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7-ACYLATED ANTHOCYANINS WITH *p*-HYDROXYBENZOIC ACID IN THE FLOWERS OF *CAMPANULA MEDIUM*

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Abstract – Three anthocyanins were isolated from the 50% acetic acid extract of the light blue-purple flowers of *Campanula medium*, 'May Blue'. Among them, a new acylated anthocyanin was elucidated to be delphinidin 3-[6-*O*-(α-rhamnopyranosyl)-β-glucopyranoside]-7-*O*-[6-*O*-(4-*O*-(β-glucopyranosyl)-*p*-hydroxybenzoyl)-β-glucopyranoside] (**5**) as a main anthocyanin, together with two known anthocyanins, violdelphin (**4**) and delphinidin 3-*O*-rutinoside-7-*O*-glucoside (**6**) as minor anthocyanins. On anthocyanin components, three other cultivars of *C. medium* were investigated by HPLC analysis, and found that campanin (**3**) was present in the purple flowers of 'May Purple' as a main anthocyanin, rubrocampanin (**1**) was present in the pink flowers of 'May Pink' as a main anthocyanin, and purprocampanin (**2**) and rubrocampanin (**1**) were present in the purple-red flowers of 'May Purple Margin' as major anthocyanins. From these results, the relations between the flower colors and anthocyanin components were discussed in the flowers of *C. medium* cultivars.

1. INTRODUCTION

Campanula medium is a popular ornamental plant with white, pink, purple-red, purple or blue-purple flowers. The floral anthocyanin study of *C. medium* cultivars was carried on due to obtaining the knowledge relating to flower colors at 1990.[1] It has been reported that three 7-*O*-acylated anthocyanins with *p*-hydroxybenzoic acid have been isolated from the flowers of *C. medium* cultivars as their main

This paper is dedicated to Professor Emeritus Keiichiro Fukumoto on the occasion of his 75th birthday.

anthocyanins, and identified to be campanin (**3**) from the purple flowers,[1] rubrocampanin (**1**) from the pink flowers,[1] and purprocampanin (**2**) from the red-purple flowers.[2] From chemotaxonomical point of view, the acyl substitution pattern based on 4-*O*-glucosyl-*p*-hydroxybenzoic acid residues at 7-hydroxyl of anthocyanidin is very unique, and only 13 anthocyanins were reported until now.[3] The distribution of these pigments is restricted in the plants of *Aconitum*,[4] *Consolida*,[5] and *Delphinium*[6-8] (Ranunculaceae), *Campanula*[1,2,9] (Campanulaceae), and *Dendrobium*[10-12] (Orchidaceae).

In this paper, we wish to report the structure elucidation of a new 7-*O*-acylated anthocyanin in the light blue-purple flowers of *C. medium* 'May Blue', and also discuss the relations between the flower colors and 7-*O*-acylated anthocyanin components in the flowers of *C. medium* cultivars.

2. RESULTS AND DISCUSSION

2-1. Anthocyanin components of *Campanula medium* 'May Blue'

Three major anthocyanin peaks were observed in the acetic acid extract of the flowers of *Campanula medium* 'May Blue' on HPLC (Table 1). The relative frequencies of their occurrence were 75% (pigment 1) (**5**), 10% (pigment 2) (**4**), and 7% (pigment 3) (**6**), respectively. These anthocyanins (pigments 1–3) (**4**-**6**) were isolated from the flowers of this cultivar with 50% HOAc, and purified using Diaion HP-20 and Sephadex LH-20 column chromatography (CC), preparative PC and HPLC, according to the procedure described previously.[2] Chromatographic and spectroscopic properties of pigments 1 - 3 (**4** - **6**) are exhibited in Section 3-4 (Experimental).

Acid hydrolysis of pigments 1 - 3 resulted in delphinidin, glucose, and rhamnose. Moreover, *p*-hydroxybenzoic acid was detected in the hydrolysates of pigments 1 (**5**) and 2 (**4**) by TLC and HPLC analyses.[1,2] Alkaline hydrolysis of pigments 1 and 2 resulted in only one deacylanthocyanin (**6**), that was identical to pigment 3 (**6**), and determined to be delphinidin 3-*O*-rutinoside-7-*O*-glucoside by the analyses of TLC and HPLC with the authentic specimen obtained from *C. medium* 'Caerulea'.[1] Furthermore, 4-*O*-glucosyl-*p*-hydroxybenzoic acid was detected in the alkaline hydrolysates of pigments 1 and 2 by TLC and HPLC analyses.[2]

