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7-*O*-METHYLATED ANTHOCYANIDIN GLYCOSIDES FROM THE REDDISH PURPLE FLOWERS OF *CATHARANTHUS ROSEUS* 'EQUATOR LAVENDER'

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Abstract – Four new anthocyanins (pigments **1** – **4**) were isolated from the reddish purple flowers of *Catharanthus roseus* 'Equator Lavender', and identified to be hirsutidin 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside]-5-*O*-galactopyranoside as pigment **4**, and 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside]s of 7-*O*-methylpetunidin as pigment **2**, of 7-*O*-methyldelphinidin as pigment **3**, and of hirsutidin as pigment **1** by chemical and spectroscopic methods. It is noteworthy that the findings of 7-*O*-methylpetunidin of pigment **2** and 7-*O*-methyldelphinidin of pigment **3** are the first report in plants. Furthermore, hirsutidin 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside] of pigment **1** was observed as a main anthocyanin in the reddish purple flower of this plant.

INTRODUCTION

Recently, we isolated two 7-*O*-methylcyanidin glycosides from orange-red flowers of *Catharanthus roseus* 'Equator Deep Apricot', and identified to be 3-rhamnosyl-galactosides of rosinidin and 7-*O*-methylcyanidin[1]. For further study of anthocyanin occurrence in *Catharanthus roseus*, we have investigated a constituent of a reddish purple cultivar 'Equator Lavender'. As a result of our extensive studies, four novel 7-*O*-methylanthocyanidin glycosides were found in the aqueous acetic acid extract of

its flower petals. In this paper, we wish to report the structure elucidation of these anthocyanins isolated from the reddish purple flowers of *C. roseus* 'Equator Lavender'.

RESULTS AND DISCUSSION

Fourteen anthocyanin peaks were observed in the 5% HOAc extract from the reddish purple flowers of *C. roseus* 'Equator Lavender' on HPLC. Among these anthocyanin peaks, six anthocyanin pigments **1** (65.4% of its relative frequency of occurrence, and 23.3 min of its retention time), **2** (5.1%, 19.2 min), **3** (3.9%, 15.0 min), **4** (7.5%, 16.1 min), **5** (2.4%, 17.3 min), and **6** (5.9%, 21.6 min) were obtained by using the isolation process described previously.[1] The chromatographic and spectroscopic properties of these pigments are summarized in Table 1. The structures of pigments **5** and **6** among these anthocyanin pigments were identified to be 3-robinobiosides of 7-*O*-methylcyanidin and rosinidin, respectively, in comparison with authentic samples obtained from the flowers of *C. roseus* 'Equator Deep Apricot'.[1]

Table 1. Chromatographic and spectroscopic properties of anthocyanins from *Catharanthus roseus*

Anthocyanins*	Rf value(x100)						HPLC	Spectral data in 0.1% HCl-MeOH			FAB-MS
	Forestal**	Formic**	BAW**	BuHCl**	1%HCl**	HOAc-HCl**	Rt(min)	λ_{max}(nm)	E_{440}/E_{max}	$AlCl_3$	$[M]^+$
Pigment 1			51	17	11	50	23.3	536,354,279	17	0	653
Pigment 2			38	12	7	32	19.2	538,352,279	15	+	639
Pigment 3			30	8	4	23	15.0	539,352,279	16	+	625
Pigment 4			38	10	47	78	16.1	537,357,283	10	0	815
Aglycone of **Pigments 1 and 4**	89	46					35.1	539,272	21	0	-
Aglycone of **Pigment 2**	65	32					28.8	539,271	23	+	-
Aglycone of **Pigment 3**	41	18					21.4	541,271	20	+	-
Malvidin	68	33					30.1	547,276	30	0	-
Petunidin	43	19					23.8	547,276	31	+	-
Delphinidin	25	11					17.4	548,275	23	+	-

* Pigment **1** ; Hirsutidin 3-*O* -robinobioside, Pigment **2** ; 7-*O* -Methylpetunidin 3-robinobioside.
Pigment **3**; 7-*O* -Methyldelphinidin 3-*O* -robinobioside, Pigment **4**; Hirsutidin 3-*O* -robinobioside-5-*O* -galactoside.
** See EXPERIMENTAL

