"Cyclamen Red" Colors Based on a Macrocyclic Anthocyanin in Carnation Flowers

Jean-François Gonnet*,† and Bernard Fenet‡

Laboratoire BMP and Centre de RMN, UCB-ESCPE, UPRESA CNRS 5012, Université Claude-Bernard Lyon I, 43 boulevard du 11 Novembre 1918, F-69622 Villeurbanne, France

The "cyclamen" red (or pink) colors in carnation flowers—cultivars Red Rox and eight others—are based on the presence of a new macrocyclic anthocyanin, pelargonidin 3,5-di-*O*-*β*-glucoside(6″,6‴-malyl diester) identified by spectroscopic methods. The instability of the bridging malyl group with sugars in acidic medium readily causes the formation of the opened ring form, 3-*O*-(6″-*O*-malylglucoside)-5-*O*-glucoside. The issue of cyclamen colors based in carnations on this original acylated pelargonidin derivative simulating those based on simpler cyanidin glycosides in *Rosa* cultivars is discussed using CIELAB colorimetric coordinates.

Keywords: *Dianthus caryophyllus; Caryophyllaceae; carnation; flower anthocyanins; malylated pelargonidin diglucoside; CIELAB*

INTRODUCTION

In cultivated plant species, colors are frequently described using those displayed by other species. This trend typically affects the flowers of ornamental cultivars sharing a basic magenta hue, currently reported in breeders' communications as "cyclamen" or "fuchsia" reds or pinks. Perceptually, and on the basis of a colorimetric assessment of codes using the CIELAB scale (Gonnet, 1995), these colors are in the range of those of chips 57A−57B and 66A−66B in the RHS color chart. In ornamental species, these shades are generally based on disubstituted B ring anthocyanins: peonidin and cyanidin glycosides in *Cyclamen* (Chapoutier, personal communication) and mainly cyanidin 3,5-diglucoside in many *Rosa* varieties (such as Lancôme, Maria Callas, or an unidentified cultivar in one of the authors' garden, for instance). By contrast, a flavonoid survey of >200 cultivars revealed that "cyclamen" tonalities in carnations (cultivars Castellaro or Sarazis, among others) were based on pelargonidin glycosides, that is, a monosubstitued B ring anthocyanidin. Only one anthocyanidin was found as the color basis in each class of the intensely colored cultivars examined: red and garnish-red, "cyclamen" and purple-violet (Lédemé, 1991; Lédemé et al., 1988). In the first two classes, pelargonidin or cyanidin, respectively, was present as a mixture of the 3-*O*-glucoside (minor constituent) and its malylated derivative (major constituent) (Lédemé, 1991; Lédemé et al., 1988; Terahara et al., 1986). Similarly, in the patterns of cyclamen and purple-violet (or red-mauve) flowers, pelargonidin or cyanidin, respectively, was consistently present as a comparable mixture of four 3,5-diosides, one being largely predominant (>60% of the total). The 3,5-diglucoside was one of the three minor molecules, but each major pigment could not be identified completely (Lédemé, 1991). Recently, an original macrocyclic anthocyanin, cyanidin 3,5-di-*O*-glucoside (6″,6‴-malyl diester), was identified as the molecule responsible for the red-mauve coloration in carnations (Bloor, 1998). Because of the many correspondences observed in the pigment patterns of purple and cyclamen flowers, it appeared that the structure of the major anthocyanin in the latter probably could present a comparable macrocyclic conjugation scheme, based on pelargonidin (Figure 1). The structural reinvestigation of the pigments of cyclamen carnations was carried out on the Red Rox variety, presenting the highest relative accumulation level of the major molecule.

