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TRIACYLATED ANTHOCYANINS FROM *AJUGA REPTANS* FLOWERS AND CELL CULTURES

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Key Word Index—*Ajuga reptans*; Lamiaceae; cell culture anthocyanins; triacylated anthocyanins; delphinidin and cyanidin 3-sophoroside-5-glucosides; *p*-coumaric; ferulic; malonic acid.

Abstract—Four anthocyanins were isolated from *Ajuga reptans* flowers and one from the cell cultures. By FAB mass spectrometry measurements, the structures of these pigments were determined as delphinidin and cyanidin glucosides acylated with two cinnamic acids, while three of them were also malonylated. A delphinidin-based pigment in the crude extract from cell cultures was identical to the major flower pigment as shown by HPLC co-chromatography. Moreover, by application of ^{1}H and ^{13}C NMR consisting of DQF–COSY, NOESY, ROESY, 2D-HOHAHA, HSQC and HMBC methods, the structures of two new anthocyanins were identified as delphinidin and cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaryl-β-D-glucopyranosyl)-(6-*O*-(*E*)-*p*-coumaryl)-β-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside). The deacylated anthocyanins were confirmed as delphinidin and cyanidin 3-sophoroside-5-glucosides.

INTRODUCTION

Ajuga reptans L. has purplish blue flowers and purplish leaves coloured with anthocyanin pigments. Callebaut and co-workers demonstrated that the cells and tissues of the young flowers could be cultured and produce anthocyanins under the appropriate physiological conditions [1–3]. The leaf tissue cultures of the plant were also demonstrated to produce these pigments by Callebaut *et al.* [1] and Ohba *et al.* [4] independently. HPLC and UV–VIS examination showed that the major pigment of the cultured cells was cyanidin-based and resembled the pigments of *A. reptans* leaves more than those of the flowers (delphinidin based) [1, 3]. Since chemical structures of the anthocyanins of *A. reptans* flowers and the flower cell cultures were only partially determined, we have established the complete structures by chemical and spectroscopic methods.

RESULTS AND DISCUSSION

The crude extract from *A. reptans* flowers mainly contained six anthocyanins. Preliminary acid hydrolysis of the crude extract gave predominantly delphinidin powders of the trifluoroacetic acid (TFA) salts. The deacylated anthocyanin (**8**) was prepared from **4** by alkaline hydrolysis and purification by preparative HPLC. Moreover, the major pigment of the flower cell cultures was also isolated by preparative HPLC, giving the TFA salt (**7**), and its deacylated derivative (**9**) was prepared by alkaline hydrolysis.

FAB mass spectra of anthocyanins **1**–**4** and **7** were measured under a positive mode with a linked scan of the flavylium glycoside cations. A molecular weight, molecular formula and construction of partial structures of each anthocyanin were obtained as shown in Fig. 1. Each anthocyanin molecular ion decomposes to the aglycone through two routes. One is the route releasing the hexosyl-hexoside (GG) fragment diacylated with *p*-coumaric acid (P) or P and ferulic acid (F) (**2**, **4** and **7**: GGPP; **1** and **3**; GGPF) and followed by the release of a hexose (**1** and **2**: G) or malonylhexoside (**3**, **4** and **7**: Gm) fragment; the second route is similar, but in reverse order. Each anthocyanin has GGPF/P and G(m) units in the molecule. Thus, the structures of **1**–**4** and **7** can be deduced as DpGGGPF, DpGGGPP, DpGmGGPF, DpGmGGPP and CyGmGGPP, respectively. Moreover **3**, **4** and **7** were measured in high

