Floral Pigments from the Blue Flowers of *Nemophila menziesii* ‘Insignis Blue’ and the Purple Flower of Its Variants

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Two anthocyanins (pigments 1 and 2) were detected from the blue flowers of *Nemophila menziesii* ‘Insignis blue’ and the purple flowers of its variants as the main floral anthocyanins. These two anthocyanins were isolated from the blue flowers and elucidated to be petunidin 3-O-[6-O-(cis-p-coumaroyl)-β-glucopyranoside]-5-O-[6-O-(malonyl)-β-glucopyranoside] (1) and petunidin 3-O-[6-O-(trans-p-coumaroyl)-β-glucopyranoside]-5-O-[6-O-(malonyl)-β-glucopyranoside] (2), respectively, by chemical and spectroscopic means, and pigment 1 was confirmed as a new anthocyanin in plants. Two flavonol glycosides (pigments 3 and 5) and two flavone glycosides (pigments 4 and 6) were also isolated from the blue flowers, and were identified to be kaempferol 3-(6-rhamnosyl)-glucoside-7-glucoside (3), apigenin 7,4′-di-glucoside (4), kaempferol 3-(2,6-di-rhamnosyl)-glucoside (5), and apigenin 7-glucoside-4′-(6-malonyl)-glucoside (6) as major flavonoids. Among these four flavonoids, however, pigments 4 and 6 (flavones) were not detected in the purple flowers. These results might be attributed to color production in blue and purple flowers.

**Key Words:** acylated petunidin glycoside, Boraginaceae, *Nemophila menziesii*, purple and blue flower color.

**Introduction**

*Nemophila menziesii* Hook. & Arn. (Boraginaceae), which is native to northern America, is a popular ornamental plant. It is often cultivated in flower gardens and pots in Japan. The flowers have five petals, blue, violet, black, or white in color, and it is called Baby Blue Eyes in English. *N. menziesii* ‘Insignis blue’ is one of the main cultivars with blue flowers, and its variants with purple flowers are bred in Japan. However, the mechanism of color production in the purple flowers is not clear.

In our series of investigations on flavonoid-based flower color variations in ornamental plants, we were interested in the chemical investigation of the blue floral pigments of *N. menziesii* ‘Insignis blue’, although another group has already reported the chemical structures of acylated anthocyanin and flavones (Yoshida et al., 2009). They reported that the relatively stable blue flower color was produced by the co-pigmentation between anthocyanin and flavone with the assistance of metal ions in an *in vitro* study. However, there has been no report on the chemical structures of other major anthocyanins and flavonols until now. Moreover, no comparative study has been conducted between flower color and the *in vivo* distribution of pigments. In this paper, we report the isolation and structure elucidation of flavonoids from the flowers of *N. menziesii* ‘Insignis blue’, and the difference in pigment components between purple flowers of its variants and standard blue flowers.

**Materials and Methods**

**General procedures**

Thin layer chromatography (TLC) was carried out on plastic sheets or glass plates coated with cellulose (Merck) using nine mobile phases: BAW (n-BuOH-HOAc-H₂O, 4 : 1 : 2), BuHCl (n-BuOH-2N HCl, 1 : 1), AHW (HOAc-HCl-H₂O, 15 : 3 : 82), 1% HCl for flavonoids, and BAW, EAA (EtOH-HOAc-H₂O, 3 : 1 : 1),
ETN (EtOH-NH₂OH-H₂O, 16 : 1 : 3), and EFW (EtOAc-HCO₂H-H₂O, 5 : 2 : 1) for sugars with aniline hydrogen phthalate spray reagent, BAW and 15% HOAc for hydroxycinnamic acids with UV light, and Forestal (HOAc-HCl-H₂O, 30 : 3 : 10) for aglycone (Harborne, 1984).

