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Acylated malvidin 3-rutinosides in dusky violet flowers of *Petunia integrifolia* subsp. *inflata*

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Abstract

A new acylated anthocyanin was isolated from a strain of *Petunia integrifolia* subsp. *inflata* with dusky violet flowers (B1204d), and identified as malvidin 3-*O*-[6-*O*-(4-*O*-(4-*O*-(6-*O*-(*trans*-caffeoyl)-*β*-D-glucopyranosyl)-*trans*-*p*-coumaroyl)-α-L-rhamnopyranosyl)-*β*-D-glucopyranoside] as a major pigment. Also, two known pigments were found in these flowers, and determined to be malvidin 3-caffeoylrutinoside and 3-*p*-coumaroylrutinoside.

Keywords: *Petunia integrifolia* subsp. *inflata*; Solanaceae; Dusky violet flower; Acylated anthocyanins; Malvidin 3-rutinosides; Caffeic acid; *p*-coumaric acid; Flower color

1. Introduction

In the investigation of floral anthocyanins in the various color forms of *Petunia* species, we have already reported the occurrence of 3-glucoside of delphinidin, 3-rutinosides of cyanidin, delphinidin and petunidin, monoacyl 3-rutinoside-5-glucosides of delphinidin, petunidin and malvidin and diacyl 3-rutinoside-5-glucosides of petunidin and malvidin in the flowers of 20 natural taxa occurring in South America (Tatsuzawa et al., 1997; Ando et al., 1998). After, Fukui et al. (1998) reported the presence of same diacylated malvidin 3-rutinoside-5-glucosides in flowers of garden petunia. As part of our ongoing program, we researched the pigment components of a new flower color strain, which was selected as a dusky violet one from a native population of *P. integrifolia* subsp. *inflata* possessing red–purple flower color (Ando et al., 1998). In this paper, we report the isolation and the structure determination of a new acylated anthocyanin from the dusky violet flowers along with two known anthocyanins.

2. Results and discussion

In a survey of the dusky violet flowers of *P. integrifolia* subsp. *inflata* (B1204d), by HPLC analysis, three major anthocyanin peaks were observed. These anthocyanins were isolated and identified as **1** (frequency ~23.3%), **2** (~15.7%) and **3** (~23.1%). The isolation and the structure determination of these anthocyanins were performed as described previously (Tatsuzawa et al., 1997; Ando et al., 1998). Their R_f values, R_t (min) and spectral data are shown in Table 1. Alkaline hydrolysis of **1**–**3** yield only one pigment (**4**) as their dea-

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Table 1
Chromatographic and spectral properties of anthocyanins from dusky violet flowered *Petunia integrifolia* subsp. *inflata* (B1204d). See Section 3 for solvent abbreviations. **1** is malvidin 3-caffeoylrutinoside, **2** malvidin 3-caffeoylglucosyl *p*-coumaroylrutinoside, **3** malvidin 3-*p*-coumaroylrutinoside, **4** malvidin 3-rutinoside (deacylanthocyanin)

Anthocyanin	R_f values (×100)				Spectral data in 0.1% HCl–MeOH				R_t[a] (min)	FAB-MS $[M]^+$
	BAW	BuHCl	1% HCl	AHW	λ_{max} (nm)	E_{acyl}/E_{max}(%)	E_{440}/E_{max}(%)	$AlCl_3$		
1	57	31	13	35	542, 322, 284	68	19	0	32.7	801
2	49	11	4	18	545, 324, 287	80	20	0	33.3	1109
3	63	37	16	42	542, 320, 283	76	21	0	36.1	785
4	38	6	23	43	539, 282	–	24	0	24.5	639

[a] Column: Waters C18 (4.6ϕ × 250 mm); other details, see Section 3. .

cyl anthocyanins, and **1** gave caffeic acid, **3** gave *p*-coumaric acid, and **2** glucosyl *p*-coumaric acid and caffeic acid as their acyl moieties. Acid hydrolysis of **4** gave malvidin, glucose and rhamnose. The E_{440}/E_{max} values (24%) of **4** indicated that the 5-OH of malvidin is free from glycosylation Table 1. The FAB mass spectrum of **4** gave its $[M]^+$ at 639 m/z, corresponding to the mass calculated for $C_{29}H_{35}O_{16}$ (639.19). Then, the structure of **4** was identified to be malvidin 3-rutinoside. This structure was confirmed by analysis of its ^1H NMR spectra including ^1H–^1H COSY (Table 2).