MATERIALS AND METHODS

Plant Material. Flowers of Red Rox (Obt. Di Gorgio, Italy) carnation were collected from plants cultivated in greenhouses at the Sophia-Antipolis station of GIP-GEVES (France). From the same source, other cyclamen carnation varieties examined were Lipari, Castellaro (Di Gorgio), Handy (A. Baratta, Italy), Doge (N. Baratta), Stanex (Van Staveeren, The Nederlands), Sarazis, Katia (Barberet-Blanc, France), and Lanasar (Lanari, France). *Rosa* cultivars Lancôme (Delbard, France) and Maria Callas (Meilland, France) were grown in one of the authors' garden.

Extraction and Isolation of Anthocyanins. Liquid nitrogen frozen fresh petals (165 g) were extracted with a boiling MeOH/EtOH (1:1 v/v) mixture (800 mL, 20 min, twice). After concentration under reduced pressure and refrigeration (−18 °C, 24 h), the crude methanolic extract was centrifuged (to remove lipophilic petal surface substances) and then adsorbed on an Extrelut (Merck, Darmstadt, Germany) column. Most of the flavonoids and phenolic acids were eluted stepwise by hexane, EtOAc, and EtOAc/1-BuOH (2:3 v/v), and the anthocyanin fraction was finally eluted by MeOH. Further purification was achieved by multiple succesive separations on Sephadex LH-20 (Pharmacia) columns (40 × 3.5 cm), alternatively eluted by MeOH or H_2O and monitored by TLC and HPLC. After concentration of the fractions containing the pure pigment, the residue was dissolved in refrigerated MeOH/0.01% HCl (8 mL) and the pigment immediately precipited

* Author to whom correspondence should be addressed (telephone 33 4 7244 8000; fax 33 4 7884 4847; e-mail gonnet@cismsun.univ-lyon1.fr).

† Laboratoire BMP.

‡ Centre de RMN.

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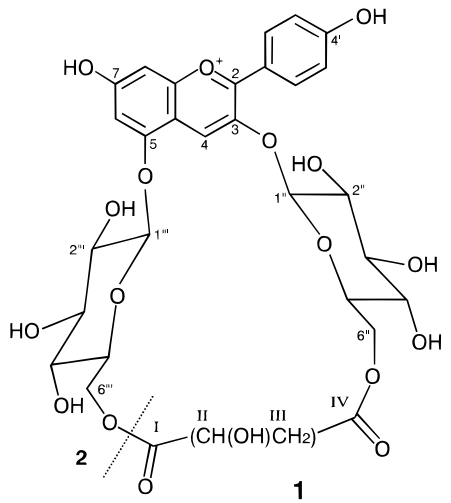

Figure 1. Structures of the native (**1**) and opened ring (**2**) forms of the major anthocyanin in petals of Red Rox carnation.

with diethyloxide, washed (diethyloxide), dried, and frozen. This procedure finally yielded 146 mg of the major anthocyanin (**1**).

Chromatographic Analysis. TLC was performed on cellulose plates (Merck 5577) with the solvents HAW (HCl/ HOAc/ H_2O, 3:15:82 v/v) and BAW (1-butanol/ HOAc/H_2O, 4:1:5 v/v, upper phase) in the presence of authentic samples of pelargonidin 3-*O*-, 3-*O*-malyl-, and 3,5-di-*O*-glucosides, isolated and identified in a previous investigation (Lédemé, 1991; Lédemé et al., 1988). HPLC analyses were performed using a Kontron 322/360-MT450 system coupled to a Shimadzu SPD6AV photodetector. Methanolic pigment extracts were diluted with MAW (MeOH/HOAc/H_2O, 25:4:21 v/v) just before their injection (20 μL) on a C_{18} Kromasil KR225 (Shandon HPLC) column (5 μm, 4.6 $\varnothing$ × 250 mm). The elution was run at 21 °C and at a 0.8 mL min^{-1} flow rate using two successive linear gradients of MeOH in 5% aqueous HCOOH (15−40% in 22 min, then 40−50% in 10 min). Anthocyanin signals were detected by absorbance readings at 510 nm. Electrophoresis was performed (4 h at 40 mV cm^{-1}) on Whatman No. 1 paper in acetate buffer at pH 4.4 (Harborne, 1986) in the presence of pelargonidin 3-*O*- and 3-*O*-malylglucosides.

