals of deep violet in *P. hybrida* are known, no reports on the pigments of their petals have been published. More recently, we carried out analyses of the pigments in petals of Titan Mid Blue and Falcon Blue using HPLC equipped with a photodiode-array detector. The results demonstrated that diacylated anthocyanins distribute in deep purple cultivars of *Petunias* as major pigments and in addition, vacuolar pH values of their petals are higher than those of reddish cultivars [Y. Fukui, unpublished results].

The reasons why the flower of VM showed a violet colour are as follows: (1) diacylated anthocyanins (**1** and **2**) are major components in petals of VM as well as in those of deep purple cultivars in *Petunias*. These anthocyanins play an important role in the change of flower colour from reddish purple to deep violet, because flower colours change to a more bluish hue with increasing number of acyl residues [5] and diacylated anthocyanins are more stable than monoacylated anthocyanins in neutral solution [3]; (2) It is well known that some genes in *Petunia* play a role in determining pH and that pH value is closely correlated with flower colour. A higher pH makes colour more bluish and a lower one makes it more reddish [2]. The vacuolar pH of flower petals of VM (pH, 5.65) is higher than that of PM (pH, 5.21) and in addition, the pH of petals in Titan Mid Blue and Falcon Blue

Fig. 1. Photographs of *Petunia hybrida* cv. Surfinia Purple Mini (left) and *Petunia hybrida* cv. Surfinia Violet Mini (right).

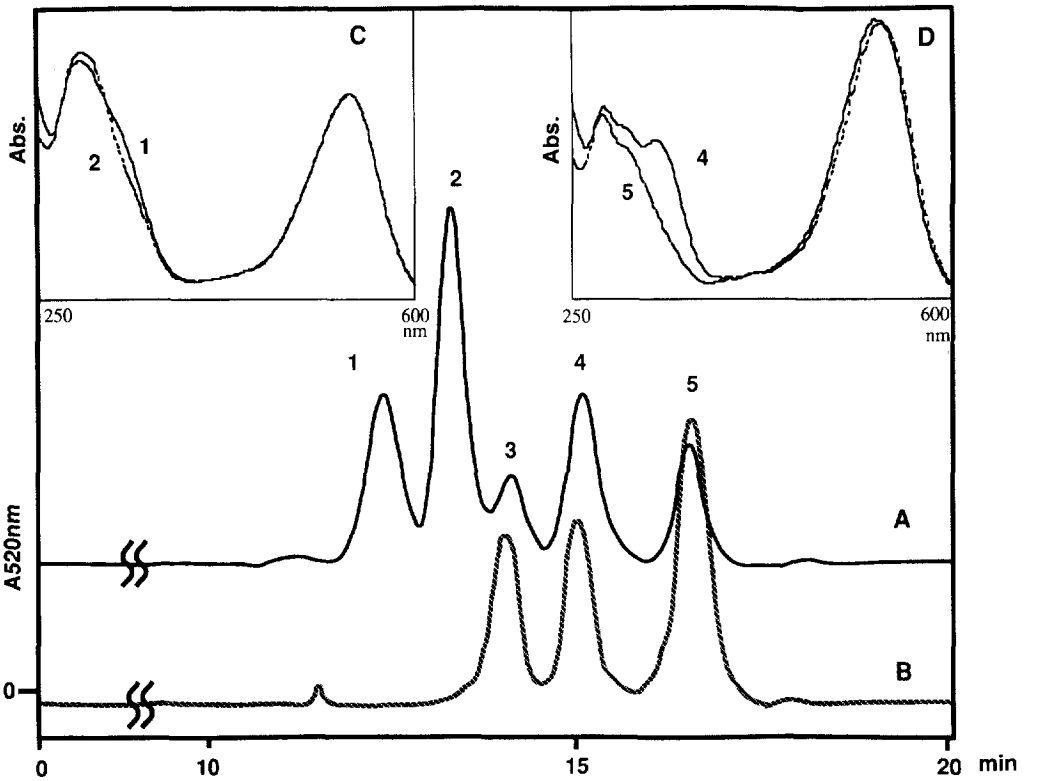


Fig. 2. HPLC analyses of *Petunia hybrida* cv. Surfinia Purple Mini (B) and *Petunia hybrida* cv. Surfinia Violet Mini (A) with a Photodiode-array Detector. A and B; Absorbance at 520 nm vs retention time. Numbers indicated the acylated anthocyanins. C; Absorbance vs wavelength of **1** and **2**. D; Absorbance vs wavelength of **4** and **5**.

are 6.07 and 5.93, respectively; (3) The concentration of anthocyanins in petals of VM are about three times higher than in PM.

