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AN ACYLATED CYANIDIN 3-RUTINOSIDE-7-GLUCOSIDE WITH *p*-HYDROXYBENZOIC ACID FROM THE RED-PURPLE FLOWERS OF *CAMPANULA MEDIUM*

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Abstract – A new acylated anthocyanin was isolated from the red-purple flowers of *Campanula medium* as a major pigment together with a known anthocyanin. The new pigment was determined to be cyanidin 3-*O*-[6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-7-*O*-{4-[6-*O*-(4-(6-*O*-(*p*-hydroxybenzoyl)-β-D-glucopyranosyl)-oxybenzoyl)-β-D-glucopyranosyloxy-benzoyl]-β-D-glucopyranoside} based on spectroscopic analyses. The known pigment was also identified to be rubrocampanin, pelargonidin 3-rutinoside-7-(*p*-hydroxybenzoylglucopyranosyl-*p*-hydroxybenzoylglucopyranosyl-*p*-hydroxy-benzoylglucopyranoside).

In 1990, we reported the isolation and structure determination of the flower anthocyanins of *Campanula medium,* and described that its purple and pink cultivars contained campanin and rubrocampanin as their main anthocyanins, respectively.[1] Both pigments were determined to be 3-rutinoside-7-(*p*-hydroxybenzoylglucopyranosyl-*p*-hydroxybenzoylglucopyranosyl-*p*-hydroxybenzoylglucopyranosides) of delphinidin (campanin) and pelargonidin (rubrocampanin), respectively.[1]

Two analogous of campanin, such as monodeacylcampanin lacking of a molecule of *p*-hydroxybenzoic acid, and violdelphin lacking of one molecule of glucosyl-*p*-hydroxybenzoic acid, were also found in the flowers of *C. isophyla*, *C.carpatica* and *C. proskarshyana* by Goto and his coworkers in 1993.[2] As a part of our continuing studies on flower color variation,[3] we report here the isolation and structure elucidation of a new anthocyanin along with a known anthocyanin, rubrocampanin, from the red-purple flowers of *C. medium* cv. 'May Purple Margin'.[4] For the sake of convenience, we named this new compound as

purprocampanin (PC).

Air-dried petals (50g) of *C. medium* were extracted with 50% AcOH. Two major and at least 8 minor peaks of anthocyanins were observed by HPLC analysis of the crude extract. Among these anthocyanins, two main compounds (**1**) (*ca*.50mg) and (**2**) (*ca* 70mg) were obtained as scarlet and dark red powders, respectively, by the process described previously.[1]

On acid hydrolysis,[5] both pigments gave glucose, rhamnose, and *p*-hydroxybenzoic acid as their sugar and hydroxyacid moieties. As their aglycones, pelargonidin and cyanidin were observed in pigments (**1**) and (**2**), respectively. On alkaline hydrolysis,[5] pigments (**1**) and (**2**) gave *p*-hydroxybenzoic acid, 4-glucosylhydroxybenzoic acid,[6] and a deacylanthocyanin. The deacylanthocyanin of pigment (**1**) was identified as pelargonidin 3-rutinoside-7-glucoside by direct comparison of TLC and HPLC[7] with authentic specimen,[8] which was derived from rubrocampanin on the alkaline hydrolysis.[1] By HPLC analysis, pigment (**1**) was identified to be rubrocampanin, and its structure was further confirmed based on the analysis of FAB mass and NMR spectra.[10] On the other hand, the deacylanthocyanin[9] of pigment (**2**) were not identical with both 3-rutinoside-7-glucosides of pelargonidin and delphinidin, obviously. Based on the examination of chemical and spectral properties of pigment (**2**),[11] it was presumed to be a new acylated cyanidin glycoside, an analogue of campanin and rubrocampanin.

The HR FAB mass spectrum of pigment (**2**) gave its molecular ion $[M]^+$ at *m/z* 1441.3906, in agreement with the mass calculated for $C_{66}H_{73}O_{36}$, which was composed of cyanidin with four molecules of glucose, three molecules of *p*-hydroxybenzoic acid and one molecule of rhamnose.

