Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 66 (2005) 1844–1851
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Acylated anthocyanins from the violet-blue flowers of *Orychophragonus violaceus*

Toshio Honda [a,*], Fumi Tatsuzawa [b], Nao Kobayashi [a], Hiroko Kasai [a], Seiji Nagumo [a], Atsushi Shigihara [a], Norio Saito [c]

[a] *Faculty of Pharmaceutical Sciences, Hoshi University, Ebara 2-4-41, Shinagawa, Tokyo 142-8501, Japan*
[b] *Department of Agro-Environmental Science, Hokkaido Junior College, Takushoku University, Fukagawa, Hokkaido 074-8585, Japan*
[c] *Chemical Laboratory, Meiji-Gakuin University, Totsuka, Yokohama 244-8539, Japan*

Received 4 February 2005; received in revised form 11 April 2005
Available online 14 July 2005

Abstract

Three acylated cyanidin 3-sambubioside-5-glucosides (**1–3**) were isolated from the violet-blue flowers of *Orychophragonus violaceus*, and their structures were determined by chemical and spectroscopic methods. Two of those acylated anthocyanins (**1** and **3**) were cyanidin 3-*O*-[2-*O*-(2-*O*-(4-*O*-(6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-acyl)-β-D-glucopyranoside]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside)s, in which the acyl groups were *p*-coumaric acid for **1**, and sinapic acid for **3**, respectively. The last anthocyanin **2** was cyanidin 3-*O*-[2-*O*-(2-*O*-(4-*O*-(6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-feruloyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside. In these flowers, the anthocyanins **2** and **3** were present as dominant pigments, and **1** was obtained in rather small amounts.

Keywords: *Orychophragonus violaceus*; Cruciferae; Violet-blue flower color; Acylated cyanidin 3-sambubioside-5-glucoside; Caffeic acid; *p*-Coumaric acid; Ferulic acid; Sinapic acid; Malonic acid

1. Introduction

Orychophragonus violaceus (Murasakihanana in Japanese) is native in China, and a common ornamental plant with the violet-blue flowers in China and Japan. Recently this plant was adopted as the symbolic flower plant of the author's university (Hoshi University), since this flower was thought to be originally introduced into Japan by Prof. S. Yamaguchi, the first president of Hoshi University, and also due to promote the friendship between Japan and China. There has been no report of anthocyanin study on *Orychophragonus violaceus*, except for some relatives such as *Matthiola incane* (Saito et al., 1995, 1996), *Arabidopsis thaliana* (Bloor and Abrahams, 2002), and *Sinapis alba* (Takeda et al., 1988).

In continuing our work on flower color variations due to acylated anthocyanins, we decided to elucidate anthocyanin pigments of this plant flowers whose anthocyanins were presumed to be heavily acylated with hydroxycinnamic acids. In this paper, we would like to report the structure elucidation of three acylated cyanidin 3-sambubioside-5-glucosides (Orychophragonus violet-blue anthocyanins, OVAs) in the violet-blue flowers of *Orychophragonus violaceus*.

* Corresponding author. Tel.: +81 3 5498 5791; fax: +81 3 3787 0036.
E-mail address: honda@hoshi.ac.jp (T. Honda).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.05.026

2. Results and discussion

Four major anthocyanin peaks were observed in the acetic acid extract of the violet-blue flowers of *Orychophragonus violaceus* on high performance liquid chromatography (HPLC). The relative frequencies of their occurrence were 13.6% (pigment **1**), 28.9% (pigment **2**), 38.0% (pigment **3**), and 3.1% (pigment **4**), respectively. Among them, three anthocyanin pigments (**1**–**3**) were isolated from the flowers of this plant with 5% HOAc solvent, and purified using Diaion HP-20 (Mitsubushi Chemical's Ion Exchange Resins) column chromatography (CC), preparative HPLC and TLC, according to the procedures described previously (Saito et al., 1995, 2002, and Tatsuzawa et al., 2004). Pigment **4** could not be obtained in a pure form, unfortunately, due to its small amount available. The chromatographic and spectral properties of these anthocyanins are summarized in Table 1.

