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Structure of an anthocyanin–anthocyanin dimer molecule in anthocyanin-producing cells of a carrot suspension culture

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ABSTRACT

A novel anthocyanin, an anthocyanin–anthocyanin dimer, was isolated from the cells of an anthocyanin-producing carrot cell-line culture, and its structure was elucidated using spectroscopic methods. It consists of two molecules of the anthocyanin, cyanidin 3-[xylosyl-(sinapoyl-glucosyl)-galactoside], with a CH–CH_3 linkage at the 8–8 position. This is the first report of the identification and isolation of an anthocyanin–anthocyanin dimer with a CH–CH_3 linkage from intact plant cells.

Anthocyanins are one of many subclasses of flavonoids and are responsible for the color of petals, pericarps and of other organs of many plant species,[1] and that of many types of food and beverage.[2,3] Anthocyanins are the glycosides of anthocyanidins (aglycons), which contain A-, B-, and C-ring moieties.[1] Novel anthocyanins with unique aglycon moieties have recently been identified. A C4-substituted anthocyanin containing an additional pyran ring between C4 and the hydroxyl group at C5 has been isolated from red wine,[3,4] black carrot juice,[5] and petals of *Rosa hybrida* cv. M'me Violet.[6] An ethyl-linked anthocyanin dimer containing anthocyanin and flavonol or two molecules of anthocyanin linked in a CH–CH_3 linkage have been isolated from red wine,[3,7] rosé cider,[8] and model alcoholic solutions.[9,10] C4-substituted anthocyanins have been isolated both in vitro and in vivo, but the ethyl-linked anthocyanin dimer has only been identified in vitro.

The carrot (*Daucus carota* L.) produces anthocyanins in intact plants and in cultured cells. Known carrot anthocyanins include cyanidin 3-galactoside (Cya 3-Gal), 3-xylosyl-Gal (3-Xyl-Gal), 3-triglycoside (3-Xyl-glucosyl-Gal) (3-Xyl-Glc-Gal), and feruloyl, 4-coumaroyl, sinapoyl, 4-hydroxybenzoyl derivatives of Cya triglycoside.[11–13] The minor component, Cya 3-Glc, was recently isolated,[14] and anthocyanins possessing a peonidin or pelargonidin type of aglycon have been detected using high-performance liquid chromatography/mass spectrometry (HPLC/MS) analysis.[15]

Our laboratory has established a variant carrot cell line (*D. carota* L. cv. Kurodagosun).[16] HPLC/MS analysis indicated that the

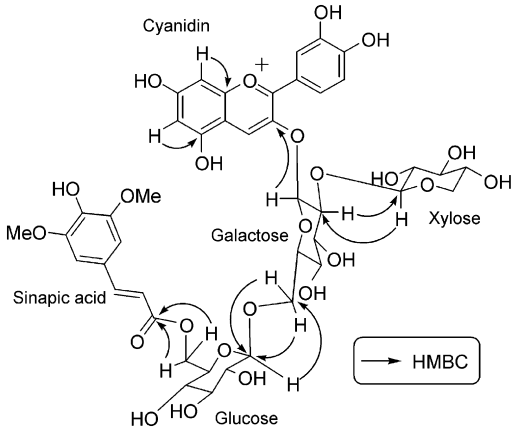

Figure 1. Structure of anthocyanin **1**. The arrows show HMBC correlations.

Abbreviations: Cya, cyanidin; DQF-COSY, double quantum filter correlation spectroscopy; Gal, galactose (galactoside); Glc, glucose (glucoside, glucosyl); HMBC, heteronuclear multiple bond connectivity; HMQC, heteronuclear multiple quantum coherence; HPLC, high-performance liquid chromatography; HR, high-resolution; MS, mass spectrometry; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; TOCSY, totally correlated spectroscopy; TOF, time-of-flight; Xyl, xylose (xyosyl).