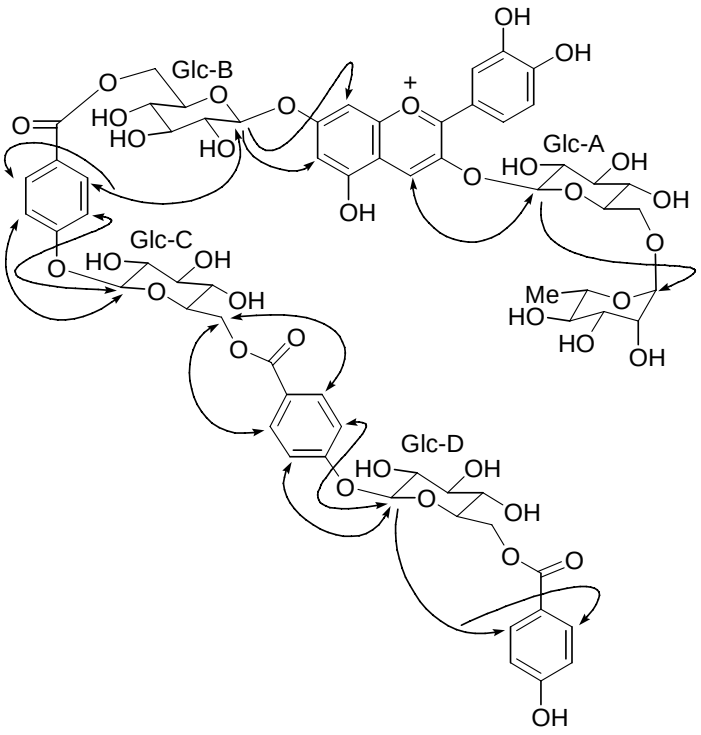

Figure 1. Purprocampanin from *Campanula medium*.
Observed NOE's are indicated by arrows.

In order to determine the structure of pigment (**2**), its ^{1}H and ^{13}C NMR spectroscopic measurements including 2D COSY, negative difference NOE (NOEDIF), HMQC, HMBC and NOESY spectra were carried out in CF_3CO_2D-DMSO-d_6 (1:9). The chemical shifts of its protons were assigned as shown in Table 1. Regarding the sugar moieties of pigment (**2**), their signals were observed in the region of δ 5.39-1.20, and five anomeric proton signals were assigned to be δ 5.37 (d, *J*=7.7 Hz, Glc-A), 5.39 (d, *J*=7.7 Hz, Glc-B), 5.13 (d, *J*=6.7 Hz, Glc-C), 5.00 (d, *J*=7.7 Hz, Glc-D), and 4.70 (s, Rhamnose). The coupling constants of the glucose moieties were in the region of *J*=6.7-7.7 Hz, suggesting that all glucose units must be β-glucopyranose form (Figure1). Furthermore, six characteristic proton signals shifted to the lower magnetic field at δ 4.73 and 4.38 (Glc-B, H-6a and –6b), δ 4.66 and 4.60 (Glc-C, H-6a and –6b), and δ 4.43 and 4.15 (Glc-D, H-6a and –6b), were observed and assigned to the methylene protons of glucose moieties by the analysis of 2D COSY and NOESY spectra, indicating that Glc-B, Glc-C, and Glc-D were acylated with *p*-hydroxybenzoic acids (I, II, and III) at OH-6 groups of three glucose moieties.

Table 1. ^{1}H NMR data for Purprocampanin of *Campanura medium* cv 'May Purple Margin'.
(500 MHz in DMSO-d_6-CF_3CO_2D, TMS as an internal standard)
Coupling constants (*J* in Hz) in parentheses.

Cyanidin		Glucose A		Glucose D	
4	8.68 s	1	5.37 d (7.7)	1	5.00 d (7.7)
6	6.80 brs	2	3.70 m	2	3.39 m
8	7.45 brs	3	3.20-3.50	3	3.20-3.60
2'	8.19 d (2.2)	4		4	
5'	7.10 d (8.9)	5		5	3.80 m
6'	8.42 dd (2.2, 8.9)	6a	4.01 d (10.1)	6a	4.43 d (10.7)
		6b	3.54 m	6b	4.15 dd (7.5, 10.7)
p-Hydroxybenzoic acid (I)		Glucose B		Rhamnose	
2, 6	7.87 d (8.2)	1	5.39 d (7.7)	1	4.70 s
3, 5	7.16 d (8.2)	2	3.59 m	2	3.74 brs
p-Hydroxybenzoic acid (II)		3	3.20-3.75	3	3.20-3.70
2, 6	7.61d (8.6)	4		4	
3, 5	6.84m	5	4.10 m	5	3.48 s
		6a	4.73 d (12.5)	Me	1.20 s
p-Hydroxybenzoic acid (III)		6b	4.38 dd (7.3, 12.5)		
2, 6	7.51 d (8.6)	Glucose C			
3, 5	6.63 d (8.6)	1	5.13 d (6.7)		
		2	3.43 m		
		3	3.36 m		
		4	3.89 t (7.8)		
		5	4.27 m		
		6a	4.66 m		
		6b	4.60 m		

