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Acylated delphinidin 3-rutinoside-5-glucosides in the flowers of *Petunia reitzii*

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Abstract

Two acylated anthocyanins were isolated from selected individuals of *Petunia reitzii*, and identified to be delphinidin 3-*O*-[6-*O*-(4-*O*-(4-*O*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-*p*-coumaroyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside] and delphinidin 3-*O*-[6-*O*-(4-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-*p*-coumaroyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside]. Nine known anthocyanins were also identified.

Keywords: *Petunia reitzii*; Solanaceae; Acylated anthocyanins; Delphinidin 3-rutinoside-5-glucoside; Caffeic acid; *p*-Coumaric acid; Flower colour

1. Introduction

We have already reported 24 anthocyanins in the flowers of 20 natural taxa of *Petunia* (Solanaceae) occurring in South America (Tatsuzawa et al., 1997; Ando et al., 1999). As shown in Ando et al. (1999), the floral anthocyanins of *Petunia reitzii* L. B. Sm. & Downs are characterized by the presence of 3-rutinoside-5-glucosides of delphinidin (61%) and petunidin (6%), and their mono-acylated ones, 3-*trans* and *cis*-*p*-coumaroylrutinoside-5-glucoside of delphinidin (24%) and petunidin (4%), as major pigments. However, in a more detailed survey of floral anthocyanins of *P. reitzii* from different sources using HPLC analysis, several individuals of slightly bluish colour were found. We have isolated two new acylated delphinidin glycosides from the flowers of these individuals as major pigments, in addition to nine known compounds, 3-rutinoside of delphinidin, 3-rutinoside-5-glucoside, 3-caffeoylrutinoside-5-glucoside, 3-*trans*-*p*-coumaroylrutinoside-5-glucoside, 3-*cis*-*p*-coumaroylrutinoside-5-glucoside of delphinidin and petunidin. The structural determination of these new pigments is given in this paper.

2. Results and discussion

2.1. Standard anthocyanin analysis

The isolated new anthocyanins are referred to as pigments **25** and **26** here, as in our previous paper (Ando et al., 1999). The relative concentrations of all

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Table 1
Chromatographic and spectral properties of anthocyanins from *P. reitzii* (B162)

Anthocyanin	R_f values (×100)					Spectral data in 0.1% HCl–MeOH			R_t[a](min)	FAB MS $[M]^+$
	BAW[e]	BuHCl[e]	1% HCl	AHW[e]	λ_{max} (nm)	E_{acyl}/E_{max} (%)	E_{440}/E_{max} (%)	$AlCl_3$		
25[b]	8	4	5	16	543,325,284	61	10	+	25.2	1243
26[c]	6	4	30	47	542,305,280	75	9	+	19.7	1081
deacyl anthocyanin	14	2	28	47	540,276	–	11	+	11.2	773
1 (delphinidin 3-rutinoside-5-glucoside)[d]	14	2	28	47	540,276	–	11	+	11.2	773
2 (petunidin 3-rutinoside-5-glucoside)[d]	17	2	32	54	538,275	–	10	+	14.6	787
4 (delphinidin 3-rutinoside)[d]	19	5	19	30	543,277	–	18	+	16.7	611
10 (delphinidin 3-*trans*-caffeoylrutinoside-5-glucoside)[d]	26	7	30	43	541,326,280	71	14	+	22.7	935
12 (delphinidin 3-*cis*-*p*-coumaroylrutinoside-5-glucoside)[d]	33	11	18	45	541,305,280	59	7	+	24.1	919
14 (petunidin 3-*trans*-caffeoylrutinoside-5-glucoside)[d]	30	7	24	44	542,326,280	71	11	+	25.3	949
15 (delphinidin 3-*trans*-*p*-coumaroylrutinoside-5-glucoside)[d]	35	8	12	39	541,305,280	82	11	+	25.4	919
18 (petunidin 3-*cis*-*p*-coumaroylrutinoside-5-glucoside)[d]	39	8	38	53	541,308,283	95	4	+	26.9	933
21 (petunidin 3-*trans*-*p*-coumaroylrutinoside-5-glucoside)[d]	41	7	13	47	540,310,283	106	13	+	28.2	933

