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Structure of anthocyanin from the blue petals of *Phacelia campanularia* and its blue flower color development

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Abstract

The dicaffeoyl anthocyanin, phacelianin, was isolated from blue petals of *Phacelia campanularia*. Its structure was determined to be 3-*O*-(6-*O*-(4′-*O*-(6-*O*-(4′-*O*-β-D-glucopyranosyl-(*E*)-caffeoyl)-β-D-glucopyranosyl)-(*E*)-caffeoyl)-β-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-β-D-glucopyranosyl)delphinidin. The CD of the blue petals of the phacelia showed a strong negative Cotton effect and that of the suspension of the colored protoplasts was the same, indicating that the chromophores of phacelianin may stack intermolecularly in an anti-clockwise stacking manner in the blue-colored vacuoles. In a weakly acidic aqueous solution, phacelianin displayed the same blue color and negative Cotton effect in CD as those of the petals. However, blue-black colored precipitates gradually formed without metal ions. A very small amount of Al^{3+} or Fe^{3+} may be required to stabilize the blue solution. Phacelianin may take both an inter- and intramolecular stacking form and shows the blue petal color by molecular association and the co-existence of a small amount of metal ions. We also isolated a major anthocyanin from the blue petals of *Evolvulus pilosus* and revised the structure identical to phacelianin.

Keywords: *Phacelia campanularia*; *Evolvulus pilosus*; Hydrophyllaceae; Convolvulaceae; Phacelianin; Diacylated anthocyanin; Blue flower color development; Circular dichroism

1. Introduction

Most flower colors, especially blue colors, are due to anthocyanins (Goto and Kondo, 1991; Brouillard and Dangles, 1994). However, little is known about the mechanism of blue color development of anthocyanins; the blue dayflower (Kondo et al., 1992) and the blue cornflower (Kondo et al., 1994, 1998) acquire their color by a supramolecular metal complex, and the blue morning glory by an increase in vacuolar pH to 7.7 (Yoshida et al., 1995). Since Goto et al. (1982) reported the structure of gentiodelphin from the blue petals of *Gentiana makinoi*, many polyacylated anthocyanins, which contain two or more aromatic acyl residues, were isolated and structurally elucidated (Goto, 1987; Goto and Kondo, 1991). Now, many hundreds of polyacylated anthocyanins are known, and a fair percentage of them is from blue petals; therefore, polyacylated anthocyanins may contribute greatly to blue flower color development. Although the stability and blue color development of polyacylated anthocyanins are expected to be established with the intramolecular stacking of aromatic acyl residues to the anthocyanidin nucleus (Goto and Kondo, 1991; Yoshida et al., 1992, 2000; Brouillard and Dangles, 1994), their stacking structure and the mechanism of intramolecular charge-transfer, which is thought to derive from the bathochromic shift in the absorption spectrum, are obscure.

Recently, we have been focusing our research on flower color development in colored cells and established several methodologies such as the simultaneous measurement of the absorption spectrum of a single cell (Yoshida et al.,

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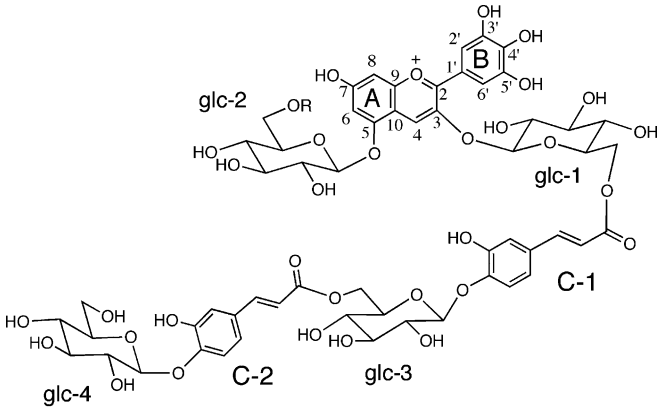
2003a,b) and vacuolar pH (Yoshida et al., 1995, 2003a,b), and the reproduction of cell color by mixing components in colored cells (Yoshida et al., 1995, 2003a,b). In this report, we describe the structure of a newly isolated polyacylated anthocyanin, phacelianin (**1**), in the blue petals of *Phacelia campanularia*. An interesting mechanism for blue color development of **1** is also discussed. We revised the structure of the major anthocyanin in blue petals of *Evolvulus pilosus* to be the same as **1**.