Acid hydrolysis of all pigments **1** – **4** gave galactose and rhamnose as the same sugars. However, hirsutidin (7,3',5'-*O*-trimethyldelphinidin) was confirmed in the hydrolysates of pigments **1** and **4** for the aglycone, whereas two unknown anthocyanidins were detected in the hydrolysates of pigments **2** and **3**.
By measuring FABMS of these pigments, the molecular ions $[M]^+$ were observed at 653 *m/z* (calc. for $C_{30}H_{37}O_{16}$, 653.208) for pigment **1**, at 639 *m/z* (calc. for $C_{29}H_{35}O_{16}$, 639.192) for pigment **2**, at 625 *m/z* (calc. for $C_{28}H_{33}O_{16}$, 625.177) for pigment **3**, and at 815 *m/z* (calc. for $C_{36}H_{47}O_{21}$, 815.261) for pigment **4**. From these results, the structures of pigments **1**–**4** were presumed to be hirsutidin 3-rhamnosylgalactoside for pigment **1**, monomethyl ether of delphinidin 3-rhamnosylgalactoside for pigment **2**, dimethyl ether of delphinidin 3-rhamnosylgalactoside for pigment **3**, and hirsutidin 3-rhamnosylgalactosyl-5-galactoside for pigment **4**, respectively.

Detailed structures of pigments **1** – **4** were further elucidated on the basis of the analysis of their ^{1}H and ^{13}C NMR spectra [500 MHz for ^{1}H and 125.78 MHz for ^{13}C spectra in CD_3OD-DCl (1:9), including 2D COSY, 2D NOESY, HMQC and HMBC spectra.

Table 2. NMR spectroscopic data of anthocyanins from *Catharanthus roseus*.

	1		**2**		**3**		**4**	
	^{13}C (ppm)*	**^{1}H (ppm)***	**^{13}C (ppm)***	**^{1}H (ppm)***	**^{13}C (ppm)***	**^{1}H (ppm)***	**^{13}C (ppm)***	**^{1}H (ppm)***
Anthocyanindin								
2	164.0		164.3		162.7		164.8	
3	146.7		146.5		146.4		147.2	
4	135.5	8.89 s	135.1	8.84 s	135.0	8.86 s	134.5	9.00 s
5	157.9		158.1		157.3		155.8	
6	103.0	6.74 d(2.1)	103.2	6.70 d(1.9)	103.2	6.74 brs	105.5	7.07 d(1.8)
7	170.5		170.6		164.6		170.1	
8	93.5	7.32 d(2.1)	93.2	7.15 d(1.9)	93.1	7.15 brs	95.6	7.45 d(1.8)
9	157.4		157.4		158.0		157.0	
10	113.8		113.7		113.6		113.9	
1'	119.6		119.7		119.8		119.5	
2'	110.9	8.00 s	109.6	7.93 d(2.1)	112.9	7.81 s	111.0	8.02 s
3'	149.7		149.7		147.3		149.7	
4'	146.5		145.8		145.1		146.6	
5'	149.7		147.4		147.3		149.7	
6'	110.9	8.00 s	114.1	7.79 d(2.1)	112.9	7.81 s	111.0	8.02 s
7-*O*-Me	58.0	4.07 s	57.8	4.05 s	57.8	4.04 s	58.1	4.10 s
3'-*O*-Me	57.5	4.02 s	57.4	3.99 s	-	-	57.5	4.02 s
5'-*O*-Me	57.5	4.02 s	-	-	-	-	57.5	4.02 s
3-Galactose								
1	104.0	5.43 d(7.7)	103.9	5.38 d(7.6)	103.7	5.32 d(7.7)	102.8	5.63 d(7.6)
2	72.1	3.99 dd(7.7, 9.6)	72.0	4.01 dd(7.6, 9.8)	71.7	4.04 m	71.9	4.03 dd(7.6, 9.5)
3	74.8	3.77 dd(3.4, 9.6)	74.8	3.77 dd(3.4, 9.8)	74.5	3.77 brd(9.8)	70.1	3.84 dd(4.0, 9.5)
4	70.2	3.93 brd(3.4)	70.3	3.94 brd(3.4)	69.7	3.97 brd(3.1)	74.8	3.79 brd(4.2)
5	76.2	4.06 m	76.2	4.06 dd(3.7, 8.3)	76.0	4.04 m	76.3	4.14 dd(4.6, 7.7)
6a	68.0	3.64 brd(10.7)	68.0	3.66 brd(11.0)	68.0	3.67 m	67.2	3.80 - 3.70
6b		3.87 dd(4.0, 10.7)		3.88 dd(3.7, 11.0)		3.87 brd(8.0)		3.85 - 3.77
Rhamnose								
1	102.1	4.62 d(1.2)	102.2	4.63 d(1.5)	102.0	4.64 brs	101.9	4.61 d(1.3)
2	71.8	3.78 dd(1.2, 3.7)	71.8	3.79 dd(1.5, 3.7)	71.7	3.80 brs	71.8	3.66 brd(3.4)
3	72.3	3.64 dd(3.7, 9.6)	72.4	3.65 dd(3.7, 9.8)	72.2	3.66 m	77.6	3.61 m
4	73.8	3.33 t(9.6)	73.9	3.36 t(9.8)	73.8	3.35 dd(8.6, 9.5)	73.7	3.29 t(9.5)
5	69.7	3.59 dd(6.1, 9.6)	69.8	3.60 dd(6.5, 9.8)	70.2	3.61 dd(6.1, 8.6)	69.7	3.56 m
Me	18.2	1.23 d(6.1)	18.0	1.23 d(6.5)	17.9	1.23 d(6.1)	17.9	1.20 d(6.1)
5-Galactose								
1							102.6	5.26 d(7.6)
2							74.5	3.73 dd(7.6, 9.3)
3							72.1	3.60 dd(3.8, 9.3)
4							70.1	3.65 brd(3.8)
5							78.4	3.71 m
6a							62.0	3.85 - 3.77
6b								3.95 m