Analytical high performance liquid chromatography (HPLC) was performed on an LC 10A system (Shimadzu, Kyoto, Japan), using a Spherisorb C18 column (4.6 × 250 mm; Waters, Milford, CT, USA) at 40°C with a flow rate of 1 mL·min⁻¹ and monitoring at 530 nm. The eluant was applied as a linear gradient elution for 40 min from 20 to 85% solvent B (1.5% H₂PO₄, 20% HOAc, 25% MeCN in H₂O) in solvent A (1.5% H₂PO₄ in H₂O) with 5 min of re-equilibration at 20% solvent B, for flavonoids and hydroxycinnamic acid (method 1). The other eluant for malonic acid was applied as an isocratic elution of solvent A for 10 min and monitored at 210 nm (method 2) (Tatsuzawa et al., 2013).

UV-Vis spectra were recorded on a UV-Vis spectrophotometer MPS-2450 (Shimadzu) in 0.1% HCl-MeOH for anthocyanins and in MeOH for flavonols and flavones. High-resolution fast atom bombardment mass (HR-FABMS) spectra were determined on a JMS-700 mass spectrometer (JEOL, Tokyo, Japan) operating in the positive ion mode using a 1 : 1 mixture of dithio-reitol and 3-nitrobenzyl alcohol as a matrix.

Nuclear magnetic resonance (NMR) spectra (AL-400; JEOL) were acquired at 400 MHz for ¹H spectra and 100 MHz for ¹³C spectra in dimethyl sulfoxide (DMSO)-d₆ for flavonoids and flavones and DMSO-d₄-deuterium chloride (DCl) (9 : 1) for anthocyanins. Chemical shifts are reported relative to a tetramethylsilane (TMS) internal standard (δ), and coupling constants are in Hz.

Plant materials
Seeds of N. menziesii ‘Insignis blue’ were purchased from Sakata Seed (Yokohama, Japan). The plants were grown in the greenhouses of Minami-Kyushu University and Iwate University. The variant plants with purple flowers were bred and grown in the greenhouses of Chiba University. The fresh flowers were collected in spring, dried at 45°C, and stored in desiccators until use. The flower colors of these plants were recorded by comparing them directly with the Royal Horticultural Society (R.H.S.) Colour Chart (Blue 100B for ‘Insignis blue’ and Purple-Violet N80C for the variant by R.H.S. Colour Chart). The chromaticity value of these fresh flowers, b* /a* = -31.43/-0.60 = 52.38 for ‘Insignis blue’ and b* /a* = -23.16/18.63 = -1.24 for the variant was measured by CM-700d spectrophotometer (Konica-Minolta, Tokyo, Japan). The absorption spectrum of the fresh petals of N. menziesii ‘Insignis blue’ exhibited characteristic absorption maxima at 715, 630, 590, and 555sh nm in the visible region of 360–750 nm. In contrast, the absorption spectrum of the fresh petals of variants exhibited absorption maxima at 621sh, 568sh, and 537 nm in the visible region of 360–750 nm.

Isolation of flavonoids, flavonols, and anthocyanin
The flavonoids in the dried blue flowers (200 g) of N. menziesii ‘Insignis blue’ were extracted with 5% HOAc (30 L). Flavonoids in the extract were absorbed on a Diaion HP-20 column and, after re-extracting with 5% HOAc-MeOH, the eluant was concentrated into a small volume. The concentrated extract was fractionated with Sephadex LH-20 Column chromatography (CC) using MeOH-HOAc-H₂O (6 : 1 : 12). The fractions were further purified with paper chromatography (PC) (BAW and 15% HOAc) and preparative HPLC. Prep. HPLC was performed on a LC 10A system (Shimadzu) using a μBondapak C18 column (19 × 150 mm, Waters) at 40°C with a flow rate of 4 mL·min⁻¹. Six pigments were obtained: 1 (10 mg) and 2 (30 mg) as dark violet powders (anthocyanins), 3 (380 mg) and 5 (150 mg) as pale yellow powders (flavonoids), and 4 (30 mg) and 6 (45 mg) as pale pink powders (flavones).