2.1. Pigment 2

The FAB mass spectrum of **2** showed a molecular ion peak $[M]^+$ at 1109 m/z, corresponding to the molecular formula $C_{53}H_{57}O_{26}$ (1109.314). The ^1H NMR spectrum of **2** showed the presence of two molecules of glucose and one molecule of malvidin, rhamnose, *p*-coumaric acid and caffeic acid, respectively. The protons of malvidin, caffeic acid and *p*-coumaric acid in this pigment were assigned by analysis of its ^1H–^1H COSY spectrum Table 2. All four olefinic proton signals of *p*-coumaric and caffeic acids of **2** had large coupling constants (J = 16.1 Hz), indicating these two hydroxycinnamic acids to have the *trans* configurations. The signals of three anomeric protons of **2** appeared at δ 5.42 (d, J = 7.0 Hz, Glc A, Fig. 1), δ 5.00 (d, J = 7.7 Hz, Glc B) and δ 4.56 (s, rhamnose), and the assigned glucose protons had coupling constants J = 7.0–10.9 Hz, indicating these glucose residues must be of β-D-glucopyranose form. In the rhamnose moiety, one singlet signal of anomeric proton (δ 4.56) and doublet signals of methyl protons (δ 0.90, d, J = 6.0) at C-5 suggested the existence of α-L-rhamnopyranose form. The proton signals of H-4 of

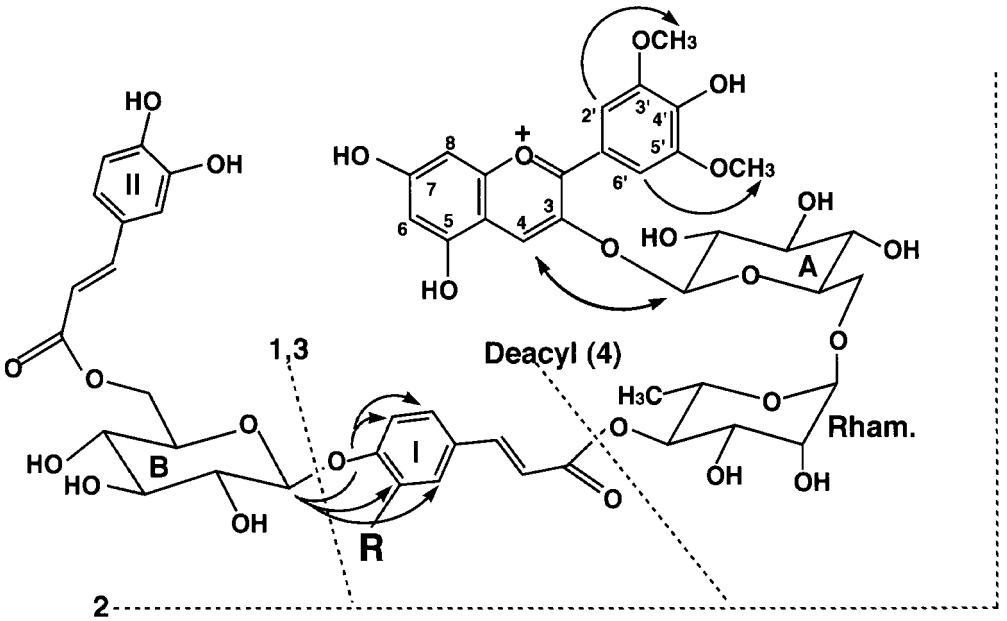

Fig. 1. Anthocyanins from dusky violet flowered *Petunia integrifolia* subsp. *inflata* (B1204d). Pigment 1:R=OH, 2,3:R=H, 4:deacylanthocyanin (malvidin 3-rutinoside). Main NOEs are indicated by arrows.

Table 2
^{1}H NMR spectral data of anthocyanins from dusky violet flowered *Petunia integrifolia* subsp. *inflata* (B1204d) (500 and 600 MHz, DCl-DMSO-d6, 1:9 at 25°C, standard TMS). Coupling constants (*J* in Hz) in parantheses

