Acetylated Anthocyanin 3-\(O\)-di-glycosides in Red-purple Flowers and Grayed-purple Leaves of *Saintpaulia* ‘Tomoko’

Fumi Tatsuzawa\(^1\)*, Sayumi Matsuda\(^2\), Kazuhisa Kato\(^1\) and Munetaka Hosokawa\(^2\)

\(^1\)Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan
\(^2\)Graduate School of Agriculture, Kyoto University, Kyoto 606-8520, Japan

A new acetylated anthocyanin was extracted from the red-purple flowers of *Saintpaulia* ‘Tomoko’ with 5% HOAc-H\(_2\)O or 5% formic acid-H\(_2\)O, and determined to be peonidin 3-\(O\)-[6-\(O\)-(4-\(O\)-(acetyl)-\(\alpha\)-rhamnopyranosyl)-\(\beta\)-glucospyranoside] (1), by chemical and spectroscopic methods. In addition, two known acetylated cyanidin glycosides, cyanidin 3-\(O\)-[6-\(O\)-(4-\(O\)-(acetyl)-\(\alpha\)-rhamnopyranosyl)-\(\beta\)-glucospyranoside] (2) and cyanidin 3-\(O\)-[2-\(O\)-(\(\beta\)-xylopyranosyl)-6-\(O\)-(acetyl)-\(\beta\)-glucopyranoside] (3), were also identified in the red-purple flowers and grayed-purple leaves of *S.* ‘Tomoko’, respectively. These three anthocyanins have not been reported hitherto in plant tissues in the genus *Saintpaulia*.

**Key Words:** cyanidin 3-acetyl-rutinoside, cyanidin 3-acetyl-sambubioside, peonidin 3-acetyl-rutinoside.

**Introduction**

*Saintpaulia* cultivars (Gesneriaceae) are widely cultivated as one of the most popular ornamental indoor plants bred from some wild species native to Southern Africa, with white, pink, red, red-purple, purple, and violet-blue flowers. Recently, we reported the structural determination of malvidin, peonidin, and pelargonidin 3-acetyl-rutinoside-5-glucosides in the violet-blue, purple, and pink flowers of *Saintpaulia* ‘Thamires’ (Saintpaulia sp.) along with apigenin 4’-glucuronide as 5% HOAc-H\(_2\)O extract (Tatsuzawa et al., 2012). However, there seem to be no previous reports on acylated peonidin and/or cyanidin 3-\(O\)-di-glycosides in the flowers and leaves of *Saintpaulia*, except some studies of non-acylated cyanidin 3-sambubioside in the leaves of *Saintpaulia ionantha* (Harborne, 1966). As part of our ongoing research on various flower colors in *Saintpaulia* cultivars, we studied the anthocyanin components of the red-purple flowers of *S.* ‘Tomoko’ along with grayed-purple leaves of *S.* ‘Tomoko’. In this paper, we report the isolation and structural elucidation of a new acylated anthocyanin and two known ones from the flowers and/or leaves of *S.* ‘Tomoko’.

**Materials and Methods**

**General procedures**

Thin-layer chromatography (TLC) was performed on cellulose-coated plastic sheets (Merck, Darmstadt, Germany) using six mobile phases: BAW (\(n\)-BuOH/\(HOAc/H_2O\), 4:1:2, v/v/v), BuHCl (\(n\)-BuOH/2N HCl, 1:1, v/v, upper layer), AHW (\(HOAc/HCl/H_2O\), 15:3:82, v/v/v), 1% HCl for anthocyanins and organic acid, BAW and ETN (EtOH/N\(_4\)OH/H\(_2\)O, 16:1:3, v/v/v) for sugars with detection using UV light and aniline hydrogen phthalate spray reagent (AHP), and Forestal (\(HOAc/HCl/H_2O\), 30:3:10, v/v/v) for anthocyanidins (Harborne, 1984).