Piｇment 1 (Petunidin 3-O-[6-O-(cis-p-coumaroyl)-β-glucopyranoside]-5-O-[6-O-(malonyl)-β-glucopyranoside])
Dark violet powder; UV-Vis (in 0.1% HCl-MeOH): λₘₐₓ 543, 305, 278 nm, E₄₁₅/E₃₄₅ (max) = 59, E₄₁₅/E₃₄₅ (max) = 14, AlCl₃ shift +; TLC (Rf values × 100): BAW 12, BuHCl 11, 1% HCl 20, AHW 50; HPLC: Rf (min) 27.3; HR-FABMS calc. C₄₀H₄₁O₂₂: 873.2089. Found: 873.2057.

Piｇment 2 (Petunidin 3-O-[6-O-(trans-p-coumaroyl)-β-glucopyranoside]-5-O-[6-O-(malonyl)-β-glucopyranoside])
Dark violet powder; UV-Vis (in 0.1% HCl-MeOH): λₘₐₓ 541, 306, 282 nm, E₄₁₅/E₃₄₅ (max) = 76, E₄₃₄/E₃₄₅ (max) = 13, AlCl₃ shift +; TLC (Rf values × 100): BAW 23, BuHCl 13, 1% HCl 5, AHW 34; HPLC: Rf (min) 33.4; HR-FABMS calc. C₄₀H₄₁O₂₂: 873.2089. Found: 873.2072. ¹H NMR [400 MHz, DMSO-d₆-DCI (9 : 1), an internal standard of TMS]; δ Petunidin: 8.88 (s, H-4), 7.05 (brs, H-6), 7.19 (brs, H-8), 7.91 (d, J = 2.2 Hz, H-2'), 7.86 (d, J = 2.2 Hz, H-5'), 3.91 (s, -OCH₃), Glc A: 5.62 (d, J = 7.8 Hz, H-1), 3.60 (t, J = 8.5 Hz, H-2), 3.51 (t, J = 8.6 Hz, H-3), 3.39 (t, J = 8.2 Hz, H-4), 3.99 (m, H-5), 4.29 (m, H-6a), 4.33 (brd, J = 12.0 Hz, H-6b), Glc B: 5.18 (d, J = 7.8 Hz, H-1), 3.53 (t, J = 8.9 Hz, H-2), 3.41 (m, H-3), 3.25 (t, J = 9.1 Hz, H-4), 3.79 (m, H-5), 4.30 (m, H-6a), 4.38 (brd, J = 11.7 Hz, H-6b), trans-p-Coumaric acid: 7.35 (d, J = 8.8, H-2'), 6.37 (d, J = 8.8, H-3.5), 6.27 (d, J = 15.8, H-α), 7.38 (d, J = 15.8, H-β), Malonic acid: 3.33 (s, -CH₂-), ¹³C NMR [100 MHz, DMSO-d₆-DCI (9 : 1), an internal standard of TMS]; δ Petunidin: 162.0 (C-2), 145.1 (C-3), 133.0 (C-4), 155.2 (C-5), 111.6 (C-10), 112.9 (C-1'), 108.5 (C-2'), 104.6 (C-3'), 103.8 (C-4'), 101.7 (C-5'), 97.7 (C-8), 95.2 (C-9), 152.9 (C-10).
Pigment 3 (Kaempferol 3-O-(6-O-rhamnosyl)-glucoside-7-O-glucoside)

Pale yellow powder; TLC (R, values × 100); BAW 45, BuHCl 60, 1% HCl 72, AHW 80, UV dark brown, UV/NH2 yellow, UV (λ_max nm); MeOH: 348, 324sh, 299sh, 285, +NaOMe 363, 299, 272, +AlCl3 395, 352, 304, 273, +AlCl3/HCl 393, 349, 302, 274, +NaOAc 348, 325sh, 266, +NaOAc/H2BO3: 348, 326sh, 266, HPLC (R, min) 16.3, HR-FABMS calc. for C34H45O13 [M + H]+

75% 21791, Found 75% 21791. *

1H NMR (400 MHz, DMSO-d6, an internal standard of TMS); δ Kaempferol: 157.4 (C-2), 133.5 (C-3), 177.6 (C-4), 160.9 (C-5), 99.4 (C-6), 162.9 (C-7), 94.7 (C-8), 156.1 (C-9), 105.7 (C-10), 120.7 (C-1′), 131.0 (C-2′,6′), 115.2 (C-3′,5′), 160.1 (C-4′), Glc C: 101.2 (C-1), 74.2 (C-2), 76.4 (C-3), 70.0 (C-4), 76.4 (C-5), 66.9 (C-6), Glc D 99.9 (C-1), 73.1 (C-3), 75.9 (C-5), 69.6 (C-4), 77.2 (C-5), 60.6 (C-6), Rha A: 100.8 (C-1), 70.4 (C-2), 70.6 (C-3), 71.8 (C-4), 68.3 (C-5), 17.7 (-CH3).