2. Results and discussion

2.1. Isolation and structure of anthocyanins in petals of *P. campanularia*

Blue petals of *P. campanularia* were extracted with aqueous acetonitrile (CH_3CN) containing trifluoroacetic acid (TFA), and the extract was analyzed by HPLC. As shown in Fig. 1, the composition of the petal extract is relatively simple, containing only two anthocyanins (**1** and **2**) with a small amount of a UV-absorbing compound (**3**). The results of photodiode array detection-HPLC indicated that **1** and **2** may have the same chromophore and two aromatic acyl residues. Compound **3** may not be a flavonoid but a cinnamic acid derivative. The components from the petals (300 g) were purified according to our general procedure (Yoshida et al., 1992, 1996, 2002, 2003b) to give **1** (170 mg), **2** (24 mg) and **3** (24 mg).

The structures of **1** and **2** were determined by FABMS and various 1D and 2D NMR experiments combined with degradation reactions (see Scheme 1). FABMS of **1** gave a molecular ion peak at $m/z = 1361$. The ^{1}H NMR spectrum of **1** showed the existence of a delphinidin nucleus, four sugars and two *E*-caffeoyl residues (Table 1). The molecular weight was larger by 86 Da than that estimated by ^{1}H NMR analysis, strongly suggesting that the remaining residue is malonyl group. The molecular ion peak of **2** was 1275, which was 86 mass units smaller than that of **1**, corresponding to a loss of a malonyl residue. The ^{1}H NMR spectrum of **2** was very similar to that of **1**, but only the signals of one sugar residue (glc-2) were different. Furthermore, acidic methanol treatment of **1** gave **2** via methyl ester of **1** being very similar in behavior to malonylshisonin (Kondo et al., 1989; Yoshida et al., 1997). These data strongly suggest that **2** is a demalonylated pigment from **1**.



Scheme 1. Structure of **1** and **2**.

By 1D- and 2D TOCSY experiments (Kondo et al., 1990), the signals of all sugar residues of **1** were assigned so that all were β-glucopyranosides (Table 1). The linkage of glc-1 to the 3 position and glc-2 to the 5 position of the delphinidin nucleus was confirmed by the NOEs between the anomeric proton of glc-1 and H-4, and that of glc-2 and H-6, respectively (Fig. 2). The methylene protons of the sugar residues (glc-1, 2 and 3) showing a downfield shift indicated that the 6-positions of glc-1, 2 and 3 were acylated. To determine the linkage of the acyl groups, an HMBC experiment was conducted (Fig. 2). One of the *E*-caffeoyl residues (C-1) was esterified to the 6-OH of the glc-1 because of the correlation between the α proton of C-1 and H-6 of glc-1, and the other caffeoyl residue (C-2) was connected to 6-OH of the glc-3 because of the correlation between the α proton of C-2 and H-6 of glc-3 (Fig. 2). Therefore, the substituted position of the malonic acid was determined to be the 6-OH of glc-2. This was confirmed from the correlation between H-6 of glc-2 and the ^{13}C signal of 168.5 ppm assigned to be the carbonyl carbon of the malonyl residue.

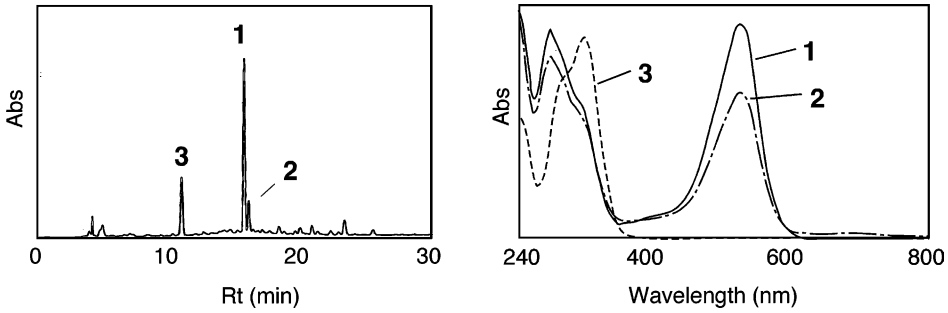


Fig. 1. HPLC chromatograms (detection: 280 nm) of the extract from the blue petals of *Phacelia campanularia* (left) and the spectra of each peak obtained with 3D-detection HPLC (right).