FAB mass spectra of pigments 1 and 2 gave their molecule ions at 1055 *m/z* and 1175 *m/z* in agreement with the masses calculated for $C_{46}H_{55}O_{28}$ (1055.2880) as pigment 1 (**5**) and for $C_{53}H_{59}O_{30}$ (1175.3091) as pigment 2 (**4**). These elemental components were confirmed by measuring their high resolution FAB-MS (HRMS) (see in Experimental). From these results, their structures were presumed to be glucosyl-*p*-hydroxybenzoyl delphinidin 3-*O*-rutinoside-7-*O*-glucoside as pigment 1 (**5**), and violdelphin (**4**)[6] as pigment 2. The detailed structures of pigments 1 and 2 were further elucidated based on the analyses of their ^{1}H NMR spectra (500 MHz) and ^{13}C NMR (125.78 MHz) in CD_3OD-DCl (9:1) including 2D COSY, NOESY, ^{1}H-^{13}C HMQC, and ^{1}H-^{13}C HMBC spectra.

Table 1 The flower colors, spectral data of intact petals and anthocyanin distribution in crude extract of *Campanula medium* cultivars

Cultivars	Flower colors[2]			λ max(nm)[3]				Absorbance[3]				Major anthocyanins(%)[4]					
	L	b/a	HVC	I	II	III	IV	I	II	III	IV	1	2	3	4	5	6
May Pink	55.2	-0.3	2.67RP 6.03/3.48	570	530	500s	452	0.24	0.24	0.13	0.07	82					
May Purple Margin[1]	43.7	-0.7	8.01P 4.93/6.91	567	530			0.19	0.20			40	38				
May Purple	29.4	-1.2	4.08P 3.43/9.76	622	569	538		0.42	0.58	0.62				83			
May Blue	55.2	-1.4	3.49P 6.04/4.00	618	569	538s		0.14	0.18	0.15					10	75	7

1) Full open stage

2) Hunter's (L, b/a) and Munsell's (HVC) values

3) Absorption spectral data of intact petals,[13,16] s: absprption shoulder

4) Anthocyanins; **1**: ruburocampanin, **2**: purpurocampanin, **3**: campanin, **4**: violdelphin (pigment 2), **5**: pigment 1, **6**: pigment 3 Percentage of total absorbance of all detected anthocyanins at 520nm in HPLC analysis (See Experimentals 3-3).

Pigment 1 (**5**)**:** The proton chemical shifts of pigment 1 (**5**) were assigned as shown in Table 2. Nine aromatic proton signals were assigned to delphinidin and *p*-hydroxybenzoic acid moieties. The signals of its sugar moieties were observed in the region of δ 1.20 – 5.39, and four anomeric proton signals were assigned to be δ 5.39 (*d*, *J*=7.6 Hz, Glc A), 5.31 (*d*, *J*=7.7 Hz, Glc B), 4.58 (*d*, *J*=7.4 Hz, Glc C), and 4.57 (*s*, rhamnose), respectively. The coupling constants of the anomeric proton signals were in the region of *J* = 7.4 – 7.7 Hz suggesting that all glucose units must be β-glucopyranose form (Figure 1). Furthermore, two characteristic proton signals shifted to the lower magnetic field at δ 4.32 and 4.71 (H-6a and -6b, Glc B) were assigned to the methylene protons of Glc B by the analysis of 2D COSY and NOESY spectra indicating that Glc B was acylated with *p*-hydroxybenzoic acid at its OH-6 group. The linkages and /or the positions of attachment of sugars and *p*-hydroxybenzoic acid were determined by the analysis of its NOESY spectrum, in which strong long range NOEs between H-4 of delphinidin and H-1 of Glc A, H-8 of delphinidin and H-1 of Glc B, and H-3 and H-5 of *p*-hydroxybenzoic acid and H-1 of Glc C were observed (Figure 1). These data indicated that OH-3 and OH-7 groups of delphinidin were glycosylated with Glc A and Glc B, respectively, and the OH-4 of *p*-hydroxybenzoic acid was glycosylated with Glc C. These results were confirmed by the analysis of its HMBC spectrum (Figure 1). Therefore, pigment 1 (**5**) was determined to be delphinidin 3-*O*-[6-*O*-(α-rhamnopyranosyl)-β-glucopyranoside]-7-*O*-[6-*O*-(4-*O*-(β-glucopyranosyl)-*p*-hydroxy-benzoyl)-β-glucopyranoside], which is a new anthocyanin in plants.