EXPERIMENTAL

^1H and ^{13}C NMR spectra were obtained on a JEOL GX500 spectrometer in a 5 mm ϕ tube using 10% (v/v) TFA-d/CD_3OD as solvent. Electronic spectra were measured on a Shimazu UV-265 spectrometer. FAB-MS spectra were recorded on a JEOL-DX-300/DA-5000 system using a glycerol matrix.

HPLC analysis of anthocyanins in extracts of VM and PM

The extracts of VM and PM were analyzed by HPLC using Asahipak ODP-50 (4.6 mm $\phi \times 250$ mm) with a flow rate of 0.6 ml/min monitoring at 520 mm and photodiode-array (250–600 nm) (Shimazu SPD-M6A). See Fig. 2A and 2B. The solvent system used was as follows; a linear gradient elution from 20 to 40% acetonitrile containing 0.5% TFA in H_2O. The percentage ratio of the anthocyanins present in the plants were 12% **1**, 29% **2**, 19% **4** and 17% **5**.

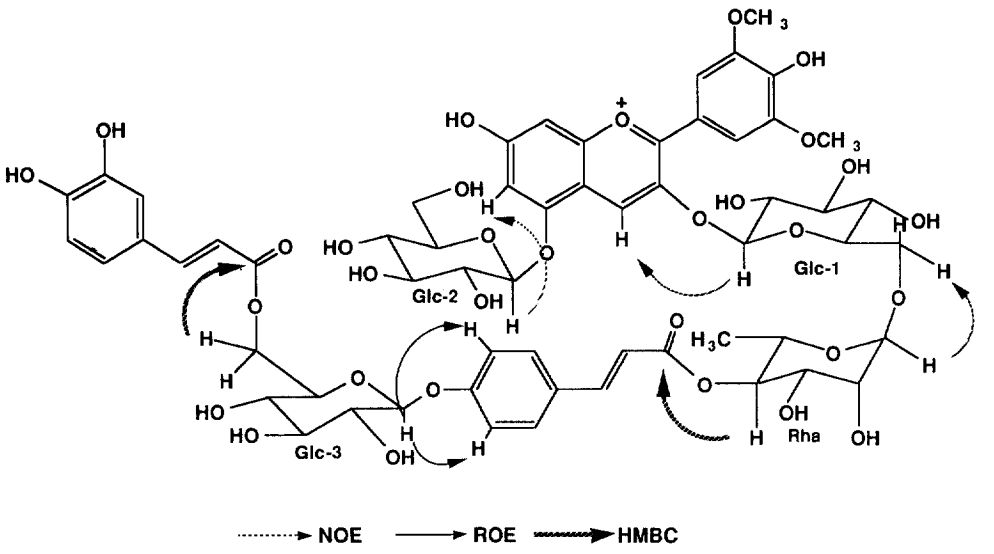
Isolation of pigments

6.9 g (fr. wt) violet flower petals of *P. hybrida* cv. Surfinia Violet Mini were frozen with liquid nitrogen and pulverized in a mortar with a pestle. The powder was extracted with 100 ml of 50% (v/v) aqueous acetonitrile containing 0.1% TFA for one day at room temp. After removing debris, the extract was evaporated to approximately half volume under reduced pressure. The concentrated extract was placed on a HP-20 column (Mitsubishi Chemical Ltd, Japan). The column was washed with water and then eluted with 50% aqueous acetonitrile containing 0.1% TFA. The fraction containing anthocyanin pigments was concentrated *in vacuo* and lyophilized. The crude pigments were purified repeatedly by prep. HPLC using ODS (Develosil-ODS 5 cm $\phi \times 30$ cm, Nomura Chemical Ltd, Japan) with a flow rate of 32 ml/min monitoring at 280 nm to give fractions I, II, III and IV, respectively. The solvent systems used were as follows; a linear gradient elution for 120 min from 10 to 60% solvent B (50% MeCN, 0.5% TFA in H_2O) in solvent A (H_2O) and further elution for 15 min 60% solvent B.