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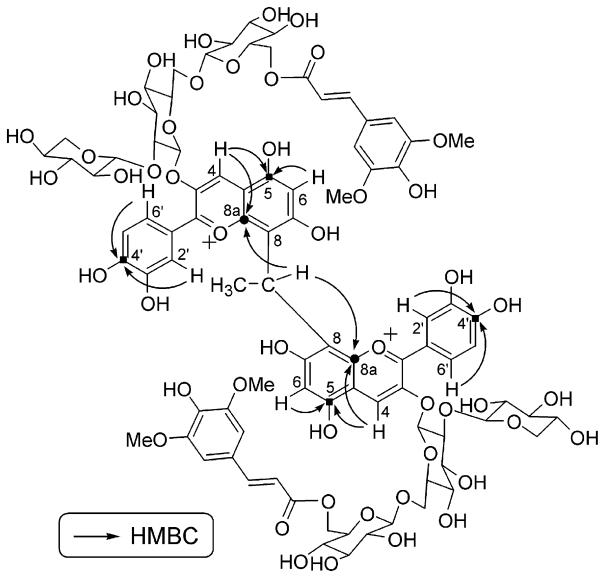

Figure 2. Structure of anthocyanin **2**. The arrows show HMBC correlations. The black squares and circles indicate carbons with signals that exhibited isotopic and non-isotopic shifts, respectively, in the H–D exchange experiment.

major anthocyanin of this cell line is Cya 3-Xyl-sinapoyl-Glc-Gal (**1**, Fig. 1) and that a novel anthocyanin, the anthocyanin–anthocyanin dimer (**2**, Fig. 2), was present as a minor component (Fig. 3). In this report, we describe the isolation and elucidation of the structures of anthocyanins **1** and **2**.

The anthocyanin-producing cells of the variant carrot cell line were grown in modified Lin and Staba liquid medium containing 5×10^{-7} M 2,4-dichlorophenoxyacetic acid and cultured for 14 days as previously reported.[16] The cells were harvested using a Buchner funnel, frozen in liquid N_2, and lyophilized. Anthocyanins were extracted over a period of 12 h from 3 g (dry weight) of cells into 1 L of 70% aqueous ethanol, after which the reddish-purple extracts were filtered and the residual solvent was removed by evaporation. The extracts were dissolved in 20% methanol containing 0.1% trifluoroacetic acid (TFA) and were applied to an ODS column (35 × 180 mm, Wakosil 25C15, Wako Pure Chemical Industries Ltd., Osaka, Japan). The anthocyanins were eluted with 20% methanol containing 0.1% TFA followed by 50% methanol containing 0.1% TFA. The solvent was removed from eluates by evaporation. Compound **1** was purified from the 20% methanol containing 0.1% TFA fraction by preparative HPLC on a Synergi 4 μ RP-80 Å (21.2 × 250 mm, Phenomenex, Torrance, CA, USA) with 0.1% aque-

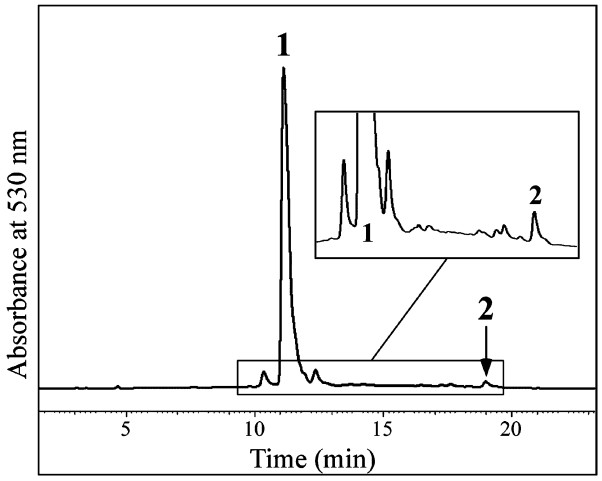

Figure 3. Elution profile of anthocyanins isolated from the cells of a variant carrot cell line by analytical HPLC using a Synergi 4 μ RP-80 Å (4.6 × 250 mm, Phenomenex) and 0.1% aqueous formic acid (A) and methanol (B) as a mobile phase. The elution program consisted of a linear gradient from 15% B to 80% B for 30 min at a flow rate of 1.0 mL/min monitored at 530 nm.

