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A MACROCYCLIC ANTHOCYANIN FROM RED/MAUVE CARNATION FLOWERS

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Key Word Index—*Dianthus caryophyllus*; Caryophyllaceae; carnation; malylated cyanidin 3,5-diglucoside.

Abstract—Flowers of the red/mauve carnation cultivars "Kortina Chanel" and "Purple Torres" contain a unique macrocyclic anthocyanin pigment, a malylated cyanidin 3,5-diglucoside in which the malyl group is linked to both sugars. The native anthocyanin readily undergoes ring opening to yield cyanidin 3-*O*-(6-*O*-malyl glucoside)-5-*O*-glucoside. This lability was found to be due to the inherent instability of the malyl interglycosidic bridge. In this paper we report the structure elucidation of the native anthocyanin, and the ring opened product.

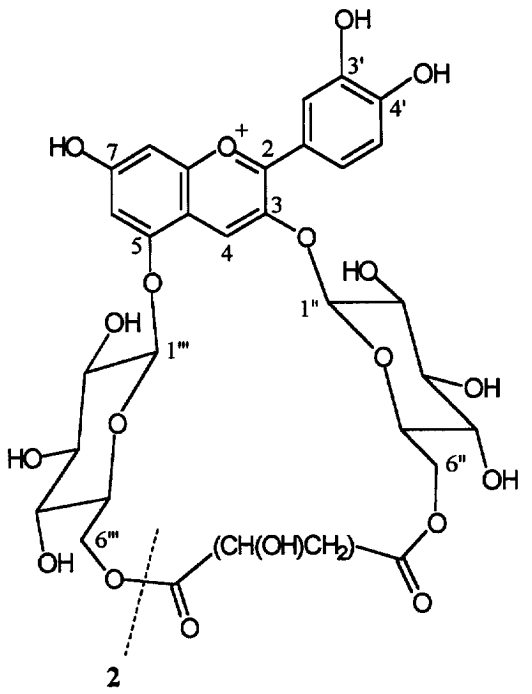
INTRODUCTION

During the investigation of the floral pigment chemistry of several carnation (*Dianthus caryophyllus*) cultivars it was observed that the major anthocyanin pigment in the light mauve "Kortina Chanel" and dark mauve/red "Purple Torres" cultivars showed a high degree of instability. Previous work on various *Dianthus* flowers has shown the major anthocyanins to be the 3-glucosides and 3,5-diglucosides of pelargonidin and/or cyanidin [1–4]. In many cases these anthocyanins are acylated with malic acid, an acyl group which is generally not encountered in non-caryophyllous pigments [3, 4]. The main area of interest in this work was in the mauve/red flowers where the 3,5-di-*O*-glucosides of cyanidin are the dominant anthocyanin type.

RESULTS AND DISCUSSION

HPLC analysis of freshly pressed juice or fresh aqueous extracts from the petals of the two mauve/red carnations showed one predominant anthocyanin (**1**). However, upon standing in acid, or on mild heating, a second anthocyanin peak, (**2**), appeared. These two anthocyanins were clearly acylated cyanidin 3,5-di-*O*-glucosides as acid and base hydrolysis produced cyanidin and cyanidin 3,5-di-*O*-glucoside respectively. For comparison, two red carnation cultivars, "Maya" and "Tasty", were also examined. Although the anthocyanin in these red cultivars had a similar HPLC R_f to **1**, it showed much greater stability and was clearly different by TLC. Analysis of base and acid hydrolysis products, and comparison with reported data [2], indicated the anthocyanin in these red cultivars was cyanidin 3-*O*-(malylglucoside).

Fresh petals of *Dianthus* "Purple Torres" were



2

1

extracted with aqueous formic acid and **1** and **2** were purified by passage of the crude extract through a column of Diaion HP-20 followed by chromatography of the crude pigment mixture on polyamide. The separation was monitored by HPLC analysis from which it was evident that partial conversion had occurred, even with the careful handling procedures employed. The analysis showed that pure compounds had been obtained from the polyamide column. The first eluted compound was **2** (HPLC R_t 12 min) and **1**, the native anthocyanin, was eluted later (HPLC R_t 18 min). The longer retention time of **1** initially suggested an additional acyl group was present, however, further evidence, detailed below, proved otherwise.