1 (7 mg) was isolated from fr. I by HPLC using Asahipak ODP-50 (2.15 cm $\phi \times 30$ cm) with a flow rate of 6 ml/min monitoring at 280 and 520 nm. Solvent

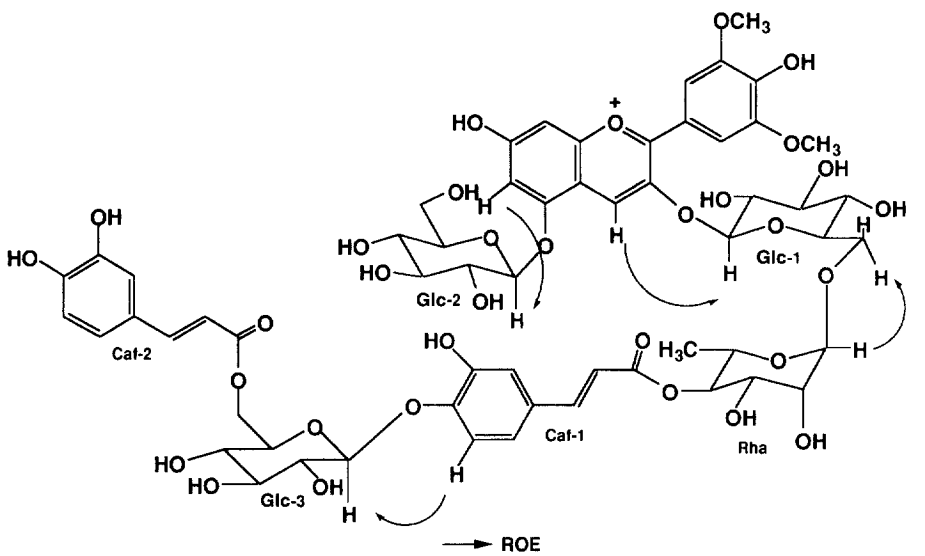
Table 1. ^{1}H and ^{13}C NMR spectral data of **1** and **2**

		2 ^{1}H			**2** ^{13}C	**1** ^{1}H		
		Chem. shift		*J*	Chem. shift	Chem. shift		*J*
Malvidin	2				163.9			
	3				146.25			
	4	8.92	*s*		133.94	8.90	*s*	
	5				157			
	6	7.00	*d*	2	105.9	7.00	*d*	2
	7				169.7			
	8	6.96	*d*	2	97.67	6.96	*d*	2
	9				156.7			
	10				112.36			
	1′				120.9			
	2′,6′	7.86	*s*		110.9	7.85	*s*	
	3′,5′				149.78			
	4′				147.39			
	OCH_3	3.93	*s*		57.26			
Glucose 1	1	5.52	*d*	7.5	102.8	5.53	*d*	9
	2	3.74	*t*	8	74.84			
	3	3.60	*t*	8	78.22			
	4	3.68	*t*	8	70.5			
	5	3.85	*m*		77.36			
	6a	4.11	*dd*	12,2	66.81			
	6b	3.85	*m*					
Glucose 2	1	5.20	*d*	7.5	102.69	5.20	*d*	9
	2	3.68	*t*	8	74.84			
	3	3.60	*t*	8	78.11			
	4	3.53	*t*	8	71.05			
	5	3.61	*m*		77.89			
	6a	3.98	*dd*	12,2	62.24			
	6b	3.81	*dd*	12,5				
Glucose 3	1	4.91	*d*	7.5	101.74	4.79	*d*	8
	2	3.52	*m*		74.84			
	3	3.52	*m*		78.22			
	4	3.40	*t*	9	72.26			
	5	3.85	*t*	9	75.6			
	6a	4.54	*dd*	12,2	64.91			
	6b	4.39	*dd*	12,9				
Rhamnose	1	4.78	*d*	1.3	102.23	4.79	*d*	1
	2	3.85	*m*		72.02			
	3	3.86	*m*		70.37			
	4	4.88	*t*	9.6	75.6	4.87	*t*	10
	5	3.83	*m*		67.7			
	6	0.98	*d*	6	19.92			
Couma (caf1)	1				129.77			
	2	7.23	*d*	9	130.72	6.94	*d*	2
	3	7.00	*d*	9	118.18			
	4				160.53			
	5	7.00	*d*	9	118.18	7.04	*d*	9
	6	7.23	*d*	9	130.72	6.61	*dd*	9,2
	α	5.88	*d*	16	116.88	5.86	*d*	16
	β	7.33	*d*	16	145.87	7.25	*d*	16
	C=O				168.47			
caf2	1				125.56			
	2	6.76	*d*	2	114	6.75	*d*	2
	3				149.1			
	4				146.84			
	5	6.53	*d*	8	116.5	6.53	*d*	9
	6	6.68	*dd*	8,2	123.12	6.68	*dd*	9,2
	α	6.12	*d*	16	115.22	6.12	*d*	16
	β	7.40	*d*	16	146.47	7.43	*d*	16
	C=O				168.73			



