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An unusual acylated malvidin 3-glucoside from flowers of *Impatiens textori* Miq. (Balsaminaceae)

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ABSTRACT

Acylated malvidin 3-glucoside was isolated from the purple flowers of *Impatiens textori* Miq. as a major anthocyanin component along with malvidin 3-(6-malonyl-glucoside). Its structure was elucidated to be malvidin 3-*O*-[6-*O*-(3-hydroxy-3-methylglutaryl)-β-glucopyranoside] by chemical and spectroscopic methods.

1. Introduction

Impatiens textori Miq. (Balsaminaceae) is a native plant to Japan, Korea and northeast China, and has an attractive purple flower color. The plant is also used as a Chinese traditional medicine for detoxification and for treatment of carbuncles and contusions, and its alcoholic extract has been shown to contain several flavones and flavonols (Ueda et al., 2003). With the anthocyanin components in *I. textori*, the occurrence of malvidin 3,5-diglucoside and a malvidin hexoside in the flowers has been observed by a chromatographic survey of anthocyanins in the flora of Japan (Ueno et al., 1969). However, there has been no report on the chemical investigation of acylated anthocyanins in this plant.

In continuing work on flower color variation due to acylated anthocyanins in ornamental plants (Honda and Saito, 2002; Tatsuzawa et al., 2006), an investigation of *I. textori* anthocyanins was carried out. In this study, a novel acylated anthocyanin with 3-hydroxy-3-methyl-glutaric acid was isolated from this plant species.

In this paper, we report its isolation and structural elucidation as a major floral anthocyanin of *I. textori* along with a known anthocyanin.

2. Results and discussion

Fresh flowers of *I. textori* were immersed in 5% HOAc for 2 h at room temperature to extract anthocyanin pigments of the flowers. Two main peaks (pigments **1** and **2**) were found in the extract by HPLC analysis. The relative frequencies of occurrence were pigment **1** (62.1%, R_t 28.8) and pigment **2** (22.9%, R_t 27.6). From the dry flowers of this plant, pigments **1** and **2** were isolated as dark-violet powders by using the process described previously (Tatsuzawa et al., 2006). The chromatographic and spectroscopic properties of pigments **1** and **2** are shown in Sections 4.4.1. and 4.4.2.

Acid hydrolysis of pigments **1** and **2** resulted in release of malvidin and glucose. In addition, pigment **1** contained 3-hydroxy-3-methylglutaric acid and pigment **2** contained malonic acid as acyl components. On alkaline hydrolysis, both pigments gave the same deacylated anthocyanin; 3-hydroxy-3-methylglutaric acid was also released from pigment **1** and malonic acid from pigment **2** in the hydrolysates. Both acid components were identified in comparison with authentic samples by analysis of HPLC and TLC (see Section 4.4.). The deacylated anthocyanin was unambiguously identified to be malvidin 3-glucoside by direct comparison with an authentic sample, malvidin 3-glucoside, obtained from the flowers of *Hibiscus syriacus* (Kim et al., 1989) (see Section 4.4).

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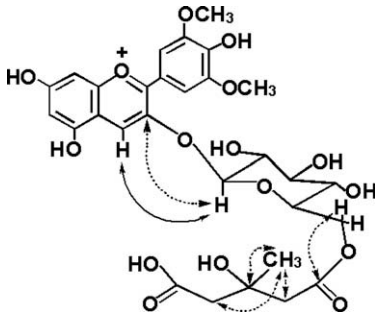

Fig. 1. Acylated anthocyanin (pigment **1**) isolated from the flower of *Impatiens textori*. Observed main NOEs are indicated by arrows. Observed important HMBCs are indicated by dotted arrows.

Pigment **2** was similarly identified to malvidin 3-[6-(malonyl)-glucoside] by analysis of HPLC and TLC in comparison with the authentic sample, malvidin 3-[6-(malonyl)-glucoside], obtained from the flowers of *H. syriacus* (Kim et al., 1989) (see Section 4.4.2).