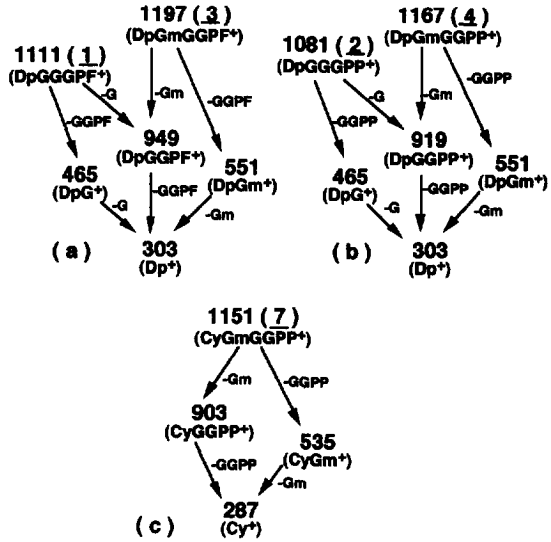
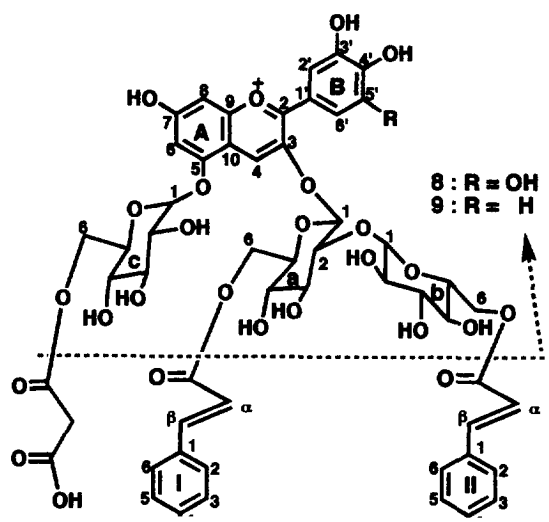


Fig. 1. FABMS fragmentation patterns of *A. reptans* flower and the cell culture anthocyanins. The values in the scheme show mass numbers (*m/z*) of the flower pigments **1** and **3** (a), **2** and **4** (b), and the cell culture pigment **7** (c). Abbreviations: Dp = delphinidin, Cy = cyanidin, G = hexose, m = malonic acid, P = *p*-coumaric acid, and F = ferulic acid.

errors +9 ppm. The basic structures of **4** and **7** were supported as Dp and Cy trihexosides by the fact that their deacylated anthocyanins **8** and **9** gave *m/z* 789 and 773 corresponding to $C_{33}H_{41}O_{22}^{+}$ and $C_{33}H_{41}O_{21}^{+}$, respectively. Since pigments **1–3** were in insufficient amounts for further characterization, only **4** and **7–9** could be used for further structural elucidation. Though the delphinidin-based pigment [3] of cell cultures could not be isolated, it was identical to **4** as shown by HPLC co-chromatography.

On the acidic hydrolysis, **4** and **7** gave Dp and Cy as aglycones, respectively, and only D-glucose (G). In the UV–VIS spectra of **4** and **7**, the presence of the characteristic absorptions around 316 nm suggested that these were acylated with cinnamic acid(s). The number of bound cinnamic acid(s) in **4** and **7** was estimated to be two due to the values (*ca* 130%) of $E_{acid.max}/E_{vis.max}$ (a ratio of absorbances at cinnamic and visible absorption maxima) [5]. On alkaline hydrolysis, the aromatic acids were identified to be P and m by TLC and HPLC, as depicted in Table 1. Apart from organic acids, the alkaline hydrolysis of **4** and **7** gave the deacylated anthocyanins **8** and **9**, respectively. The 3,5-disubstitution was also confirmed from the fact that values (**4** and **7–9**: about 13%) of $E_{440}/E_{vis.max}$ (a ratio of absorbances at 440 nm and visible maxima) were similar to those of common Dp and Cy 3,5-diglucosides (10 and 13%, respectively) [5, 6].



Detailed chemical structures of **4**, **7** and **9** were established through ^{13}C and ^{1}H NMR measurements, and the assignment data are summarized in Table 2. One-dimensional ^{13}C NMR spectra were assigned with the aid of DEPT, HSQC and HMBC spectra, and ^{1}H NMR spectral singals with aid of DQF-COSY, HOHAHA, NOESY and ROESY spectra. In the low magnetic field (δ_H 6–9 ppm) of ^{1}H NMR spectra, aglycone and *p*-coumaryl protons occur as well separated signals, and in the high magnetic field (δ_H 3–6 ppm), sugars and malonyl protons are observed as overlapping signals. Four singlets of **4** are assignable to protons of the Dp-ring having a symmetrical B-ring, while four singlets and two doublets of **7** and **9** are assignable to Cy-ring protons. In **4** and **7**, two pairs of characteristic doublets with large coupling constants (J = 16 Hz) are allocated to two pairs of olefinic α and β protons of trans (*E*)-*p*-coumaryl moieties. Though the glucosyl and malonyl protons are somewhat superimposed, the assignment was perfectly carried out by HOHAHA spectra. As three anomeric protons of a-, b- and c-glucoses (G_a-1, G_b-1 and G_c-1) in each pigment are characteristic doublets shifted to lower field with large coupling constants (7 Hz), and the sugar ring protons have large coupling constants (7–9 Hz), all sugars have the β-D-glucopyranosyl configuration. Each G_a-2 is shifted to lower magnetic field (chemical shift differences ($\Delta\delta_H$): from +0.51 to +1.08 ppm) from those of G_b-2 and G_c-2, indicating G_a-2-OH is glycosylated with G_b. Thus, the connectivity of G_a and G_b is deduced as 2-*O*-(β-D-glucopyranosyl)-β-D-glucopyranoside, a sophoroside. In **4** and **7**, 6-methylene protons of all sugars are also observed in lower magnetic field ($\Delta\delta_H$: from +0.39 to +0.79 ppm) as compared with those of **9**, indicating 6-OHs of G_a, G_b and G_c are acylated. The tendency is clearer in the ^{13}C NMR data ($\Delta\delta_C$ of C-2: from +6.54 to +10.09 ppm; $\Delta\delta_C$ of C-6: from +1.26 to +4.63) as demonstrated in Table 2. Malonyl methylene protons and carbons are observed at δ_H 3.33 (δ_C 41.43) and δ_H 3.37 (δ_C 41.51) ppm in **4** and **7**, respectively. In lower magnetic field (δ_C 166–169 ppm) of **4** and **7**, four ^{13}C signals can