*CD_3OD/DCl = 9:1

The molecular ion $[M]^+$ of pigment **1** was observed at 653 *m/z*, indicating that pigment **1** is composed of hirsutidin with one molecule each of rhamnose and galactose. The elemental components were confirmed by measuring its high-resolution FABMS (See EXPERIMENTAL).

The structure of pigment **1** was elucidated based on the analysis of its ^{1}H NMR spectra. The chemical

shifts of five aromatic protons of anthocyanidin moiety were assigned as shown in Table 2. Nine proton signals corresponding to three methyl groups of 7,3',5'-*O*-trimethyldelphinidin were observed at δ 4.07 (s, 3H at 7-*O*-methyl group) and at δ 4.02 (s, 6H at 3'- and 5'-*O*-methyl groups). By the measurement of its NOESY spectrum, strong long range NOEs between both signals of δ 6.74 (H-6) and δ 7.32 (H-8) and three proton signals at δ 4.07 (7-*O*-methyl group), and also between two proton signals at δ 8.00 (s, H-2' and -6') and six proton signals at δ 4.02 (3'- and 5'-*O*-methyl groups) were observed (Figure 1), supporting that OH-7, OH-3' and OH-5' groups of aglycone were methylated, respectively. Thus, the structure of this aglycone was confirmed to be 7,3',5'-*O*-trimethyldelphinidin, hirsutidin (Figure 1). The chemical shifts of the sugar moieties were observed in the region of δ 5.43 – 1.23, where the two anomeric protons resonated at δ 5.43 (d, J=7.7 Hz, Galactose H-1) and δ 4.62 (d, J=1.2 Hz, Rhamnose H-1). Based on the observed coupling constants (Table 2), galactose was assumed to be in the β-pyranose form and rhamnose to be in the α-pyranose form. By the analysis of its NOESY spectrum, the correlation between H-4 (δ 8.89) of aglycone and H-1 (δ 5.43) of galactose, and also H-1 (δ 4.62) of rhamnose and H-6b (δ 3.87) of galactose were observed, respectively, indicating that OH-3 of anthocyanidin moiety was glycosylated with galactose and OH-6 of galactose was bonded to OH-1 of rhamnose moiety.