Spectroscopic Analysis. Low-resolution MS spectra (API-ES, positive mode) were recorded on a Hewlett-Packard 1100 MSD system. NMR spectra (500 MHz for ^1H and 125.77 MHz for ^{13}C without an internal standard) were recorded and experiments performed at 20 °C on a Bruker Avance DRX500 spectrometer using the Bruker software. Measurements of the solution (15 mg of **1** in 0.5 mL of CD_3OD/2% TFA) were run instantly and repeated after the conversion of the native cyclic molecule **1** to the opened ring form **2** had occurred completely (monitored by TLC and electrophoresis).

Color Specifications. Color measurements of the flower petals and of the RHS chart were performed with a Datacolor International ChromaSensor 5 spectrocolorimeter using the CIE d/8 viewing/illuminating geometry with specular component included, according to a previously described procedure (Gonnet, 1993). From the spectral reflectance curves, the CIELAB coordinates were calculated for the D_{65}/10° illuminant/observer condition (CIE, 1986) using the Datacolor ACS-QC software. Colors are numerically specified using the LCH notation, in terms of hue angle (h_{ab}), metric chroma (C^*), and lightness (L^*) representing the psychometric correlates of the natural attributes of the color perception by the human visual system, chromatic tonality, saturation, and lightness, respectively (CIE, 1986). The RHS color chart employed was a brand new copy of the 1986 edition. Colorimetric assessment of RHS codes was performed with the help of a computer-aided system using visual color differences calculations (Gonnet, 1995), coupled with visual assessment under matched reference conditions for the observation: diffuse reference daylight simulating the CIE D_{65} source (color temperature = 6500 K), at a 1000 lx illuminance level and on an 18% reflective gray background. Consequently, color code determination is valid only for these light conditions.

RESULTS AND DISCUSSION

HPLC of an acid-free alcoholic extract from petals of Red Rox carnation showed the presence of four pigments: the predominant one (**1**, retention time, t_R = 28 min) and pelargonidin 3,5-di-*O*-glucoside (t_R = 20.2 min, identified by spectroscopic and hydrolytic methods and by comparison with a reference coumpound from ExtraSynthèse, Genay, France) represented 85 and 9% of the pigment pattern, respectively; the other compounds (t_R = 22.7 and 24.8 min) were present as only traces (<3% each). All appeared as orange-red spots on the TLC, the major one exhibiting a bright orange-yellow fluorescence under UV light (365 nm). No constituent with electrophoretic mobility at pH 4.4 was clearly detectable. In the HPLC profile of the same extract after standing for 48 h in MAW, the signal of the major pigment decreased to 64% while a simultaneous increase up to 20% resulted for the signal of the minor compound at 24.8 min; in addition, a minor mobile spot was present on the electrophoregram of this sample. These observations were in accordance with two particularities reported for the major cyclic cyanidin glycoside in red-mauve carnations: no electrophoretic mobility and instability of the bond of the bridging acyl group with sugars in acidic medium and a subsequent conversion of the native cyclic molecule into an "opened"—and electrophoretically mobile—form (Bloor, 1998). Therefore the similarities between the pelargonidin (**1**) and the cyanidin derivatives in carnations became more evident; in particular, the HPLC signal at 24.8 min was probably attributable to the opened ring form **2** of the native anthocyanin **1**.