Table 1
Assignment of the ^{1}H and ^{13}C NMR spectra of **1** and **2** (^{1}H: 600 MHz,^{13}C: 150 MHz, 23 °C, 10% TFA *d*-CD_3OD)

		1		**2**		**1**	**2**
		^{1}H δ (ppm)	*J* (Hz)	^{1}H δ (ppm)	*J* (Hz)	^{13}C δ (ppm)	^{13}C δ (ppm)
2						164.3	164.1
3						146.2	146.3
4		8.70 *s*		8.66 *s*		132.8	133.2
5						156.4	156.6
6		7.00 *d*	2.0	7.00 *d*	2.0	106.2	105.9
7						168.7	168.9
8		6.76 *d*	2.0	6.68 *d*	2.0	97.6	97.6
9						156.3	156.3
10						112.8	112.7
1′						119.7	119.6
2′,6′		7.64 *s*		7.57 *s*		113.3	113.3
3′,5′						147.5	147.4
4′						146.4	146.1
C-1	1					130.9	130.8
	2	6.91 *d*	2.0	6.98 *d*	2.0	117.5	116.8
	3					148.7	148.6
	4					148.5	148.5
	5	7.03 *d*	8.5	7.03 *d*	8.5	118.6	119.1
	6	6.75 *dd*	8.5, 2.0	6.69 *dd*	8.5, 2.0	121.5	122.3
	α	6.37 *d*	16.0	6.35 *d*	16.0	117.3	117.2
	β	7.31 *d*	16.0	7.27 *d*	16.0	146.5	146.4
	C=O					168.8	168.7
C-2	1					130.4	130.4
	2	6.81 *d*	2.0	6.79 *d*	2.0	115.3	115.3
	3					148.3	148.3
	4					148.7	148.7
	5	6.84 *d*	8.5	6.82 *d*	8.0	117.2	117.2
	6	6.43 *dd*	8.5, 2.0	6.36 *dd*	8.0, 2.0	122.0	122.0
	α	6.17 *d*	16.0	6.15 *d*	16.0	117.2	117.2
	β	7.25 *d*	16.0	7.20 *d*	16.0	145.8	145.8
	C=O					168.5	168.5
glc-1	1	5.36 *d*	8.0	5.37 *d*	8.0	102.6	102.8
	2	3.90 *dd*	9.0, 8.0	3.90 *dd*	9.0, 8.0	74.1	74.1
	3	3.63 *t*	9.0	3.64 *t*	9.0	78.3	78.3
	4	3.54 *dd*	9.5, 9.0	3.56 *t*	9.0	72.7	72.6
	5	4.03 *td*	9.5, 2.5	4.01 *td*	9.0, 3.0	75.4	75.5
	6a	4.58 *dd*	11.5, 9.5	4.64 *dd*	11.5, 9.0	65.0	64.8
	6b	4.53 *dd*	11.5, 2.5	4.48 *dd*	11.5, 3.0		
glc-2	1	5.09 *d*	7.5	5.12 *d*	7.5	103.4	103.5
	2	3.78 *dd*	9.0, 7.5	3.78 *dd*	9.0, 7.5	74.9	74.9
	3	3.53 *t*	9.0	3.55 *t*	9.0	78.1	78.0
	4	3.42 *t*	9.0	3.37 *t*	9.0	70.9	71.4
	5	3.73 *ddd*	9.0, 6.5, 2.5	3.61 *ddd*	9.0, 7.0, 1.5	76.1	79.2
	6a	4.53 *dd*	11.5, 2.5	4.01 *dd*	11.5, 1.5	65.1	62.8
	6b	4.23 *dd*	11.5, 6.5	3.64 *dd*	11.5, 7.0		
glc-3	1	4.92 *d*	7.5	4.92 *d*	7.5	103.2	103.2
	2	3.57 *dd*	8.5, 7.5	3.58 *dd*	9.0, 7.5	74.7	74.7
	3	3.56 *dd*	9.0, 8.5	3.55 *t*	9.0	77.7	77.8
	4	3.40 *dd*	9.5, 9.0	3.39 *dd*	9.5, 9.0	72.4	72.5
	5	3.84 *td*	9.5, 2.0	3.82 *td*	9.5, 2.0	75.5	75.5
	6a	4.67 *dd*	11.5, 2.0	4.65 *dd*	11.5, 2.0	65.4	65.4
	6b	4.21 *dd*	11.5, 9.5	4.19 *dd*	11.5, 9.5		
glc-4	1	4.90 *d*	8.5	4.91 *d*	7.5	102.6	102.5
	2	3.54 *t*	8.5	3.53 *dd*	9.0, 7.5	74.7	74.7
	3	3.55 *dd*	9.0, 8.5	3.57 *t*	9.0	77.8	77.5
	4	3.44 *dd*	9.5, 9.0	3.47 *dd*	9.5, 9.0	71.2	71.1
	5	3.56 *m*		3.58 *m*		77.6	78.0
	6a	3.89 *dd*	12.5, 2.5	3.89 *dd*	12.5, 2.0	62.4	62.3
	6b	3.74 *dd*	12.5, 5.0	3.75 *dd*	12.5, 5.0		
Malonyl	C=O (carboxylate)					168.5	
	C=O (carboxylic acid)					170.6	