2

Fig. 3. Structure and the NOE, ROE and HMBC networks of **2**.



1

Fig. 4. Structure and the ROE network of **1**.

systems used were as follows; a linear gradient elution for 100 min from 35–45% solvent B (50% MeCN, 0.5% TFA in H_2O) in solvent A (50 mM KH_2PO_4, 0.5% TFA in H_2O).

2 (15 mg) was isolated from fr. II, **4** (6 mg) and **5** (5 mg) were isolated from fr. III and IV, respectively.

1: UV_{max} nm, 290, VIS_{max} nm, 540, MS *m/z*; 1287 $[M]^+$, 1125 $[M\text{-}162]^+$. The ^1H NMR data are summarized in Table 1. **2**: UV_{max} nm, 292, VIS_{max} nm, 540, MS *m/z*; 1271 $[M]^+$, 1109 $[M\text{-}162]^+$. The ^1H and ^{13}C NMR data are summarized in Table 1. **4**: UV_{max} nm, 280, VIS_{max} nm, 532, MS *m/z*; 963 $[M]^+$, 801 $[M\text{-}162]^+$. **4** was identical with authentic specimens by co-chromatography on HPLC. **5**: UV_{max} nm, 280, VIS_{max} nm, 536, MS *m/z*; 947 $[M]^+$, 785 $[M\text{-}162]^+$. **5** was

identified by co-chromatography on HPLC with authentic sample. HPLC conditions were the same as those used in the isolation of **4** and **5**.

Identification of anthocyanidin and the acyl and sugar components in **2**

A solution of **2** (0.5 mg) in 6N-HCl (1 ml) was heated in a boiling water bath for 20 min. After cooling, the reaction mixture was extracted with 1-pentanol. HPLC analysis of organic layer showed the presence of caffeic acid (retention time, 14.3 min) and *p*-coumaric acid (retention time, 16.3 min), which were identified by co-chromatography on HPLC with authentic specimens. HPLC condition; Asahipak ODP-50 (4.6 mm $\phi \times 250$ mm) column, a flow rate of solvent, 0.6 ml/min, monitor, 330 nm. Solvent systems used were as follows; a linear gradient elution from 20 to 90% acetonitrile containing 0.5% TFA in H_2O.

HPLC analysis of the organic layer indicated the presence of malvidin (retention time, 6.5 min), which was identified by co-chromatography on HPLC with an authentic sample. HPLC condition; YMC ODS-A312 (6 mm $\phi \times 150$ mm) column, a flow rate of solvent, 1 ml/min, monitor, 520 nm. Solvent system used was as follows; CH_3COOH:CH_3OH:H_2O = 15:20:65.

HPLC analysis of the aqueous layer showed the presence of glucose (retention time, 6.9 min) and rhamnose (retention time, 4.8 min), which were identified by co-chromatography on HPLC with authentic specimens. HPLC condition; Asahipak NH_2P-50 (4.6 mm $\phi \times 250$ mm) column, a flow rate of solvent, 1 ml/min, monitor, RI (Shodex SE61, range 2). Solvent system used was 70% CH_3CN.

pH measurement of vacuole

About 2 g (fr. wt) of the petals were frozen at −80°C in 1 h and the frozen petals were homogenized. After centrifugation, pH of the obtained supernatant was measured by using Horiba pH Meter F-22 with electrode 6069-10C. The vacuolar pH values of PM and VM were 5.21 and 5.65, respectively.

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