Table 1. Chromatographic properties of acylating acids of anthocyanins from *A. reptans* flower (**4**) and cell culture (**7**)

	TLC					
	$R_f \times 100$			Detection		HPLC
Organic acid	ETN	BAW	PNW	UV	BCG	t_R (min)
From **4**	63	96	34	Violet	nd	16.2
	13	75	14	nd	Yellow	—
From **7**	64	97	36	Violet	nd	16.1
	13	76	14	nd	Yellow	—
p-Coumaric acid	63	98	37	Violet	nd	16.1
Ferulic acid	54	95	27	Blue, fl	nd	18.6
Caffeic acid	65	91	50	Blue, fl	nd	10.0
Malonic acid	13	76	14	nd	Yellow	—
Succinic acid	23	81	18	nd	Yellow	—
Malic acid	17	68	16	nd	Yellow	—

Abbreviations: fl = fluorescence; nd = not detected; t_R = retention time.
TLC solvent definitions and HPLC conditions: see Experimental.

sugars in **4**, **7** and **9** were clearly determined by NOESY and/or ROESY spectra. Cross-peaks of intense NOEs between Ad-4 and G_a-1, Ad-6 and G_c-1, and G_a-2 and G_b-1 proved that G_a, G_c and G_b were glycosylated to Ad-3-, 5-OH and G_a-2-OH, respectively. Hence, these basic structures are Ad-3-$\{G_b(1 \rightarrow 2)G_a\}$-5-$G_c$. The connectivity is also supported by the observation of weak NOEs between Ad-B-ring protons (2'-, 6'- and 5'-H) and G_b protons, and between Ad-6 and G_c protons, showing that G_b and G_c were spatially near to the Ad-B-ring and A-ring, respectively. Similarly, connecting positions on sugars of three acyl residues in **4** and **7** were determined by NOESY spectra. Thus malonic acid was linked to G_c-6-OH due to the presence of week NOEs between G_c and malonyl-methylene protons, and two *p*-coumaric acids might be linked to G_a and G_b, respectively.

In conclusion, the structure of **9** is cyanidin 3-*O*-(2-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl)-5-*O*-(β-D-glucopyranoside). The major anthocyanins **4** and **7** were identified as delphinidin and cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaryl-β-D-glucopyranosyl)-(6-*O*-(*E*)-*p*-coumaryl-β-D-glucopyranosyl)-5-*O*-(6-*O*-malonyl-β-D-glucopyranoside). These anthocyanins are relatively stable in weakly acidic or neutral aqueous solution because of the cinnamic acid diacylation which prevents the fading of colour [7, 8]. Therefore they are potential colourants for foods or other applications.