Analytical HPLC was performed on an LC 10A system (Shimadzu, Kyoto, Japan), using a C18 (4.6 × 250 mm) column (Waters, Milford, MA, USA) at 40°C with a flow rate of 1 mL·min\(^{-1}\) and monitoring at 530 nm. The eluant by 5% HOAc-H\(_2\)O or 5% formic acid was applied as a linear gradient elution for 40 min from 20 to 85% solvent B (1.5% H\(_3\)PO\(_4\), 20% HOAc, 25% MeCN in H\(_2\)O) in solvent A (1.5% H\(_3\)PO\(_4\) in H\(_2\)O) with 5 min of re-equilibration at 20% solvent B, for anthocyanins, anthocyanidins, and hydroxycinnamic acids (method 1). The other eluant for acetic acid was applied as an isocratic elution of solvent A for 10 min and monitoring at 210 nm (Tatsuzawa et al., 2009) (method 2).

UV-Vis spectra for purified anthocyanins were recorded on an MPS-2450 (Shimadzu) in 0.1% HCl-MeOH (from 200 to 700 nm). Fast atom bombardment mass spectra (FABMS) were obtained in positive ion
mode using a 1:1 mixture of dithiothreitol and 3-nitrobenzyl alcohol as a matrix with JMS-700 (JEOL Ltd., Tokyo, Japan). NMR spectra were recorded on JNM AL-400 and JNM GX-500 (JEOL Ltd.) at 400 and 500 MHz for 1H spectra and 100 and 126 MHz for 13C spectra in DMSO-CD3OD (9:1). Chemical shifts are reported on the δ-scale from tetramethylsilane as the internal standard, and coupling constants (J) are in Hz. In addition, the alkaline and acid hydrolysis products of this anthocyanin producing deacetylanthocyanin, aglycone, acid, and sugars were analyzed by TLC (Harborne, 1984). Authentic samples, glucose, rhamnose, xylose, and acetic acid were used with commercial standards (Wako Pure Chemical Industries, Ltd., Osaka, Japan); peonidin 3-rutinoside, cyanidin 3-rutinoside, and cyanidin 3-sambubioside were obtained from Cymbidium (Tatsuzawa et al., 1996) and Corydalis ambigua (Tatsuzawa et al., 2005).

Plant materials

The red-purple petals [Red-Purple 61A by the Royal Horticultural Society Colour Chart and b*(20.06)/a*(59.79) = 0.34, L* = 16.17 by a SE-2000 Spectro Color Meter (Nippon Denshoku Industries Co., Ltd., Tokyo, Japan)] and the grayed-purple leaves [Grayed-Purple 187B and b*(-1.27)/a*(63.77) = −0.03, L* = 30.27] of S. ‘Tomoko’ (Royal Green Inc., Gifu, Japan) were grown in a greenhouse at the Experimental Farm of Kyoto University (Kyoto, Japan). Their petals and leaves were harvested from February to March, 2010. These petals and leaves were dried overnight at 40°C and kept in a refrigerator at −20°C until use.

Isolation and purification of pigments

Dried red-purple flowers (ca. 5 g) and grayed-purple leaves (ca. 50 g) of S. ‘Tomoko’ were immersed in 5% HOAc-H2O (5 L each) at room temperature for 12 h and then extracted. The extract was passed through a Diaion HP-20 (Nippon Rensui Co., Tokyo, Japan) column (90 × 150 mm), on which pigments were absorbed. Next the column was thoroughly washed with 5% HOAc-H2O (20 L) and eluted with 5% HOAc-MeOH (500 mL) to recover the pigments. After concentration, the pigments were separated and purified by paper chromatography using BAW. The separated pigments were further purified by preparative HPLC, which was performed on a Waters C18 (19 × 150 mm, Waters) column at 40°C with a flow rate of 4 mL/min and monitoring at 530 nm. The solvent used was as follows: a linear gradient elution for 15 min from 60% to 70% solvent B in solution A. The fraction was transferred to a Diaion HP-20 column, on which pig ment was adsorbed. Pigments were eluted with 5% HOAc-MeOH (5:95, v/v) followed by the addition of excess Et2O and then dried. Purified pigment 1 (ca. 25 mg), pigment 2 (ca. 5 mg), and pigment 3 (ca. 45 mg) were obtained as dried dark-red powders.