Electrophoretic mobility measurements have been widely used as a preliminary screen for the presence of diacid acyl groups mono-esterified to anthocyanins [5, 6]. Only anthocyanin **2** was electrophoretically mobile and showed similar movement to malonylawobanin, a malonylated anthocyanin from bluebell. Neither fresh flower extract nor **1** showed electrophoretic mobility indicating the absence of free acid groups. The FAB (+ve and −ve) mass spectra of **1** and **2** were particularly illuminating in that there was a clear MW difference of 18 between **1** at 710 and **2** at 728. This data was consistent with a monomalyl cyanidin diglycoside **2** and a dehydrated monomalyl cyanidin diglycoside **1**. The combined electrophoretic and MS data indicated a structure for **2** in which one of the malic carboxyl groups is a free acid and thus electrophoretically mobile, and a structure for **1** in which the malic acid has formed a diester with two hydroxyl groups.

This was further supported by the NMR spectra. Owing to the requirement for an acidic medium for anthocyanin NMR spectral determination, there was inevitably a small amount of **2** present in the NMR sample of **1**. This provided only a minor problem as the important signals were well resolved and the major signals in the spectrum of **2** could be assigned. Most of the signals in the spectrum of **2** were readily assigned by comparison with data for known compounds. **2** was clearly a cyanidin 3,5-diglucoside and the signals for a malyl group (see Table 1) were also evident. The malyl group was attached at the 6-position of one of the glucoses shown by the diagnostic downfield shift of one of the glc-6 methylene carbons (65.0 ppm). Although the anomeric proton of the 3-glucose is usually more downfield than that of the 5-glucose we confirmed that initial assignment of the anomeric proton signals via a ROESY NMR experiment. This showed NOE's between the proton at 5.41 ppm and the anthocyanidin H-4 and the proton at 5.18 and H-6 proving these were the 3- and 5-glucosyl anomeric proton signals respectively. A TOCSY NMR experiment connected the acylated glc-6 protons to the anomeric proton of the C-3 glucose (δ 5.41). Thus the structure of **2** is cyanidin 3-*O*-(6-*O*-malyl glucoside)-5-*O*-glucoside.

As expected, the NMR spectra of **1** showed considerable similarity to that of **2**. The only differences must be those due to a cyclisation of the malyl group to form an ester at a second hydroxyl. This second hydroxyl was not provided by the anthocyanidin because of the absence of significant differences in that part of the NMR spectra. Initially the second attachment point was not clearly evident; however, a C-H COSY (HMQC) NMR experiment showed the four proton signals which correlated to the two glucose-6 carbons (62.0 and 65.6 ppm) were all at higher field than 4.1 ppm. This is somewhat unusual in that the shift of one of the carbon signals (62.0 ppm) suggests it is not substituted (c.f. **2**). But, since the large downfield shift of the signals of the protons attached to the carbon at 62 ppm comprises the major difference between the ^{1}H NMR spectra of **1** and **2**, the malyl group must be linked to the 6 position of both sugars. The lack of an acylation-induced shift of the glc-6 carbon must be due to steric hindrance or strained bond effects due to the bridging acyl group. This strain is probably responsible for the lack of stability of this acyl bridge. Also, the ^{1}H NMR signals of the two malyl methylene protons are well separated in **1**, indicating a relatively rigid conformation for the cyclic structure, whereas in **2** these protons are a overlapped multiplet. The orientation of the malyl group (i.e. 2-hydroxybutanedioic or 3-hydroxybutanedioic acid) was not determined in previous work and could not be deduced from the spectral data for **1** or **2**. The unusual difference between the glc-6 carbon shifts in **1** is apparently not ascribable to the effect of the malyl hydroxyl as the ^{13}C NMR shifts of the methyl groups in dimethyl malate differ by less than 1 ppm. The structure of **1** is then cyanidin 3,5-di-*O*-glucoside(6″,6‴-malyl diester).

