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CYANIDIN 3-MALONYLGLUCURONYLGLUCOSIDE IN *BELLIS* AND CYANIDIN 3-MALONYLGLUCOSIDE IN *DENDRANTHEMA*

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Key Word Index–*Dendranthema morifolium*; *Bellis perennis*; Compositae; FABMS; ^{13}C NMR; ^{1}H NMR; acylated anthocyanin; cyanidin 3-*O*-(6-*O*-malonylglucoside); cyanidin 3-*O*-(6-*O*-malonyl-4-*O*-(β-D-glucuronyl)-β-D-glucoside); flower colour.

Abstract–The major anthocyanin of three red cultivars of *Dendranthema morifolium* has been identified as cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside) by FABMS and NMR spectroscopy. The major anthocyanin of red flowers of *Bellis perennis* has been identified as cyanidin 3-*O*-(6-*O*-malonyl-4-*O*-(β-D-glucuronyl)-β-D-glucopyranoside). Both malonylanthocyanins were more stable in neutral solution than cyanidin 3-glucoside, but less stable than cyanidin 3-glucuronylglucoside.

INTRODUCTION

Recently the occurrence of several malonyated cyanidin glycosides have been reported in the Compositae [1–3], e.g. cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside) in *Cichorium intybus* [1]. We have previously reported the presence of cyanidin 3-glucoside derivative with unknown acylation as major pigments in red flowers of *Dendranthema* cultivars [4]. Further structural studies have now been made using new techniques particularly on the major *Dendranthema* pigment [1–3]. Another unusual malonylcyanidin 3-glycoside was observed in red English daisy (*Bellis perennis* cv 'Super Siberius Crimson') during a survey of the occurrence of acylated anthocyanins in red flowers, and it has now been identified using similar methods.

RESULTS AND DISCUSSION

Anthocyanin pigments were isolated from three *Dendranthema morifolium cvs* by extraction with acetic acid–methanol–water and purified as described previously [1,3]. Anthocyanin pigments were obtained similarly from *Bellis perennis* 'Super Siberius Crimson'. R_f values and spectra of both plant pigments (Table 1) were in good agreement with those of Takeda *et al.* [3]. Both major pigments were purified using Sephadex LH-20, PC and TLC (solvents, BAW and 15% HOAc). The visible λ_{max} of the major *Bellis* pigment was 528nm, and identical with those of the major *Dendranthema* pigment and cyanidin 3-glucoside. The major *Bellis* pigment had an additional minor λ_{max} at 335 nm, similar to that obtained earlier for the anthocyanin of *Heleuium autumnale* [3].

On acid hydrolysis, the major *Bellis* pigment produced cyanidin, glucose, glucuronic acid and malonic acid, and the major *Dendranthema* pigment yielded glucose and malonic acid. Partial hydrolysis gave cyanidin 3-glucoside (both pigments) and cyanidin 3-glucuronylglucoside (the *Bellis* pigment). The deacylated pigment from *Bellis* produced glucuronylglucose by hydrogen peroxide degradation.

FABMS measurement of the major *Dendranthema* pigment showed the $[M]^+$ 535 *m/z*, $[M - \text{malonic acid}]^+$ 449 and aglycone 287. Also, FABMS of the major *Bellis* pigment gave its M_r as 711 *m/z*, with fragments corresponding to cyanidin 3-glucuronylglucoside (625 *m/z*), cyanidin 3-malonylglucoside (535 *m/z*) and cyanidin (287 *m/z*). This result confirms that the *Bellis* pigment is identical to the malonated cyanidin 3-glucuronosyl glucoside described earlier from *Helenium* [3]. The M_r (711 *m/z*) is in good agreement with the mass calculated for $C_{30}H_{31}O_{20}$ (711.139 *m/z*).