2.1. Pigment *1*

The FAB mass spectrum of pigment **1** gave a molecular ion $[M]^+$ of *m*/*z* 637 (calc. for $C_{29}H_{33}O_{16}$), indicating that it was composed of malvidin with one molecule each of glucose and 3-hydroxy-3-methylglutaric acid. The elemental components were confirmed by measuring the high-resolution FAB MS (calc. $C_{29}H_{33}O_{16}$: 637.1769, found: 637.1780). The structure of pigment **1** was further elucidated on the basis of analyses of its ^1H and ^{13}C NMR spectra [500 MHz for ^1H and 125.78 MHz for ^{13}C spectra in DCl–DMSO-d_6 (1:9) or DCl–CD_3OD (1:9)], including 2D COSY, 2D NOESY, HMQC and HMBC spectra. The chemical shifts of four aromatic protons and two methoxyl protons (6H, 2 × OMe) of malvidin were assigned by analysis of the 2D COSY spectrum as summarized in Section 4.4.1. Chemical shifts of the methyl protons [δ 1.25, (3H)] and two methylene protons [4H, 2 × CH_2; δ 2.53 (2H), 2.62 (1H) and 2.68 (1H)] of 3-hydroxy-3-methylglutaric acid were also assigned by the same technique (Section 4.4.2.). Chemical shifts of the sugar moiety were observed in the region of δ 5.37–3.41, where the anomeric proton resonated at δ 5.37 (d, J = 7.6 Hz). Based on the observed coupling constants (see Section 4.4.1.), the sugar was assumed to be in a β-pyranose form. By analysis of the 2D COSY spectrum, two characteristic proton signals (δ 4.21 and 4.46 for H-6a and 6b) that were shifted to a lower magnetic field were assigned to methylene protons of the glucose residue. This indicated that the OH-6 group of the glucose residue was acylated with 3-hydroxy-3-methylglutaric acid. The linkages and/or positions of attachments of the sugar and acid groups were determined by analysis of 2D NOESY and HMBC spectra (Fig. 1). A through-space correlation between H-4 (δ 8.96) of malvidin and H-1 of glucose was observed in its NOESY spectrum indicating the OH-3 of malvidin was glycosylated with glucose. This result was confirmed by analysis of the HMBC spectrum. Furthermore, a long-range correlation between H-6a (δ 4.21) of glucose and CO (δ 172.0) of 3-hydroxy-3-methylglutaric acid was observed by analysis of its HMBC spectrum, supporting the result indicating that the OH-6 group of glucose was acylated with this acid (Fig. 1). Therefore, the structure of pigment **1** was determined to be malvidin 3-*O*-[6-*O*-(3-hydroxy-3-methylglutaroyl)-β-glucopyranoside], a new anthocyanin.

3. Concluding remarks

To the best our knowledge, this is the first report of the presence of an anthocyanin acylated with 3-hydroxy-3-methylglutaric acid in plants, although there are some reports on the distributions of flavones and flavonols acylated with this acid, such as in *Citrus* (Berhow et al., 1994; Kumamoto et al., 1985; Horie et al., 1986), *Rheum* (Iwashina et al., 2004), *Rubus* (Wald et al., 1986), *Viscum* (Harborne and Baxter, 1999) and *Frullania* (Kraut et al., 1993). The occurrence of a new type of acylated anthocyanin provides further interesting information for researchers in this field of chemistry.

4. Experimental

4.1. General procedures

TLC was carried out on cellulose-coated plastic sheets (Merck) using eight mobile phases: BAW (*n*-BuOH–HOAc–H_2O, 4:1:2), BuHCl (*n*-BuOH–2 N HCl, 1:1, upper layer), AHW (HOAc–HCl–H_2O, 15:3:82), 1% HCl and Forestal (HOAc–HCl–H_2O, 30:3:10) for anthocyanins, and BAW, 15% HOAc, EAA (EtOAc–HCOOH–H_2O, 5:2:1) and ETN (EtOH–NH_4OH-H_2O, 16:1:3) for sugars and organic acids with aniline hydrogen phthalate (AHP) spray reagent and bromocresol green (BCG) spray reagent (Harborne, 1984).