Acid hydrolysis of all three pigments resulted in cyanidin, glucose, xylose, and caffeic acid. Moreover, another molecule of hydroxycinnamic acid was detected in the hydrolysates of pigment **1** (*p*-coumaric acid), pigment **2** (ferulic acid), and pigment **3** (sinapic acid), respectively. Malonic acid was also detected in the hydrolysates of pigments **1** and **3**. Alkaline hydrolysis of these three anthocyanins resulted in only one deacylated anthocyanin, whose structure was identified to be cyanidin 3-sambubioside-5-glucoside by the analyses of TLC and HPLC with the authentic sample obtained from Matthiola violet anthocyanins by alkaline hydrolysis (Saito et al., 1995). This structure was also confirmed by the analysis of FAB mass spectrum as shown in Table 1. In the alkaline hydrolysates of three pigments, 4-glucosylcaffeic acid was detected as the acylated sugar, and its structure was identified by direct comparison with the authentic sample obtained from Cinerarin and Evolvulus anthocyanins by the same treatment (Toki et al., 1994).

2.1. Pigment 3 (OVA 3)

The molecular ion [M^+] of pigment **3** was observed at *m/z* 1845 ($C_{82}H_{93}O_{48}$), indicating that the pigment is composed of cyanidin with five molecules of glucose, two molecules of caffeic acid, and one molecule each of xylose, sinapic acid and malonic acid. The elemental components were confirmed by measuring its high-resolution FABMS (HRMS), and the mass data obtained were summarized in Section 3.5. The structure of pigment **3** was further elucidated based on the analysis of its ^{1}H NMR spectra [500 MHz in CF_3COOD-DMSO-d_6 (1:9)], including 2D COSY, 2D NOESY, and negative difference NOE (NOEDIF) spectral experiments (Kondo et al., 1987). The chemical shifts of 14 aromatic protons of cyanidin, caffeic acid, and sinapic acid moieties with their coupling constants were assigned as shown in Table 2. The ^{1}H NMR spectrum exhibited six proton signals corresponding to two methoxy groups on sinapic acid, in addition to six olefinic proton signals of hydroxycinnamic acid (J = 15.6–15.9 Hz) with the *trans* configuration (Table 2). The chemical signals of the sugar moieties were observed in the region of δ 5.67–3.12, where the six anomeric protons exhibited at δ 5.67 (d,J = 7.4 Hz, Glc A), δ 5.18 (d, J = 7.7 Hz, Glc B), δ 5.16 (d, J = 8.2 Hz, Xyl C), δ 4.91 (d,J = 8.4 Hz, Glc D), δ 4.93 (d, J = 7.9 Hz, Glc D), and δ 4.35 (d, J = 7.9 Hz, Glc F), respectively. Based on the observed coupling constants (Table 2), these six sugars were assumed to have β-pyranose forms. The linkages and/or positions of the attachments of the sugar and acyl groups in this pigment were mainly determined by using 2D COSY, ROESY and NOEDIF experiments (Fig. 1). A proton signal (δ 4.02, t, J = 8.3 Hz) detected at a lower magnetic field was assigned to H-2 of Glc A by the analysis of its 2D COSY spectrum. These results supported that xylose C was linked to 2-OH of Glc A by forming sambubioside group at the 3-OH of cyanidin. Seven characteristic proton signals shifted to a lower magnetic field were also assigned to the methylene protons of Glc A (δ 4.20 and 4.43, H-6a and b), Glc B (δ 3.82 and 4.41, H-6a and b), and Glc E (δ 4.32 and 4.48, H-6a and b), and to the methine proton (δ 4.65, t, J = 8.6 Hz, H-2) of Xyl C. Thus, the four hydroxy groups of the sugars, 6-OHs of Glc A, Glc B, Glc E and 2-OH of Xyl C, were assumed to be acylated with three molecules of hydroxycinnamic acids and one

Table 1
Chromatographic and spectral data for the main Orychophragonus violet-blue anthocyanins

Anthocyanins[a]	R_f values (×100)				HPLC (R_t) (min)	Spectral data in 0.1% HCl–MeOH		FAB-MS [M^+]
	BAW[b]	*n*-BuOH–HCl[b]	1% HCl	HOAc–HCl[b]		λ_{max} (nm)	E_{acyl}/E_{vis} (%)	
1	20	4	39	76	29.9	532, 316, (292), 285	150	1785
2	11	0	50	82	30.9	531, 320, (293), 284	165	1729
3	5	0	34	74	32.2	532, 321, 293, (286)	124	1845
deacyl compound	18	5	33	65	13.1	528, 273	–	743
Cy 3-sam-5-Glc	18	5	33	65	13.1	528, 273	–	743