The connections between glc-3 to 4-OH of C-1 and glc-4 to 4-OH of C-2 were strongly indicated by the NOEs between the anomeric proton of glc-3 to 5-H of C-1 and that of glc-4 to 5-H of C-2 (Fig. 2). However, the latter connection was not confirmed by the HMBC experiments because it was difficult to differentiate the ^{13}C signals of

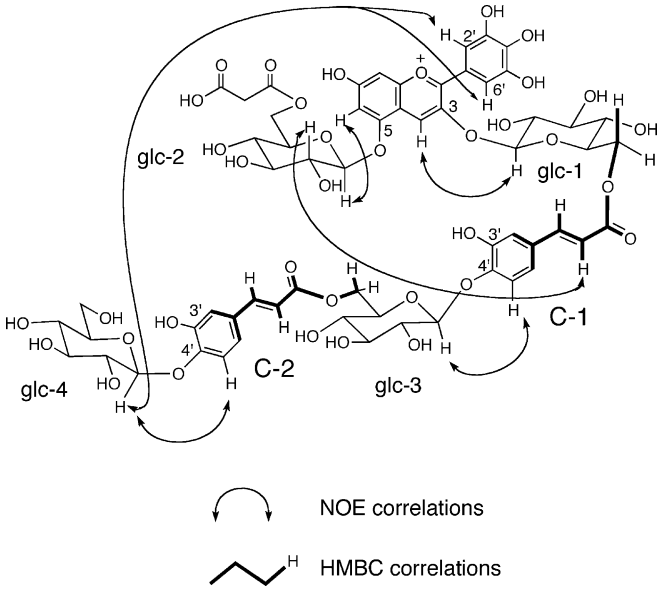


Fig. 2. NOE and HMBC correlation of **1**.

the 3 and 4 positions of C-2. Therefore, we conducted alkaline hydrolysis of **1**. Reaction of **1** in aq. NaOH-methanol under an argon (Ar) atmosphere gave a mixture of 4-*O*-glucosyl-(*E*)-caffeic acid (**6**) and methyl 4-*O*-glucosyl-(*E*)-caffeate (**7**). The structure was confirmed by NMR, MS experiment and comparison with authentic sample (Goto et al., 1981). The combined yield of **6** and **7** was quantitative (195%) to **1**; therefore, the linkage of glc-3 and 4 was determined to be at the 4-OH of caffeoyl residues, C-1 and C-2, respectively. Thus, **1** was deduced to be 3-*O*-(6-*O*-(4′-*O*-(6-*O*-(4′-*O*-β-D-glucopyranosyl-(*E*)-caffeoyl)-β-D-glucopyranosyl)-(*E*)-caffeoyl)-β-D-glucopyranosyl)-5-*O*-(6-*O*-malonylβ-D-glucopyranosyl)delphinidin.

The structure of **2** was determined by using the same MS and NMR techniques (Table 1) to be 3-*O*-(6-*O*-(4′-*O*-(6-*O*-(4′-*O*-β-D-glucopyranosyl-(*E*)-caffeoyl)-β-D-glucopyranosyl)-(*E*)-caffeoyl)-β-D-glucopyranosyl)-5-*O*-β-D-glucopyranosyl-delphinidin.

By NOESY and ROESY experiments long range NOEs between the anomeric proton of glc-4 and the protons at 2′ and 6′ of the delphinidin nucleus were observed (Fig. 2) Furthermore, the protons at 2, 5 and 6 positions in the both caffeoyl residues (C-1 and C-2) shifted toward 0.16–0.61 ppm upfield as compared with the corresponding protons of methyl 4-*O*-glucosyl-(*E*)-caffeate (**7**) (Table 1 and data in Section 3.2.2). These data indicate that the caffeoyl residues of **1** and **2** stack to the anthocyanidin nucleus intramolecularly in TFA *d*-CD_3OD just as the same as gentiodelphin (Yoshida et al., 1991, 2000). Since intramolecular stacking of acyl residues of polyacylated anthocyanins to the anthocyanidin nucleus becomes stronger in neutral aqueous condition than that in acidic methanol (Yoshida et al., 1991, 2000), the caffeoyl residues of phacelianin (**1**) and its demalonylated pigment (**2**) should stack to the delphinidin nucleus in the petal cells.