Analyses of pigment

The identification of anthocyanin was performed by standard procedures involving deacetylation with acid, and both alkaline and acid hydrolyses (Harborne, 1984).

1. Pigment 1

Dark-red powder: UV-VIS (in 0.1% HCl-MeOH): λmax 529, 294sh, 281 nm, Eε298/Emax = 31, AlCl3 shift 0; TLC: (Rf-values) BAW 0.52, BuHCl 0.53, 1% HCl 0.69, AHW 0.83; HPLC (method 1): Rt (min) 28.1; high-resolution FAB mass spectra (HR-FABMS) calc. for C39H32O16: 651.1925. Found: 651.1930; 1H NMR (400 MHz) δ Peonidin: 8.93 (s, H-4), 6.79 (d, J = 1.9 Hz, H-6), 7.05 (d, J = 1.9 Hz, H-8), 8.20 (d, J = 2.2 Hz, H-2'), 7.13 (d, J = 8.8 Hz, H-5'), 8.30 (dd, J = 2.2, 8.8 Hz, H-6'), 3.97 (s, -OCH3). Glucose: 5.47 (d, J = 7.6 Hz, H-1), 3.51 (t, J = 8.3 Hz, H-2), 3.45 (t, J = 8.9 Hz, H-3), 3.29 (t, J = 9.3 Hz, H-4), 3.72 (dd, J = 1.5, 6.1, 10.0 Hz, H-5), 3.57 (dd, J = 5.4, 11.3 Hz, H-6a), 3.91 (dd, J = 1.2, 11.3 Hz, H-6b). Rhamnose: 4.62 (d, J = 1.5 Hz, H-1), 3.69 (dd, J = 1.5, 3.3 Hz, H-2), 3.64 (dd, J = 3.3, 9.8 Hz, H-3), 4.75 (t, J = 9.8 Hz, H-4), 3.55 (m, H-5), 0.91 (d, J = 6.4 Hz, -CH3). Acetic acid: 1.97 (s, -CH3). 13C NMR (100 MHz) δ Peonidin: 161.9 (C-2), 144.2 (C-3), 135.6 (C-4), 156.3 (C-5), 102.7 (C-6), 169.0 (C-7), 94.7 (C-8), 157.8 (C-9), 112.2 (C-10), 119.7 (C-1'), 114.5 (C-2'), 148.4 (C-3'), 155.2 (C-4'), 117.5 (C-5'), 128.0 (C-6'), 56.2 (-OCH3). Glucose: 102.2 (C-1), 73.6 (C-2), 76.6 (C-3), 69.8 (C-4), 76.1 (C-5), 66.4 (C-6). Rhamnose: 100.6 (C-1), 70.5 (C-2), 68.5 (C-3), 74.5 (C-4), 66.2 (C-5), 17.5 (-CH3). Acetic acid: 21.0 (-CH3), 170.3 (COOH).

