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NEW DIACYLATED DELPHINIDIN 3-RUTINOSIDE-5-GLUCOSIDES ISOLATED FROM THE BLUE-PURPLE FLOWERS OF *BROWALLIA SPECIOSA*

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Abstract – Two new diacylated delphinidin 3-rutinoside-5-glucosides were isolated from the blue-purple flowers of *Browallia speciosa* cv. 'Purple'. As a major anthocyanin (**1**), delphinidin 3-*O*-[6-*O*-(4-*O*-(*trans*-caffeoyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[2-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside] was determined by chemical and spectroscopic methods. Another one was tentatively assigned to be the *cis*-*p*-coumroyl isomer of the major anthocyanin due to its small amount available. In the pigment **1**, the 5-glucose residue of the anthocyanin is acylated with *p*-coumaric acid at 2-OH group of the sugar moiety, and this acylation pattern is the first report in plants.

INTRODUCTION

Browallia speciosa (the Solanaceae) is native in tropical America, and a popular ornamental plant with rich blue-purple or white flowers. Regarding the anthocyanin in the cyanic flowers of *B. speciosa*, delphinidin 3-(di-*p*-coumaroylglucoside)-5-glucoside was isolated by Harborne,[1] however, its detailed structure remained still unknown. Although 35 acylated anthocyanins with hydroxy cinnamic acids were so far found in the Solanaceae,[1, 2] the acylation pattern of these anthocyanins was restricted to the 3-rutinoside residues of anthocyanidins in the plants of the Solanaceae except for *B. speciosa*.[2]

In continuing work on the flower color variation, we found that the flowers of blue-purple cv. of *B. speciosa* contained new anthocyanins acylated with aromatic acids.

In this paper we wish to report the isolation and structure determination of two new acylated delphinidin 3-rutinoside-5-glucosides from the blue-purple flowers of *B. speciosa* cv. 'Purple'.

RESULTS AND DISCUSSION

The fresh flowers exhibiting blue-purple of *Browallia speciosa* were immersed in 5% AcOH for 24 h at room temperature. Four anthocyanin peaks were found in the extract by HPLC analysis. Their relative frequencies of occurrence were as follows: pigment **1** (42%, Rt 25.7), **2** (29%, Rt 26.0), **3** (13%, Rt 23.2), and **4** (5%, Rt 22.0). Pigments **1** and **2** were isolated as purplish red powder by using the process described previously.[3,4] The chromatographic and spectral properties of pigments **1** and **2** were summarized in Table 1.

Table 1. Chromatographic and spectral data of anthocyanins from *Browallia speciosa*.

Anthocyanins[a]	Rf values (x100)				Spectral data in 0.1% HCl-MeOH				Rt
	BAW	BuHCl	1% HCl	HOAc-HCl	λmax (nm)	Eacyl / Emax	E_{440} / Emax	$AlCl_3$	(min)
Pigment 1	75	71	15	57	309, 546	147	11	+	25.7
Pigment 2	75	72	26	62	310, 546	133	10	+	26.0
Dp3GR5G	13	7	25	50	276, 540	—	11	+	18.8

[a] Pigment **1**: delphinidin 3-caffeoylrutinoside-5-*trans*-*p*-coumaroylglucoside
Pigment **2**: delphinidin 3-caffeoylrutinoside-5-*cis*-*p*-coumaroylglucoside
Dp3GR5G: delphinidin 3-rutinoside-5-glucoside

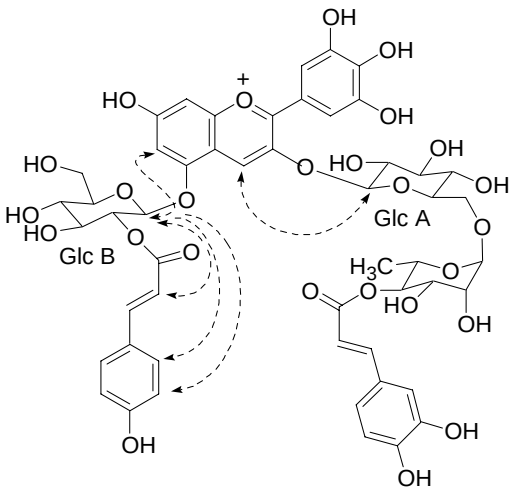

Figure 1. Browallia blue-purple anthocyanin **1**.
Observed major NOE's are indicated by dashed arrows.