Analytical HPLC was performed on an LC 10A system (Shimadzu) using a Waters C18 (4.6φ × 250 mm) column at 40 °C with a flow rate of 1 mL/min and monitoring at 530 nm for anthocyanins and monitoring at 210 nm for organic acids. The eluants for anthocyanins were applied as linear gradient elutions for 40 min 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). The other eluant for organic acids was applied as an isocratic elution of solvent A for 10 min.

UV–Vis spectra were recorded on a 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 measured at 500 MHz for ^1H spectra and at 125.78 MHz for ^{13}C spectra in DCl–DMSO-d_6 (1:9) and DCl–CD_3OD (1:9). Chemical shifts are reported relative to a TMS internal standard (δ), and coupling constants are in Hz.

4.2. Plant materials

Wing petals (*ca.* 200 g) of purple flowers [Purple 78B by Royal Horticultural Society color chart and chromaticity value, b^* (−12.85)/a^* (41.09)] of *I. textori* were collected from summer to autumn (from 2000 to 2005) in Fukagawa City, Hokkaido, Japan, and were dried at 45 °C and kept in a refrigerator at about 4 °C. The chromaticity values were recorded on a CM-2002 Spectro Color Meter (Minolta Co., Ltd.). Identification of species depends on that of Satake (1982), with the plant identification confirmed by Yuki Mikanagi. A voucher specimen (CBM-BS-239748) is deposited at the Natural History Museum and Institute, Chiba, Japan.

4.3. Isolation of anthocyanin

Dried flowers (*ca.* 20 g) were immersed in 5% HOAc (3L; HOAc–H_2O, 1:19) at room temperature overnight and extracted. The extracted pigments were adsorbed on a Diaion HP-20 (Mitsubishi Chemical's Ion Exchange Resins) column (90φ × 150 mm), and the absorbed pigments were washed with H_2O. The pigments were then eluted with HOAc–MeOH (5:95, v/v, 500 mL) to recover anthocyanins. After concentration, the eluates were separated and purified using paper chromatography (PC) using BAW. The separated pigments were further purified by TLC (developed by 15% HOAc) and prep. HPLC. Prep. HPLC was performed on a Waters C18 (4.6φ × 250 mm) column at 40 °C with a flow rate of 1 mL/min and monitoring at 530 nm with the same solvent system of

analysis. Each fraction was transferred to a Diaion HP-20 column, on which pigments were adsorbed. Anthocyanin pigments were eluted with 5% HOAc–MeOH (5:95, v/v) followed by addition of excess Et_2O and then dried. The purified pigments **1** (*ca.* 5 mg) and **2** (*ca.* 1 mg) were obtained as dried dark-violet powders.

4.4. Chemical and spectroscopic analyses of purified anthocyanins

Acid hydrolyses of pigments **1** (*ca.* 1 mg) and **2** (*ca.* 1 mg) were carried out using 2 N HCl (2 mL) at 100 °C for 2 h, to provide malvidin, glucose and 3-hydroxy-3-methylglutaric acid from pigment **1**, and malvidin, glucose and malonic acid from pigment **2**. Moreover, alkaline hydrolyses of pigments **1** (*ca.* 1 mg) and **2** (*ca.* 1 mg) were carried out with 2 N NaOH solution (1 mL) under N_2 gas at ambient temperature for 15 min to give the same deacylated anthocyanin, whose structure was identified to be malvidin 3-glucoside in comparison with authentic sample obtained from *H. syriacus* (Kim et al., 1989).