The presence of both malonyl and glucuronyl groups was confirmed by NMR spectroscopy [1,5,6] which also defined their position of attachment. The ^{1}H NMR spectra (400 MHz) of both major anthocyanins were measured in 10% TFA-90% DMSO-d_6 following Bridle *et al.* [1] (Table 2). The major *Bellis* anthocyanin showed two doublets δ 3.32 (J = 16.0) and 3.36 (J = 16.0), and the major *Dendranthema* anthocyanin also had two doublets δ 3.36 (J = 15.5) and 3.40 (J = 15.5) which can be assigned to the malonyl CH_2 as shown in Table 2. The characteristic two protons at δ 4.15, 4.42 (the *Bellis* anthocyanin) and 4.13, 4.47 (*Dendranthema* anthocyanin) with the geminal coupling (J = 11.0) were assigned to C-6″ methylene, indicating that the malonyl moiety was attached to C-6″ of glucose part [1, 5, 6]. The anomeric proton δ 5.41 (Table 2) of the major *Dendranthema* anthocyanin was strongly coupled to H-2″ (J = 7.0 Hz), indicating that it is a β-D-glucopyranoside. The other protons of glucose moiety were assigned as shown in Table 2. In the *Bellis* anthocyanin, the anomeric protons at δ 5.66 (J = 7.0 Hz) and 4.66 (J = 7.6) suggest that the compound has a β-D-glucopyranoside and a β-D-glucuronide structure. Most of the glucuronyl protons were observed at δ

Table 1. R_f values and spectral properties of malonylcyanidin 3-glycosides and related pigments

Pigment	R_f values (x 100)*				Spectral data in		
	BAW	BuHCl	1%HCl	AcOHCl	MeOH–HCl	$AlCl_3$	$E_{440}/E_{vis\ max}$
Bellis pigments							
†Cyanidin 3-malonylglucuronylglucoside	48	43	43	72	282,335,528	+	24
Cyanidin 3-malonylglucoside	51	32	4	23	281,528	+	38
Pelargonidin 3-malonylglucoside	68	52	8	35	272,512	–	39
Dendranthema pigments							
†Cyanidin 3-malonylglucoside	51	32	4	23	281,528	+	38
Cyanidin 3-dimalonylglucoside	51	43	5	29	281,528	+	
Related pigments							
Cyanidin 3-glucoside	43	19	3	19	282,528	+	25
Cyanidin 3-glucuronylglucoside	39	26	36	62	281,528	+	28
Pelargonidin 3-glucoside	64	37	7	31	272,512	–	39

*Solvent key: BAW, *n*-BuOH–HOAc–H_2O (4:1:5) ; BuHCl, *n*-BuOH–1N HCl (1:1); 1%HCl, H_2O–conc HCl (97:3); AcOHCl, HOAc–HCl–H_2O (82:3:15).

† major pigments of the plants.

Table 2. ^1H NMR data of malonylcyanidin 3-glycosides using DMSO-d_6 with CF_3COOD (chemical shifts in ppm from TMS)

	Cyanidin 3-malonylglucoside		Cyanidin 3-malonylglucuronylglucoside (*Bellis* pigment)
	Cichorium pigment†	*Dendranthema* pigment	
H4	8.86 (*s*)	8.82 (*s*)	8.79 (*s*)
H6	6.75 (*d*, J = 2.0)	6.74 (*d*, J = 2.0)	6.72 (*d*, J = 2.0)
H8	6.93 (*br s*)	6.92 (*d*, J = 2.0)	6.90 (*d*, J = 2.0)
H2′	8.04 (*d*, J = 2.0)	7.99 (*d*, J = 2.4)	7.96 (*d*, J = 2.0)
H5′	7.07 (*d*, J = 8.3	7.04 (*d*, J = 8.5)	7.10 (*d*, J = 9.0)
H6′	8.27 (*dd*, J = 2.5, 8.3)	8.25 (*dd*, J = 2.4,8.5)	8.19 (*dd*, J = 2.0,9.0)
Glucose anomeric H1″	5.44 (*d*, J = 6.8)	5.41 (*d*, J = 7.0)	5.66 (*d*, J = 7.0)
Glucose-CH_2-H6″$_a$	4.17 (*dd*, J = 7.8,9.7)	4.13 (*dd*, J = 7.8,12.2)	4.15 (*dd*, J = 8.0,11.0)
H6″$_b$	4.51 (*d*, J = 11.2)	4.47 (*d*, J = 10.5)	4.42 (*d*, J = 11.0)
H2″		3.53 (*t*, J = 8.5)	3.65 (*t*, J = 8)
H3″	3.20–4.0 (*m*)	3.43 (*t*, J = 9.1)	3.26 (*t*, J = 8)
H4″		3.25 (*t*, J = 9.0)	3.96 (*t*, J = 8)
H5″		3.48 (*dt*, J = 9.3)	3.90 (*dt*, J = 2,8)
Glucuronic acid anomeric H			4.66 (*d*, J = 7.6)
H2‴			3.32–3.7 (*m*)
H3‴			3.02 (*t*, J = 9)
H4‴			3.06 (*t*, J = 9)
H5‴			3.18 (*d*, J = 9)
Malonyl CH_2	3.42 (*s*)	3.40 (*d*, J = 15.5)	3.32 (*d*, J = 16.0)
		3.36 (*d*, J = 15.5)	3.36 (*d*, J = 16.0)

† Ref. [1].