Table 1
^{1}H (800 MHz) and ^{13}C (200 MHz) assignments of anthocyanin **1** and **2** in CD_3OD/CF_3COOD (9:1) at 25 °C

Position	1		2	
	δ^1H (ppm)	δ^{13}C (ppm)	δ^1H (ppm)	δ^{13}C (ppm)
Ethyl-linkage				
CH	–	–	5.19 (q, 8)	28.4
CH_3	–	–	1.95 (3H, d, 8)	19.3
Cyanidin				
2	–	162.3	–	162.4, 164.1
3	–	145.6	–	144.85, 144.92
4	8.34	132.9	8.51[c], 8.60	133.3, 133.7
4a	–	112.4	–	112.3, 113.4
5	–	158.7	–	156.9, 157.3
6	6.61	103.3	6.61,6.63	102.4, 103.1
7	–	169.3	–	167.3, 167.7
8	6.39	95.1	–	110.4, 112.0
8a	–	156.6	–	153.1, 155.7
1′	–	120.8	–	121.4, 121.7
2′	7.69	118.7	8.06, 8.15	118.5, 119.6
3′	–	147.0	–	147.4, 147.6
4′	–	156.0	–	155.2, 156.1
5′	6.92 (d, 9)	117.5	7.14 (2H, d, 9)	117.1, 117.9
6′	8.08 (d, 9)	129.8	8.29 (d, 9), 8.51[c]	129.1, 130.1
Galactose				
1	5.16 (d, 7)	102.3	5.44 (d, 7), 5.50 (d, 7)	101.0, 101.6
2	4.35 (d, 7)	80.6	4.29 (t, 8), 4.35 (t, 8)	80.7, 81.1
3	4.20 (dd, 3,10)	75.6	4.21–4.27 (4H)[d]	75.37, 75.44
4	3.99 (d, 3)	70.7	3.98 (2H)	70.6, 70.7
5	4.48 (d, 10)	77.5	4.55 (d, 9), 4.56 (d, 8)	77.3, 77.5
6a	3.79 (d, 12)	73.6	3.70–3.79 (6H)[e]	73.7, 73.8
6b	4.32 (d, 10)		4.21∼4.27 (4H)[d]	
Xylose				
1	4.88 (d, 8)	105.9	4.89 (d, 8), 4.95[l]	106.1, 106.2
2	3.21 (t, 9)	76.2	3.32[l]	76.2, 76.3
3	3.40 (t, 9)	78.2	3.38–3.47 (10H)[f]	78.2, 78.3
4	3.27 (dd, 6, 9)	71.2	3.38–3.47 (10H)[f]	71.26,71.31
5a	3.19 (d, 12)	67.2	3.17 (t, 11), 3.27 (t,11)	67.4, 67.5
5b	3.63 (dd, 6, 12)		3.70–3.79 (6H)[e]	
Glucose				
1	4.53 (d, 7)	107.0	4.47 (2H,t, 7)	107.18, 107.23
2	3.52 (d, 7)[a]	77.9	3.38–3.47 (10H)[f]	77.87, 77.90
3	3.52 (d, 7)[a]	74.7	3.38–3.47 (10H)[f]	74.7
4	3.74 (t,9)	70.1	3.70–3.79 (6H)[e]	70.0
5	3.46 (d,10)	75.3	3.38–3.47 (10H)[f]	75.6
6a	4.12 (d, 11)	61.0	4.14, (2H, t, 12)	61.1
6b	5.33 (d,10)		5.37 (2H,t, 11)	
Sinapic acid				
1	–	125.4	–	125.8
2	6.04 (2H)[b]	105.0[h]	6.31 (2H), 6.36 (2H)[g]	105.6[j]
3	–	148.9[i]	–	149.1[k]
4	–	139.2	–	139.3
5	–	148.9[i]	–	149.1[k]
6	6.04 (2H)[b]	105.0[h]	6.31 (2H), 6.36 (2H)[g]	105.6[j]
7	7.20 (d, 16)	147.8	7.36 (d, 16), 7.45 (d, 16)	144.7, 148.1
8	6.07 (d, 16)	116.2	6.26 (d, 16), 6.32 (d, 16)	116.3, 116.5
9	–	169.2	–	169.3, 169.4
OMe	3.37 (6H)	56.2	3.52 (6H), 3.60 (6H)	56.4, 56.5