	2 δH (600 MHz)	**1** δH (500 MHz)	**3** δH (500 MHz)	**4** (Deacyl 1–3) δH (600 MHz)
Malvidin				
4	8.87 s	8.87 s	8.87 s	8.91 s
6	6.94 brs	6.96 brs	6.96 brs	7.01 brs
8	7.08 brs	7.09 brs	7.08 brs	7.16 brs
2′,6′	7.89 s	7.91 s	7.90 s	7.95 s
3′,5′ (O–CH_3)	3.88 s	3.89 s	3.89 s	3.93 s
Hydroxycinnamic acid				
[I]				
2	7.54 d(8.4)	6.96 d(2.0)	7.48 d(8.9)	
3	7.02 d(8.4)	–	6.80 d(8.9)	
5	7.02 d(8.4)	6.78 d(8.2)	6.80 d(8.9)	
6	7.54 d(8.4)	6.94 dd(2.0, 8.2)	7.48 d(8.9)	
α	6.28 d(16.1)	6.17 d(15.9)	6.24 d(16.2)	
β	7.50 d(16.1)	7.43 d(15.9)	7351 d(16.2)	
[II]				
2	7.05 d(1.8)			
5	6.75 d(8.4)			
6	6.92 dd(1.8, 8.4)			
α	6.21 d(16.1)			
β	7.41 d(16.1)			
Glucose				
[A]				
1	5.42 d(7.0)	5.40 d(7.3)	5.41 d(7.3)	5.42 d(6.6)
2	3.44 t(8.8)	3.43 m	3.43 m	3.45 m
3	3.42 t(8.8)	3.41 m	3.41 m	3.10–3.91
4	3.20 t(9.2)	3.17 t(9.5)	3.18 t(8.9)	3.10–3.91
5	3.73 m	3.70 m	3.71 m	3.10–3.91
6a	3.45 m	3.43 m	3.45 m	3.10–3.91
6b	3.87 m	3.89 m	3.89 m	3.10–3.91
[B]				
1	5.00 d(7.7)			
2	3.27 t(8.4)			
3	3.33 t(8.8)			
4	3.24 t(9.2)			
5	3.70 m			
6a	4.17 dd(6.2, 10.9)			
6b	4.36 brd(10.9)			
Rhamnose				
1	4.56 s	4.55 s	4.55 s	4.51 s
2	3.66 brs	3.66 brs	3.66 brs	2.25 m
3	3.67 m	3.66 m	3.66 m	3.10–3.91
4	4.83 t(9.7)	4.78 t(7.3)	4.79 t(7.8)	3.10–3.91
5	3.61 dd(6.2, 9.7)	3.60 m	3.58 m	3.36 m
6 (-CH_3)	0.90 d(6.0)	0.92 d(6.1)	0.90 d(6.0)	1.05 d(6.0)

rhamnose (δ 4.83, t, J = 9.7 Hz) and H-6a (δ 4.17, dd, J = 6.2, 10.9 Hz) and b (δ 4.36, br d, J = 10.9 Hz) of Glc B were shifted to lower magnetic fields Table 2, indicating that the OH-4 of rhamnose and OH-6 of Glc B are acylated with hydroxycinnamic acids. In order to determine the attachments and positions between sugar and hydroxycinnamic acid units in this pigment, the negative difference nuclear Overhauser (DIFNOE) spectra were measured and analyzed (Kondo, Kawai, Tamura, & Goto, 1987). By irradiation at the anomeric proton of Glc A, a strong NOE was observed at H-4 of malvidin, indicating Glc A is bonded with 3-OH of malvidin (Fig. 1). Furthermore, by irradiation at H-1 of Glc B, a strong NOE was observed at H-3,5 of *p*-coumaric acid moiety and a rather weak NOE at H-2,6 of *p*-coumaric acid moiety, supporting that Glc B was glycosylation with OH-4 of *p*-coumaric acid and esterified with caffeic acid at OH-6 of Glc B.

Therefore, **2** is malvidin 3-*O*-[6-*O*-(4-*O*-(4-*O*-*trans*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-*p*-coumaroyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside], which is a new anthocyanin (Harborne & Grayer, 1988; Strack & Wray, 1994).

2.2. Pigment 1 and 3

The FAB mass spectra of **1** and **3** gave their molecular ion $[M]^+$ at 801 and 785 m/z, respectively, in good agreement with the mass calculated for $C_{38}H_{41}O_{19}$ and $C_{38}H_{41}O_{18}$. The ^1H NMR spectrum of **1** was superimposable on that of **3**, except for signals of hydroxycinnamic acid moieties (Fig. 1 and Table 2). The detailed structures of **1** and **3** were elucidated by ^1H–^1H COSY spectral method as described for that of **2**. Analyses of ^1H NMR spectra of **1** and **3** revealed the presence of malvidin 3-rutinoside as their partial structures in both pigments, and the presence of an additional molecule of caffeic acid in **1** and also *p*-coumaric acid in **3** Table 2. In both spectra of **1** and **3**, the H-4 protons of both rhamnose moieties of **1** and **3** were shifted to lower magnetic fields at δ 4.78 (t, J = 7.3, pigment **1**) and δ 4.79 (t, J = 7.8, pigment **3**) supporting that OH-4 of the rhamnose moiety in **1** is acylated with caffeic acid and OH-4 of the rhamnose moiety in **3** is acylated with *p*-coumaric acid. Therefore, **1** is malvidin 3-*O*-[6-*O*-(4-*O*-(*trans*-caffeoyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside] and **3** is malvidin 3-*O*-[6-*O*-(4-*O*-(*trans*-*p*-coumaroyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]. The occurrence of **1** and **3** in *Petunia* has already been reported by Griesbach (1996). However, this is the first time that **1** and **3** have been fully determined by FAB mass and NMR spectra (Harborne & Grayer, 1988; Strack & Wray, 1994; Ando et al., 1998).