As far as we know this is the first report of an anthocyanin with a cyclic structure involving interglycosidic diacid acylation. This sort of linkage occurs only rarely in other secondary metabolites such as saponins. The occurrence of such compounds suggests that caution should be applied when using electrophoretic surveys for the detection of aliphatic diacid acylation in anthocyanidin 3,5-diglucosides.

EXPERIMENTAL

NMR experiments were run at 500 MHz on a Varian Unity instrument or at 300 MHz (75 MHz for ^{13}C) on a Bruker AC 300 instrument using manufacturer supplied software. Anthocyanin samples were dissolved in 1% CF_3COOD in CD_3OD. Mass spectra were obtained using a VG 70-250S instrument. HPLC analysis was performed as described previously [7] using a Waters 600 solvent delivery system coupled to a Waters 994 PDA detector. Electrophoresis was performed on cellulose acetate strips using a Gelman 51156 electrophoresis unit (pH 4.5 acetate buffer). Dimethyl malate was prepared from DL-malic acid

Table 1. NMR Data for **1** and **2** (MeOH-d_4/TFA-*d*)

	1		**2**	
2	164		164.7	
3	145.4		146.0	
4	132.05	8.80 *s*	134.9	8.92 *s*
5	156.6		157.1	
6	104.4	6.93 *s*	105.5	7.01 *s*
7	169.2		169.7	
8	97.2	7.05 *s*	97.5	7.06 *br s*
9		156.5	156.7	
10	112.6		113.1	
1′	120.8		121.0	
2′	118.3	8.02 *d* 2.2	118.6	8.03 *d* 2.2
3′	147.5		147.6	
4′	156.0		156.6	
5′	117.6	7.03 *d* 8.8	117.6	7.03 *d* 8.7
6′	128.8	8.26 *dd* 8.8, 2.2	129.0	8.28 *dd* 8.7, 2.2
3-*O*-glc				
1″	101.2	5.36 *d* 7.4	102.8	5.41 *d* 7.7
2″	74.5		74.4	
3″	77.9		78.1	
4″	71.4		71.5	
5″	76.1		75.7	
6″	65.6	4.18–4.30 *m*	65.0	4.3–4.6 *m*
5-*O*-glc				
1‴	100.5	5.59 *d* 7.6	102.5	5.18 *d* 7.7
2‴	74.6		74.8	
3‴	78.0		78.0	
4‴	69.5		71.1	
5‴	76.1		78.8	
6‴	62.0	5.03 *dd* 12, 1; 4.17 *d* 12	62.4	3.95 *m*
malyl				
	174.4		176.3	
	40.0	2.80 *dd* 17, 10; 2.50 *dd* 17, 2	39.9	2.7 *m*
	69.8	4.40 *dd* 10, 2	68.2	4.37 *m*
	171.5		172.2	

and diazomethane. ^{13}C NMR ($CDCl_3$) 173.6, 170.9, 67.1, 52.7 (Me), 51.9 (Me), 38.3.

Plant material

Flowers were from plants grown at Florigene (Kortina Chanel) or purchased from J. Dekker, Levin, N.Z. (Purple Torres statorli, Maya stadofab, Tasty kgr).

Isolation of anthocyanins

The fresh flowers (170 g) were extracted by blending with 5% aq. formic acid and standing at room temp. for 4 h. The extract was adsorbed onto a Diaion HP-20 column and washed with H_2O. The pigments were eluted with EtOH–HOAc–H_2O (10:1:9), concentrated on a rotary evaporator and the aqueous pigment mixture freeze-dried. Approximately half of the pigment fr. was applied to a polyamide column (MN-SC-6). **2** (30 mg) was eluted with 0.1% TFA in water and **1** (95 mg) with EtOH–HCO_2H–H_2O 20:5:75. Frs were evaporated by rotary evaporation and freeze drying.

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