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Triacylated cyanidin 3-(3^X-glucosylsambubioside)-5-glucosides from the flowers of *Malcolmia maritima*

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Abstract

Three acylated cyanidin 3-(3^X-glucosylsambubioside)-5-glucosides (**1**–**3**) and one non-acylated cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside (**4**) were isolated from the purple-violet or violet flowers and purple stems of *Malcolmia maritima* (L.) R. Br (the Cruciferae), and their structures were determined by chemical and spectroscopic methods. In the flowers of this plant, pigment **1** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-(β-D-glucopyranoside) as a major pigment, and a minor pigment **2** was determined to be the *cis*-*p*-coumaroyl isomer of pigment **1**. In the stems, pigment **3** was determined to be cyanidin 3-*O*-[2-*O*-(2-*O*-(*trans*-sinapoyl)-3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) as a major anthocyanin, and also a non-acylated anthocyanin, cyanidin 3-*O*-[2-*O*-(3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) was determined to be a minor pigment (pigment **4**). In this study, it was established that the acylation-enzymes of malonic acid has important roles for the acylation of 5-glucose residues of these anthocyanins in the flower-tissues of *M. maritima*; however, the similar enzymatic reactions seemed to be inhibited or lacking in the stem-tissues.

Keywords: *Malcolmia maritima* (L.) R. Br; Cruciferae; Acylated anthocyanins; Cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside; *p*-Coumaric acid; Sinapic acid; Malonic acid; Flower color

1. Introduction

Malcolmia maritima (L.) R. Br. (Virginia stock in English) is a plant species native mainly to the Mediterranean region, and cultivated as a popular annual garden plant with white, pink, purple-violet, and violet flowers.

In the continuing work on flower color variation due to acylated anthocyanins of the ornamental plants in the Cruciferae, we have already reported the distribution of structurally complicated acylated anthocyanins in the flowers of *Matthiola incana* (Saito et al., 1995, 1996), *Orychophragonus violaceus* (Honda et al., 2005) and *Cheiranthus cheiri*, *Lobularia maritima*, and *Lunaria annua* (Tatsuzawa et al., 2006, 2007). As part of our continuing work, we are interested in the structures of the floral anthocyanins of *M. maritima*, since anthocyanins of this plant have not been thoroughly studied.

In this paper, we report the structure elucidation of cyanidin 3-(3^X-glucosylsambubioside)-5-glucoside, a novel cyanidin glycoside pattern, and also its three acylated anthocyanin derivatives in *M. maritima* of the Cruciferae.

2. Results and discussion

Four anthocyanin pigments (**1** –**4**) (Figs. 1 and 2) were found in the methanol–acetic acid–water (MAW: 4:1:5,

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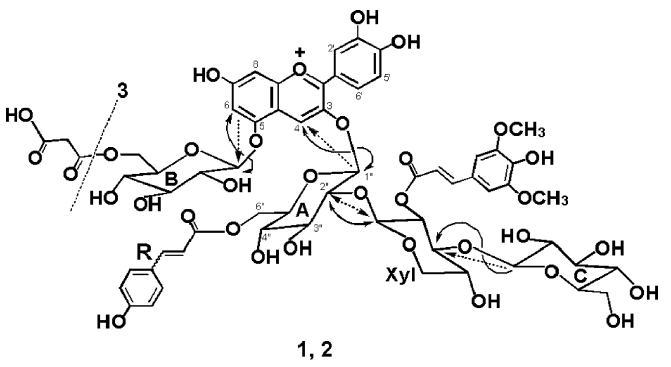
v/v/v) extracts from purple-violet to violet flowers, and also purple stems of *M. maritima* by HPLC analysis (Fig. 2). Their frequencies as estimated by HPLC were pigment **1** (64.4%), pigment **2** (8.6%) and pigment **3** (2.3%) in the MAW extract from their flowers. On the other hand, in the MAW extract from its stems pigment **1** (1.5%), pigment **3** (42.3%) and pigment **4** (3.8%) were obtained together with three other small pigment peaks.

These four pigments (**1**–**4**) were extracted from the mixture of flowers and stems with 5% HOAc, and purified using Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column chromatography (CC), preparative HPLC and TLC, according to the procedures described previously (Tatsuzawa et al., 2006, 2007). The chromatographical data and spectral properties of these pigments are shown in Table 1.

