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ANTHOCYANINS ACYLATED WITH MALIC ACID IN *DIANTHUS CARYOPHYLLUS* AND *D. DELTOIDES*

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Key Word Index—*Dianthus caryophyllus*; *D. deltoides*; Caryophyllaceae; carnation; pelargonidin 3-malylglucoside; cyanidin 3-malylglucoside.

Abstract—The major anthocyanin in pink and red forms of *Dianthus caryophyllus* has been identified as pelargonidin 3-malylglucoside. The corresponding cyanidin 3-malylglucoside has been found in red flowers of *D. deltoides*. This is the first complete characterization in plants of anthocyanins substituted with malic acid.

INTRODUCTION

During a survey of the flowering plants for zwitterionic anthocyanins [1], such pigments were provisionally detected in petals of all three members of the Caryophyllaceae examined, in *Dianthus deltoides* L., *D. sylvestris* Wulf. and *Lychnis flos-juvis* Desv. [1]. It thus appeared that anthocyanins with aliphatic dicarboxylic acids attached might be common in this family. This survey also suggested that the pigments of the ornamentally important carnation *D. caryophyllus* L., previously reported as containing the 3-glucosides and 3,5-diglucosides of pelargonidin and cyanidin [2–6], might bear reinvestigation. As already pointed out [1], acyl groups based on malonic or related organic acids are labile and are lost during conventional extraction procedures, so they may have been overlooked during earlier studies.

We here report the first complete identification of malylated anthocyanins in nature, through their isolation and characterization from *D. caryophyllus* and *D. deltoides* petals.

RESULTS AND DISCUSSION

Dried petals of Scania, a typical red Japanese carnation cultivar, or fresh petals of commercially available British pink carnations were extracted with ethanolic aqueous acetic acid and the major pigment purified by preparative PC and by chromatography on Sephadex LH-20. This pigment **1** indeed differed in R_f from pelargonidin 3-glucoside, reputedly the main anthocyanin of the red carnation (see Table 1), but on deacylation it was converted to the 3-glucoside [7]. That the acyl group was not an aromatic hydroxycinnamic acid was clear from the UV–visible spectrum, by the absence of strong absorption in the 300–340 nm region [8]. That the acyl group was not malonic acid was apparent from the fact that **1** was different in R_f from pelargonidin 3-(6″-malonyl-

Table 1. Chromatographic and electrophoretic properties of *Dianthus* anthocyanins

Pigment	R_f (× 100) in BAW	BuHCl	1% HCl	HOAc–HCl*	Electrophoretic mobility† (cm)	HPLC *RR*,‡
Pelargonidin						
3-glucoside	46	33	09	30	0.0	1.00
3-malonylglucoside	48	45	11	39	2.1	2.11
3-malylglucoside (**1**)	34	38	13	42	1.2	1.91
Cyanidin						
3-glucoside	29	20	04	19	0.0	1.00
3-malonylglucoside	36	35	07	24	1.3	2.14
3-malylglucoside (**2**)	21	26	07	28	0.6	1.97

*TLC on microcrystalline cellulose. BAW = *n*-BuOH–HOAc–H_2O (4:1:5); BuHCl = *n*-BuOH–2 N HCl (1:1); HOAc–HCl = HOAc–HCl–H_2O (15:3:82).

†On Whatman No. 3 paper at pH 4.4 at 40 V/cm for 1.5 hr.

‡On reversed phase Spherisorb-hexyl (5 μm) column at 35° with gradient elution with increasing amounts of MeOH in 0.6% aq. perchloric acid and detection at 520 nm and a flow rate of 1 ml/min.

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Table 2. Chromatographic properties of organic acids and acylated sugars from *Dianthus* anthocyanins

Substance	R_f* × 100				Colour with	
	ETN	BAW3	EAA	BAW1*	BCG	AHP†
Organic acids						
Deacylated product Malic acid	18	59	66	60	yellow	n.d.
Malonic acid	13	71	73	69	yellow	n.d.
Succinic acid	23	80	82	81	yellow	n.d.
Sugars						
Malylglucose‡	29	20	27	25	yellow	brown
Glucose	53	21	24	26	n.d.	brown

*TLC on microcrystalline cellulose in ETN, EtOH–H_2O–NH_4OH (16:3:1); BAW3, *n*-BuOH–HOAc–H_2O (4:1:1); EAA, EtOAc–HOAc–H_2O (3:1:1) and BAW1, *n*-BuOH–HOAc–H_2O (4:1:5).

†BCG, AHP, bromocresol green and aniline hydrogen phthalate spray reagents, respectively; n.d. = not detected.

‡Mobility was 0.40 relative to salicylic acid on electrophoresis at pH 4.4, and 40 V/cm on Whatman no. 1 paper.

glucoside), which was available for comparison [9].