[a] **1**: cyanidin 3-[2-(2-(4-(6-(4-glucosylcaffeoyl)-glucosyl)-caffeoyl)-xylosyl)-6-(4-glucosyl-*p*-coumaroyl)gIucoside]-5-gjucoside, **2**: cyanidin 3-[2-(2-(4-(6-(4-glucosylcaffeoyl)-glucosyl)-caffeoyl)-xylosyl)-6-(4-glucosyl-feruloyl)glucoside]-5-glucoside, **3**: cyanidin 3-[2-(2-(4-(6-(4-glucosylcaffeoyl)-glucosyI)-caffeoyl)-xylosyl)-6-(4-glucosyl-sinapoyl)glucoside]-5-(6-malonylglucoside).

[b] See Section 3 for abbreviations.

Table 2
NMR data of Orychophragonus violet-blue anthocyanins[a]

	3		**1**	**2**
	δC	δH	δH	δH
Cyanidin				
2	162.4			
3	144.4			
4	131.6	8.77 s	8.71 s	8.75 s
5	155.0			
6	104.6	6.96 d (1.6)	7.00 brs	6.97 d (1.9)
7	167.5			
8	96.2	7.04 d (1.6)	7.02 brs	7.02 d (1.9)
9	155.3			
10	111.8			
1′	117.6			
2′	117.2	8.01 d (2.1)	8.02 d (2.2)	8.02 d (2.1)
3′	146.7			
4′	155.5			
5′	116.7	7.08 d (8. 7)	7.07 d (8.7)	7.08 d (8.8)
6′	128.9	8.51 dd (2.1, 8.7)	8.49 dd (2.2, 8.7)	8.49 dd (2.1, 8.8)
Hydroxycinnamic acid				
(I) 1	124.1			
2	106.5	6.80 brs	7.29 d (8.3)	7.09 s
3	148.1		6.72 d (8.3)	
4	138.7			
5	148.1		6.72 d (8.3)	6.81 d (8.3)
6	106.5	6.80 brs	7.29 d (8.3)	7.04 m
α	113.7	6.36 d (15.6)	6.25 d (15.6)	6.33 d (15.9)
β	145.9	7.44 d (15.6)	7.35 d (15.6)	7.39 d (15.9)
CO_2H	166.9			
Me	56.2	3. 75 brs		3.79 s
Me	56.2	3. 75 brs		
(II) 1	129.2			
2	114.5	7.26 s	7.26 brs	7.27 brs
3	144.8			
4	147.4			
5	116.0	7.16 d (8.6)	7.16 d (8.6)	7.16 d (8.9)
6	119.8	7.09 m	7.08 in	7.08 m
α	115.2	6.49 d (15.6)	6.49 d (15.6)	6.50 d (15.6)
β	144.4	7.52 d (15.6)	7.53 d (15.6)	7.53 d (15.6)
CO_2H	166.7			
(III) 1	129.4			
2	121.2	7.24 s	7.24 brs	7.24 brs
3	147.2			
4	147.7			
5	116.3	7.18 m	7.18 m	7.18 m
6	121.2	7.18 m	7.18 m	7.18 m
α	114.6	6.49 d (15.9)	6.49 d (15.6)	6.50 d (15.9)
β	144.8	7.60 d (15.9)	7.60 d (15.6)	7.60 d (15.9)
CO_2H	166.1			
Malonic acid				
$–CH_2–$	41.2	3.42–3.40	3.45–3.40	
COOH	167.0			
COOH	168.3			
Sugars				
Glucose-A				
1	98.4	5. 67 d (7.4)	5.70 d (7.3)	5.70 d (7.9)
2	77.6	4.02 t (8.3)	3.98 m	4.02 t (8.3)
3	75.7	3.61 m	3.61 m	3.62 t (8.3)
4	74.8	3.46 m	3.42 m	3.45 m
5	73.2	3.97 m	4.04 m	3.93 m

Table 2 (*continued*)