These results were also confirmed by the analysis of its ^{13}C NMR and HMBC spectra (Figure 1). Therefore, the structure of pigment **1** was determined to be 7,3',5'-*O*-trimethyldelphinidin 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside], which is a new anthocyanin in plants.[1-4]

The molecular ion $[M]^+$ of pigment **4** was observed at 815 *m/z* (calc. 815.261, $C_{36}H_{47}O_{21}$), indicating that pigment **4** is composed of hirsutidin with one molecule of rhamnose and two molecules of galactose. The elemental components were confirmed by measuring its HRMS (See EXPERIMENTAL). The structure of pigment **4** was further elucidated on the basis of the analysis of its NMR spectra according to the same process as described above for the structure determination of pigment **1**.

The ^1H NMR of pigment **4** was similar to that of pigment **1** except for the signals of galactose moiety at the OH-5 group. The proton chemical shifts of 5-*O*-galactose moiety were observed in δ 5.26 – δ 3.60, and its anomeric proton signal appeared at δ 5.26 (δ, J=7.6 Hz). Based on the observed coupling constants (Table 2), the galactose moiety was assumed to have β-pyranose form. By the analysis of NOESY and HMBC spectra, the glycosylation of galactose was confirmed at OH-5 of hirsutidin as well as linkages between OH-3 of hirsutidin and OH-1 of galactose A and OH-6 of galactose A and OH-1 of rhamnose (Figure 1). Therefore, pigment **4** was determined to be hirsutidin 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside]-5-*O*-β-galactopyranoside (Figure 1), which is a new anthocyanin in plants.

The molecular ion $[M]^+$ of pigment **3** was observed at 625 *m/z* (calc. 625.177, $C_{28}H_{33}O_{16}$), indicating that pigment **3** is composed of a delphinidin monomethyl ether with one molecule each of rhamnose and

galactose. The elemental components were confirmed by measuring its HRMS (See EXPERIMENTAL). The ^{1}H NMR spectrum of pigment **3** was similar to that of pigment **1** except for signals of 3'- and 5'-*O*-methyl groups of pigment **1**. The spectrum of pigment **3** lacked the signals of 3'- and 5'-*O*-methyl groups. All proton chemical shifts of pigment **3** were assigned by the same process for pigment **1** as shown in Table 2. The linkages between aglycone and galactose moieties and also rhamnose and galactose moieties in this molecule were confirmed by the analysis of its NOESY and HMBC spectra (Figure 1). Consequently, pigment **3** was determined to be 7-*O*-methyldelphinidin 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside], which is a new anthocyanin in plants. It is noteworthy that the finding of 7-*O*-methyldelphinidin as the aglycone of pigment **3** is the first report in plants.

The molecular ion $[M]^+$ of pigment **2** was observed at 639 *m/z* (calc. 639.192, $C_{29}H_{35}O_{16}$), indicating that pigment **2** is composed of a delphinidin dimethyl ether with one molecule each of rhamnose and galactose. The elemental components were confirmed by measuring its HRMS (See EXPERIMENTAL). On ^{1}H NMR spectrum of pigment **2** the chemical shifts of five aromatic proton signals of aglycone moiety were assigned as shown in Table 2. Particularly, the signals of H-2' and H-6' of aglycone were observed separately at δ 7.93 and δ 7.79, supporting that OH-3' group is bonded with a methyl group. Moreover, the signals of 3'-*O*-methyl group are observed at δ 3.99 (3 x H, s) as well as the signals of 7-*O*-methyl group at δ 4.05 (3 x H, s). The other chemical shifts of pigment **2** were identical with those of pigments **1** and **3** (Table 2). Therefore, the structure of pigment **2** was determined to be 7-*O*-methylpetunidin 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-galactopyranoside], which is a new anthocyanin in plants. This structure was further confirmed by the analysis of ^{13}C NMR spectra (Table 2 and Figure 1). Again, the finding of 7-*O*-methylpetunidin as the aglycone of pigment **2** is the first report in plants.