[a] Column: Waters C18 (4.6 ϕ × 250 mm); for other details see Section 3.
[b] **25**: delphinidin 3-caffeoylglucosyl *p*-coumaroylrutinoside-5-glucoside.
[c] **26**: delphinidin 3-glucosyl-*p*-coumaroylrutinoside-5-glucoside.
[d] Pigment no. and names are the same as in Ando et al. (1999).
[e] BAW: *n*-BuOH–HOAc–H_2O (4:1:2); AHW: HOAc–HCl–H_2O (15:3:82); BuHCl: *n*-BuOH–2N HCl (1:1).

described anthocyanins determined by procedures similar to those reported previously (Ando et al., 1999), were **25** (22.7–42.6%), **26** (15.7–28%), delphinidin 3-rutinoside (0–2%), delphinidin 3-rutinoside-5-glucoside (18.2–22.2%), delphinidin 3-caffeoylrutinoside-5-glucoside (0–5%), delphinidin 3-*trans*-*p*-coumaroylrutinoside-5-glucoside (0–5%), delphinidin 3-*cis*-*p*-coumaroylrutinoside-5-glucoside (0–3%), petunidin 3-rutinoside-5-glucoside (0–3%), petunidin 3-caffeoylrutinoside-5-glucoside (0–2%), petunidin 3-*trans*-*p*-coumaroylrutinoside-5-glucoside (0–2%) and petunidin 3-*cis*-*p*-coumaroylrutinoside-5-glucoside (0–2%).

Table 2
^{1}H-NMR spectral data of *P. reitzii* (B162) anthocyanins (600 MHz, DCl-DMSO-d_6, 1:9 at 25°C, standard TMS)

H	25	26
Delphinidin		
4	8.84 *s*	8.85 *s*
6	7.02 *brs*	7.03 *brs*
8	7.19 *brs*	7.21 *brs*
2′, 6′	7.83 *s*	7.84 *s*
Hydroxycinnamic acid[a,b]		
(I)		
2,6	7.58 *d* (8.5)	7.62 *d* (8.1)
3,5	7.06 *d* (8.5)	7.05 *d* (8.1)
a	6.34 *d* (15.8)	6.40 *d* (15.5)
b	7.51 *d* (15.8)	7.56 *d* (15.5)
(II)		
2	7.10 *brs*	
5	6.80 *d* (8.0)	
6	6.98 *brd* (8.0)	
a	6.26 *d* (15.8)	
b	7.46 *d* (15.8)	
Glucose[a,b]		
(A)		
1	5.59 *d* (7.9)	5.60 *d* (7.7)
2	3.66 *t* (8.5)	3.67 *t* (8.6)
3	3.20–3.60	3.30–3.75
4	3.20–3.60	3.30–3.75
5	3.20–3.60	3.30–3.75
6a	3.62 *brd* (8.9)	3.62 *m*
6b	3.84 *brd* (10.9)	3.85 *brd* (11.4)
(B)		
1	5.04 *d* (7.3)	5.10 *d* (7.3)
2	3.32 *t* (8.5)	3.46 *t* (8.5)
3	3.20–3.86	3.30–3.75
4	3.20–3.86	3.30–3.75
5	3.20–3.86	3.30–3.75
6a	3.20–3.86	3.30–3.75
6b	3.20–3.86	3.30–3.75
(C)		
1	4.95 *d* (8.3)	4.95 *d* (7.7)
2	3.71 *t* (8.4)	3.26 *t* (8.4)
3	3.50 *m*	3.33 *t* (9.2)
4	3.30 *m*	3.38 *m*
5	3.73 *m*	3.61 *m*
6a	4.21 *m*	3.42 – 3.75
6b	4.40 *brd* (11.0)	3.42 – 3.75
Rhamnose[a,b]		
1	4.61 *s*	4.60 *s*
2	3.66 *m*	3.67 *m*
3	3.68 *m*	3.69 *m*
4	4.81 *t* (9.6)	4.81 *t* (9.9)
5	3.63 *m*	3.61 *m*
–CH_3	0.91 *d* (6.0)	0.91 *d* (6.0)

[a] Assigned by ^{1}H–^{1}H COSY.
[b] Assigned by DIFNOE coupling constants (*J* in Hz) are given in parentheses.