TMS was used as internal standard.
Values in parentheses indicate integral, multiplicity and coupling constants (*J* in Hz).
[a–g] Overlapped with each other.
[h–k] Assignments with the same letters are interchangeable.
[l] Overlapped with solvent peak. Signals were detected by HMQC and HMBC analyses.

ous TFA as solvent A and methanol as solvent B. The elution program consisted of a linear gradient from 10% B to 70% B for 20 min and from 70% B to 80% B for the next 10 min at a flow rate of 10 mL/min monitored at 530 nm. Compound **2** was purified from the 50% methanol containing 0.1% TFA fraction of ODS column using preparative HPLC and an elution program consisting of a linear gradient from 40% B to 80% B for 20 min at a flow rate of 10 mL/min monitored at 330 nm to separate other phenolic compounds including flavonoids from anthocyanin **2**. The fraction containing **2** was subjected to further purification by preparative HPLC using an elution program consisting of a linear gradient from 30% B to 80% B for 20 min at a flow rate of 10 mL/min monitored at 530 nm. After repeating these purification processes for three times, we obtained 54.2 mg of **1** and 10.9 mg of **2**.

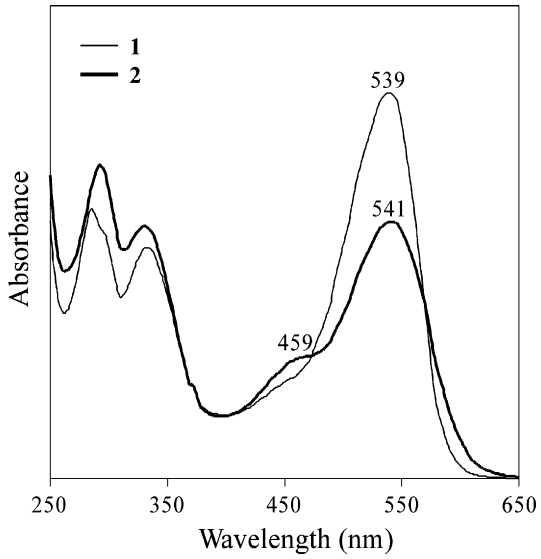

Figure 4. UV–visible spectra of compounds **1** and **2** in methanol containing 1% hydrochloric acid.

Purified anthocyanin **1** was subjected to high-resolution time-of-flight (HR-TOF)-MS analysis, which showed that its molecular formula was $C_{43}H_{49}O_{24}^+$ (*m/z* 949.2573 $[M]^+$, calculated 949.2608). The 1D (^1H and ^{13}C) and 2D (heteronuclear multiple bond connectivity (HMBC), heteronuclear multiple quantum coherence (HMQC), double quantum filter correlation spectroscopy (DQF-COSY) and totally correlated spectroscopy (TOCSY)) nuclear magnetic resonance (NMR) analyses showed that **1** was a Cya 3-Xyl-(sinapoyl-Glc)-Gal, one of the main carrot anthocyanins (Fig. 1, Table 1).[12] HMBC correlations between Gal and Cya, Xyl and Gal, Glc and Gal, and Glc and sinapic acid for structure **1** are shown in Figure 1.