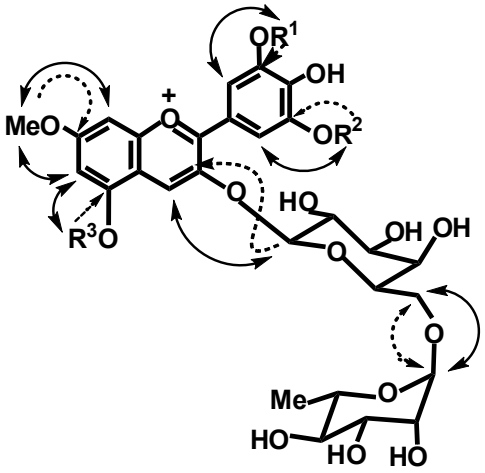

Figure 1. Hirsutidin 3-*O*-robinobioside (pigment 1; R^1 and R^2 = Me, R^3 = H), 7-*O*-methylpetunidin 3-*O*-robinobioside (pigment 2; R^1 = Me, R^2 and R^3 = H), 7-*O*-methyldelphinidin 3-*O*-robinobioside (pigment 3; R^1, R^2 and R^3 = H), and hirsutidin 3-*O*-robinobioside-5-*O*-galactoside (pigment 4; R^1 and R^2 = Me, R^3 = galactose). Observed NOE's are indicated by arrows. Observed HMBC correlations are indicated by dotted arrows.

The anthocyanidin distribution of three unique 7-*O*-methylanthocyanidins such as rosinidin, 7-*O*-methylcyanidin, and hirsutidin, was reported in the flowers and callus of *C. roseus*.[1,5,6] In this study, two more unique 7-*O*-methylanthocyanidins were found in the reddish purple flowers of *C. roseus* 'Equator Lavender' such as 7-*O*-methyldelphinidin 3-robinobioside and 7-*O*-methylpetunidin 3-robinobioside. Therefore, there are five 7-*O*-methylanthocyanidins, 7-*O*-methylcyanidin, rosinidin, 7-*O*-methyldelphinidin, 7-*O*-methylpetunidin and hirsutidin, in *C. roseus*. Moreover, it was revealed in this study that the 7-*O*-methylation of anthocyanidin A-ring is dominant in this plant, and also expected to occur prior to the other methylations of 3'- and 5'- hydroxyl groups in the B-ring during the anthocyanidin biosynthesis of *C. roseus*.

On the glycosidic pattern in *C. roseus*, the distribution of 3-robinobiosides of anthocyanidins is a very rare case, and limited to only five families, Apocynaceae, Cornaceae, Epacridaceae, Gentianaceae and Polygonaceae.[2,4,7] Of course, the pattern of 3-robinobioside-5-galactoside of hirsutidin is the first report in plants.

EXPERIMENTAL

General procedures

TLC was carried out on plastic coat cellulose sheets (Merck) using nine mobile phases: BAW (*n*-BuOH-HOAc-H_2O, 4:1:5, upper layer), BuHCl (*n*-BuOH-2N HCl, 1:1, upper layer), HOAc-HCl (HOAc-HCl-H_2O, 15:3:82), 1% HCl for anthocyanins, Forestal (HOAc-HCl-H_2O, 30:3:10) and Formic (HCl-HCO_2H-H_2O, 2:5:3) for anthocyanidins, and BAW, *i*-PrOH-H_2O (4:1), *i*-PrOH-*n*-BuOH-H_2O (7:1:2) and PhOH-H_2O (4:1) for sugars.[8]

Analytical HPLC was performed on a Hitachi 6200 system, using an Inertsil ODS-2 (4.6 ϕ x 250 mm) column at 35 ^oC with a flow rate of 0.8 mL/min and monitoring at 520 nm. The eluants were applied as linear gradient elutions for 40 min from 25 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 UV-Vis multi purpose spectrophotometer (MPS-2450, Shimadzu) in 0.1% HCl-MeOH (from 200 to 700 nm). FAB mass spectra were obtained in the positive ion mode using the magic bullet (5:1 mixture of dithiothreitol and dithioerythritol) as a matrix. NMR spectra were acquired at 500 MHz for ^1H spectra and at 125.78 MHz for ^{13}C spectra in CD_3OD-DCl (95:5). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants are in Hz.