EXPERIMENTAL

General procedures. TLC was carried out on microcrystalline cellulose plates (Funacell SF, Funakoshi) using solvents such as ETN (EtOH–conc. NH_4OH–H_2O, 16:1:3), BAW (*n*-BuOH–HOAc–H_2O = 4:1:2), PNW (*i*-PrOH–conc. NH_4OH–H_2O, 8:1:1) for organic acids. The sample spots on chromatograms were detected by UV and BCG (Hitachi). Analyt. HPLC was run on an Inertsil ODS-2 (4.6 × 250 mm, GL Sciences) column at 35° with a flow rate of 1 ml min^{-1} and monitoring at 312 nm for UV-absorbing compounds and at 520 or 530 nm for anthocyanins. Solvent systems used were as follows: a linear gradient elution for 45 min from 25 to 70% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O). Prep. HPLC was performed on an Inertsil ODS (20 × 250 mm, GL Sciences) column with isocratic elution (7–10 ml min^{-1}) using a mixt. of solvent A (15% HOAc in H_2O) and solvent B (15% HOAc, 30% MeCN in H_2O), A:B = 7:3 at 530 nm. UV–VIS spectra were recorded on an MPS-2000 (Shimadzu) spectrophotometer in 0.01% HCl–MeOH. FABMS were measured on a JMS SX102 (JEOL) in MeOH with *m*-nitrobenzyl alcohol as matrix with a positive mode and a linked-scan method. High resolution FABMS were also measured on the same apparatus under a positive ion mode in DMSO–TFA with PEG for a mass calibration (accelerating voltage: 10 kV; scan mass range: m/z 900–1300). ^{13}C (150.8 MHz) and ^1H (600.1 MHz) NMR were run on an alpha-600 (JEOL) in DMSO-d_6–CF_3CO_2D (9:1).

Plant materials. Flowers of *A. reptans* were collected in April 1994 and 1995 in Kumamoto prefecture. Anthocyanin producing cultures were induced from young flowers according to the previous reports [1, 3].

Isolation and preparation of pigments. Reddish-purple flowers (183 g) were soaked in 1.75 l of 15% HOAc for 3 days and filtered. The crude extract contained anthocyanins **1**–**6** at retention times (contents in %): **1**, 22.9 (6); **2**, 23.3 (11); **3**, 27.2 (18); **4**, 27.8 (51); **5**, 28.6 (4); **6**, 29.0 (6%) min respectively, by HPLC analysis. The extract was applied on an adsorption resin (Diaion HP-20 or Amberlite XAD-2000, 45 × 375 mm) column, and the column was washed with 1% HOAc (2 l) and then eluted with 1% HOAc in 70% EtOH. The pigment eluate was sepd into 2 frs on a Sephadex LH-20

Table 2. ^{13}C and ^{1}H NMR data for *Ajuga reptans* flower and cell culture anthocyanins (δ ppm)