	3		**1**	**2**
	δC	δH	δH	δH
6a	63.6	4.20 m	4.25 m	4.30 m
6b		4.43 brd (11. 2)	4.40 brd (10.4)	4.36 m
Glucose-B				
1	101.9	5.18 d (7.7)	5.18 d (7.7)	5.09 d (7.6)
2	74.8	3.76 m	3.52 m	3.51 m
3		3.52 m		
4		3.41 m	3.75–3.20	
5		3.42 m		3.85–3.36
6a	64.2	3.82 m	3.83 m	
6b		4.41 brd (11.6)	4.41 m	
Xylose-C				
1	101.9	5.16 d (8.2)	5.15 d (7.6)	5.15 d (7.9)
2	73.7	4.65 t (8.6)	4.66 t (9.2)	4.66 t (8.9)
3	74.2	3.46 m	3.44 m	3.50–3.40
4		3.181 (9.2)	3.32 m	3.21 m
5a	66.5	3.44 m	3.50–3.40	3.50–3.40
5b		3.90 m	3.91 m	3.90 m
Glucose-D				
1	101.9	4.93 d (7.9)	4.93 d (8.3)	4.94 d (7.6)
2	76.7	3.44 m	3.42 m	345 m
3		3.80–3.40	3.80–3.35	3.65–3.35
4				
5				
6a	60.4			
6b		3.83 brd (11.0)		
Glucose-E				
1	101.5	4.91 d (8.4)	4.91 d (7.9)	4.92 d (7.7)
2	75.5	3.42 m	3.42 m	3.45 m
3		3.42–3.35	3.60–3.40	3.50–3.40
4				
5	69.8	3.72 m	3.75 m	3.77 m
6a	63.3	4.32 m	4.30 m	4.32 m
6b		4.48 brd (11.0)	4.48 brd (10.9)	4.49 brd (10.7)
Glucose-F				
1	103.5	4.35 d (7.9)	4.35 d (7.9)	4.36 d (7.9)
2	73.5	3.081 (8.3)	3.081 (8.3)	3.08 t (8.3)
3	70.3	3.121 (9.2)	3.121 (9.5)	3.131 (8.4)
4		3.21 t (9.2)	3.27 m	3.21 m
5	80.3	3.26 m		3.26 m
6a	60.4	3.45–3.35	3.50–3.20	3.55–3.35
6b		3.50 m		

[a] ^{1}H NMR (500 MHz) and ^{13}C NMR (125.78 MHz) (DMSO-d_6-CF_3COOD, 1:9), an internal standard of TMS. Coupling constants (*J* in Hz) in parentheses.

molecule of malonic acid. By irradiation at H-1 of Glc A, a strong NOE was observed at H-4 of cyanidin together with rather weak NOEs at 2-,6- and α-H of sinapic acid (I), supporting that Glc A was attached to 3-OH of cyanidin through a glycosidic bond. Moreover, Glc A was presumed to be acylated with sinapic acid (I) at 6-OH of Glc A based on the consideration of its NOESY spectrum (Fig. 1). In the NOESY spectrum, the correlations between 6-H of cyanidin and 1-H of Glc B, 5-H of caffeic acid (II) and 1-H of Glc E, 5-H of caffeic acid (III) and 1-H of Glc E, and both methoxy proton signals of sinapic acid (I) and 1-H of Glc F were observed, respectively, revealing the glycosylation patterns; such as 5-OH of cyanidin was glycosylated with Glc B, 4-OH of caffeic acid (II) with Glc E, 4-OH of caffeic acid (III) with Glc D, and 4-OH of sinapic acid (I) with Glc F. Irradiation of 2-H of Xyl C gave NOEs at α- and β-H of caffeic acid (II) as well as 1- and 3-H of Xyl C. Therefore, Xyl C was acylated with caffeic acid (II) at the 2-position. Further irradiation of 6a-H (δ 4.48) of Glc E afforded the NOEs at α-, β-, and 2-H signals of caffeic acid (III), indicating that Glc E was acylated with caffeic