2. Pigment 2

Dark-red powder: UV-VIS (in 0.1% HCl-MeOH): λmax 530, 293sh, 281 nm, Eε298/Emax = 28, AlCl3 shift 0; TLC: (Rf-values) BAW 0.47, BuHCl 0.38, 1% HCl 0.33, AHW 0.60; HPLC (method 1): Rt (min) 24.1; high-resolution FAB mass spectra (HR-FABMS) calc. for C39H32O16: 637.1679. Found: 637.1786; 1H NMR (400 MHz) δ Cyanidin: 8.86 (s, H-4), 6.76 (d, J = 2.0 Hz, H-6), 6.94 (d, J = 2.0 Hz, H-8), 8.04 (d, J = 2.2 Hz, H-2'), 7.07 (d, J = 8.8 Hz, H-5'), 8.27 (dd, J = 2.2, 8.8 Hz, H-6'). Glucose: 5.45 (d, J = 7.6 Hz, H-1), 3.54 (t, J = 8.4 Hz, H-2), 3.43 (t, J = 9.0 Hz, H-3), 3.28 (t, J = 9.4 Hz, H-4), 3.73 (dd, J = 1.2, 6.2, 9.3 Hz, H-5), 3.58 (m, H-6a), 3.90 (dd, J = 10.2 Hz, H-6b). Rhamnose: 4.61 (brs, H-1), 3.69 (dd, J = 1.5, 3.4 Hz, H-2), 3.64 (dd, J = 3.4, 9.8 Hz, H-3), 4.74 (t, J = 9.8 Hz, H-4), 3.55 (m, H-5), 0.91 (d, J = 6.3 Hz, -CH3). Acetic acid: 1.97 (s, -CH3). 13C NMR (100 MHz) δ Cyanidin: 161.9 (C-2), 144.3 (C-3), 134.5 (C-4), 155.9 (C-5), 102.5 (C-6), 168.4 (C-7), 94.3 (C-8), 157.6 (C-9), 111.8 (C-10), 119.8 (C-1'), 114.4 (C-2'), 146.2 (C-3'), 154.4 (C-4'), 117.5 (C-5'), 127.1 (C-6'). Glucose: 101.7 (C-1), 73.1 (C-2), 76.3 (C-3), 69.8 (C-4), 75.8 (C-5), 66.2 (C-6). Rhamnose: 100.5 (C-1), 70.4 (C-2), 68.4 (C-3), 74.1 (C-4), 66.1 (C-5), 17.4 (-CH3). Acetic acid: 21.0
(-CH$_3$), 170.2 (COOH).

3. Pigment 3

Dark-red powder: UV-VIS (in 0.1% HCl-MeOH): λmax 528, 270 nm, $E_{440}/E_{	ext{max}} = 34$, AlCl$_3$ shift +; TLC: ($R_f$-values) BAW 0.46, BuHCl 0.35, 1% HCl 0.18, AHW 0.55; HPLC (method 1): Rt (min) 28.4; high-resolution FAB mass spectra (HR-FABMS) calc. for C$_{35}$H$_{32}$O$_{16}$: 623.1612. Found: 623.1619; 1$^H$ NMR (500 MHz) δ Cyanidin: 8.79 (s, H-1), 7.40 (d, $J = 6.9$ Hz, H-6), 6.92 (d, $J = 1.9$ Hz, H-8), 8.00 (d, $J = 2.5$ Hz, H-2), 7.04 (d, $J = 8.6$ Hz, H-5$'$), 8.32 (dd, $J = 2.5$, 8.6 Hz, H-6$'$). Glucose: 5.66 (d, $J = 7.7$ Hz, H-1), 3.93 (t, $J = 8.2$ Hz, H-2), 3.68 (t, $J = 8.9$ Hz, H-3), 3.32 (t, $J = 9.5$ Hz, H-4), 3.90 (ddd, $J = 2.2$, 8.6, 10.5 Hz, H-5), 4.05 (dd, $J = 7.8$, 10.4 Hz, H-6a), 4.43 (brd, $J = 10.4$ Hz, H-6b). Xylose: 4.71 (d, $J = 7.6$ Hz, H-1), 3.01 (t, $J = 8.4$ Hz, H-2), 3.14 (t, $J = 8.9$ Hz, H-3), 3.62 (dd, $J = 5.5$, 10.4 Hz, H-4), 3.52 (dd, $J = 5.5$, 11.3 Hz, H-5a), 2.94 (t, $J = 11.0$ Hz, H-5b). Acetic acid: 2.00 (s, -CH$_3$). 13$^C$ NMR (126 MHz) δ Cyanidin: 161.9 (C-2), 143.7 (C-3), 133.8 (C-4), 154.7 (C-5), 102.4 (C-6), 168.3 (C-7), 94.2 (C-8), 157.6 (C-9), 111.6 (C-10), 119.7 (C-1'), 116.7 (C-2'), 146.3 (C-3'), 155.9 (C-4'), 117.6 (C-5'), 127.7 (C-6'). Glucose: 99.0 (C-1), 80.8 (C-2), 76.7 (C-3), 69.6 (C-4), 74.4 (C-5), 63.6 (C-6). Xylose: 104.7 (C-1), 74.4 (C-2), 76.4 (C-3), 69.5 (C-4), 66.2 (C-5). Acetic acid: 20.6 (-CH$_3$), 170.5 (COOH).