Pigment 4 (Apigenin 4′-O-(6-O-malonyl)-glucoside-7-O-glucoside)

Pale pink powder; TLC (R, values × 100); BAW 14, BuHCl 12, 1% HCl 39, AHW 54, UV dark brown, UV/NH2 yellow, UV (λ_max nm); MeOH: 335sh, 316, 269, +NaOMe 335sh, 316, 269, +AlCl3 378, 336, 297, 278, +AlCl3/HCl 378, 332, 297, 278, +NaOAc 335sh, 316, 269, +NaOAc/H2BO3: 335sh, 316, 269, HPLC (R, min) 18.7, HR-FABMS calc. for C34H45O13 [M + H]+

595.1633, Found 595.1633. *

1H NMR (400 MHz, DMSO-d6, an internal standard of TMS); δ Apigenin: 7.00 (s, H-3), 6.46 (d, J = 2.2 Hz, H-6), 6.88 (d, J = 2.2 Hz, H-8), 8.08 (d, J = 9.0 Hz, H-2′,6′), 7.21 (d, J = 9.0 Hz, H-3′,5′), Glc E: 5.04 (d, J = 7.3 Hz, H-1), 3.29 (t, J = 8.4 Hz, H-2), 3.36 (m, H-5), 3.42 (m, H-6a), 3.46 (m, H-6b), Glc F: 5.08 (d, J = 7.3 Hz, H-1), 3.18 (t, J = 8.4 Hz, H-2), 3.36 (m, H-3), 3.20 (m, H-4), 3.41 (m, H-5), 3.49 (m, H-6a), 3.71 (m, H-6b). *C NMR (100 MHz, DMSO-d6, an internal standard of TMS); δ Apigenin: 164.3 (C-2), 104.1 (C-3), 181.4 (C-4), 160.5 (C-5), 99.5 (C-6), 163.5 (C-7), 94.9 (C-8), 157.0 (C-9), 105.5 (C-10), 123.8 (C-1′), 128.3 (C-2′,6′), 116.9 (C-3′,5′), 158.9 (C-4′), Glc E: 99.8 (C-1), 73.0 (C-2), 76.5 (C-3), 69.4 (C-5), 77.3 (C-5), 60.6 (C-6), Glc F: 99.8 (C-1), 73.1 (C-2), 76.5 (C-3), 69.4 (C-5), 77.2 (C-5), 60.6 (C-6).
Aglycones, sugars, and acids

Acid hydrolysis of pigments (ca. 0.5 mg each) was achieved by 2N HCl (1 mL) at 90°C for 2 h. Moreover, alkaline hydrolysis of pigments (ca. 0.5 mg each) was achieved by 2N NaOH (1 mL) using a degassed syringe to stir for 15 min. The solution was then acidified with 2N HCl (1.1 mL). These solutions were used for TLC and HPLC (Tatsuzawa et al., 2012). Products in these hydrolysates were identified in comparison with the authentic standards of glucose, rhamnose, p-coumaric acid, malonic acid, kaempferol, and apigenin, which were of commercial origin. Petunidin and petunidin 3,5-di-glucoside were obtained from the flowers of *Torenia fournieri* (Tatsuzawa and Shinoda, 2005).

**Petunidin 3,5-di-glucoside (deacetylanthocyanin)**

UV-Vis: \( \lambda_{\text{max}} = 537, 274 \text{ nm}, \ E_{280}/E_{\text{max}} = 12\% \), AlCl₃ shift +; TLC (\( R_t \) values × 100); BAW 6, BuHCl 1, 1% HCl 7, AHW 24; HPLC (method 1): \( R_t \) (min) 15.0.