The isolation and structure determination of pigments **25** and **26** were accomplished as described previously (Tatsuzawa et al., 1997; Ando et al., 1999). Their R_f values, R_t (min) and spectral data are given in Table 1. Alkaline hydrolysis of pigments **25** and **26** yielded only delphinidin 3-rutinoside-5-glucoside as their deacyl anthocyanins, as determined by HPLC analysis; pigment **25** gave glucosyl-*p*-coumaric and caffeic acids as acyl moieties, while pigment **26** gave only glucosyl*p*-coumaric acid. The nine known anthocyanins were identified as 3-rutinoside of delphinidin, 3-rutinoside-5-glucoside, 3-caffeoylrutinoside-5-glucoside, 3-*trans*-*p*-coumaroylrutinoside-5-glucoside, 3-*cis*-*p*-coumaroylrutinoside-5-glucoside of delphinidin and petunidin, by direct comparison with authentic samples which were isolated from the normal flowers of *P. reitzii* as described previously (Ando et al., 1999).

2.2. Pigment 25

The FAB mass spectrum of pigment **25** showed a molecular ion peak $[M]^+$ at 1243 *m*/*z*, corresponding to the molecular formula $C_{57}H_{63}O_{31}$ (1243.335). The ^{1}H-NMR spectrum of pigment **25** showed the presence of three molecules each of glucose and one molecule each of delphinidin, rhamnose, *p*-coumaric acid and caffeic acid. The aromatic protons of delphinidin, caffeic acid and *p*-coumaric acid in this pigment were assigned (Table 2) by analysis of its ^{1}H–^{1}H COSY spectrum. All four olefinic proton signals of the *p*-coumaric and caffeic acid moieties of pigment **25** had large coupling constants (J = 15.8 Hz), indicating these two hydroxycinnamic acids to have *trans*-configuration. The signals of four anomeric protons of pigment **25** appeared at δ 5.59 (*d*, J = 7.9 Hz, glucose A, Fig. 1), δ 5.04 (*d*, J = 7.3 Hz, glucose B), δ 4.95 (*d*, J = 8.3 Hz, glucose C) and δ 4.61 (*s*, rhamnose); the glucose protons (including those mentioned above) had coupling constants of J = 7.3–11.0 Hz, suggesting that these glucose residues comprise a β-D-glucopyranose. In the rhamnose moiety, singlet anomeric proton signal (δ 4.61), and doublet signals of methyl protons (δ 0.91, *d*, J = 6.0) at C-5, suggested the existence of an α-L-rhamnopyranose. The proton signals of H-4 of rhamnose (δ 4.81, *t*, J = 9.6 Hz) and H-6a (δ 4.21, *m*) and b (δ 4.40, *brd*, J = 11.0 Hz) of glucose C were shifted to a lower magnetic field (Table 2), indicating that the OH-4 of rhamnose and OH-6 of glucose C are acylated with hydroxycinnamic acids. In order to

determine the attachments and positions between sugar and hydroxycinnamic acid units in this pigment, the DIFNOE spectra were recorded and analyzed (Kondo et al., 1987). Upon irradiation of the anomeric proton of glucose A, a strong NOE was observed at H-4 of delphinidin, indicating glucose A is bound to 3-OH of delphinidin (Fig. 1). Furthermore, upon irradiation of H-1 of glucose C, a strong NOE was observed at H-3,5 of the *p*-coumaric acid moiety and a rather weak NOE at H-2,6 of the *p*-coumaric acid moiety, indicating that glucose C was attached to OH-4 of the *p*-coumaric acid, and also esterified with caffeic acid at OH-6. An NOE was observed between the anomeric proton of the rhamnose moiety and the methylene protons of glucose A, supporting rhamnose bound to 6-OH of glucose A. Therefore, pigment **25** was determined to be delphinidin 3-*O*-[6-*O*-(4-*O*-(4-*O*-*trans*-(6-*O*-(*trans*-caffeoyl)-β-D-glucopyranosyl)-*p*-coumaroyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside], which is a new anthocyanin (Harborne and Grayer, 1988; Strack and Wray, 1994; Harborne and Baxter, 1999).