		4		**7**		**9**	
Assignment		δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Aglycone	2	161.90	—	162.59	—	162.76	—
	3	145.31	—	144.48	—	144.91	—
	4	144.82	8.66 *s*	132.65	8.80 *s*	132.20	8.87 *s*
	5	154.93	—	155.46	—	155.31	—
	6	105.36	6.88 *brs*	105.12	6.96 *s*	104.27	7.02 *s*
	7	167.43	—	167.85	—	167.48	—
	8	96.22	6.86 *brs*	96.50	6.96 *s*	96.24	7.14 *s*
	9	155.14	—	155.46	—	155.20	—
	10	111.79	—	111.84	—	111.68	—
	1′	118.34	—	119.77	—	119.82	—
	2′	111.58	7.73 *s*	117.84	8.03 *s*	117.93	8.11 *s*
	3′	146.76	—	146.60	—	146.42	—
	4′	147.62	—	155.21	—	155.20	—
	5′	146.76	—	117.30	7.11 *d* (9)	117.19	7.13 *brd* (8)
	6′	111.58	7.73 *s*	127.89	8.27 *d* (9)	127.76	8.29 *brd* (8)
Glucose-a	1	98.57	5.72 *d* (7)	99.30	5.69 *d* (7)	99.76	5.62 *d* (7)
	2	83.38	4.09 *t* (7)	81.74	4.08 *t* (8)	81.18	4.08 *t* (8)
	3	74.03	3.70 *t* (9)	74.32	3.75 *m*	76.05	3.69 *t* (9)
	4	69.75	3.43 *t* (9)	69.84	3.46 *t* (9)	69.66	3.42 *t* (9)
	5	75.13	3.94 *ddd* (3, 8, 8)	76.10	3.98 *m*	76.03	3.59 *m*
	6a	64.23	4.24 *dd* (7, 12)	65.35	4.27 *dd* (7, 12)	60.72	3.77 *m*
	6b	64.23	4.37 brd (10)	65.35	4.43 brd (12)	60.72	3.79 m
Glucose-b	1	104.99	4.73 *d* (8)	104.76	4.83 *d* (7)	103.89	4.70 *d* (7)
	2	73.95	3.06 *t* (8)	74.18	3.13 *t* (8)	74.64	3.00 *t* (8)
	3	74.32	3.14 *t* (9)	75.00	3.26 *m*	76.32	3.11 *t* (9)
	4	69.11	2.94 *m*	69.67	3.16 *m*	69.26	3.05 *t* (9)
	5	75.90	3.15 *m*	76.30	3.26 *m*	77.48	2.70 *m*
	6a	61.86	3.58 *brd* (11)	62.72	3.92 *brd* (11)	60.60	3.19 *m*
	6b	61.86	3.81 *brd* (9)	62.72	3.98 *m*	60.60	3.19 *m*
Glucose-c	1	101.96	5.03 *d* (7)	104.99	5.12 *d* (7)	101.54	5.16 *d* (7)
	2	73.29	3.50 *t* (8)	73.40	3.57 *t* (8)	73.21	3.52 *brt* (8)
	3	74.32	3.35 *t* (9)	74.47	3.41 *t* (8)	76.61	3.41 *brt* (7)
	4	69.97	3.22 *t* (9)	70.03	3.29 *t* (9)	69.76	3.32 *t* (9)
	5	75.90	3.69 *m*	76.30	3.75 *m*	77.63	3.52 *brt* (8)
	6a	63.46	4.04 *dd* (7, 12)	64.34	4.10 *dd* (6, 12)	60.72	3.62 *m*
	6b	63.46	4.36 *brd* (10)	64.34	4.41 *brd* (12)	60.72	3.62 *m*
p-Coumaryl-I	1	125.11	—	125.26	—	—	—
	2 & 6	130.48	7.33 *d* (8)	130.57	7.36 *d* (8)	—	—
	3 & 5	116.05	6.74 *d* (9)	116.15	6.78 *d* (8)	—	—
	4	160.04	—	160.13	—	—	—
	a	113.99	5.98 *d* (16)	114.11	6.06 *d* (16)	—	—
	b	144.48	7.13 *d* (16)	145.00	7.26 *d* (16)	—	—
	C=O	166.39	—	166.65	—	—	—
p-Coumaryl-II	1	125.21	—	125.29	—	—	—
	2 & 6	130.57	7.28 *d* (9)	130.67	7.32 *d* (8)	—	—
	3 & 5	115.82	6.68 *d* (9)	115.93	6.73 *d* (8)	—	—
	4	160.11	—	160.23	—	—	—
	a	113.83	6.20 *d* (16)	113.98	6.23 *d* (16)	—	—
	b	144.56	7.32 *d* (16)	145.40	7.36 *d* (16)	—	—
	C=O	166.82	—	166.95	—	—	—
Malonyl	$-CH_2-$	41.43	3.33 *s*	41.51	3.37 *s*	—	—
	C=O	167.08	—	167.21	—	—	—
	C=O	168.18	—	168.33	—	—	—

Abbreviations: *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet, *brd* = broad doublet and *brt* = broad triplet.

in vacuo, dissolved in a small amount of TFA, and pptd with excess Et_2O to give TFA salts of **1**–**4** as red powders in amounts of 1, 2, 2 and 16 mg, respectively. Anthocyanins from cell cultures were extracted with MAW (MeOH–HOAc–H_2O, 10:1:9). The crude extract contained 19 or more anthocyanins in which the major anthocyanin (**7**) appeared at 33.7 min (24%) in the HPLC chromatogram. After filtration, the crude extract was purified on a polyamide column. The pigments eluting with MeOH–10% HCO_2H (1:1) were collected. The major anthocyanin fr. was purified and isolated by prep. HPLC using solvent A–B (7:3), giving (**7**) TFA salt. The 2nd anthocyanin in the crude cell extract (delphinidin based) could not be isolated in pure form. Deacylated anthocyanins **8** and **9** were respectively prepd from **4** and **7** by hydrolysis in aq. 2N NaOH for 15 min under N_2 and the reaction mixt. was applied to an adsorption resin column, washed with 1% HOAc and eluted 1% HOAc–70% EtOH. The evapn residue was purified by prep. HPLC, giving pure **8** and **9** TFA salts.