In the survey of polyacylated anthocyanins in blue flower petals, we found that the HPLC data of the major pigment of the blue petals of *E. pilosus* were identical to those of **1**. However, Toki et al. (1994) reported a different structure for the petal anthocyanin. Therefore, we isolated anthocyanin from the blue petals of *E. pilosus* and compared it with **1** and the authentic pigment. The HPLC data and ^{1}H NMR spectral data were identical to those of **1**, resulting in the structure of the pigment in *E. pilosus* and *P. campanularia* being identical to **1**.

The structure of **3** was deduced to be 3-*O*-(*E*)-caffeoylquinic acid (chlorogenic acid) by comparison with an authentic sample.

2.2. VIS absorption spectra and CD of petals and colored cells of P. campanularia

To help understand how pigment molecules are present in a living petal cell, we measured the VIS absorption spectra and CD of a living petal and colored cells. The CD can provide very important information on molecular association, especially the stacking manner of the chromophores (Goto and Kondo, 1991; Kondo et al., 1992). Thus, the CD recording of intact flower petals were also conducted by Hoshino (1986). However, CD measurement of intact petals has some difficulties; the colorless intercellular space filled with air causes diffuse reflections following low sensitivity with a noisy spectrum. To overcome these problems, we applied a single cell measurement using the microspectrophotometric method for recording the VIS spectrum. As another solution to reduce scattered reflection, we evacuated the petals, filling the intercellular space with water. The obtained petals became more transparent, and the VIS spectrum and CD measurements could then be conducted more noiselessly with higher sensitivity. We also purified the blue-colored cells and CD of the gathered cells was recorded in a quartz cuvette.

Fig. 3 shows the VIS absorption spectra and CD of fresh petals and colored cells from the blue petals of *P. campanularia*. The absorption spectrum of the petals showed three λmax around 638, 578 and 546 nm, coinciding with that of a single protoplast. In the CD, a negative Cotton effect was observed at the absorption maximum at the VIS region, and there was no difference between the CD of the evacuated petal and the suspension of the colored protoplasts (Fig. 3). The negative Cotton effect strongly suggests chiral self-association of the anthocyanidin nuclei of 1 in an anti-clockwise stacking manner. Until now, many polyacylated anthocyanins have been isolated and their CD spectra recorded. However, no pigment showed such a CD with Cotton effects. Generally, aromatic acyl residues of polyacylated anthocyanins are stacked from both sides of the anthocyanidin nucleus to stabilize the chromophores, preventing hydration of the nucleus. Only simple anthocyanins without aromatic acyl residues (Hoshino et al., 1981a,b; Goto et al., 1987) or with one acyl residue (Yoshida et al., 1991; Kondo et al., 1992; Kondo et al.,

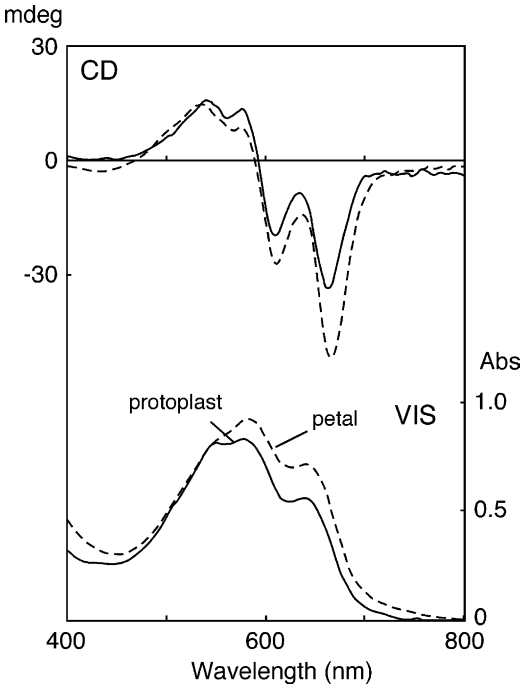


Fig. 3. VIS absorption spectra (lower) and CD (upper) of the blue petal and suspension of the blue cells of phacelia. -----: petal, ——: suspension of protoplasts.