2.3. Pigment *26*

The FAB mass spectrum of pigment **26** gave a molecular ion $[M]^+$ at 1081 *m*/*z*, in good agreement with the mass calculated for $C_{48}H_{57}O_{28}$ (1081.304). Analysis of the ^{1}H-NMR and ^{1}H–^{1}H COSY spectra indicated that the structure was composed of three glucose, one rhamnose, *p*-coumaric acid and delphinidin. The structure of **26** was almost the same as **25** apart from the absence of the terminal caffeic acid moiety (Table 2). Analysis of the ^{1}H-NMR and ^{1}H–^{1}H COSY spectra revealed that the methylene protons of glucose C (δ 3.42–3.75) were shifted to a higher field than in **25** (δ 4.21, H-6a and 4.40, H-6b). Other proton signals of **26** were assigned as for **25**, and were in good agreement with those expected for **25** without the terminal caffeic acid moiety (Table 2). Therefore, pigment **26** is delphinidin 3-*O*-[6-*O*-(4-*O*-(4-*O*-*trans*-(β-D-glucopyranosyl)-*p*-coumaroyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[β-D-glucopyranoside], which is also a new anthocyanin (Harborne and Grayer, 1988; Strack and Wray, 1994; Harborne and Baxter, 1999).

3. Experimental

3.1. Plant materials

Forty individuals were established from the seeds of a dried specimen, collected in a natural population of *P. reitzii* located at 4 km N.W. of Paraiso da Serra to route BR282, Mun. Bom Retiro, Santa Catarina State, Brazil. (The herbaria samples were named G. Hashi-

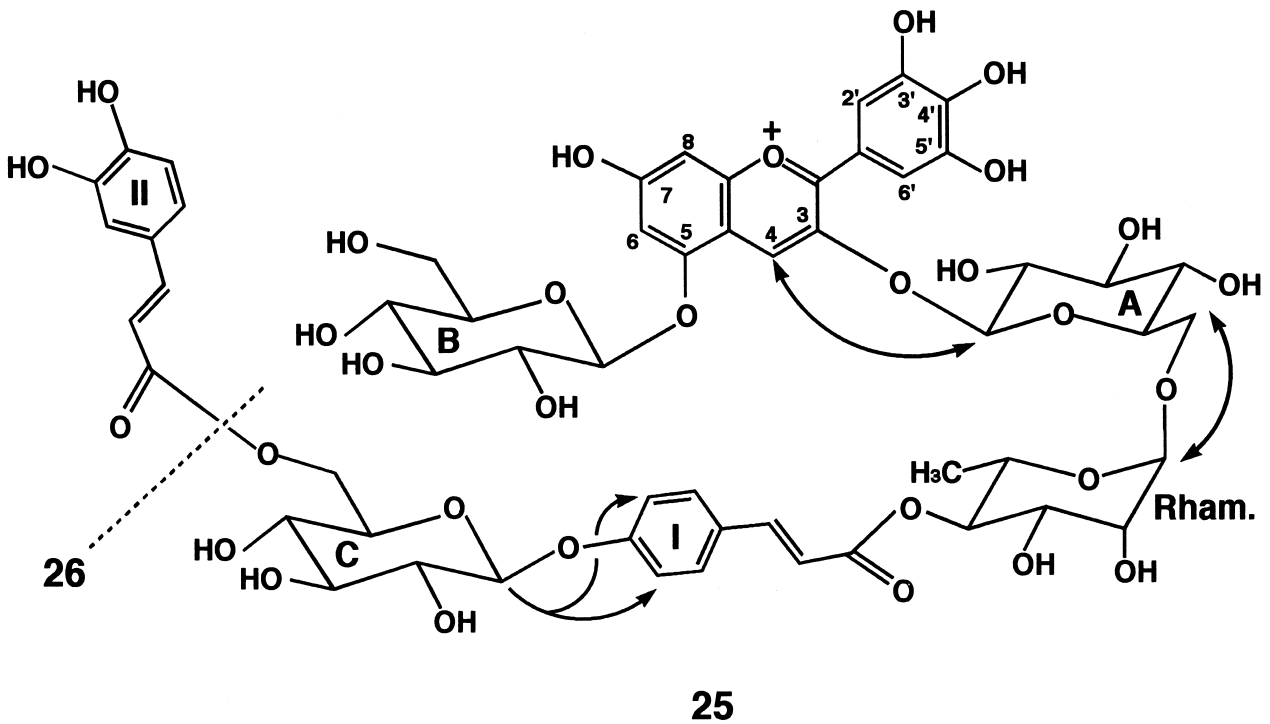

Fig. 1. Two novel anthocyanins from *P. reitzii*. NOEs are indicated by arrows.

moto et al. B162 and deposited in the Natural History Museum, London, the Museu Botânico Municipal, Curitiba, Brazil, and in the Centro de Pesquisas de História Natural, São Paulo, Brazil.) We separated four individuals having a slightly bluish flower colour (RP 80A, after R.H.S. colour chart & hue = −0.1; individual code, B162-5, 7, 14 and 18) compared to the 'normal' ones (RP 61C & hue = 0.1). Individuals with this abnormal flower colour were propagated vegetatively in order to provide flower samples for anthocyanin analysis.