**Petunidin**

UV-Vis: \( \lambda_{\text{max}} = 547, 272 \text{ nm}, \ E_{280}/E_{\text{max}} = 20\% \), AlCl₃ shift +; TLC (\( R_t \) values × 100); Forestal 47; HPLC (method 1): \( R_t \) (min) 26.9.

**Kaempferol**

UV-Vis: \( \lambda_{\text{max}} = 368, 268 \text{ nm}, \ TLC (\( R_t \) values × 100); Forestal 56; HPLC (method 1): \( R_t \) (min) 38.0.

**Apigenin**

UV-Vis: \( \lambda_{\text{max}} = 336, 269 \text{ nm}, \ TLC (\( R_t \) values × 100); Forestal 84; HPLC (method 1): \( R_t \) (min) 39.2.

**Glucose**

TLC (\( R_t \) values × 100); BAW 24, EAA 18, ETN 62, EFW 49; Color (aniline hydrogen phthalate (AHP)) Brown.

**Rhamnose**

TLC (\( R_t \) values × 100); BAW 41, EAA 37, ETN 71, EFW 52; Color (AHP) Brown.

**p-Coumaric acid**

TLC (\( R_t \) values × 100); BAW 91 and 91 (trans and cis), 15% HOAc 49 and 74 (trans and cis); Color (under UV) Violet; HPLC (method 1): \( R_t \) (min) 17.2 and 16.2 (trans and cis).

**Malonic acid**

HPLC (method 2): \( R_t \) (min) 4.1.

### Results and Discussion

Two major HPLC peaks were detected in each group of anthocyanins, flavones, and flavonols, respectively, in 5% HOAc_H₂O extract from the blue flowers of *N. menziesii* ‘Insignis blue’ by HPLC analysis with monitoring at 530 nm for anthocyanins and at 350 nm for both flavonols and flavones, respectively. The proportions of these anthocyanin peaks were 9.3% (pigment 1) and 78.3% (pigment 2) based on the percentage of the total absorbance of peaks (530 nm), those of flavonol peaks were 28.6% (pigment 3) and 8.1% (pigment 5), and those of flavone peaks were 8.1% (pigment 4) and 16.7% (pigment 6), respectively, based on the percentage of the total absorbance of peaks (350 nm) (Fig. 1). In contrast, the proportions of flavonoid peaks of purple flowers were 18.1% (pigment 1) and 59.3% (pigment 2) based on the percentage of the total absorbance of peaks (530 nm), and those of flavonol peaks were 28.5% (pigment 3) and 13.4% (pigment 5) based on the percentage of the total absorbance of peaks (350 nm), respectively (Fig. 1).

The six pigments 1–6 were purified using Diaion HP-20 resin CC, PC, Sephadex LH-20 CC, and preparative HPLC, as described previously (Toki et al., 2009). Chromatographic and spectroscopic properties of these pigments are shown in Table 1 and in the Experimental Section.

Acid hydrolysis of anthocyanins (pigments 1 and 2) resulted in petunidin, malonic acid, and glucose. Moreover, cis-p-coumaric acid and trans-p-coumaric acid were detected in the hydrolysates of pigments 1 and 2, respectively, by analysis of TLC and HPLC. Acid hydrolysis of flavonols and flavones (pigments 3–6) resulted in kaempferol for pigments 3 and 5, and apigenin for pigment 4 and 6 as their aglycones. In their sugar components, glucose and rhamnose for pigments 3 and 5 and glucose for pigments 4 and 6 were observed in hydrolysates, but malonic acid was detected only in the hydrolysate of pigment 6. Furthermore, alkaline hydrolysis of pigments 1 and 2 resulted in one deacetylanthocyanin, which was identical to petunidin 3,5-diglucoside on the basis of the comparison of TLC and HPLC profiles (Tatsuzawa and Shinoda, 2005).