Acid hydrolysis of these pigments resulted in the same components; delphinidin, glucose, rhamnose, caffeic and *p*-coumaric acids. On alkaline hydrolysis, these pigments gave the same compounds, caffeic and *p*-coumaric acids and a deacylated anthocyanin. These compounds were determined by analyses of HPLC and TLC in comparison with authentic samples. The deacylated anthocyanin was unambiguously identified as delphinidin 3-rutinoside-5-glucoside by direct comparison with the authentic anthocyanin obtained from the flowers of *Petunia*.[5]

Table 2. NMR spectral data for Browallia anthocyanins. [^{1}H NMR 500 MHz and ^{13}C NMR 125.78 MHz in CF_3CO_2D-DMSO-d_6 (1:9). TMS as an internal standard, *Coupling constants (J* Hz) in parentheses]

		Pigment 1		Pigment 2
		δC	δH	δH
delphinidin	2	164.5		
	3	147.1		
	4	131.1	8.69 s	8.67 s
	5	156.1		
	6	104.4	6.95 s	6.94 s
	7	169.1		
	8	97.2	6.95 s	6.94 s
	9	156.6		
	10	112.1		
	1'	119.9		
	2'	113.0	7.75 s	7.75 s
	3'	145.9		
	4'	147.7		
	5'	145.9		
	6'	113.0	7.75 s	7.75 s
p-coumaric acid	1	127.1		
	2	131.3	7.38 d (8.5)	7.61 d (8.8)
	3	116.9	6.80 d (8.5)	6.72 d (8.6)
	4	161.3		
	5	116.9	6.80 d (8.5)	6.72 d (8.6)
	6	131.3	7.38 d (8.5)	7.61 d (8.8)
	α	114.9	6.21 d (15.9)	5.51 d (12.8)
	β	146.7	7.55 d (15.9)	6.76 d (12.8)
	COOH	169.9		
caffeic acid	1	127.4		
	2	115.6	7.05 d (2.2)	7.05 m
	3	146.5		
	4	149.9		
	5	116.5	6.72 d (8.2)	6.71 d (8.7)
	6	123.5	7.00 dd (2.2, 8.2)	7.01 m
	α	114.5	6.31 d (15.9)	6.32 d (15.9)
	β	148.3	7.69 d (15.9)	7.70 d (15.9)
	COOH	168.8		
glucose A	1	101.2	5.58 d (7.6)	5.58 d (7.6)
	2	72.2	3.87 t (9.5)	3.86 m
	3	78.4	3.75 t (8.9)	3.40-4.00
	4	71.4	3.53 t (9.8)	
	5	77.5	4.39 ddd (2.1, 6.1, 9.8)	4.33 m
	6a	66.8	3.76 m	3.60-4.00
	6b		4.13 br dd (2.1, 11.6)	4.06 m
glucose B	1	101.3	5.41 d (8.0)	5.41 d (8.0)
	2	75.4	5.25 dd (8.0, 9.8)	5.24 m
	3	74.4	3.84 t (8.6)	3.50-4.00
	4	71.6	3.65 t (9.8)	
	5	78.8	3.69 ddd (1.8, 5.2, 9.8)	
	6a	62.1	3.81 m	
	6b		3.97 dd (1.9, 12.2)	
rhamnose	1	101.7	4.75 d (1.2)	4.74 br s
	2	75.2	3.86 d (3.4)	3.70-4.00
	3	70.5	3.92 dd (3.4, 9.8)	
	4	75.5	4.91 t (9.8)	4.91 t (9.5)
	5	67.7	3.82 m	3.875 m
	Me	17.9	0.97 d (6.1)	0.97 d (6.4)