Deacylanthocyanin and acetic acid

Pigments 1, 2, and 3 (ca. 0.5 mg each) were dissolved in 2N NaOH (1 mL) using a degassed syringe to stir for 15 min. The solution was then acidified with 2N HCl (1.1 mL). This solution was used for TLC and HPLC with authentic peonidin 3-rutinoside and cyanidin 3-rutinoside from Cymbidium (Tatsuzawa et al., 1996) and cyanidin 3-sambubioside from Corydalis ambigua (Tatsuzawa et al., 2005) and acetic acid (Wako Pure Chemical Industries).

1. Peonidin 3-rutinoside

UV-vis (in 0.1% HCl-MeOH): λmax 528, 279 nm, $E_{440}/E_{	ext{max}} = 29$%, AlCl$_3$ shift 0; TLC: ($R_f$-values) BAW 0.38, BuHCl 0.11, 1% HCl 0.09, AHW 0.43; HPLC (method 1): Rt (min) 19.9.

2. Cyanidin 3-rutinoside

UV-vis (in 0.1% HCl-MeOH): λmax 530, 280 nm, $E_{440}/E_{	ext{max}} = 25$%, AlCl$_3$ shift +; TLC: ($R_f$-values) BAW 0.30, BuHCl 0.36, 1% HCl 0.08, AHW 0.33; HPLC (method 1): Rt (min) 16.0.

3. Cyanidin 3-sambubioside

UV-vis (in 0.1% HCl-MeOH): λmax 529, 282 nm, $E_{440}/E_{	ext{max}} = 22$%, AlCl$_3$ shift +; TLC: ($R_f$-values) BAW 0.28, BuHCl 0.14, 1% HCl 0.14, AHW 0.47; HPLC (method 1): Rt (min) 17.7.

4. Acetic acid

HPLC (method 2): Rt (min) 5.0.

Acetyl, glucose, rhamnose, xylose, and acetic acid

Acid hydrolysis of pigments 1, 2, and 3 (ca. 0.5 mg each) was performed with 2N HCl (1 mL) at 90°C for 2 h. The hydrolysates were used for the analysis of UV-vis, TLC, and HPLC with authentic peonidin, cyanidin, acetic acid, glucose, rhamnose, and xylose (Wako Pure Chemical Industries).

1. Peonidin

UV-vis (in 0.1% HCl-MeOH): λmax 528, 270 nm, $E_{440}/E_{	ext{max}} = 25$%, AlCl$_3$ shift 0; TLC: ($R_f$-values) Forecastle 0.60; HPLC (method 1): Rt (min) 34.0.

2. Cyanidin

UV-vis (in 0.1% HCl-MeOH): λmax 536, 273 nm, $E_{440}/E_{	ext{max}} = 44$%, AlCl$_3$ shift +; TLC: ($R_f$-values) Forecastle 0.42; HPLC (method 1): Rt (min) 25.5.

3. Glucose

TLC: ($R_f$-values) BAW 0.15, ETN 0.24, color with AHP: Brown.

4. Rhamnose

TLC: ($R_f$-values) BAW 0.23, ETN 0.34, color with AHP: Yellow-Brown.

5. Xylose

TLC: ($R_f$-values) BAW 0.19, ETN 0.28, color with AHP: Reddish-Brown.

6. Acetic acid

See above.