Purified anthocyanin **2** was subjected to HR-TOF-MS analysis, which showed that the molecular formula of **2** was $C_{88}H_{99}O_{48}^+$ (*m/z* 1923.5339 $[M]^+$, calculated 1923.5306). Other signals were observed at *m/z* 1717, 1555, 1261, 962, 893, 631, and 599. The ion peak at *m/z* 962 and 631 had an isotopic ion peak at 0.5 mmu intervals, which suggests that anthocyanin **2** is a double charged ion, possibly an anthocyanin–anthocyanin dimer because monomeric anthocyanins generally have molecular ion peaks $[M]^+$ and isotopic ion peaks at 1 mmu intervals. Comparison of our results with those of a report in which an anthocyanin–anthocyanin dimer was generated from a model alcoholic solution[10] suggests that the ion peak at *m/z* 962 is caused by a di-flavylium cation (Fig. 2) and the ion peak at *m/z* 1923 is caused by a neutral quinoidal base and a flavylium cation in the dimeric anthocyanin. The MS signals at *m/z* 1717, 1555, 1261, 893, and 599 corresponded to losses of 206 (sinapic acid), 162 (Glc), 294 (Xyl and Gal), 368 (sinapic acid and Glc), and 294 (Xyl and Gal) from the ion peak signal at 1923, respectively, indicating that anthocyanin **2** has two anthocyanin **1** molecules. The signal at *m/z* 599 indicates that there are two cyanidin moieties and one C_2H_4 linkage. Comparison of the

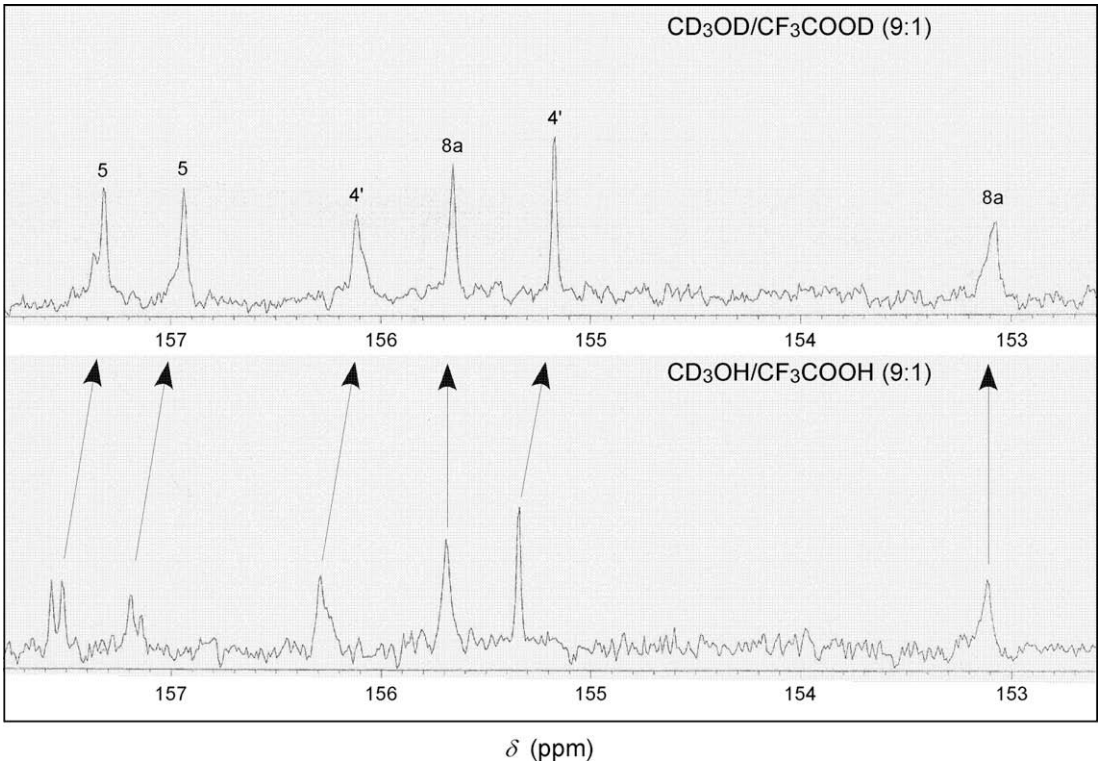

Figure 5. ^{13}C NMR chart for CD_3OD/CD_3COOD (9:1) and CD_3OH/CD_3COOH (9:1) in the range δ_C153–158 ppm.