3.0–4.0 as multiplets, and the glucosyl protons were essentially identical with those of cyanidin 3-malonylglucoside [1]. The six aromatic protons of the major *Bellis* anthocyanin were in good agreement with the major *Dendranthema* anthocyanin and cyanidin 3-malonylglucoside of *Cichorium* anthocyanin [1] (Table 2).

Further confirmation of the structures came from ^{13}C NMR spectroscopy (Table 3). The identification of ^{13}C NMR signals of each monosaccharide residue relied mostly on comparison with those of model compounds, since it has been well established that the chemical shifts of monosaccharide units within polysaccharide chains are similar to those of monosaccharides except for substituent effect. In the ^{13}C NMR spectrum of cyanidin 3-malonylglucuronylglucoside in 10% TFA-90% DMSO-d_6, an additional nine signals corresponding to glucur-

Table 3. ^{13}C NMR data of malonylcyanidin 3-glycosides (DMSO-d_6 + CF_3COOD)

C	Cyanidin 3-glucoside	‡Cyanidin 3-malonylglucoside	Cyanidin 3-malonyl-glucuronylglucoside
Anthocyanidin			
2	158.12	158.98	161.82
3	157.52	157.54	157.74
4	144.12	136.45	143.49
4_a	146.08	145.49	146.17
5	168.34	170.33	167.62
6	94.05	(97.5)	94.16
7	155.78	155.84	155.77
8	102.34	103.46	102.43
8_a	161.67	164.04	166.70
1′	111.79	113.11	111.52
2′	116.71	117.35	117.00
3′	154.24	147.35	154.51
4′	145.94	145.49	146.17
5′	117.42	118.20	117.14
6′	134.89	128.46	134.02
Glucose			
1″	102.05	95.25	99.62
2″	73.06	74.56	71.21
3″	76.43	77.83	73.82
4″	69.63	71.26	75.47
5″	77.61	75.87	73.98
6″	60.72	65.46	64.07
Glucuronic acid			
1			103.64† (103.56)
2			75.53 (77.18)
3			81.41 (83.38)
4			69.10 (68.93)
5			75.67 (77.76)
6			168.29 (175.05)
Malonic acid		168.56	168.32
		45.10	41.18
		170.09	169.79

†( ) D-glucurono-3,6-lactone.
‡ Ref. [1].

onyl C and malonyl C moieties were observed in comparison with the ^{13}C chemical shifts of cyanidin 3-glucoside. The malonyl methylene C as appeared at 41.18 ppm [1] and the three signals of low fields (δ 168.29, 168.32 and 169.79) were attributed to the carbonyls C of the glucuronic acid and malonate moieties. The chemical shifts of five peaks attributable to glucuronic acid C were quite similar to those of D-glucurono-3, 6-lactone (Table 3) and this fact indicated that the *Bellis* pigment might be present in the lactone form in this solvent system.

The glycosidic linkage between glucuronic acid and glucose was determined by the characteristic downfield shift (δ 75.47) of the C-4″ in the glucosyl moiety in comparison with cyanidin 3-glucoside (C-4″, δ 69.63). Thus the glucuronyl moiety is attached to the C-4″ position of the glucose residue. Although the same pigment was earlier reported in *Helenium* [3], the interglucoside link was not then determined. This paper reports for the first time that the malonyl residue in the cyanidin 3-malonylglucuronylgucoside is located at the 6″-position that the glucuronyl residue is linked with the 4″-position of the glucose residue. The major *Dendranthema* anthocyanin is determined as the known cyanidin 3-*O*-(6-*O*-malonyl-β-D-glucopyranoside).

As minor anthocyanin components, two malonylanthocyanins were isolated from the red flowers of *Bellis perennis* 'Super Siberius Crimsom'. These anthocyanins were identified as pelargonidin 3-malonylglucoside and cyanidin 3-malonylglucoside by TLC analysis and spectral data [3,7] (Table 1).