Results and Discussion

Pigments from red-purple flowers and grayed-purple leaves of Saintpaulia

HPLC analyses of 5% HOAc-H$_2$O or 5% formic acid-H$_2$O extracts from red-purple flowers and grayed-purple leaves of S. ‘Tomoko’ revealed that two major anthocyanins (pigments 1 and 2) (81.3% and 10.4%, of the total anthocyanin contents calculated from the HPLC peak area at 530 nm) and one major anthocyanin (pigment 3) (85.9%), respectively, were observed in extract at retention times of 28.1 min (pigment 1), 24.1 min (pigment 2), and 28.3 min (pigment 3), along with some other minor peaks.

1. Pigment 1

Pigment 1 was isolated from the 5% HOAc-H$_2$O extract of dried petals, and purified using Diaion HP-20 column chromatography, preparative HPLC, and paper chromatography, according to the procedure described previously (Tatsuzawa et al., 2012). The chromatographic and spectroscopic properties of pigment 1 are shown in Materials and Methods.

Acid hydrolysis of pigment 1 yielded peonidin as its aglycone, glucose, rhamnose, and acetic acid. Alkaline hydrolysis of pigment 1 yielded a deacylanthocyanin and acetic acid. The deacylanthocyanin was identified as peonidin 3-rutinoside by HPLC, TLC, and UV-vis spectroscopy in comparison with authentic peonidin 3-rutinoside.

The molecular ion [M]+ of pigment 1 was observed at m/z 651 (C$_{30}$H$_{28}$O$_{16}$) using FABMS, indicating that
pigment 1 is composed of peonidin with one molecule each of glucose, rhamnose, and acetic acid. The elemental components of pigment 1 were confirmed by high-resolution FABMS.

The structure of pigment 1 was further elucidated by investigation of its $^1$H and $^{13}$C NMR spectra, including 2D COSY, 2D NOESY, HMQC, and HMBC spectra (Fig. 1).

The chemical shifts of six aromatic protons of the peonidin moiety with their coupling constants were assigned on the basis of analysis of the 2D COSY spectrum. The signals of two anomic protons of sugar moieties in pigment 1 appeared at $\delta$ 5.47 ($d, J = 7.6$ Hz, Glucose) and $\delta$ 4.62 ($d, J = 1.5$ Hz, Rhamnose), and the chemical shifts of other sugar protons were assigned by analysis of the 2D COSY spectrum with their coupling constants, indicating that the glucose residues of pigment 1 must be $\beta$-glucopyranosyl. In the rhamnose moiety, the doublet signal corresponds to an anomic proton ($\delta$ 4.62, $d, J = 1.5$ Hz) and doublet signals of methyl protons ($\delta$ 0.91, $d, J = 6.4$ Hz) at C-5 suggested the presence of $\alpha$-rhamnopyranosyl. The signal of the anomic proton of glucose correlated with that of the C-3 carbon ($\delta$ 144.2) of peonidin in the HMBC spectrum and also to the signal of H-4 proton ($\delta$ 8.39) in the NOESY spectrum of peonidin. The characteristic feature revealed that the OH-3 position of peonidin is glycosylated by glucose. The signal of the anomic proton of rhamnose correlated with that of the C-6 carbon ($\delta$ 66.4) in the HMBC spectrum and to the signal of H-6a and b protons ($\delta$ 3.57 and 3.91) in the NOESY spectrum of glucose. Therefore, rhamnose was bonded with glucose at OH-6 of glucose forming rutinoside. The proton signal of the H-2 proton ($\delta$ 3.93) in the NOESY spectrum of pigment 1 was observed at OH-2 of glucose forming sambubiose in the pigment. The proton signal of the H-4 of rhamnose ($\delta$ 4.75, $t, J = 9.8$ Hz) was shifted downfield, indicating that the OH-4 of rhamnose is acylated with acetic acid. This linkage was further confirmed by HMBC correlation (Fig. 1). Consequently, the structure of pigment 1 was elucidated to be peonidin 3-($O_2$-(acetyl)-$\alpha$-rhamnopyranosyl)-$\beta$-glucopyranoside (Fig. 1), which was found in Saintpaulia for the first time (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Veitch and Gray, 2008, 2011), although this pigment has been found in the berries of Eurya japonica (Terahara et al., 1988). 3. Pigment 3