Acid hydrolysis of all four pigments (**1**–**4**) resulted in cyanidin, glucose, and xylose. Moreover, *p*-coumaric acid and sinapic acid were detected in the hydrolysates of pigments **1**, **2** and **3**, and also malonic acid was detected in the hydrolysates of pigments **1** and **2** by TLC (Harborne, 1984). Alkaline hydrolysis of pigments **1**–**3** resulted in only one deacylated anthocyanin, whose structure was identified to be pigment **4** by the analyses of TLC and HPLC (Table 1). The deacylanthocyanin was hydrolyzed with 2 N HCl by heating in a water bath of about 80–100 °C for 1, 5, and 10 min. By the analysis of TLC and HPLC, the four intermediary glucosides (3-glucoside, 5-glucoside, 3,5-diglucoside, 3-sambubioside-5-glucoside of cyanidin) as well as deacylanthocyanin were detected in three partial hydrolysates. From these results, the structure of the deacylanthocyanin (pigment **4**) was presumed to be a glucosyl cyanidin 3-sambubioside-5-glucoside. The structures of the four anthocyanins (pigments **1**–**4**) were further elucidated based on the analyses of their ^{1}H NMR spectra (500 MHz) and ^{13}C NMR (125.78 MHz) in DMSO-d_6–CFCOOD (9:1), including 2D COSY, NOESY, ^{1}H–^{13}C HMQC, ^{1}H–^{13}C HMBC and negative difference NOE (DIFNOE) spectra.



1, 2

Fig. 1. Pigment **1**, **2** and **3** in the flowers and stems of *Malcolmia mantima*. Pigment **1** and **3** R = *trans*, Pigment **2**, R = *cis*. Observed main NOE's are indicated by arrows. Observed HMBCs are indicated by dotted arrows.

2.1. Pigment 4 and deacyl pigments 1, 2 and 3

The molecular ions $[M]^{+}$ of pigment **4** and deacyl pigments **1**, **2** and **3** were observed at *m*/*z* 905 ($C_{38}H_{49}O_{25}$), indicating that these pigments are composed of cyanidin with three molecules of glucose and one molecule of xylose. The elemental components of pigment **4** were confirmed by measuring its high-resolution FABMS (HRMS), and the mass data obtained are summarized in Section 4.4. As mentioned before deacyl pigments **1**, **2** and **3** are identical with pigment **4**. Therefore, only the structure elucidation of pigment **4** was carried out as follows.

From the analysis of the ^{1}H NMR spectra of pigment **4**, the chemical shifts of six aromatic protons of cyanidin were assigned as shown in Table 2. Also four signals of anomeric protons were observed at δ 5.59 (*d*, $J = 7.6$ Hz, glu A), δ 5.09 (*d*, $J = 7.6$ Hz, glu B), δ 4.80 (*d*, $J = 8.0$ Hz, xylose) and δ 4.34 (*d*, $J = 7.7$ Hz, glu C). Based on the observed coupling constants (Table 2), the four sugars were assumed to have a β-pyranose form.

The linkages and/or positions of the attachment of the sugar groups in this pigment whose structure was presumed to be glucosyl cyanidin 3-sambubioside-5-glucoside by the analysis of its partial hydrolysate were mainly determined by using 2D COSY, NOEDIF and HMBC experiments. By NOEDIF experiments, strong NOEs were observed between H-4 of cyanidin and H-1 of glu A, and H-6 of cyanidin and H-1 of glu B indicating that cyanidin was glycosylated with glu A at OH-3 of cyanidin and also glycosylated with glu B at OH-5 of cyanidin. A proton signal (δ 3.94, *t*, $J = 8.2$ Hz) shifted to a lower magnetic field was assigned to H-2 of glu A by the analysis of 2D COSY spectrum of pigment **4**. This resonance was correlated to the ^{13}C-1 (δ 103.9) of xylose and also to the resonance of H-1 (xylose, δ 4.80) was correlated to the ^{13}C-2 (δ 80.5) of glu A in the HMBC spectrum, supporting that xylose was linked to OH-2 of glu A forming sambubiose. Furthermore, a strong NOE was observed at H-3 of xylose by the irradiation of NOEDIF experiment at H-1 of glu C indicating that glu C was linked to the OH-3 of xylose. This result was confirmed by the analysis of HMBC spectrum (Fig. 1).

Therefore, pigment **4** and deacyl pigments **1**–**3** were determined to be cyanidin 3-*O*-[2-*O*-(3-*O*-(β-D-glucopyranosyl)-β-D-xylopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside, which is a new cyanidin glycoside in plants (Harborne and Baxter, 1999; Andersen and Jordheim, 2006).

2.2. Pigment 1

The molecular ion $[M]^{+}$ of pigment **1** was observed at *m*/*z* 1343 ($C_{61}H_{67}O_{34}$) indicating that pigment **1** is composed of cyanidin with three molecules of glucose, one molecule each of xylose, as well as sinapic, *p*-coumaric and malonic acids. The elemental components were confirmed by measuring its HRMS, and the mass data obtained were