From these results, the structures of pigments 1–6 were presumed to be cis-p-coumaroyl-malonyl-petunidin 3,5-diglucoside (pigment 1), trans-p-coumaroyl-malonyl-petunidin 3,5-diglucoside (pigment 2), di-glucosyl-mono-rhamnosyl-kaempferol (pigment 3), di-glucosyl-apigenin (pigment 4), mono-glucosyl-di-rhamnosyl-kaempferol (pigment 5), and malonyl-di-glucosyl-apigenin (pigment 6), respectively.
two olefinic proton signals of the \( p \)-coumaric acid moiety, indicated a cis configuration for the acid on the basis of their coupling constants (\( J = 13.2 \text{ Hz} \)) (Table 1). The chemical shifts of the sugar moiety protons were observed in the region of \( \delta 5.70–3.27 \), with the two anomeric proton resonances at 5.70 (\( \delta \), \( J = 7.8 \text{ Hz} \), Glc A) and 5.18 (\( \delta \), \( J = 7.6 \text{ Hz} \), Glc B). Based on the observed coupling constants (Table 1), these two sugars were assumed to be in their \( \beta \)-pyranose forms. The linkages and/or positions of the attachments of the sugar and acyl groups were determined based on 2D COSY and NOESY experiments. By application of a NOESY experiment, NOEs between H-1 of Glc A and H-4 (\( \delta 8.72 \)) of petunidin, H-1 of Glc B and H-6 (\( \delta 6.96 \)) of petunidin were observed (Fig. 2), supporting the presence of the glycosylation of C-3 and C-5 petunidin hydroxyl groups with Glc A and Glc B, respectively.

Four characteristic downfield shifted proton signals were assigned to the methylene protons of Glc A (\( \delta 4.38 \) and 4.27, H-6a and b) and Glc B (\( \delta 4.30 \) and 4.47, H-6a and b), indicating acylation of C-6 OHs (Glc A and B) with two acid molecules. HMBC spectra were studied to identify the attachment sites of acid moieties (Fig. 2), supporting the presence of the glycosylation of C-3 and C-5 petunidin hydroxyl groups with Glc A and Glc B, respectively.

The elemental components of these pigments were also confirmed by measuring their HR-FABMS spectra (see Materials and Methods). The structures of these pigments were further elucidated as follows based on the analysis of their \(^1\)H and \(^{13}\)C NMR spectra [400 MHz for \(^1\)H and 100 MHz for \(^{13}\)C spectra in DCl-DMSO-\( d_6 \) (1 : 9) or DMSO-\( d_6 \), including 2D correlation spectroscopy (COSY), 2D nuclear Overhauser enhancement spectroscopy (NOESY), hetero-nuclear multiple quantum coherence (HMQC), and hetero-nuclear multiple bond correlation (HMBC) spectra] (see Materials and Methods).

1. Pigment 1 (anthocyanin)

The FABMS of pigment 1 gave a molecular ion [M]+ at 873 \( m/z \) (calc. for \( \text{C}_{40}\text{H}_{41}\text{O}_{22} \)), indicating that pigment 1 is composed of petunidin with two molecules of glucose and one molecule each of \( p \)-coumaric acid and malonic acid. The elemental components were confirmed by measuring its HR-FABMS (calc. \( \text{C}_{40}\text{H}_{41}\text{O}_{22} \) : 873.2089, found 873.2057).

The \(^1\)H NMR spectrum of pigment 1 exhibited nine aromatic protons identified with petunidin and \( p \)-coumaric acid moieties, together with their coupling constants, and assigned as shown in Table 1. Three protons were assigned to a methoxyl group of petunidin. A set of one pair of doublet resonance, assigned to the two olefinic proton signals of the \( p \)-coumaric acid moiety, indicated a cis configuration for the acid on the basis of their coupling constants (\( J = 13.2 \text{ Hz} \)) (Table 1). The chemical shifts of the sugar moiety protons were observed in the region of \( \delta 5.70–3.27 \), with the two anomeric proton resonances at 5.70 (\( d, J = 7.8 \text{ Hz} \), Glc A) and 5.18 (\( d, J = 7.6 \text{ Hz} \), Glc B). Based on the observed coupling constants (Table 1), these two sugars were assumed to be in their \( \beta \)-pyranose forms. The linkages and/or positions of the attachments of the sugar and acyl groups were determined based on 2D COSY and NOESY experiments. By application of a NOESY experiment, NOEs between H-1 of Glc A and H-4 (\( \delta 8.72 \)) of petunidin, H-1 of Glc B and H-6 (\( \delta 6.96 \)) of petunidin were observed (Fig. 2), supporting the presence of the glycosylation of C-3 and C-5 petunidin hydroxyl groups with Glc A and Glc B, respectively.