Acid hydrolysis of pigment 3 yielded cyanidin as its aglycone, glucose, xylose, and acetic acid. Alkaline hydrolysis of pigment 3 yielded a deacylanthocyanin and acetic acid. The deacylanthocyanin was identified as cyanidin 3-sambubioside by HPLC, TLC, and UV-vis spectroscopy in comparison with authentic cyanidin 3-sambubioside.

The molecular ion [M]$^+$ of pigment 3 was observed at m/z 623 ($C_{27}H_{45}O_{22}$) using FABMS, indicating that pigment 3 is composed of cyanidin with one molecule each of glucose, xylose, and acetic acid. The elemental components of pigment 3 were confirmed by high-resolution FABMS.

The structure of pigment 2 was further elucidated by investigation of its $^1$H and $^{13}$C NMR spectra, including 2D COSY, 2D NOESY, HMQC, and HMBC spectra (Fig. 1).

The $^1$H and $^{13}$C NMR spectra of pigment 2 were similar to those of pigment 1 except for cyanidin instead of peonidin. Thus, pigment 2 was identified as cyanidin 3-($6-O$-(4-O-($\alpha$-rhamnopyranosyl)-$\beta$-glucopyranoside) (Fig. 1), which was found in Saintpaulia for the first time (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Veitch and Gray, 2008, 2011), although this pigment has been found in the berries of Eurya japonica (Terahara et al., 1988).

The molecular ion [M]$^+$ of pigment 3 was observed at m/z 637 ($C_{30}H_{35}O_{16}$) using FABMS, indicating that pigment 2 is composed of cyanidin with one molecule each of glucose, rhamnose, and acetic acid. The elemental components of pigment 2 were confirmed by high-resolution FABMS.

The chemical shifts of six aromatic protons of the cyanidin moiety with their coupling constants were assigned on the basis of analysis of the 2D COSY spectrum. The signals of two anomic protons of sugar moieties in pigment 2 appeared at $\delta$ 5.66 ($d, J = 7.7$ Hz, Glucose) and $\delta$ 4.71 ($d, J = 7.6$ Hz, Xylose), and the chemical shifts of other sugar protons were assigned by analysis of the 2D COSY spectrum with their coupling constants, indicating that those glucose and xylose residues of pigment 3 must be $\beta$-pyranose forms. The signal of the anomic proton of glucose A correlated with that of the C-3 carbon ($\delta$ 143.7) of cyanidin in the HMBC spectrum and also to the signal of H-4 proton ($\delta$ 8.79) in the NOESY spectrum of cyanidin. The characteristic feature revealed that the OH-3 position of cyanidin is glycosylated by gluoses. The signal of the anomic proton of xylose correlated with that of the C-2 carbon ($\delta$ 80.8) in the HMBC spectrum and to the signal of H-2 proton ($\delta$ 3.93) in the NOESY spectrum of glucose. Therefore, xylose was bonded with glucose at OH-2 of glucose forming sambubiose in the pigment. On the basis of the result of analysis of its COSY spectra, two characteristic methylene proton signals ($\delta$ 4.05 and 4.43) being shifted to lower magnetic fields were assigned to H-6a and b protons of glucose. This result indicated that the OH-6 of glucose must be esterified.
with acetic acid. This linkage was further confirmed by HMBC correlation (Fig. 1). Consequently, the structure of pigment 3 was elucidated to be cyanidin 3-O-[2-O-(β-xylopyranosyl)-6-O-(acetyl)-β-glucopyranoside] (Fig. 1), which was found in Saintpaulia for the first time (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Veitch and Grayer, 2008, 2011), although this pigment has been found in the flowers of Camellia ’Dalicha’ (Li et al., 2008).