Pigment 2 (**4**)**:** The structure of pigment 2 (**4**) was identified to be violdelphin by direct comparison of TLC and HPLC behaviors with the authentic specimen isolated from the flowers of *Delphinium hybridum*.[6]

2-2. Distribution of anthocyanins and flower colors in four cultivars of *C. medium*

Owing to know the relations between flower colors and anthocyanin components in the flowers of *C. medium* cultivars, four different flower color cultivars were picked up such as 'May Pink' being pink flower, 'May Purple Margin' being red-purple, 'May Purple' being purple, and 'May Blue' being light blue-purple, and analyzed their anthocyanin components, flower colors, and light absorption patterns of their intact flowers (Table 1). From the results obtained, it was revealed that their flower colors were approximately settled by their anthocyanin types as follows; purple to blue purple flowers contain delphinidin as 'May Purple' and 'May Blue', pink flowers contain pelargonidin as 'May Pink', and red-purple flowers, an intermediate flower color between both cultivars, contain pelargonidin and cyanidin as 'May Purple Margin'.

On the light absorption responsible for flower color variation, absorption spectra of intact petals of four cultivars were measured, and their absorption maxima are given in Table 1. Absorption maxima at 622, 569 and 538 nm of 'May Purple' were approximately indicated with those at 620, 570 and 540 nm of *Platycodon grandiflorum*[13] and at 610, 566 and 538 nm of *Aconitum japonicum*[13] whose structures are typical 7-*O*-acylated delphinidin 3-*O*-rutinoside-7-*O*-glucosides.[14]

Table 2 NMR spectroscopic data of a new anthocyanin from *Campanula medium

	^{13}C δ (ppm)	^{1}H δ (ppm)	
Delphinidin			
2	164.8		
3	146.9		
4	133.5	8.69	s
5	156.1		
6	104.3	6.76	d (2.2)
7	166.8		
8	95.6	7.08	d (2.2)
9	158.1		
10	113.5		
1'	119.6		
2'	113.6	7.60	s
3'	147.5		
4'	147.4		
5'	147.5		
6'	113.6	7.60	s
p-Hydroxybenzoic acid			
1	124.7		
2	132.3	7.71	d (8.9)
3	117.2	6.69	d (8.9)
4	162.6		
5	117.2	6.69	d (8.9)
6	132.3	7.71	d (8.9)
-COOH	167.5		
Glucose A			
1	102.7	5.39	d (7.6)
2	74.7	3.74	m
3	77.6	] 3.30 - 3.60	
4	71.1		
5	77.5	3.74	m
6a	67.8	3.57	m
6b		4.00	d (11.6)
Glucose B			
1	100.9	5.31	d (7.7)
2	77.7	3.52	dd (7.7, 9.5)
3	77.9	] 3.20 - 3.60	
4	71.4		
5	75.8	4.00	m
6a	65.2	4.23	dd (7.7, 11.9)
6b		4.71	d (11.9)
Glucose C			
1	101.3	4.58	d (7.4)
2	74.5	3.30	m
3	78.0	] 3.50 - 3.62	
4	72.4		
5	74.8		
6a	62.3	3.57	m
6b		3.71	dd (3.1, 12.0)
Rhamnose			
1	102.1	4.57	s
2	71.8	3.73	d (4.9)
3	72.0	3.59	t (9.2)
4	73.9	3.23	t (9.8)
5	69.7	3.49	dd (6.1, 9.5)
-CH3	17.9	1.08	d (6.1)

* 125.78 MHz for ^{13}C NMR and 500 MHz for ^{1}H, CD_3OD-DCl (9:1) and TMS as an internal standard.

