Tetrahedron Letters 49 (2008) 7330–7333

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

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

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ARTICLE INFO

Article history:
Received 3 September 2008
Revised 8 October 2008
Accepted 10 October 2008
Available online 14 October 2008

Keywords:
Anthocyanin
Carrot
Dimer
Ethyl-linked
Daucus carota

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

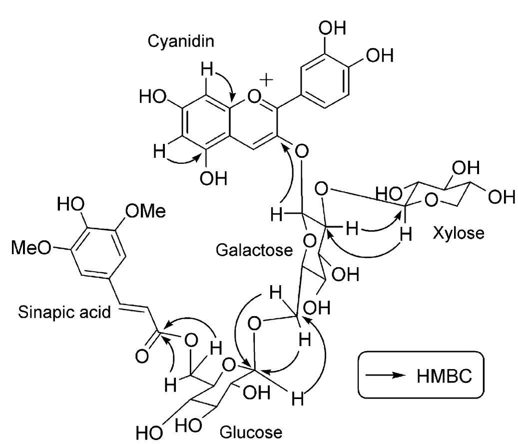

Figure 1. Structure of anthocyanin **1**. The arrows show HMBC correlations.

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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doi:10.1016/j.tetlet.2008.10.041

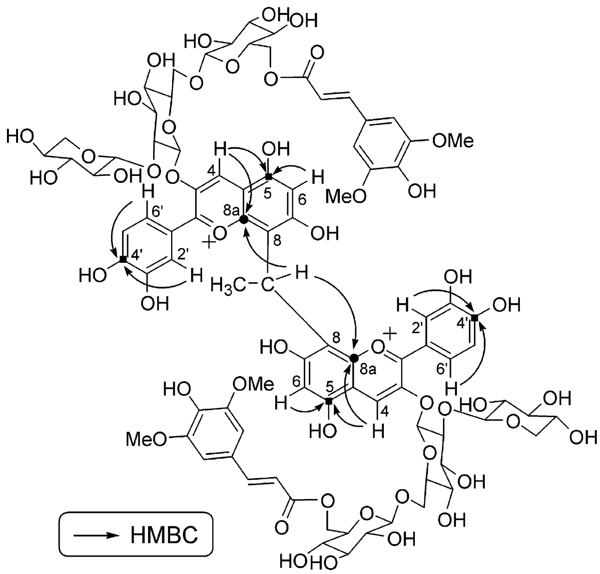

Figure 2. Structure of anthocyanin **2**. The arrows show HMBC correlations. The black squares and circles indicate carbons with signals that exhibited isotopic and non-isotopic shifts, respectively, in the H–D exchange experiment.

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-

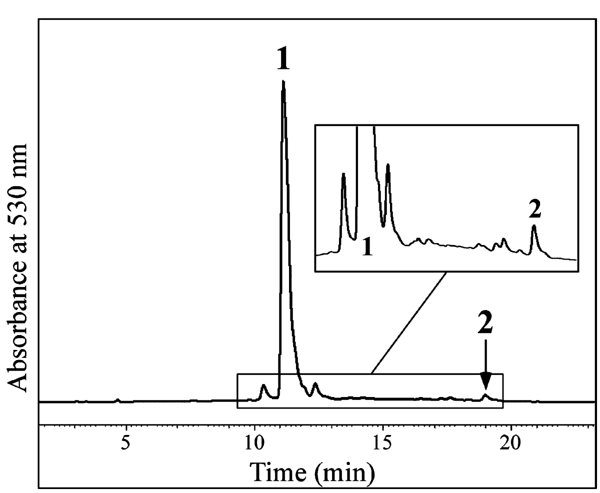

Figure 3. Elution profile of anthocyanins isolated from the cells of a variant carrot cell line by analytical HPLC using a Synergi 4 μ RP-80 Å (4.6 × 250 mm, Phenomenex) and 0.1% aqueous formic acid (A) and methanol (B) as a mobile phase. The elution program consisted of a linear gradient from 15% B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