The authentic sample of 3-hydroxy-3-methylglutaric acid is commercially available (Wako Pure Chemical Industries, Ltd.). TLC, Yellow coloration with BCG (Bromocresol green (0.1 g) were dissolved in 100 mL of ethanol. To this solution, 2 N NaOH was added until the color of the solution become blue.), BAW 0.75, 15% HOAc 0.82, HPLC, R_t (min) 5.6 min. Malonic acid: TLC, Yellow coloration with BCG, BAW 0.63, 15% HOAc 0.78, HPLC, R_t (min) 3.2 min. Glucose: TLC, Brown coloration with AHP (*o*-Phthalic acid (1.66 g) and 0.91 mL of aniline were dissolved in 100 mL of *n*-BuOH saturated with H_2O.), BAW 0.21, EAA 0.33, ETN 0.83. Malvidin: TLC, mauve coloration in visible light, Forestal 0.56, HPLC R_t (min) 32.3 min. Malvidin 3-glucoside: UV–Vis (in 0.1% HCl–MeOH), λ_{max} 538, 280 nm, E_{440}/E_{max} (%) 23, +$AlCl_3$ 0 shift; TLC (R_f x100) BAW 27, BuHCl 14, 1% HCl 4, AHW 14; HPLC (R_t (min)) 22.0 min.

4.4.1. Malvidin 3-[6-(3-hydroxy-3-methylglutaroyl)-glucoside] (pigment **1**)

UV–Vis (in 0.1% HCl–MeOH), λ_{max} 540, 274 nm, E_{440}/E_{max} (%) 22, +$AlCl_3$ 0 shift; TLC (R_f x100) BAW 52, BuHCl 41, 1% HCl 10, AHW 36; HPLC (R_t (min)) 28.8 min; FAB-MS *m/z* 637 $[M]^+$ (calc. for $C_{29}H_{33}O_{16}$), HR-FAB MS (calc. 637.1769, found: 637.1780); ^1H NMR (500 MHz, DCl–CD_3OD = 1:9): δ for malvidin: 8.96 (s, H-4), 6.70 (br d, J = 1.9 Hz, H-6), 7.01 (br d, J = 1.9 Hz, H-8), 7.96 (2H, s, H-2′, 6′), 4.00 (s, 2 *x*-CH_3). For glucose: 5.37 (d, J = 7.6 Hz, H-1), 3.64 (t, J = 8.6 Hz, H-2), 3.5 (t, J = 8.9 Hz, H-3), 3.41 (t, J = 8.9 Hz, H-4), 3.79 (ddd, J = 2.1, 7.2, 8.9 Hz, H-5), 4.21 (dd, J = 7.2, 12.2 Hz, H-6a), 4.46 (dd, J = 2.1, 12.2 Hz, H-6b). For 3-hydroxy-3-methylglutaric acid: 2.53 (d, J = 4.3 Hz, H-2a or 4a), 2.53 (d, J = 4.3 Hz, H-2b or 4b), 2.62 (d, J = 14.7 Hz, H-4a or 2a), 2.68 (d, J = 14.7 Hz, H-4b or 2b), 1.25 (s, –CH_3). ^{13}C NMR (125.78 MHz, DCl–CD_3OD = 1:9): δ for malvidin: 163.4 (C-2), 148.0 (C-3), 137.5 (C-4), 156.3 (C-5), 103.8 (C-6), 170.9 (C-7), 98.3 (C-8), 158.1 (C-9), 113.6 (C-10), 119.7 (C-1′), 110.8 (C-2′), 149.8 (C-3′), 145.6 (C-4′), 149.8 (C-5′), 110.8 (C-6′), 57.4 (–CH_3). For glucose: 103.8 (C-1), 75.0 (C-2), 77.9 (C-3), 71.4 (C-4), 76.0 (C-5), 64.5 (C-6). For 3-hydroxy-3-methyl-glutaric acid: 172.3 (CO), 46.1 (C-2 or 4), 70.7 (C-3), 46.1 (C-4 or 2), 173.3 (COOH), 27.8 (–CH_3).

4.4.2. Malvidin 3-(6″-malonyl)-glucoside (pigment **2**)

UV–Vis (in 0.1% HCl–MeOH), λ_{max} 539, 275 nm, E_{440}/E_{max} (%) 24, +$AlCl_3$ 0 shift; TLC (R_f x100) BAW 33, BuHCl 24, 1% HCl 5, AHW 19; HPLC (R_t (min)) 27.6 min.

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