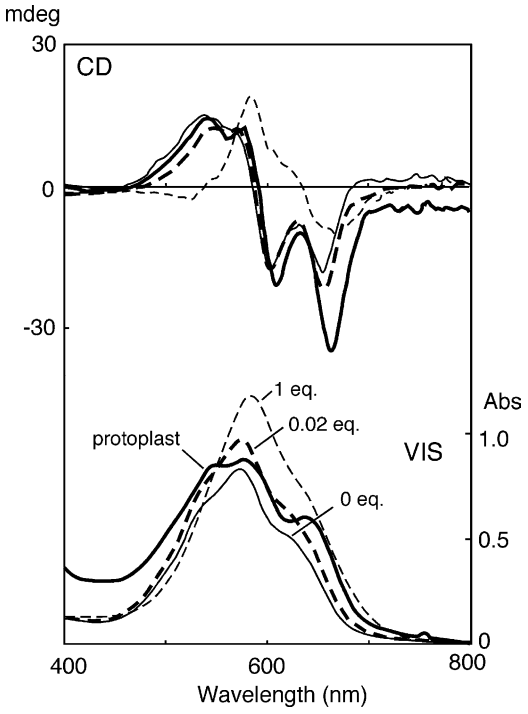


Fig. 4. Reproduction of the blue color of the protoplast of *Phacelia campanularia* by mixing **1** (100 μM) with/without Al^{3+} in buffered solution (100 mM acetate buffer, pH 5.5), path length, 1.0 mm. ——: suspension of protoplasts, ——: without Al^{3+}, – – –: 0.02 eq. Al^{3+}, -----: 1 eq. Al^{3+}.

1994; Kondo et al., 2001) are supposed to be able to stack together with an anthocyanidin nuclei. Therefore, the Cotton effect of the blue petal and cells is very interesting and important information on blue color development.

2.3. Reproduction of blue color with combined petal components

To clarify the mechanism of the blue flower color development of *P. campanularia*, we first analyzed the components of colored cells. The HPLC detected at 280 nm showed that only **1** with a small amount of **2** and **3** existed in the colored cells. The content of metals was analyzed, and Mg, Al and Fe were detected. The molar ratio of Mg, Al and Fe to the combined anthocyanins (**1** and **2**) was 0.2–0.5 eq., 0.01–0.02 eq. and 0.01–0.02 eq., respectively.

By referring to the above-mentioned data, we tried to reproduce the same blue color of phacelia petals by mixing petal components in various pH aqueous solutions. As shown in Fig. 4, **1** (5×10^{-4} M) in aqueous solution at pH 5.5–6.0 gave the same VIS spectrum and CD as those of petals. However, the stability of the blue solution was low at this pH and after 24 h, blue precipitates were formed. By the addition of Al^{3+} or Fe^{3+} (>1/3 eq. to **1**), a blue solution with different VIS spectra and CD from that of petals and protoplasts was obtained. The addition of an excessive amount of Mg^{2+} (1–10 eq.) to **1** in buffer (pH 5.5) affects neither the spectrum nor the stability of the solution. However, by the addition of a small amount of Al^{3+} or Fe^{3+} to **1** (<0.02 eq.), a stable blue solution, the same as that of petals and colored protoplasts, was obtained and after 24 h, no precipitates were formed. Compound **2** showed the same behavior and color development as **1**. Since the bathochromic shift of anthocyanins by addition of trivalent metal ions are well known (Harborne, 1958; Bayer, 1958; Bayer et al., 1966; Takeda et al., 1990; Kondo et al., 1994; Dangles et al., 1994; Yoshida et al., 2003a; Kondo et al., 2005) metal complexation of Al^{3+} and/or Fe^{3+} may involve in the blue coloration of phacelia petals in some extent.

In conclusion, phacelianin (**1**) was the first structural elucidated polyacylated anthocyanin that takes two kinds of molecular association, self-association and intramolecular stacking. In the colored vacuole, the anthocyanidin nuclei of **1** stacked each other in an anticlockwise stacking manner, and intramolecular caffeoyl residues stacked to the other side of the nucleus. Furthermore, a small amount of trivalent metal ions may solubilize **1** in weakly acidic vacuoles without any co-pigments and the blue petal color is developed.