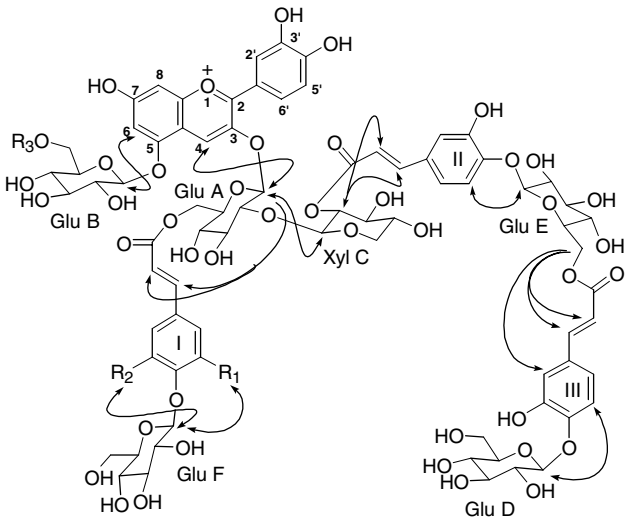

Fig. 1. Orychophragonus violet-blue anthocyanins. OVA 1: R_1 = R_2 = H, R_3 = malonyl; OVA 2: R_1 = H, R_2 = OCH_3, R_3 = H; OVA 3: R_1 = R_2 = OCH_3, R_3 = malonyl. Observed NOEs are indicated by arrows.

acid (III) at 6-OH of Glc E. Since both methylene proton signals of Glc D and Glc F were not shifted to a lower magnetic field, both 6-OHs of those sugars should be free from acyl moiety, and supposed to be the terminal residues. These results led to the conclusion that Glc B was acylated with malonic acid at the 6-position. Thus, pigment **3** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(4-*O*-(6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-sinapyl)-β-D-glucopyranoside]-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside). This structure was further confirmed by the analysis of HMQC and HMBC spectra, in which ^{13}C chemical shifts of malonic acid were assigned at δ 41.2 (–CH_2–), δ 167.0 (–CO–), and δ 168.3 (–CO–).

2.2. Pigment 2 (OVA 2)

The FAB mass spectrum of pigment **2** gave its molecular ion [M^+] at *m/z* 1729 in good agreement with the mass calculated for $C_{78}H_{89}O_{44}$, which was composed of cyanidin with five molecules of glucose, two molecules of caffeic acid, and one molecule each of xylose and ferulic acid. By contemplation of the mass data, the structure of pigment **2** is thought to have ferulic acid instead of sinapic acid as in pigment **3**. The ^1H NMR spectrum of pigment **2** was closely related to that of pigment **3** except for the signals of sinapic and malonic acid moieties. Detailed structure of **2** was further elucidated by the analysis of ^1H NMR spectra including 2D COSY, NOESY and NOEDIF spectral measurements as described above. Fifteen aromatic protons in cyanidin, caffeic acid, and ferulic acid, and three protons for the methoxy group on ferulic acid were easily assigned with their coupling constants as shown in Table 2. Six olefinic protons of caffeic acid and ferulic acid exhibited large coupling constants (*J* = 15.9, 15.9, and 15.9 Hz), indicating the presence of *trans*-hydroxycinnamic acids. The signals of the sugar moieties were observed in the region of δ 5.70–3.31, where six anomeric protons were assigned to be at δ 5.70 (d, *J* = 7.9 Hz, Glc A), δ 5.09 (d, *J* = 7.6 Hz, Glc B), δ 5.15 (d, *J* = 7.9 Hz, Xyl C), δ 4.94 (d, *J* = 7.6 Hz, Glc D), δ 4.92 (d, *J* = 7.7 Hz, Glc E), and δ 4.36 (d, *J* = 7.9 Hz, Glc F), respectively, with the coupling constants showing the presence of β-pyranose forms. Five characteristic proton signals shifted to a lower magnetic field were also assigned to four methylene protons of Glc A (δ 4.36 and 4.30) and Glc E (δ 4.49 and 4.32), and one methine proton of Xyl C (δ 4.66, H-2). Thus, 6-OHs of Glc A and Glc E, and 2-OH of Xyl C were acylated with three molecules of hydroxycinnamic acids, such as caffeic acid and ferulic acid. The linkages and/or attachment positions of these acids were elucidated by the same process as described for pigment **3** using NOESY and DIFNOE spectral methods. By irradiation of 1-H of Glc A, a strong NOE was observed at 4-H of cyanidin, together with rather weak NOEs at 2-, 6-, α-,and β-H signals of ferulic acid (I), supporting that Glc A attached to 3-OH of cyanidin through a glycosidic bond, was acylated with ferulic acid (I) at 6-OH. Irradiation of 2-H of Xyl C gave a NOEDIF spectrum, in which NOEs were observed at the signals for α- and β-protons of caffeic acid (II) in addition to the signals for 1-H and 3-H of Xyl C. These data supported that Xyl C was acylated with caffeic acid (II) at the 2-position. By further irradiation of 6a-H (δ 4.49) of Glc E exhibiting NOEs at the signals for α-, β-, and 2-H of caffeic acid (III), Glc E was confirmed to be acylated with caffeic acid (III) at the 6-position. In the NOESY and NOEDIF spectra, the correlations between 5-H of cyanidin and H-1 of Glc B, 5-H of caffeic acid (II) and 1-H of Glc D, 5-H of caffeic acid (III) and 1-H of Glc D, and 5-H of ferulic acid and 1-H of Glc F were observed, respectively, revealing the glycosylation patterns; such as 5-OH of cyanidin was glycosylated with Glc B, 4-OH of caffeic acid (II) with Glc E, 4-OH of caffeic acid (III) with Glc D, and 4-OH of ferulic acid with Glc F. Based on these results, the structure of pigment **2** was determined as cyanidin 3-*O*-[2-*O*-(2-*O*-(4-*O*-(6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-feruloyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside.