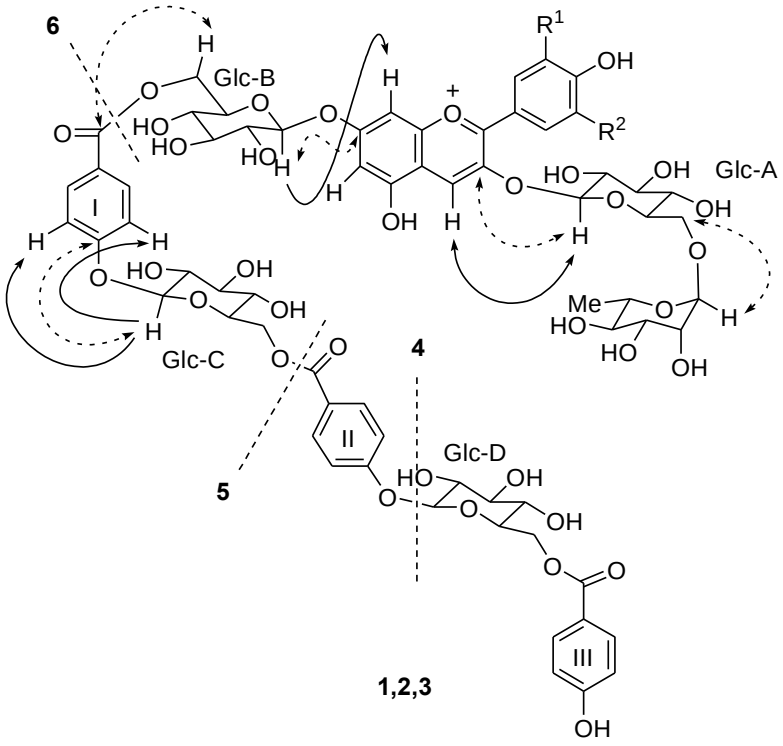

Figure 1. Anthocyanins in the flowers of *Campanula medium* cultivars.

anthocyanins
1: ruburocampanin ($R^1 = R^2 = H$)
2: purprocampanin ($R^1 = OH$, $R^2 = H$)
3: campanin ($R^1 = R^2 = OH$)
4: pigment 2 (violdelphin; $R_1 = R^2 = OH$)
5: pigment 1 ($R^1 = R^2 = OH$)
6: pigment 3 (deacylanthocyanin; $R^1 = R^2 = OH$)

observed main NOEs are indicated by arrows.
observed HMBC correlations are indicated by dotted arrows.

Moreover, absorption maxima at 570, 530, (500) and 452 nm of 'May Pink' were similar to those at 564, 530, 500 and (465) nm of rubrocampanin (**1**) in the phosphate buffer solution at pH 6.86.[14] These results supported that the flower colors of both cultivars were formed by the intramolecular co-pigmentation of anthocyanins between their anthocyanidin and aromatic acid moieties in their flowers.[14] Absorption maxima at 570 and 530 nm of 'May Purple Margin' were identical with the values at 570 and 530 nm of 'May Pink', however their absorption curves were quite different with each other. The absorption curve of 'May Purple Margin' exhibited two broad maxima at 570 and 530 nm, and especially the absorption curve of the maximum at 570 nm exhibited rather strong absorbance up to near 600 nm due to take part purprocampanin (**2**) (cyanidin type) into rubrocampanin (**1**) (pelargonidin type) forming its flower color. Therefore, the flower color of 'May Purple Margin' shows red-purple, and is more bluish than that of 'May Pink'.

On the other hand, the absorption curve of ‘May Blue’ exhibited two broad maxima at 618 and 569 nm, and their peaks of the maxima were not as sharp as those of ‘May Purple’. For the reason, it may be considered that the absorption curve of ‘May Blue’ is produced from the incomplete intramolecular co-pigmentation of anthocyanins in this flowers,[14] because its main anthocyanin, pigment 1, is composed of only one molecule of *p*-hydroxybenzoic acid. However, ‘May Blue’ has more bluish flowers than ‘May Purple’, albeit only slightly (Table 1). This is thought to mean that the other factor(s), pH of cell sap, intermolecular co-pigmentation and so on, contributed to blue flower color of this cultivar.

3. EXPERIMENTAL

3-1. General procedures

TLC was carried out on plastic coated cellurose sheets (Merck) using seven 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), and 1% HCl for anthocyanins, BAW, *i*-PrOH-H_2O (4:1), *i*-PrOH-*n*-BuOH-H_2O (7:1:2) and PhOH-H_2O (4:1) for sugars.[15]

Analytical HPLC was performed on a Hitachi 6200 system, using an Inertsil ODS-2 (4.6 ϕ x 250 mm) column at 35 °C with a flow rate of 0.8 mL/min and monitoring at 520 nm. The eluant was applied as a linear gradient elution 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 a MPS-2000 (Shimadzu) in 0.1% HCl-MeOH (from 200 to 700 nm). Spectral absorption of the flower was directly measured on the intact petals using a recording spectrophotometer operated as a double beam instrument (Type MPS-2000).[13,16]

High resolution FAB mass (FABMS) 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 determined at 500 MHz for ^1H spectra and at 125.78 MHz for ^{13}C spectra in DCl-CD_3OD (1:9). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants (*J*) are in Hz.