Deacylation of **1** in fact yielded malic acid, $HO_2CCH_2CHOHCO_2H$, which was identified on the basis of co-chromatography with authentic material (Table 2) and of IR spectral measurements. The attachment of the malic acid through glucose was established by H_2O_2 oxidation of **1** [10], which provided a malylglucose. This structure was confirmed by its electrophoretic mobility on paper and by its acid hydrolysis to yield malic acid and glucose (Table 2).

The structure of **1** as pelargonidin 3-malylglucoside was further confirmed by fast atom bombardment mass spectrometry (FAB-MS), which showed the presence of a molecular ion at 549 ($C_{25}H_{25}O_{14}$ requires 549) and also an ion for the potassium salt at 587. Additionally, there were fragments at 433 mu, corresponding to pelargonidin 3-glucoside, and at 271, corresponding to pelargonidin. The FAB mass spectrum thus confirmed the presence of a single molecule of malic acid linked to the glucose residue in the 3-position.

Precisely similar studies on the major anthocyanin **2** of the crimson red petals of *Dianthus deltoides* established its structure as cyanidin 3-malylglucoside. Again FAB-MS confirmed this structural assignment, since a molecular ion was observed at 565 ($C_{25}H_{25}O_{15}$ requires 565) and an aglycone fragment was detected at 287 mu due to cyanidin.

Electrophoretic investigations of other *Dianthus* species suggest that these malylated pigments are not necessarily present throughout the genus. Thus, while *D. sylvestris* appears to be similar to *D. deltoides* in having cyanidin 3-malylglucoside, two other species with cyanic flowers surveyed, *D. carthusianorum* L. and *D. nitidus* Waldst. & Kit., lack zwitterionic pigments [11]. Also, the character varies within *D. caryophyllus*, because two different British cultivars of the crimson carnation flower were found to lack acylated anthocyanins, whereas the malylated cyanidin 3-glucoside was present in Japanese forms [Yamaguchi, M. and Terahara, N., unpublished results].

While anthocyanins acylated with malonic acid have been reported from a variety of plants [9], and a succinyl derivative of cyanidin 3,5-diglucoside has been found in *Centaurea* [12], anthocyanins with malic acid are much rarer. There is only one previous report of malic acid in this connection, a malvin derivative in Cabernet Sauvignon grapes [13], but the pigment was not fully characterized.

It may be observed that the two new malylated pigments do differ in R_f, electrophoretic mobility and retention time in HPLC from the corresponding malonated derivatives (Table 1). Malylation lowers the retention time and the electrophoretic mobility as compared to malonylation. It should thus be possible to directly differentiate between the two classes of acylated pigment during surveys.

Further investigation of other caryophyllous species for malylated anthocyanins is in progress. It may be noted that such pigments have not been detected by us in flowers of *Silene dioica*, well known as a source of anthocyanins carrying acylation with hydroxycinnamic acids [14]. Clearly, a range of acyl substituents may be present in the pigments of this family.

EXPERIMENTAL

Plant material. Flower petals of the red carnation, *Dianthus caryophyllus* cv. Scania were collected from the farm of Minami-Kyushu University, Miyazaki Prefecture and dried at 50° overnight and stored in a desiccator. Other cultivars of pink and deep crimson carnations and plants of *Dianthus deltoides* examined were of British horticultural origin. Flowers of *Dianthus* species were from the living collection in the University of Reading botanical garden.

Extraction and purification. The petals were extracted with EAW (EtOH–HOAc–H_2O, 10:1:10). The extract was purified by prep. PC on Toyo No. 526 or Whatman No. 3 filter paper in BAW2 (*n*-BuOH–HOAc–H_2O, 4:1:5, bottom), BAW1 and 15% HOAc, variously. After passing through a Sephadex LH-20 column with EAW, the eluate was evaporated *in vacuo* to a small vol. The concentrate was kept cool for several days, when crystalline anthocyanin was obtained.

Characterization of pigments. Pigment **1**, dried *in vacuo* over P_2O_5 overnight at room temp., had mp > 300° (uncorr.);

UV–VIS $\lambda_{max}^{0.01\%\ HCl\text{-}MeOH}$ nm: 270, 510; E_{270}/E_{max} 63%, E_{320}/E_{max} < 20%, E_{440}/E_{max} 43%; IR ν_{max}^{KBr} cm^{-1}: 3380, 1730 (ester C=O), 1640, 1335, 1172. Pigment **2** was also obtained in crystalline form with UV–VIS λ_{max} at 270 and 523 nm and absorbance ratios of a cyanidin 3-glycoside. Pigment identifications were carried out by standard procedures, involving H_2O_2 oxidation, deacylation with alkali and hydrolysis with acid [9]. FAB-MS was carried out on a JMS. DX-300 (Jeol) or on Kratos MS9/50TC apparatus. The identification of malic acid from deacylation was confirmed by IR measurements in nujol, which were identical to those made on authentic material.

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