The distributions of 3-acetyl-rutinoside-5-glucosides of malvidin, pelargonidin, and peonidin have already been reported in the violet-blue, purple, and pink flowers of S. ’Thamires’ as their main anthocyanins (Sato et al., 2011; Tatsuzawa et al., 2012). However, in this study, it was revealed that the red-purple flowers of S. ’Tomoko’ contain peonidin 3-acetyl-rutinoside and cyanidin 3-acetyl-rutinoside as the main anthocyanins. Therefore, it is presumed that S. ’Tomoko’ has two characteristic biosynthetic features different from S. ’Thamires’ (Sato et al., 2011; Tatsuzawa et al., 2012) for producing the red-purple flowers as follows: (1) it is devoid of flavonoid 3’,5’-hydroxylase activity and (2) 5-OH of anthocyanidin is free from glucosylation. On the other hand, anthocyanin in the grayed-purple colored leaves of S. ’Tomoko’ contained cyanidin 3-acetyl-sambubioside as a main anthocyanin. Therefore, it was thought that the biosynthesis of anthocyanin 3-glucoside moiety for the leaf anthocyanin depended on another biosynthesis system, not the flower one.

Acknowledgements

We thank Prof. Norio Saito (Meiji-Gakuin University), Prof. Toshio Honda (Hoshi University), and Prof. Fumio Hashimoto (Kagoshima University) for their helpful advice and Mr. James Hall (Iwate University) for his careful revision of the manuscript.
Literature Cited
Andersen, Ø. M. and M. Jordheim. 2006. The anthocyanins. p. 471–551. In: Ø. M. Andersen and K. R. Markham (eds.). Flavonoids: Chemistry, biochemistry and applications. CRC Press, Boca Raton.
Harborne, J. B. 1966. Comparative biochemistry of flavonoids-II. 3-Desoxyanthocyanins and their systematic distribution in ferns and gesnerads. Phytochemistry 5: 589–600.
Harborne, J. B. 1984. Phytochemical methods, second ed. Chapman and Hall, London.
Harborne, J. B. and H. Baxter. 1999. Anthocyanins. p. 1–114. In: J. B. Harborne and H. Baxter (eds.). The Handbook of Natural Flavonoids, vol. 2. John Wiley & Sons, Chichester.
Harborne, J. B. and M. Doi. 2011. Tissue culture-induced flower-color changes in Saintpaulia caused by excision of the transposon inserted in the flavonoid 3',5' hydroxylase (F3',5'H) promoter. Plant Cell Rep. 30: 929–939.

Tatsuzawa, F., M. Hosokawa, N. Saito and T. Honda. 2012. Three acylated anthocyanins and a flavone glycoside in violet-blue flowers of Saintpaulia ‘Thamires’. S. Afr. J. Bot. 79: 71–76.

Tatsuzawa, F., Y. Mikanagi, N. Saito, K. Shinoda, A. Shigihara and T. Honda. 2005. Cyanidin glycosides in flowers of genus Corydalis (Fumariaceae). Biochem. Syst. Ecol. 33: 789–798.

Tatsuzawa, F., N. Saito, Y. Mikanagi, K. Shinoda, K. Toki, A. Shigihara and T. Honda. 2009. An unusual acylated malvidin 3-glucoside from flowers of Impatiens textori Miq. (Balsaminaceae). Phytochemistry 70: 672–674.

Tatsuzawa, F., N. Saito and M. Yokoi. 1996. Anthocyanins in the flowers of Cymbidium. Lindleyana 11: 214–219.

Terahara, N., M. Yamaguchi and K. Shizukuishi. 1988. Cyanidin 3-acetylrutinoside in Eurya japonica berries. Phytochemistry 27: 3701–3703.

Veitch, N. C. and R. J. Grayer. 2008. Flavonoids and their glycosides, including anthocyanins. Nat. Prod. Rep. 25: 555–611.

Veitch, N. C. and R. J. Grayer. 2011. Flavonoids and their glycosides, including anthocyanins. Nat. Prod. Rep. 28: 1625–1695.