Four characteristic downfield shifted proton signals were assigned to the methylene protons of Glc A (\( \delta 4.38 \) and 4.27, H-6a and b) and Glc B (\( \delta 4.30 \) and 4.47, H-6a and b), indicating acylation of C-6 OHs (Glc A and B) with two acid molecules. HMBC spectra were studied to identify the attachment sites of acid moieties (Fig. 2). The signals of the methylene protons of Glc A and Glc B was correlated with those of the COOH carbons of \( p \)-coumaric acid (\( \delta 167.0 \)) and malonic acid (\( \delta 167.5 \)) in the HMBC spectrum (Fig. 2). Therefore, the structure of
2. Pigment 2 (anthocyanin)

The FABMS of pigment 2 gave a molecular ion [M]+ at 873 m/z (calc. for C_{40}H_{41}O_{22}), indicating that pigment 2 is composed of petunidin with two molecules of glucose and one molecule each of p-coumaric acid and malonic acid.

The 1H NMR spectrum of pigment 2 was similar to that of pigment 1, except for the signals of the p-coumaric acid moiety (see Materials and Methods). The olefinic protons of pigment 2 were shifted to lower fields of δ 6.27 and 7.38 (d, J = 15.8 Hz each) in comparison with those of pigment 1, establishing pigment 2 as petunidin 3-O-[6-O-(trans-p-coumaroyl)-β-glucopyranoside]-5-O-[6-O-(malonyl)-β-glucopyranoside], which has been found in *Hyacinthus orientalis* (Hosokawa et al., 1995). Moreover, this pigment was found in *N. menziesii* by K. Yoshida and her co-workers, and named nemophillin, whose structure was also confirmed by analysis of its 13C, HMQC, and HMBC NMR spectra.

3. Pigments 3 and 5 (flavonols)

Acid hydrolysis of pigments 3 and 5 yielded kaempferol, rhamnose, and glucose. The FABMS of pigments 3 and 5 gave their molecular ions [M + H]⁺ at 757 m/z (calc. C_{33}H_{41}O_{20}) and at 741 m/z (calc. C_{33}H_{41}O_{19}), respectively, indicating that pigment 3 is composed of kaempferol with one molecule of rhamnose and two molecules of glucose, and pigment 5 is composed of kaempferol with two molecules of rhamnose and one molecule of glucose. Their elemental components were confirmed by measuring their HR-FABMS (see Materials and Methods).

The 1H NMR spectrum of pigment 3 exhibited six aromatic proton signals of kaempferol (see Materials and Methods). The anomic protons of Glc C, Glc D, and Rha A were observed at δ 5.36 (d, J = 7.3 Hz), 5.08 (d, J = 7.3 Hz), and 4.38 (s). The binding patterns of these compounds were confirmed by NOESY and/or HMBC experiments. NOEs between H-1 of Rha A and H-6a of pigment 1 was determined to be petunidin 3-O-[6-O-(cis-p-coumaroyl)-β-glucopyranoside]-5-O-[6-O-(malonyl)-β-glucopyranoside], which is a new anthocyanin in plants (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Veitch and Grayer, 2008, 2011).

![Fig. 2. Structure of pigment 1 isolated from the blue flowers of Nemophila menziesii ‘Insignis blue’. Main NOEs observed are indicated by arrows. Main HMBC correlations observed are indicated by dotted arrows.](image-url)
pigment of glucose, and pigment Methods). Previously been reported in the plant Nemophila, although the pigment has previously been reported in the plant Equisetum palustre (Beckmann and Geiger, 1963).