The linkages and/or the positions of attachment of glucoses and *p*-hydroxylbenzoic acids were determined by the measurements of NOEDIF and NOESY spectra. By irradiation at H-1 of Glc-A, the

appearance of a strong NOE signal at H-4 of cyanidin indicated Glc-A to be attached to OH-3 of cyanidin through a glucosidic bond. Moreover, a rather weak NOE signal was observed at H-1 of rhamnose as well as at those of other four proton signals of Glc-A, supporting that Glc-A bonded with rhamnose at OH-6. The presence of this binding system was confirmed by the detection of rutinose in the products of H_2O_2 degradation of pigment (**2**).[5] By irradiation at H-1 of Glc-B, the strong NOE signals of H-6 and H-8 in cyanidin were observed. Thus, Glc-B was confirmed to attach to OH-7 of cyanidin. Furthermore, by irradiation of both H-1 protons of Glc-C and Glc-D, strong NOE signals were observed at H-3 and H-5 signals of *p*-hydroxybenzoic acid I, and also H-3 and H-5 signals of *p*-hydroxybenzoic acid II, supporting that *p*-hydroxybenzoic acid I is glycosylated with Glc-C at OH-4 of *p*-hydroxybenzoic acid I and also *p*-hydroxybenzoic acid II is glycosylated with Glc-D at OH-4 of *p*-hydroxybenzoic acid II (Figure 1). Similarly, rather weak NOEs were observed at H-2 and H-6 signals of *p*-hydroxybenzoic acid I by the irradiation at H-1 and H-6 of Glc-B indicating that Glc-B is acylated with *p*-hydroxybenzoic acid I at OH-6 of Glc-B. However, NOE signals were not observed at any proton signals of *p*-hydroxybenzoic acid III by above irradiations. Consequently, the structure of pigment (**2**), purprocampanin, was determined to be cyanidin 3-*O*-[6-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-7-*O*-{4-[6-*O*-(4-(6-*O*-(*p*-hydroxybenzoyl)-β-D-glucopyranosyl)oxybenzoyl)-β-D-glucopyranosyloxybenzoyl]-β-D-glucopyranoside, which is a new anthocyanin in plants.

Purprocampanin occurs mainly in the margin of *Campanula* 'May Purple Margin' flower petals, accompanying with rubrocampanin. The contents and distribution ratios of the pigments in the flowers are dependent on the flower stages and/or climate circumstances, and the flower color of *C.* 'May Purple Margin' is usually varied in the range of red to red-purple. Based on the above results, it is considered that increased distribution ratios of purprocampanin affect the change of the flower color of *C.* 'May Purple Margin' from red to purple.

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2. K. Brandt, T. Kondo, H. Aoki, and T. Goto, *Phytochemistry*, 1993, **33**, 209.
3. N. Saito, K. Toki, Y. Morita, A. Hoshino, S. Iida, A. Shigihara, and T. Honda, *Phytochemistry*, 2005, **66**, 1852.
4. The flowers of *Campanula medium* cv 'May Purple Margin' were obtained from the plants growing in the green house of Minami-Kyushu University, Takanabe, Miyazaki.
5. J. B. Harborne, "*Phytochemical Methods*," second ed. Chapman and Hall, London, 1984.
6. 4-Glucosyloxybenzoic acid: UV-VIS λ_{max}: 246nm: Rf values (TLC): *n*-butanol-acetic acid-water (4 :

1 : 5, BAW) 70%; 6% AcOH 76%; H_2O 91%; *n*-butanol-ethanol-water (4 : 1 : 2.2, BEW) 66%: HPLC: Rt (min) 5.4.