Pigment **1:** The HR FABMS spectrum of pigment **1** gave its molecular ion $[M]^+$ at *m/z* 1081.2800 in agreement with the mass calculated for $C_{51}H_{53}O_{26}$, which was composed of delphinidin with two molecules of glucose, and one molecule each of rhamnose, *p*-coumaric acid and caffeic acid. The structure of pigment **1** was further elucidated based on the analysis of its ^{1}H NMR spectra [500 MHz in CF_3COOD-CD_3OD (1:9) and DCl-DMSO-d_6 (1:9)], including 2D COSY, 2D NOESY and negative difference NOE (NOEDIF) experiments. By the analysis of ^{1}H NMR and 2D COSY, it was confirmed that pigment **1** has one molecule each of delphinidin, rhamnose, *p*-coumaric acid, and caffeic acid, and also two molecules of glucose (Figure 1). The chemical shifts of 12 aromatic protons of delphinidin, *p*-coumaric acid, and caffeic acid moieties with their coupling constants were assigned as shown in Table 2. The proton signals of sugar moieties were observed in the region of δ 5.58 - 0.97. The signals of two anomeric protons appeared at δ 5.58 (d, J = 7.6 Hz, Glc A) and δ 5.41 (d, J = 8.0 Hz, Glc B), and the vicinal coupling constants of both glucoses were J = 7.6 - 12.2 Hz, therefore, both glucose A and B must be β-glucopyranose form. The anomeric proton of rhamnose unit was observed at δ 4.75 (d, J = 1.2 Hz), and vicinal coupling constants (J = 1.2 - 9.8 Hz) indicated that rhamnose must be α-pyranorhamnose. Since two pairs of olefinic protons in *p*-coumaric and caffeic acids exhibited the large coupling constants (J = 15.9 and 15.9 Hz) between α and β proton signals, both olefinic double bonds of *p*-coumaric and caffeic acids have a *trans* configuration. By the analysis of its 2D COSY spectrum, the H-2 signal of Glc B was shifted to lower magnetic field (δ 5.25, dd, J = 8.0, 9.8 Hz), and also the H-4 signal of rhamnose was shifted downfield at δ 4.91 (t, J = 9.8 Hz). Therefore, the OH-2 of Glc B and the OH-4 of rhamnose were confirmed to be acylated with *p*-coumaric acid and caffeic acid.

In order to determine the attachments and / or positions of the sugars and acyl units in pigment **1**, NOESY and NOEDIF experiments were performed (Figure 1). Strong long range NOEs between the anomeric proton of Glc A and the proton H-4 of delphinidin, and also between H-1 of Glc B and H-6 of delphinidin were observed indicating that the OH-3 and OH-5 of delphinidin were glycosylated with glucoses A and B. Furthermore, by irradiations at the H-2 signal of Glc B, NOEs were observed at the proton signals of H-α, H-β, H-2, H-6, H-3 and H-5 in the *p*-coumaric acid moiety suggesting that the OH-2 of Glc B was acylated with *p*-coumaric acid. Therefore, the structure of pigment **1** was determined to be delphinidin 3-*O*-[6-*O*-(4-*O*-(*trans*-*p*-caffeoyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-[2-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside], which is a new anthocyanin in plants.[2,6] This structure was further confirmed by the analysis of ^{13}C NMR spectra containing HMBC and HMQC spectra as shown in Table 2.

Pigment **2:** The chromatographic and spectroscopic properties of pigment **2** are shown in Table 1. As mentioned before, pigment **2** gave delphinidin, glucose, rhamnose, caffeic acid and *p*-coumaric acid by acid hydrolysis. Furthermore, delphinidin 3-rutinoside-5-glucoside was obtained from the alkaline

hydrolysate as its deacyl anthocyanin.

The ^{1}H NMR spectrum of pigment **2** was similar to that of pigment **1** except for signals of its *p*-coumaric acid moiety (Table 2). The chemical shifts of olefinic protons of the *p*-coumaric acid moiety were shifted to a higher magnetic field at δ 5.51 and 6.76 with small coupling constants (J = 12.8 and 12.8 Hz) in comparison with those (δ 6.21 and 7.55, and J = 15.9 and 15.9) of pigment **1**. Thus, the configuration of *p*-coumaric acid in pigment **2** was confirmed to be a *cis*-type. Whereas the configuration of caffeic acid in pigment **2** was assumed to be *trans* as same to that of pigment **1**. Unfortunately, its further structure determination could not be carried out because of its small amounts obtained. The structure of pigment **2** was tentatively estimated to be delphinidin 3-caffeoylrutinoside-5-(*cis*-*p*-coumaroylglucoside) as a *cis*-*p*-coumaroyl isomer of pigment **1** at present.