3.2. Isolation of anthocyanins

After removing corolla-tubes, corolla limbs of plants propagated from four individuals were mixed, dried (ca. 150 g) and extracted with MeOH–HOAc–H_2O, 2:1:7, 3 l. The extract was purified by Diaion HP-20 gel CC, PC and TLC as described previously (Tatsuzawa et al., 1997; Ando et al., 1999). Solvents used were *n*-BuOH–HOAc–H_2O (4:1:2) and 15% HOAc. Preparative HPLC used a Waters C18 (19 ϕ × 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 fractions were evaporated in vacuo to dryness, dissolved in a small volume of 10% HOAc–MeOH, and precipitated by addition of excess Et_2O. The precipitated pigments were dried to give **25** (ca. 15 mg), **26** (ca. 15 mg), delphinidin 3-rutinoside (ca. 1 mg), delphinidin 3-rutinoside-5-glucoside (ca. 10 mg), delphinidin 3-caffeoylrutinoside-5-glucoside (ca. 2 mg), delphinidin 3-*trans*-*p*-coumaroylrutinoside-5-glucoside (ca. 2 mg), delphinidin 3-*cis*-*p*-coumaroylrutinoside-5-glucoside (ca. 1 mg), petunidin 3-rutinoside-5-glucoside (ca. 1 mg), petunidin 3-caffeoylrutinoside-5-glucoside (ca. 1 mg), petunidin 3-*trans*-*p*-coumaroylrutinoside-5-glucoside (ca. 1 mg) and petunidin 3-*cis*-*p*-coumaroylrutinoside-5-glucoside (ca. 1 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., 1999). Quantitative analysis of the anthocyanin in the flower extracts was performed by HPLC on a Waters C18 column (4.6 ϕ × 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. Characterization of pigments were carried out by TLC and by UV-VIS spectroscopy. Solvents used were *n*-BuOH–HOAc–H_2O (4:1:2), *n*-BuOH–2N HCl (1:1), 1% HCl and HOAc–HCl–H_2O (15:3:82) for anthocyanins, and *n*-BuOH–HOAc–H_2O (4:1:2), EtOH–NH_4OH–H_2O (16:1:3), EtOAc–HOAc–H_2O (3:1:1) and EtOAc–HCOOH–H_2O (5:2:1) for organic acids and sugars.

3.4. FAB mass and NMR measurement

FAB mass spectra were recorded on a JEOL JMS HX-110A spectrometer, operating positive mode in magic bullet. NMR spectra were recorded at 600 MHz for ^1H spectra by JEOL JNM LA 600 in DMSO-d_6-DCl (9:1). Chemical shifts (δ) are reported relative to internal TMS and coupling constants (J) are reported in Hz.

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References

Ando, T., Saito, N., Tatsuzawa, F., Kakefuda, T., Yamakage, K., Ohtani, E., Koshi-ishi, M., Matsusake, Y., Kokubun, H., Watanabe, H., Tsukamoto, T., Ueda, Y., Hashimoto, G., Marchesi, E., Asakura, K., Hara, R., Seki, H., 1999. Biochemical Systematics and Ecology 27, 623.

Harborne, J.B., 1984. Phytochemical Methods, 2nd ed. Chapman & Hall, London.

Harborne, J.B., Baxter, H., 1999. The Handbook of Natural Flavonoids, vol. 2. Wiley, Chichester, New York, Weinheim, Brisbane, Singapore, Toronto, p. 1.

Harborne, J.B., Grayer, R.J., 1988. In: Harborne, J.B. (Ed.), The Flavonoids, Advances in Research since 1980. Chapman & Hall, London, p. 1.

Kondo, T., Kawai, T., Tamura, H., Goto, T., 1987. Tetrahedron Lett. 28, 2273.

Strack, D., Wray, V., 1994. In: Harborne, J.B. (Ed.), The Flavonoids, Advances in Research since 1986. Chapman & Hall, London, p. 1.

Tatsuzawa, F., Koshiishi, M., Ohtani, E., Ando, T., Watanabe, H., Kokubun, H., Yokoi, M., Hashimoto, G., Seki, H., Saito, N., Honda, T., 1997. Heterocycles 45, 1197.