3. Experimental

3.1. General

UV–Vis spectra and reflection spectra were recorded on a JASCO V-560 spectrometer. The absorption spectra of the colored cells were measured with an inverted

microscope (IX70, OLYMPUS) equipped with a micro-spectrophotometer (MCPD-7000, Photal). Circular dichroism (CD) was measured with a JASCO J-720 spectrometer. NMR spectra were obtained with a JEOL ECA-500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) and JNM-A600 spectrometer (^{1}H: 600 MHz, ^{13}C: 150 MHz) in a 5-mm ∅ tube at variable temperature using 5–10% TFA *d*-CD_3OD as a solvent. Chemical shifts were recorded as parts per million (ppm) with the CD_2HOD resonance as a standard. FABMS data were recorded on a JEOL JMS-700 using glycerol–HCl as a matrix. Analytical and preparative HPLC were conducted according to our procedure (Yoshida et al., 1992, 1996, 2002, 2003b) using an ODS-column (Develosil ODS-HG-5 2.0 mm ∅ × 250 mm, 4.6 mm ∅ × 250 mm and 20 mm ∅ × 250 mm, Nomura Chemical). HPLC was performed at 40 °C with 30-min linear gradient elution from 10% to 30% aq. CH_3CN solution containing 0.5% TFA, or isocratic elution with various concentrations of CH_3CN aq. solution containing 0.5% TFA. Elemental analysis was conducted with a Perkin–Elmer Optima 2100 DV ICP-AES instrument.

3.2. Isolation of phacelianin (**1**) and demalonylphacelianin (**2**)

3.2.1. Isolation of **1** and **2** from *P. campanularia*

Blue petals (300 g) of *P. campanularia* cultivated at the Nagoya University Farm were extracted with 2.6 L of 35% aq. CH_3CN solution containing 0.5% TFA. The extract was evaporated under reduced pressure to half volume, and the condensed extract was purified with an Amberlite XAD-7 column. The column was eluted with a stepwise gradient elution from 0.5% TFA–H_2O to 20% aq. CH_3CN solution containing 0.5% TFA. The 15% aq. CH_3CN fraction was evaporated to give a crude anthocyanin. Further purification was conducted by a preparative ODS-HPLC eluted with 5–16% aq. CH_3CN solution containing 0.5% TFA. Compounds **1** (170 mg) and **2** (24 mg) were obtained from the 16% aq. CH_3CN fraction as dark-red amorphous TFA salts with **3** (10% aq. CH_3CN fraction, 24 mg).

3.2.2. Isolation of **1** from blue petals of *E. pilosus*

Blue petals (150 g) of *E. pilosus* cultivated at the Nagoya University Farm were extracted with 1.5 L of 50% aq. CH_3CN solution containing 1.0% TFA. The extract was evaporated under reduced pressure to one-third volume, and the condensed extract was purified with an Amberlite XAD-7 column. The column was eluted with a stepwise gradient elution from 1.0% TFA–H_2O to 50% aq. CH_3CN solution containing 1.0% TFA. In the 15–20% aq. CH_3CN fraction, phacelianin (**1**), demalonylphacelianin (**2**), chlorogenic acid (**3**), and 3,5-dicaffeoylquinic acid (**4**) were eluted. Further purification was conducted by repeated preparative ODS-HPLC to give **1** (53 mg) and **2** (19 mg) as dark-red amorphous TFA salts with **3** (7.6 mg) and **4** (73 mg).

3.3. Alkaline hydrolysis of **1**

Compound **1** (TFA salt, 5.7 mg, 3.8 mmol) was dissolved in methanol (1 mL) and 0.1 M aq. NaOH (2 mL) under an argon atmosphere and stood at 0 °C for 1 h. A small portion of 6 M aq. HCl (0.2 mL) was added to the reaction mixture, and the mixture afforded preparative HPLC to give 2.1 mg of delphin (**5**, 74%), 1.0 mg of 4-*O*-β-glucopyranosylcaffeate (**6**, 72%) and 1.7 mg of methyl 4-*O*-β-glucopyranosylcaffeate (**7**, 123%), respectively.

3.3.1. 4-*O*-Glucosyl-(*E*)-caffeic acid (**6**)

^{1}H NMR (500 MHz, CD_3OD): 3.41 (1H, *dd*, 9.5, 8.0, glc-4), 3.45 (1H, *ddd*, 9.5, 5.0, 1.5, glc-5), 3.48 (1H, *dd*, 9.5, 8.0, glc-3), 3.52 (1H, *dd*, 9.0, 7.5, glc-2), 3.72 (1H, *dd*, 12.0, 5.0, glc-6a), 3.90 (1H, *dd*, 12.0, 1.5, glc-6b), 4.85 (1H, *d*, 7.5, glc-1), 6.31 (1H, *d*, 16.0, caf-α), 7.03 (1H, *dd*, 8.5, 2.5, caf-6), 7.10 (1H, *d*, 2.5, caf-2), 7.19 (1H, *d*, 8.5, caf-5), 7.55 (1H, *d*, 16.0, caf-β).