7. HPLC was run in the same condition as described previously.[3]
8. Pelargonidin 3-rutinoside-7-glucoside; UV-VIS λ_{max} (0.1%HCl-MeOH) 268, 504nm: Rf values (TLC): BAW 27%; 1%HCl 51%; BuH(2N HCl-*n*-BuOH, 1 : 1) 17%; AcOH-HCl (H_2O-acetic acid-water, 15 : 3 : 82) 75%: HPLC: Rt (min) 10.08.
9. Cyanidin 3-rutinoside-7-glucoside; UV-VIS λ_{max} (0.1%HCl-MeOH) 283, 526 nm: Rf values (TLC): BAW 17%; 1%HCl 29%; BuH 9%; AcOH-HCl 59%: HPLC: Rt(min) 7.58.
10. Rubrocampanin: UV-VIS λ_{max} (0.1%HCl-MeOH) 518 nm, Eacyl/Evismax=163%: Rf values (TLC): BAW 29%; 1%HCl 24%; BuH 36%; AcOH-HCl 59%: HPLC: Rt (min) 24.2: High resolution FABMS calc. For $C_{66}H_{73}O_{35}$: 1425.3932 *m/z*. Found: 1425.3893. ^{1}H NMR (500MHz, CF_3CO_2D-DMSO-d_6, 1 : 9): pelargonidin; δ 8.76 (1H, s, H-4), 8.74 (2H, d, *J*=9.2 Hz, H-2', 4'), 7.51 (1H, m, H-8), 7.10 (2H, d, *J*=9.2 Hz, H-3', 5'), *p*-hydroxybenzoic acid (I); δ 7.86 (2H, d, *J*=8.9 Hz, H-2, 6), 7.18 (2H, d, *J*=8.9 Hz, H-3, 5), *p*-hydroxybenzoic acid (II); δ 7.62 (2H, d, *J*=8.9 Hz, H-2, 6), 6.86 (2H, d, *J*=8.9 Hz, H-3, 5), *p*-hydroxybenzoic acid (III); δ 7.51 (2H, d, *J*=8.9 Hz, H-2, 6), 6.63 (2H, d, *J*=8.9 Hz, H-3, 5), Sugars: Glc-A; δ 5.37 (1H, d, *J*=8.0 Hz, H-1), 4.00 (1H, d, *J*=10.4 Hz, H-6a), 3.69 (1H, m, H-5), 3.63 (1H, d, *J*=8.3 Hz, H-2), Glc-B; δ 5.37 (1H, d, *J*=8.0 Hz, H-1), 4.75 (1H, m, H-6a), 4.35 (1H, dd, *J*=7.4 Hz, 11.9 Hz, H-6b), 4.12 (1H, m, H-5), 3.55 (1H, m, H-2), Glc-C; 5.13 (1H, d, *J*=7.3 Hz, H-1), 4.62 (1H, d, *J*=12.2 Hz, H-6a), 4.27 (1H, m, H-6b), 3.87 (1H, t, *J*=9.5 Hz, H-5), 3.41 (1H, m, H-2), Glc-D; δ 5.00 (1H, d, *J*=8.0 Hz, H-1), 4.43 (1H, d, *J*=10.4 Hz, H-6a), 4.15 (1H, m, H-6b), 3.81 (1H, t, *J*=9.2 Hz, H-5), 3.39 (1H, m, H-2), Rhamnose; δ 4.69 (1H, s, H-1), 3.74 (1H, d, *J*=1.2 Hz, H-2), 3.42 (1H, m, H-5), 1.16 (3H, s, -CH3).
11. Purprocampanin (pigment **2**): UV-VIS λ_{max} (0.1%HCl-MeOH) 537 nm, Eacyl/Evismax=159%: Rf values (TLC): BAW 23%; 1%HCl 18%; BuH 26%; AcOH-HCl 50%: HPLC: Rt (min) 22.9: High resolution FABMS calc. For $C_{66}H_{73}O_{36}$; 1441.3882 *m/z*. Found: 1441.3906. ^{13}C NMR (125.65MHz, CF_3CO_2D-DMSO-d_6, 1 : 9): cyanidin; δ 162.3 (C-2), 146.0 (C-3), 133.2 (C-4), 155.2 (C-5), 103.1 (C-6), 165.2 (C-7), 96.0 (C-8), 156.9 (C-9), 112.5 (C-10), 119.6 (C-1'), 118.2 (C-1'), 141.0 (C-3'), 156.0 (C-4'), 116.0 (C-5'), 128.4 (C-6'), *p*-hydroxybenzoic acid (I); δ 123.5 (C-1), 131.1 (C-2), 115.3 (C-3), 146.3 (C-4), 115.6 (C-5), 131.1(C-6), 161.1 (COOH), *p*–ηψδροξψβενζοιχ αχιδ (II); δ 123.3(C-1), 131.0 (C-2), 115.3 (C-3), 146.6 (C-4), 115.3 (C-5), 131.0 (C-6), 160.7(COOH), *p*-hydroxybenzoic acid (III); δ 123.1(C-1), 131.4 (C-2), 114.5 (C-3), 145.1 (C-4), 114.5 (C-5), 131.4 (C-6), 162.0 (COOH), Sugars, Glc-A; δ 102.3 (C-1), 66.6 (C-6); Glc-B; δ 99.9 (C-1), 74.3 (C-5), 64.2 (C-6), Glc-C; 99.5 (C-1), 64.0 (C-6); Glc-D; δ 99.0 (C-1), 73.7 (C-5), 64.0 (C-6).