The relative colour stabilities of the four anthocyanins were compared with one another in neutral solution (Fig. 1). Each anthocyanin was dissolved in a phosphate buffer (pH 7.0), kept at room temperature (*ca* 25–30°), and then absorbances at 550 nm were measured at intervals for eight hr. The colour stabilities of these pigments increased in the order cyanidin 3-glucoside, cynidin 3-malonylglucoside, cyanidin 3-malonylglucuronylglucoside and cyanidin 3-glucuronylglucoside (deacylated from the major *Bellis* pigment). Thus the acyl groups are effective in maintaining colour stability. Cyanidin 3-malonylglucoside has been reported more stable than cyanidin 3-glucoside at pH 3 (ref. [10]). It is noteworthy that the glucuronyl group is more effective than the

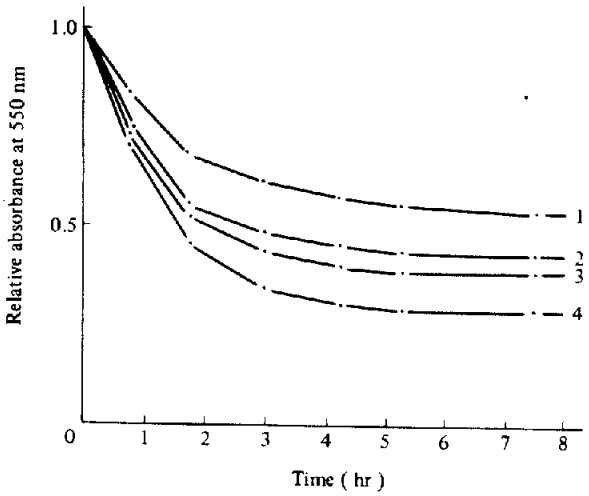

Fig. 1. Stability of malnoylcyanidin glycosides in buffer solution (pH 7.0, 2×10^{-3} M, path length 10 mm, *ca* 25–30°). (1). Cyanidin 3-glucuronylglucoside; (2). cyanidin 3-malonylglucuronylglucoside; (3). cyanidin 3-malonylglucoside; (4). cyanidin 3-glucoside.

malonyl group alone or the malonyl and glucuronyl groups together. These acyl groups help to conserve the flavylium-cation-form through ionization of free carboxyl groups of malonic acid and/or glucuronic acids, as described previously [8].

EXPERIMENTAL

Material. The air-dried colour petals of *Dendranthema morifolium* (Ramat.)Izrelev cultivars, 'Chiyono-bi' (50 g), 'Kishinoshisyu' (50 g) and 'Kokka-seizan' (50 g) were collected in the Agricultural Farm of the Kyoto University. The air-dried petals of *Bellis perennis* cv 'Super Siberius Crimson' (20 g) were collected in the garden of Minamikyusyu University. These petals were extracted with AcOH–MeOH–H_2O (1:11:9) and purified by Sephadex LH-20 gel CC, and also TLC (solvents, BAW and 15% HOAc) using processes similar to those of ref. [3].

Cyanidin 3-malonylglucoside (*ca* 30 mg) was isolated from three cvs of *Dendranthema morifolium*. A second anthocyanin component in the three cultivars is probably cyanidin 3-dimalonylglucoside [3, 7] (shown in Table 1), but because of the small quantitiy of the pigment, it was not completely characterized. Recently, Yamaguchi *et al.* also reported the occurrence of such a pigment in chrysanthemums [9].

Cyanidin 3-malonylglucuronylglucoside (40 mg) was obtained from the extract of *Bellis perennis* as a major anthocyanin; small amounts of cyanidin 3-malonylglucoside and pelargonidin 3-malonylglucoside were also obtained. These minor components were identified only by the data of TLC (cellulose) and absorption spectra due to their small amounts [3, 7]. Cyanidin 3-glucuronylglucoside was obtained by deacylation in 10% NaOH under N_2.

Detection of malonic acid. After saponification of each acylated pigment and acidification, the hydrolysate was allowed to evaporate to dryness. The organic acid was dissolved into Et_2O and this extract was chromatographed on microcrystalline cellulose against authentic markers in three solvents, with detection with BCG (in alkaline EtOH) [3]. The solvents were BAW, EtOAc–HOAc–H_2O (3:1:1) and EtOH–H_2O–NH_4OH (16:1:3).

^1H and ^{13}C NMR, and fast atom bombardment mass spectrometry. ^1H and ^{13}C NMR of anthocyanins were obtained with JEOL FX 400 spectrometer and samples were measured in 10% TFA-90% DMSO-d_6. Mass spectra were taken with JEOL JMS D-300 spectrometer.

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