2.3. Pigment 1 (OVA 1)

The FAB mass spectrum of pigment **1** gave its molecular ion [M^+] at *m/z* 1785 in good agreement with the mass calculated for $C_{80}H_{89}O_{46}$, which was composed of cyanidin with five molecules of glucose, two mole-

cules of caffeic acid, and one molecule each of xylose, *p*-coumaric acid and malonic acid. Thus, the structure of pigment **1** is assumed to have *p*-coumaric acid instead of sinapic acid in pigment **3**. The ^{1}H NMR spectrum of pigment **1** was similar to that of pigment **3** except for the signals of *p*-coumaric acid moiety. The detailed structure was determined according to the procedures as described as above for pigments **2** and **3** (Table 2). The linkages and/or attachment positions of the aglycone with sugars and acids were elucidated by the same procedures as described for pigments **2** and **3** using 2D COSY, NOESY and DIFNOE spectral methods. Finally, pigment **1** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(4-*O*-(6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-glucopyranosyl)-*trans*-caffeoyl)-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(6-O-malonyl-β-D-glucopyranoside).

To date, five reports on the occurrence of polyacylated anthocyanins in the family of Cruciferae have been published as summarized in Table 3. From the chemotaxonomical point of view, two typical anthocyanidin glycoside types have been known in this family, such as anthocyanidin 3-sophoroside and 3-sambubioside. The flower anthocyanins (OVA **1**–**3**) of *Orychophragonus violaceus* with bulky acylated side chains at their 3-positions are obviously classified into the latter group (Table 3).

Regarding the bluing effect of anthocyanins by acylation with hydroxycinnamic acid, it is rationalized that the anthocyanins having three molecules of hydroxycinnamic acids display stronger blue color than those of the anthocyanins bearing two molecules of the corresponding acids (Honda and Saito, 2002). Actually, the blue flower color (Violet-Blue 90C) by the Royal Horticultural Society (RHS). Color Chart of *O. violaceus* having three molecules of hydroxycinnamic acids, is stronger than that (Purple 76A-Violet 84A by RHS Color Chart) of *Matthiola incana* bearing two molecules of the corresponding acids (Saito et al., 1995). The blue flower colors of pigments **1–3** probably arose from the formation of intramolecular co-pigmentations between anthocyanidin and hydroxycinnamic acids presented in their molecules.

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, 4:1:2), BuH (*n*-BuOH–2N HCl, 1:1), 1% HCl and AHW (HOAc–HCl–H_2O, 15:3:82) for anthocyanins, and BAW, EAA (EtOAc–HCOOH–H_2O, 5:2:1) for organic acids and sugars. Analytical HPLC was performed on a LC-10A system (Shimadzu), using a Waters C_{18} (4.6$_\varphi$, 250 mm) column at 40 °C with a flow rate 1 mL/min and monitoring at 530 nm. The eluant was applied as a linear gradient 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 in H_2O). UV–VIS spectra were recorded on a MPS-2400 (Shimadzu) in 0.1% HCl–MeOH (from 200 to 700 nm), whereas 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 ^{1}H spectra and 125.78 MHz