3.3.2. Methyl 4-*O*-glucosyl-(*E*)-caffeate (**7**)

^{1}H NMR (500 MHz, CD_3OD): 3.41 (1H, *dd*, 9.0, 8.0, glc-4), 3.45 (1H, *ddd*, 9.0, 5.0, 1.5, glc-5), 3.48 (1H, *dd*, 9.0, 8.0, glc-3), 3.52 (1H, *dd*, 9.0, 7.5, glc-2), 3.72 (1H, *dd*, 12.0, 5.0, glc-6a), 3.76 (3H, *s*, OCH_3), 3.90 (1H, *dd*, 12.0, 1.5, glc-6b), 4.86 (1H, *d*, 7.5, glc-1), 6.35 (1H, *d*, 16.0, caf-α), 7.04 (1H, *dd*, 8.5, 2.5, caf-6), 7.10 (1H, *d*, 2.5, caf-2), 7.19 (1H, *d*, 8.5, caf-5), 7.57 (1H, *d*, 16.0, caf-β).

3.4. Acid hydrolysis of **1**

Compound **1** (TFA salt, 1.0 mg) was dissolved in 1% HCl–methanol (1 mL) and stood at 40 °C for 3 days. The reaction mixture was analyzed with HPLC (column: 2 mm ∅, solvent: 16% aq. CH_3CN solution containing 0.5% TFA) repeatedly.

3.5. Preparation of protoplasts

The preparation of colored protoplasts was conducted using a slightly modified method (Yoshida et al., 2003a,b). Fresh petals (0.5 g) were cut at a thickness of 1 mm and incubated in a medium (0.6 M mannitol, 20 mM Tris–AcOH, pH 6.0) containing 0.2% (w/v) Macerozyme R-10 (Yakult) and 2.0% (w/v) Cellulase ONOZUKA R-10 (Yakult) at 30 °C for 80 min. The reaction mixture was filtered through Miracloth (Calbiochem), and the obtained filtrate was washed 3 times. The obtained protoplast mixture was further purified by sucrose density gradient centrifugation. The protoplast mixture was suspended in 2.5 mL of buffer (1.0 M sucrose, 50 mM Mes–Tris pH 6.0). The suspension was overlaid on 2.5 mL of 0.6 M sucrose and 0.65 M mannitol gradient in 50 mM Mes–Tris, pH 6.0 and centrifuged at 320*g* for 10 min. The blue-colored cells obtained at the interface between 0.6 M sucrose and 0.65 M mannitol were collected and washed.

3.6. VIS spectrum and CD measurement of petals and colored cells

The reflection spectrum of a fresh petal was recorded according to a previous report (Yoshida et al., 2003a,b). CD of the petal was recorded with a pre-evacuated tissue. A fresh petal was cut into squares of ca. 10 mm × 10 mm, and the pieces were put into a flask with water. The flask was evacuated several times, then, the intercellular space of the petal tissue was filled with water and the transparency of the petal increased. The petal was fixed on a quartz plate and CD was measured (400–800 nm). The suspension of colored cells in a medium (0.6 M mannitol, 20 mM Tris–AcOH, pH 6.0) was poured into a quartz cuvette (path length, 1 mm) and the VIS absorption spectrum and CD were recorded.

3.7. Analysis of components in colored protoplasts

An aliquot quantity of the suspension of colored protoplasts was diluted with water containing 0.5% TFA and analyzed with HPLC. The amount of **1** was calculated directly by the calibration curve of standard solutions. Another aliquot quantity of the suspension of colored protoplasts was diluted with 0.5% (w/v) aq. HNO_3, and Al, Mg and Fe were analyzed by ICP-AES under standard operating conditions.

3.8. Reproduction of petal color

Compound **1** (TFA salt) was dissolved in water at a concentration of 5×10^{-2} M. Individual solutions were diluted to 5×10^{-4} M with 0.1 M acetate buffer (pH 5–5.5) or 0.1 M phosphate buffer (pH 6–8) with/without Mg^{2+}, Al^{3+}, Fe^{3+}, and **3**. UV–Vis spectra and CD were measured in a quartz cell (path length: 1 mm) at 25 °C.

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