The UV−visible spectral properties of **1** were those of a pelargonidin 3,5-dioside (Harborne, 1958): λ_{max} 512, 419 (sh), 321 (sh), and 268 nm, $A_{440}/A_{\lambda max}$ = 0.17 and $A_{\lambda maxUV}/A_{\lambda max}$ = 0.51 in MeOH containing 0.1% HCl; λ_{max} 497, 416 (sh), 327 (sh), and 267 nm, $A_{440}/A_{\lambda max}$ = 0.21 and $A_{\lambda maxUV}/A_{\lambda max}$ = 0.59 in aqueous 0.1 N HCl. Acid and alkaline hydrolyses produced pelargonidin, glucose, and malic acid and pelargonidin 3,5-diglucoside and malic acid, respectively. The electrospray MS (positive mode) showed a molecular ion at *m*/*z* 693, corresponding to a dehydrated monomalyl diglucosyl pelargonidin; other signals at 595, 549, 433, and 271 were attributable to pelargonidin (3,5-)diglucoside, malylglucosylpelargonidin, monoglucosylpelargonidin, and pelargonidin, respectively (Lédemé, 1991). In some spectra, the presence of an additional signal at 711 was assigned to a monomalylated diglucosyl pelargonidin, corresponding to an opened ring form of the native molecule. These data and those concerning the different electrophoretic properties of the original molecule and of its byproduct upon standing in acid medium strongly supported the structural hypothesis of a cyclic malylated diglucosylpelargonidin.

The complete structure of this pigment was determined by NMR analysis. A first set of spectra was performed immediately after the pigment was dissolved in CD_3OD/TFA; it appeared that the solution quickly contained both the native (**1**) and the putative converted (**2**) forms, in a strangely high ratio (approximately 1:1); consequently, a second set of measurements was run later on this solution, when the conversion of the native

Table 1. NMR Data for the Native (1) and Converted (2) Anthocyanins (CD_3OD/TFA)

carbon no. (see Figure 1)	1				2			
	^{13}C	^1H		J(Hz)	^{13}C	^1H		J(Hz)
2	163.5				164.0			
3	144.6				144.8			
4	131.7	8.85	s		134.4	8.97	s	
5	155.1				155.7			
6	103.4	6.93	s		104.3	7.01	s	
7	168.3				168.7			
8	96.2	7.06	s		96.4	7.10	s	
9	155.9				156.2			
10	112.3				111.9			
1′	119.6				119.6			
2′−6′	135.2	8.60−8.56	dd	9.1−1.6	135.2	8.60−8.56	dd	9.1−1.6
3′−5′	117.1	7.05	dd	9−1.6	117.1	7.05	dd	9−1.6
4′	166.3				166.3			
3-*O*-Gluc								
1″	100.3	5.39	d	7.9	101.6	5.23	d	7.9
2″	73.4	3.72	t	8.4	73.7	3.73	t	7.8
3″	75.5	3.63	t	8.8	76.8	3.60	t	8.7
4″	75.1	3.97	t	9.2	70.0	3.51	d, dd	11.2, 5.6−12
5″	68.8	3.79	t	9.9	77.8	3.61	m	
6″	60.8	4.29−5.06	d, d	11.4, 10.4	61.2	3.79−3.99	m	
5-*O*-Gluc								
1‴	99.7	5.68	d	7.5	101.3	5.50	d	7.9
2‴	73.7	3.72	t	8.1	73.4	3.72	t	8.1
3‴	77.1	3.63	t	8.1	77.1	3.60	t	8.7
4‴	70.4	3.37	t	9.8	70.5	3.44	t	9.2
5‴	75.1	3.77	m		74.6	3.90	t	7.5
6‴	64.5	4.34	m		64.0	4.39	m	
malic acid								
I	173.3				173.9			
II	68.5	4.42	d	11.7	67.2	4.39	m	
III	40.2	2.52−2.83	dd, dd	10.4−17, 17−2.8	38.8	2.71	m	
IV	170.4				171.0			

molecule was fully completed. In the first one, although the signals of both forms were superimposed in the spectra, the assignment of the specific signals to each one was achieved (Table 1) with the help of a set of 1-D ^{13}C and ^1H spectra and 2D experiments: COSY, relayed COSY (one, two relays), HSQC, HSQC-TOCSY, and HMBC.