UV-visible spectra for **1** and **2** in methanol containing 1% hydrochloric acid shows that the spectrum of **2** has a shoulder at 459 nm (Fig. 4), which is present in the absorbance spectrum of the CH–CH_3 linked anthocyanin dimer in the previous reports.[7–10] These results suggest that **2** consists of two **1** molecules and has CH–CH_3 linkage moieties. To elucidate the structure of **2**, in particular the linkage moiety and its position, 1D (^{1}H and ^{13}C) and 2D (HMBC, HMQC, and DQF-COSY) NMR analyses were performed. The ^{1}H NMR spectra of **2** showed signals characteristic of an anthocyanin dimer with a CH–CH_3 linkage[8–10] (CH; 5.19, q, J = 8 and CH_3; 1.95, d, J = 8, Table 1). To determine the position of the CH–CH_3 linkage, we first determined the 5- and 8a-position carbon signals. From the results of ^{13}C NMR analysis of anthocyanin **1** (Table 1), the six carbon signals in the range δ_C 153.1–157.3 ppm were predicted to be 5-, 8a-, and 4′-position carbon signals (Fig. 5). The HMBC correlations were observed between 4-position proton signals (δ_H 8.51 and 8.60) and four carbon signals (δ_C 153.1, 155.7, 156.9, and 157.3), and done between 2′- and 6′-position proton signals (2′, δ_H 8.06 and 8.15; 6', δ_H 8.29 and 8.51) and two carbon signals (δ_C 155.2 and 156.1) (Fig. 2). These correlations showed that four carbon signals (δ_C 153.1, 155.7, 156.9, and 157.3) were either 5- or 8a-position carbon signals and two carbon signals (δ_C 155.2 and 156.1) were 4′-position carbon signals. As the 5- and 8a-position carbon signals were not distinguishable from the aforementioned four carbon signals, we conducted a H–D exchange experiment using methanol D-3 (CD_3OH)/TFA (CF_3COOH) (9:1) and methanol D-4 (CD_3OD)/TFA D-1 (CF_3COOD) (9:1) (Fig. 5). In CD_3OD/CF_3COOD (9:1), hydroxyl groups were deuterated, leading to isotopic shift of hydroxyl carbon signals on ^{13}C NMR analysis, whereas in CD_3OH/CF_3COOH (9:1), hydroxyl groups were not deuterated and showed a non-isotopic shift in hydroxyl carbon signals on ^{13}C NMR analysis. Thus, compared with the ^{13}C NMR signals in CD_3OH/CF_3COOH (9:1) and CD_3OD/CF_3COOD (9:1), the hydroxyl carbon signals showed isotopic shift. For these four carbon signals, those with isotopic and non-isotopic shifts were attributed to 5-position and 8a-position carbon signals, respectively. The carbon signals, δ_C 156.9 and 157.3, showed isotopic shifts (Fig. 5), which were attributed to 5-position carbon signals. The other carbon signals, δ_C 153.1 and 155.7, showed non-isotopic shifts, which were attributed to the 8a-position. HMBC correlations were observed between the methine proton signal (δ_H 5.19) and the two carbon signals at the 8a-position (δ_C 153.1 and 155.7) (Fig. 2). HMBC correlations between two proton signals at the 6-position (δ_H 6.61 and 6.63) and two carbon signals at the 5-position (δ_C 156.9 and 157.3) were also observed (Fig. 2). Other HMBC correlations (between Gal and Cya, Xyl and Gal, Glc, and Gal and Glc and sinapic acid) observed for anthocyanin **1** (Fig. 1) were also observed for anthocyanin **2**. As such, anthocyanin **2** was determined to be a Cya 3-Xyl-sinapoyl-Glc-Gal dimer linked by a CH–CH_3 linkage at the 8–8 position (Fig. 2, Table 1).

This is a first report of an ethyl-linked anthocyanin–anthocyanin dimer in living cells. In vitro experiments have shown that ethyl-linked anthocyanin is formed in an acetaldehyde-mediated condensation reaction between anthocyanins and flavonols[8,9] or between anthocyanins.[10] In red wine, acetaldehyde produced by yeast metabolism during fermentation[17] or by ethanol oxidation[18] forms dimeric anthocyanins with CH–CH_3 linkages.[9] Studies to determine how anthocyanin **2** is produced in intact cells and on the isolation and identification of the other minor components detected by HPLC analysis (Fig. 3) are now in progress.

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