The 1H NMR spectrum of pigment 5 exhibited six aromatic proton signals of kaempferol (see Materials and Methods). The anomeric protons of Glc C, Rha A, and Rha B were observed at δ 5.49 (d, J = 7.1 Hz), 4.32 (s), and 5.06 (s), respectively. The binding patterns of these compounds were confirmed by NOESY and/or HMBC experiments. NOEs between H-1 of Rha A and H-6a and b of Glc C were observed. Moreover, correlations between H-1 of Glc C and C-3 of kaempferol, H-1 of Rha A and C-6 of Glc C, and H-1 of Rha B and C-2 of Glc C were observed in the HMBC spectrum. These results suggested that OH-3 of kaempferol, OH-6 of Glc C, and OH-2 of Glc C were glycosylated with Glc C, Rha A, and Rha B, respectively. Therefore, pigment 3 was determined to be kaempferol-3-O-(6-O-rhamnosyl)-glucoside-7-O-glucoside. This is the first report of the isolation of this pigment from the genus Nemophila, although the pigment has previously been reported in the plant Clitoria ternatea (Kazuma et al., 2003).

4. Pigments 4 and 6 (flavonoids)

Acid hydrolysis of pigments 4 and 6 yielded apigenin and glucose. Moreover, malonic acid was detected in the hydrolysate of pigment 6 by analysis using TLC and HPLC. The FABMS of pigments 4 and 6 gave their molecular ions [M + H]+ at 595 m/z (calc. C30H29O19) and at 681 m/z (calc. C45H33O23), respectively, indicating that pigment 4 is composed of apigenin with two molecules of glucose, and pigment 6 is composed of apigenin with two molecules of glucose and one molecule of malonic acid. Their elemental components were confirmed by measuring their HR-FABMS (see Materials and Methods).

The 1H NMR spectrum of pigment 4 exhibited seven aromatic proton signals of apigenin (see Materials and Methods). The anomeric protons of Glc E and Glc F were observed at δ 5.04 (d, J = 7.3 Hz) and 5.08 (d, J = 7.3 Hz). The binding patterns of these compounds were confirmed by NOESY experiments. NOEs between H-1 of Glc E and H-3' and 5' of apigenin and H-1 of Glc F and H-6 and 8 of apigenin were observed. These results suggest that OH-4' and 7 of apigenin were glycosylated with Glc E and Glc F, respectively. Therefore, pigment 4 was determined to be apigenin 4',7-di-O-glucoside, which has been found in some Salvia species (Yoshida et al., 2009). Moreover, this pigment has been found in N. menziesii by Yoshida et al. (2009), and its structure was also confirmed by analysis of its 13C, HMOC, and HMBC NMR spectra.

The 1H NMR spectrum of pigment 6 was identical to that of pigment 4 except for the signals of malonic acid moiety (see Materials and Methods). The methylene proton of malonic acid was assigned at 3.17 (s). By analysis of its NOESY spectrum, NOEs between CH2 of malonic acid and CH3 of Glc E were observed to support that OH-4' of apigenin was glycosylated with malonylglucose. Therefore, pigment 6 was determined to be apigenin 4'-O-(6-O-malonyl)-glucoside-7-O-glucoside, which has been found in Centaurea cyanus (Yoshida et al., 2009). This structure was also confirmed by analysis of its 13C, HMOC, and HMBC NMR spectra. Moreover, this pigment has been reported from N. menziesii by Yoshida et al. (2009) as a flavone component in the metal complex pigment (Nemophilin).

An in vitro restoration study of the blue flower color of N. menziesii was recently performed by mixing pigments 2 (anthocyanin) and 6 (flavone) with Fe and Mg (Yoshida et al., 2009). The flower color of the variants used in this study, which did not contain pigment 6, was purple like that of Commelina communis (Kondo et al., 1991). Pigment 4 (flavone) was not also accumulated in the purple flowers of N. menziesii ‘Insignis blue’ variants. Therefore, it might be considered that pigments 4 and 6 (flavones) play a role as co-pigments in producing the blue flower color of N. menziesii ‘Insignis blue’ as an important component in vivo.

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