FABMS of anthocyanins. FABMS gave *m/z* as follows: **1**: 1111 ($M = C_{52}H_{55}O_{27}$)$^+$, 949 (DpGGPF = $C_{46}H_{45}O_{22}$)$^+$, 465 (DpG = $C_{21}H_{21}O_{12}$)$^+$, 303 (Dp = $C_{15}H_{11}O_7$)$^+$, 177 (F = $C_{10}H_9O_3$)$^+$, 147 (P = $C_9H_7O_2$)$^+$; **2**: 1081 ($M = C_{51}H_{53}O_{26}$)$^+$, 919 (DpGGPP = $C_{45}H_{43}O_{21}$)$^+$, 465 (DpG = $C_{21}H_{21}O_{12}$)$^+$, 303 (Dp = $C_{15}H_{11}O_7$)$^+$, 147 (P = $C_9H_7O_2$)$^+$; **3**: 1197 ($M = C_{55}H_{57}O_{30}$)$^+$, 949 (DpGGPF = $C_{46}H_{45}O_{22}$)$^+$, 551 (DpGm = $C_{24}H_{23}O_{15}$)$^+$, 303 (Dp = $C_{15}H_{11}O_7$)$^+$, 177 (F = $C_{10}H_9O_3$)$^+$, 147 (P = $C_9H_7O_2$)$^+$; **4**: 1167 ($M = C_{54}H_{55}O_{29}$)$^+$, 919 (DpGGPP = $C_{45}H_{43}O_{21}$)$^+$, 551 (DpGm = $C_{24}H_{23}O_{15}$)$^+$, 465 (DpG = $C_{21}H_{21}O_{12}$)$^+$, 303 (Dp = $C_{15}H_{11}O_7$)$^+$, 147 (P = $C_9H_7O_2$)$^+$; **7**: 1151 ($M = C_{54}H_{55}O_{28}$)$^+$, 903 (CyGGPP = $C_{45}H_{43}O_{20}$)$^+$, 535 (CyGm = $C_{24}H_{23}O_{14}$)$^+$, 287 (Cy = $C_{15}H_{11}O_6$)$^+$, 147 (P = $C_9H_7O_2$)$^+$; **8**: 789 ($M = C_{33}H_{41}O_{22}$)$^+$; **9**: 773 ($M = C_{33}H_{41}O_{21}$)$^+$, 449 (CyG = $C_{21}H_{21}O_{11}$)$^+$, 287 (Cy = $C_{15}H_{11}O_6$)$^+$. High resolution FABMS of **3**, **4** and **7** gave *m/z* as follows: **3**: 1197.2958 (1197.2935 calc. as $C_{55}H_{57}O_{30}^+$, error +1.9 ppm); **4**: 1167.2886 (1167.2829 calc. as $C_{54}H_{55}O_{29}^+$, error +4.9 ppm), **7**: 1151.2977 (1151.2880 calc. as $C_{54}H_{55}O_{28}^+$, error +8.55 ppm).

Anthocyanin **4** (*delphinidin* 3 - (*di* - *p* - *cou* - *maryl*)*sophoroside* - 5 - *malonylglucoside*). HPLC t_R: 27.8 min; UV–VIS λ_{max} (0.01% HCl–MeOH) nm: 544 (shifted bathochromically by 36 nm with $AlCl_3$), 316, $E_{440}/E_{vis.max} = E_{440}/E_{544} = 12\%$, $E_{acid.max}/E_{vis.max} = E_{316}/E_{544} = 130\%$.

Anthocyanin **7** (*cyanidin* 3 - (*di* - *p* - *cou* - *maryl*)*sophoroside* - 5 - *malonylglucoside*). HPLC t_R: 33.7 min; UV–VIS λ_{max} (0.01% HCl–MeOH) nm: 535 (shifted bathochromically by 24 nm with $AlCl_3$), 315, $E_{440}/E_{vis.max} = E_{440}/E_{535} = 13\%$, $E_{acid.max}/E_{vis.max} = E_{315}/E_{535} = 129\%$.

Anthocyanin **8** (*delphinidin* 3 - *sophoroside* - 5 - *glucoside*). HPLC t_R: 4.0 min; UV–VIS λ_{max} (0.01% HCl–MeOH) nm: 537 (shifted bathochromically by 49 nm with $AlCl_3$), 279, $E_{440}/E_{vis.max} = E_{440}/E_{537} = 13\%$, $E_{uv.max}/E_{vis.max} = E_{279}/E_{537} = 64\%$.

Anthocyanin **9** (*cyanidin* 3 - *sophoroside* - 5 - *glucoside*). HPLC t_R: 4.8 min; UV–VIS λ_{max} (0.01% HCl–MeOH) nm: 527 (shifted bathochromically by 41 nm with $AlCl_3$), 278, $E_{440}/E_{vis.max} = E_{440}/E_{527} = 13\%$, $E_{uv.max}/E_{vis.max} = E_{278}/E_{527} = 49\%$.

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