3-2. Plant materials

The seeds of *Campanula medium* cvs ‘May Blue’, ‘May Purple’, ‘May Purple Margin’, and ‘May Pink’ were purchased from Sakata Seeds Co. Ltd (Yokohama, Japan), and grown in the green-house of Minami-Kyushu University, Takanabe, Miyazaki, Japan.

The flower colors of these plants were recorded on a SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co., Ltd.). These values are shown in Table 2. The flowers were collected in May to June, dried overnight at 45 °C, and kept in a refrigerator about 4 °C.

3-3. Distribution survey of anthocyanins in the flowers of four cultivars

Dried petal (0.02 g) or fresh petal (0.2 g) of each cultivar was extracted with 50% AcOH. Quantitative analysis of anthocyanins was performed by HPLC on an Inertsil ODS-2 (4.6 ϕ x 250 mm) column at 35 °C with a flow rate of 0.8 mL/min and monitoring at 520 nm. Solvent system was used: a linear gradient elution 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). Anthocyanins of these extracts were identified by the analysis of TLC and HPLC with the authentic anthocyanins (see in Experimental and Table 2). TLC solvents used were BAW, BuHCl, 1% HCl, and HOAc-HCl.

3-4. Isolation of anthocyanins

The dried flowers (53 g) of *C. medium* 'May Blue' were extracted with 50% HOAc (5 L) at room temperature (20 °C) overnight. Anthocyanins in the extract were adsorbed on a Diaion HP-20 column, and the column was washed with H_2O (5 L). The absorbed anthocyanins were eluted with MeOH-HOAc-H_2O (70:5:25). After concentration, the eluates were fractionated with Sephadex LH-20 CC using MeOH-HOAc-H_2O (6:1:12). The fractions were further purified with PC (*n*-BuOH-HOAc-H_2O, 4:1:2 and 15% HOAc) and preparative HPLC. Preparative HPLC was performed on a Hitachi 6200 system using an Inertsil ODS-2 column (20 ϕ x 200 mm) with a HOAc solvent. Consequently, pigment 1 (42.6 mg) and pigment 2 (6 mg) were obtained as dark red powders. Small amount of pigment 3 was also obtained by this procedure.

3-4-1. Pigment 1 **(5)**: Dark red powder; for UV-VIS λmax in 0.1% HCl-MeOH: 542, 250 nm, E_{acyl}/E_{max} (%) = 88, E_{440}/E_{max} (%) = 14, $AlCl_3$ shift +; TLC (R_f x100) BAW 9, BuHCl 4, 1% HCl 6, HOAc-HCl 58; HPLC: R_t(min) 20.7; HR-FABMS calc. for $C_{46}H_{55}O_{28}$: 1055.2880. Found: 1055.2943; for ^1H and ^{13}C NMR spectra see Table 2.

3-4-2. Pigment 2 (violdelphin : **4)**: Dark purple powder; for UV-VIS λmax in 0.1% HCl-MeOH: 548, 251 nm, E_{acyl}/E_{max} (%) = 180, E_{440}/E_{max} (%) = 14, $AlCl_3$ shift +; TLC (R_f x100) BAW 13, BuHCl 7, 1% HCl 7, HOAc-HCl 31; HPLC: R_t(min) 22.3; HR-FABMS calc. for $C_{53}H_{59}O_{30}$: 1175.3091. Found: 1175.3105.

3-4-3. Pigment 3 (deacylanthocyanin: **6)**: Dark red powder; for UV-VIS λmax in 0.1% HCl-MeOH: 539, 285 nm, E_{440}/E_{max} (%) = 11, $AlCl_3$ shift +; TLC (R_f x100) BAW 6, BuHCl 3, 1% HCl 19, HOAc-HCl 47; HPLC: R_t(min) 7.0.

3-5. Authentic pigments

Campanin, rubrocampanin and purprocampanin were obtained from cultivars of *C. medium* by the process described previously.[1,2] Violdelphin was obtained from the blue flowers of *Delphinium hybridum* by the process described.[6]

3-6. Deacylanthocyanin and 4-glucosyl-*p*-hydroxybenzoic acid

A deacylanthocyanin of pigments 1 and 2 were obtained in the following process.[1,2] Mixed pigments 1 and 2 (5 mg) were dissolved in 2N NaOH (1 mL) under N_2 gas and allowed to stand for 30 min. Then the solution was sufficiently acidified with 2N HCl and evaporated in vacuo to dryness. The residue was dissolved in 1% HCl-MeOH and subjected to TLC (BAW) to yield a deacylanthocyanin (2 mg) and 4-*O*-glucosyl-*p*-hydroxybenzoic acid (0.5 mg).

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