Plant materials

Seed of *C. roseus* 'Equator Lavender' were purchased from the Sakata Seed Co., Ltd. (Japan). The plants were grown in the greenhouses of Minami-Kyushu University and Iwate University. The fresh flowers

were collected in July – October, and dried at 45 ^{o}C, and stored in desiccators until needed. The chromaticity values of the fresh flowers of this cultivar, b/a = -21.05/35.89 = -0.587 by SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co., Ltd).

Isolation of anthocyanins and anthocyanidins

The dried flowers (*ca.* 50 g) were extracted with 5% HOAc (20 L) at room temperature (ca. 20 ^{o}C) overnight. Anthocyanins in the extract were adsorbed on a Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column, and the column was washed with H_2O (10 L). The absorbed anthocyanins were eluted with MeOH-HOAc-H_2O (75:5:20). After concentration, the eluates were fractionated with Sephadex LH-20 CC using MeOH-HOAc-H_2O (6:1:12). The frs were further purified with PC (*n*-BuOH-HOAc-H_2O, 4:1:2) and prep. HPLC. Prep. HPLC was performed on a Hitachi 6200 system using as Inertsil ODS-2 column (20 ϕ x 250 mm) with HOAc solvent. Each fraction was concentrated to dryness in vacuo. Pigments were resolved in 1% TFA-MeOH followed by addition of excess Et_2O. Then, the pptd pigments were dried to powders; pigment **1** (35 mg), pigment **2** (8 mg), pigment **3** (7 mg) and pigment **4** (13 mg).

Acid hydrolysis of anthocyanins was carried out with 2N HCl at 100 ^{o}C for 2 h. After anthocyanins were extracted with *iso*-amyl alcohol, the extracts were concentrated to dryness. Each anthocyanidin of pigments **1-4** was isolated and purified from their dried extracts by TLC with Forestal and Formic.

Analyses of anthocyanins

The identification of anthocyanins was carried out by standard procedures,[1,8] and the results were shown as follows.

7,3',5'-*O*-Trimethyldelphinidin 3-*O*-robinobioside (hirsutidin 3-*O*-robinobioside, pigment 1)

Dark violet powder; for UV-Vis and TLC, see Table 1; for ^{1}H and ^{13}C NMR spectra, see Table 2; HR-FABMS calc. for $C_{30}H_{37}O_{16}$: 653.2082, Found; 653.2091.

7,3'-*O*-Dimethyldelphinidin 3-*O*-robinobioside (7-*O*-methylpetunidin 3-*O*-robinobioside, pigment 2)

Dark violet powder; for UV-Vis and TLC, see Table 1; for ^{1}H and ^{13}C NMR spectra, see Table 2; HR-FABMS calc. for $C_{29}H_{35}O_{16}$: 639.1925, Found; 639.1891.

7-*O*-Methyldelphinidin 3-*O*-robinobioside (pigment 3)

Dark violet powder; for UV-Vis and TLC, see Table 1; for ^{1}H and ^{13}C NMR spectra, see Table 2; HR-FABMS calc. for $C_{28}H_{33}O_{16}$: 625.1769, Found; 625.1796.

Hirsutidin 3-*O*-robinobioside-5-*O*-galactoside (pigment 4)

Dark violet powder; for UV-Vis and TLC, see Table 1; for ^{1}H and ^{13}C NMR spectra, see Table 2; HR-FABMS calc. for $C_{36}H_{47}O_{21}$: 815.2610, Found; 815.2615.

Anthocyanidins of pigments 1-4

The data of UV-Vis, HPLC and TLC of pigments **1** – **4** see Table 1.

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