Table 3
Polyacylated anthocyanins with aromatic acid in the Cruciferae

Species	Anthocyanins	Deacylaanthocyanii	Aromatic acid	Molecular numbers of hydroxycinnamic acid
Brassica oleracea[a]	Brassica anthocyanins	Cy 3-sopho-5-glu	*p*-coumaric, ferulic, sinapic acid	2
Raphanus sativus[b]	Radish anthocyanins	Pel 3-sopho-5-glu	*p*-coumaric, caffeic, ferulic acid	2
Arabidopsis thaliana[c]	Arabidopsis anthocyanins	Cy 3-sam-5-gIu	*p*-coumaric, sinapic acid	2
Matthiola incana[d]	Matthiola red anthocyanins	Pel 3-sam-5-glu	*p*-couinaric, caffeic, ferulic, sinapic acid	2
Matthiola incana[e]	Matthiola violet-blue anthocyanins	Cy 3-sam-5-glu	p-couniaric, caffeic, ferulic, sinapic acid	2
Orychophragonus violacens	Orychophragonus violet-blue anthocyanins	Cy 3-sam-5-glu	*p*-coumaric, caffeic, ferulic, sinapic acid	3
Sinapis alba[f]	Sinapis anthocyanins	Cy 3-sam-5-glu Cy 3-sam-5-sopho	*p*-coumaric, ferulic, sinapic acid	2

Pel = Pelaigonidin, Cy = Cyanidin, soplio = sophoroside, glu = glucoside, sam = sambubioside.
[a] Hrazdina et al. (1977), Idaka et al. (1987).
[b] Otsuki et al. (2002), Ishikura and Hayashi (1963).
[c] Bloor and Abrahams (2002).
[d] Saito et al. (1996).
[e] Saito et al. (1995).
[f] Takeda et al. (1988).

for ^{13}C spectra in DMSO–CF_3COOD (9:1). Chemical shifts are reported relative to a TMS internal standard, and coupling constants are in Hz.

3.2. Plant materials

The seeds of *Orychophragonus violaceus* were purchased from the Takii nursery (Japan), and grown in the experimental garden of Hoshi University. Fresh violet-blue flowers (Violet-Blue 90C by RHS color chart and its chromaticity values $L^* = 63.0$, $b^*/a^* = 28.4/-22.2$) were collected in spring. The chromaticity values were recorded on a ZE 2000 color difference meter (Nippon Denshoku Co., Ltd.).

3.3. Extraction and purification of anthocyanins

Fresh flowers (2.1 kg) were immersed in 0.5% HOAc (10 L) for 4 days at room temperature (ca. 20 °C). During the extraction, any partial hydrolysis was not observed by monitoring with HPLC. The extract was subjected to Diaion HP-20 (Mitsubushi Chemical's Ion Exchange Resins) column chromatography (CC) and washed with H_2O (15 L). The pigments were eluted with 5% HOAc–MeOH (500 mL). After concentration, the eluate was fractionated with paper chromatography (PC) using BAW. The crude fractionated pigments obtained, were further purified by TLC (15% HOAc) and prep. HPLC. Prep. HPLC was performed on a Waters C_{18} (19_{φ}, 150 mm) column at 40 °C with a flow rate of 4 mL/min monitoring at 530 nm. The solvent system used was as follows: a linear gradient elution for 18 min from 55% to 85% solvent B in solvent A. Each fraction was transformed to a Diaion HP-20 column, and its anthocyanin was absorbed on the column. Anthocyanins were eluted with 5% HOAc–MeOH from the column, and evapn anthocyanin residues were dissolved in a small volume of 5% HOAc–EtOH, respectively, followed by addition of an excess of Et_2O. Anthocyanin powders, pigment **1** (Orychophragonus blue anthocyanin 1; OVA 1) (5 mg), pigment **2** (OVA 2) (7 mg), and pigment **3** (OVA 3) (15 mg), were obtained from the extract.

3.4. Acid and alkaline hydrolysis of pigments *1–3*

Acid hydrolysis of pigments **1–3** (ca. 3 mg) was carried out with 2 N HCl (15 mL) at 80–100 °C for 1 h to give cyanidin, glucose, xylose, and caffeic and malonic acids by the standard procedure (Harborne, 1984). Those compounds were confirmed by direct comparison of TLC and HPLC with the authentic samples. Hydroxycinnamic acids obtained from pigment **1** (*p*-coumaric acid), pigment **2** (ferulic acid), and pigment **3** (sinapic acid), respectively, were also identified by the analyses of their spectral data in addition to the comparison of TLC and HPLC with the authentic samples. Malonic acid from pigments **1** and **3** was also identified by direct comparison of the authentic sample. Alkaline hydrolysis of pigments **1–3** (ca. 3 mg) was carried out with 2 N NaOH solution (20 mL) at ambient temperature for 3 h to provide only one deacylated anthocyanin, whose structure was identified to be cyanidin 3-sambubioside-5-glucoside by the analyses of TLC and HPLC with the authentic sample obtained from Matthiola violet anthocyanins by the same alkaline hydrolysis.