As reported earlier (Ando et al., 1998), the red–purple flowers of normal *P. integrifolia* subsp. *inflata* contained di- and mono-acylated 3-rutinoside-5-glucosides of malvidin and petunidin as main pigments. On the other hand, this dusky violet strain has only 3-acylated rutinosides of malvidin as its main pigments such as **1**–**3**. The enzyme reaction of anthocyanin 5-glucosyltransferase is completely locking or inhibited in this dusky violet flowers in comparison with that in the red–purple flowers of normal form. Therefore, these 3-acylated rutinosides of malvidin in this dusky violet strain may be considered to play an important role to produce the dusky or gray color such as the cases of pelargonidin 3-caffeoylglucosides in the maroon flowers of *Pharbitis nil* (L.) Choisy (Saito et al., 1994), peonidin 3-glucosylcaffeoylglucoside in the slate flowers of *Pharbitis nil* (Saito et al., 1996), and cyanidin 3-di(glucosylcaffeoyl)glucoside and cyanidin 3-glucoside in the brown–red flowers of *Ipomoea purpurea* (L.) Roth. (Saito, Tatsuzawa, Kasahara, Iida, & Honda, 1998).

3. Experimental

3.1. Plant material

In a large native population of *P. integrifolia* (Hook.) Schinz and Thell. subsp. *inflata* (R.E. Fr.) Wijsman of several hectares established in a finished wheat field (24 km southeast of Garruchos on the way to Santo Antônio das Missões, Mun. São Borja, Rio Grande do Sul State, Brazil), authors (G.H. and T.A.) found a possible mutant of abnormal flower color, dusky violet, on 28th November, 1996. From the second generation of the seed collected from the plant, we obtained a strain named B1204d fixed for the flower color (Violet 86D by R.H.S. color chart, chromaticity value $b/a = -0.40$). As already reported (Ando et al., 1998), the normal color of this taxon is red–purple (Red Purple 74A, $b/a = -0.30$).

3.2. Isolation of anthocyanins

Mixed dried corolla limbs (ca. 100 g) were extracted with MAW (MeOH–HOAc–H_2O, 2:1:7) (2 l). The extract was purified by Diaion HP-20 gel CC, PC and TLC as described previously (Tatsuzawa et al., 1997; Ando et al., 1998). Solvents used were BAW (*n*-BuOH–HOAc–H_2O, 4:1:5) and 15% HOAc. Prep. HPLC was run on a Waters C18 (19ϕ × 150 mm) column at 40°C with a flow rate of 4 ml min^{-1} monitoring at 530 nm for anthocyanins. Solvent systems used were as follows; a linear gradient elution for 40 min 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). The pigment frs were evapd in vacuo to dryness. After these processes each fr. of these pigments was dissolved in small vol. of 10% HOAc–MeOH, and pptd by addition of excess Et_2O. Then the pptd pigments were dried to powders **1** (ca. 10 mg), **2** (ca. 10 mg), **3** (ca. 20 mg) and **4** (ca. 10 mg).

3.3. Standard analysis of anthocyanins

Pigment identifications were carried out by standard procedures involving alkaline deacylation and acid hydrolysis (Harborne, 1984; Tatsuzawa et al., 1997; Ando et al., 1998). The anthocyanin quantitative analysis of the flower extracts was performed by HPLC on an Inertsil ODS-2 column (4.6ϕ × 250 mm) at 40°C with a flow rate of 1 ml min^{-1} and monitoring at 530 nm for anthocyanins. A linear gradient within 40 min from 20 to 85% solvent B in solvent A was applied. TLC was carried out on microcrystalline cellulose (Avicel SF, Funakoshi) using BAW, BuHCl (*n*-BuOH–2 M HCl, 1:1), 1% HCl and AHW (HOAc–HCl–H_2O, 15:3:82) for anthocyanins and BAW, ETN (EtOH–NH_4OH–H_2O, 16:1:3), EAA (EtOAc–HOAc–

H_2O, 3:1:1) and EFW (EtOAc–HCOOH–H_2O, 5:2:1) for organic acids and sugars.

3.4. FAB mass and NMR measurements

FAB mass spectra were recorded in the positive mode with magic bullet. NMR spectra were recorded at a frequency of 500 or 600 MHz for ^1H spectra in DMSO-d6-DCl (9:1) and DMSO-d6-TFA (9:1). Chemical shifts are reported relative to a TMS int. standard (δ) and coupling constants are reported in Hz.

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