CONCLUSION

Harborne (1967) mentioned the distribution of delphinidin 3-(di-*p*-coumaroylglucoside)-5-glucoside in the flower of *Browallia speciosa*.[1] However, its detailed structure elucidation has not been reported until to date. In this study, the structure of a major anthocyanin of *B. speciosa* was unambiguously elucidated to be delphinidin 3-[6-(4-*trans*-caffeoylrhamnosyl)-glucoside]-5-[2-(*trans*-*p*-coumaroyl)-glucoside], and a minor one was estimated to be a *cis*-*p*-coumaroyl isomer of the major anthocyanin. Regarding the patterns of glycosylation and acylation in these anthocyanins, the 3-rutinoside-5-glucoside is common in the Solanaceae, but the acylation at both 3- and 5-sugar residues with hydroxycinnamic acids is not reported until now.[1, 2] The distribution of 5-acylated sugar substitutions with hydroxycinnamic acids in acylated anthocyanins is very restricted in plants, and only reported in *Gentiana*,[7,8,9] and *Eustoma*.[10] The finding of acylation at 2-OH group of 5-glucose moiety with *p*-coumaric acid in these anthocyanins is the first report in plants.[2,6]

EXPERIMENTAL

General procedures

TLC was carried out on plastic coated cellulose sheets (Merck) using eight mobile phases: BAW (*n*-BuOH-AcOH-H_2O, 4:1:5), BuH (*n*-BuOH-2N HCl, 1:1), 1% HCl and AcOH-HCl (AcOH-HCl-H_2O, 15:3:82) for anthocyanins, and BAW, *i*-PrOH-*n*-BuOH-H_2O (7:1:2), and PhOH-H_2O (4:1) for sugars and BAW, H_2O and 6% AcOH for organic acids.[11] Analytical HPLC was performed on a Hitachi 6200 system, using an Inertsil ODS-2 (4.6ϕ×250mm) column at 35 °C with a flow rate of 0.8 mL / min and monitoring at 520nm. The eluant was applied as a linear gradient for 40 min from 25% to 85% solvent B (1.5% H_3PO_4, 20% AcOH, 25% MeCN in H_2O). UV-VIS spectra were recorded on an MPS-2400 (Shimadzu) in 0.1% HCl-MeOH (200-700 nm), 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 125.78 MHz for ^{13}C spectra in DMSO-d_6-DCl (9: 1) and CD_3OD-CF_3CO_2D (9:1). Chemical shifts are reported relative to a TMS internal standard, and coupling constants are in Hz.

Plant materials

The seeds of *Browallia speciosa* cv. 'Purple' were purchased from Sakata Seed Co., Ltd. (Japan). The plants were grown in the greenhouse of Minami-Kyushu University. The fresh petals (b / a = - 56.07 / 37.69 = - 1.49) of this plant were collected, and dried at 45°C.

Isolation of anthocyanins

The dried petals (ca. 80 g) were extracted with 5% AcOH at rt overnight. The extract was adsorbed on a Diaion HP-20 column, and the absorbed pigments were washed with H_2O. The pigments were eluted with AcOH-MeOH-H_2O (5:75:20). After concentration, the eluates were fractionated with Sephadex LH-20 CC using AcOH-MeOH-H_2O (1:6:12). The frs were further purified with PC (*n*-BuOH-AcOH-H_2O, 4:1:2 and 15% AcOH), and preparative HPLC. Prep. HPLC was performed on a Hitachi 6200 system using on Inertsil ODS-2 column (20ϕ×250mm) with AcOH solvent. The pigment fractions were evaporated *in vacuo* to dryness. After these processes pigments **1** and **2** were dissolved in small volume of 1% TFA-MeOH, and precipitated by addition of excess Et_2O. Then pigments **1** (25 mg) and **2** (5 mg) were dried to powder.

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