3.5. 4-Glucosylcaffeic acid

UV–Vis λ_{max} (MeOH): 283, 312; R_f values (TLC) BAW 0.55; 6% HOAc 0.46; R_t 7.5 min.

3.6. High resolution FABMS

Orychophragonus violet-blue anthocyanin 1 (OVA 1; pigment **1**): HR-FABMS calc. for $C_{80}H_{89}O_{46}$: 1785.4625. Found: 1785.4619.

OVA 2 (pigment **2**): HR-FABMS calc. for $C_{78}H_{89}O_{44}$: 1729.4727. Found: 1729.4757.

OVA 3 (pigment **3**): HR-FABMS calc. for $C_{82}H_{93}O_{48}$: 1845.4837. Found: 1845.4810.

References

Bloor, S.J., Abrahams, S., 2002. The structure of the major anthocyanin in *Arabidopsis thaliana*. Phytochemistry 59, 343–346.

Harborne, J.B., 1984. Phytochemical Methods, second ed. Chapman and Hall, London.

Honda, T., Saito, N., 2002. Recent progress in the chemistry of polyacylated anthocyanins as flower color pigments. Heterocycles 56, 633–692.

Hrazdina, G., Iredala, H., Mattick, L.R., 1977. Anthocyanin composition of *Brassica oleracea* cv. Red Danish. Phytochemistry 16, 297–299.

Idaka, E., Yamakita, H., Ogawa, T., Kondo, T., Yamamoto, M., Goto, T., 1987. Structure of three diacylated anthocyanins isolated from Red Cabbage, *Brassica oleracea*. Chem. Lett., 1213–1216.

Ishikura, N., Hayashi, K., 1963. Chromatographic separation and characterization of the component anthocyanins in radish root. Bot. Mag. Tokyo 76, 6–13.

Kondo, T., Kawai, H., Tamura, H., Goto, T., 1987. Structure determination of Heavenly Blue Anthocyanin, a complex monomeric anthocyanin from the morning glory *Iomoea tricolor*, by means of the negative NOE method. Tetrahedron Lett. 28, 2273–2276.

Otsuki, T., Matsufuji, H., Takeda, M., Toyoda, M., Goto, T., 2002. Acylated anthocyanins from red radish (*Raphanus sative*L.). Phytochemistry 60, 79–87.

Saito, N., Tatsuzawa, F., Nishiyama, A., Yokoi, M., Shigihara, A., Honda, T., 1995. Acylated cyanidin 3-sambubioside-5-glucoside in Matthiola incana. Phytochemistry 38, 1027–1032.

Saito, N., Tatsuzawa, F., Hongo, A., Khin, W.W., Yokoi, M., Shigihara, A., Honda, T., 1996. Acylated pelargonidin 3-sambubioside-5-glucoside in Matthiola incana. Phytochemistry 41, 1613–1620.

Saito, N., Toki, K., Moriyama, H., Shigihara, A., Honda, T., 2002. Acylated anthocyanins from the blue-violet flowers of *Anemone coronaria*. Phytochemistry 60, 365–373.

Takeda, K., Fischer, D., Grisebach, H., 1988. Anthocyanin composition of *Sinapis alba*, light induction of enzymes and biosynthesis. Phytochemistry 27, 1351–1353.

Tatsuzawa, F., Saito, N., Miyoshi, K., Shinoda, K., Shigihara, A., Honda, T., 2004. Diacylated 8-C-glucosylcyanidin 3-glucoside from the flowers of *Tricyrtis formosama*. Chem. Pharm. Bull. 52, 631–633.

Toki, K., Saito, N., Kawano, K., Lu, T.S., Shigihara, A., Honda, T., 1994. An acylated delphinidin glycoside in the blue flowers of *Evolvulus pilosus*. Phytochemistry 36, 609–612.