Signals in the downfield part of the ^1H NMR spectrum [chemical shifts (δ) between 6.9 and 9.0 ppm] were clearly attributable to the aromatic protons (A and B rings) of the anthocyanidin nucleus. The four signals (doublets) between 5.20 and 5.70 ppm corresponded to the protons on the anomeric carbon from the four glucose residues (two in each form **1** and **2**); their large coupling constant (J = 7.5−7.9 Hz, ^1H NMR spectra) showed that the hydrogens at the C-1 and C-2 of each sugar subunit were in the axial configuration, revealing that all glucoses were in the β-configuration (Giusti et al., 1998). Except for three (in the 4.3−4.5 ppm spectral interval), all of the proton resonances from the non-anomeric sugar groups appeared grouped in the 3.35−4.0 ppm spectral area, with a severe overlapping of signals. However, the complete spin system of each glucose ring was finally established with 2D-COSY and HSQC-TOCSY. For each glucose unit, starting from H-1, the signals of each successive H (-2, -3, and -4) on the ring were sequentially assigned with COSY and relayed COSY. The assignment of H-5 and H-6 was more difficult, but the task was completed by a HSQC-TOCSY experiment, in which the ^1H data are spread in the carbon dimension. The same approach was employed for the assignment of the resonances of ^{13}C and ^1H (2.5−2.8 and 5.5−5.7 ppm) of the malyl residues and those of the aromatic rings of the aglycon, too.

All the subunits were then linked together with the results of an HMBC experiment. First, the ^{13}C-3 and ^{13}C-5 of each pelargonidin nucleus were determined by the connectivity of their signals with the ^1H on their respective adjacent C-4: signals at 144.6 and 155.1 ppm were correlated with the ^1H at 8.85 ppm for **1** and those at 144.8 and 155.7 ppm with 8.97 ppm for **2**. The attachment of the two glucose units on each aglycon was then determined by the correlation of their H-1 with the carbons of the aglycon: H-1″ (5.39 and 5.23 ppm for **1** and **2**, respectively) appeared linked with the C-3 (144.6 and 144.8 ppm, respectively) and H-1‴ (5.68 and 5.50 ppm, respectively) with the C-5 (155.1 and 155.7 ppm, respectively). The attachment of the malyl units was finally determined by the correlation between the carbonyl carbons and the protonated ones, all but one carbonyl carbon showing correlations with a proton of a glucose unit. For **1**, the correlations were observed between the carbonyl carbon at 173.3 ppm (C-I) and the glucose H-6‴ (4.34 ppm) and between the carbonyl carbon at 170.4 ppm (C-IV) and the glucose H-6″ (4.29 ppm), confirming the interglucosidic linkage by malic acid and its esterification to both glucoses through their OH at C-6. The downfield shift observed for the first carbonyl carbon is attributable to its vicinity with the CHOH group. Consequently, the cyclic structure of the native molecule **1** definitively corresponds to the one presented in Figure 1. For **2**, HMBC revealed that only one carbonyl carbon (171 ppm, C-IV) was coupled with a sugar proton (H-6″, 3.8−4.0 ppm), confirming that the malyl interglucosidic bridge opening occurs at the bond with the glucose at the C-5 of the anthocyanin. The 1D-NMR spectral data (^{13}C and ^1H) recorded later on the "pure" converted form match those recorded for this molecule in the mixture with the native one and confirmed the attributions of the two species.

Finally, the basic pigment of the cyclamen color of Red Rox appears to be pelargonidin 3,5-di-*O*-β-glucoside

(6″,6‴-malyl diester) (**1**) accompanied by 3-*O*-(6″-*O*-malyl-*β*-glucoside)-5-*O*-*β*-glucoside (**2**), pelargonidin-3,5-di-*O*-*β*- glucoside, and an unidentified pelargonidin 3,5-dioside, present as minor constituents.

The correspondences previously observed among the global anthocyanin patterns of carnations displaying the four "intense" cyanic colors are confirmed and amplified with the identification of pelargonidin 3,5-di-*O*-*β*-glucoside(6″,6‴-malyl diester) as the main pigment in flowers of Red Rox. This molecule also remains the very predominant anthocyanin in the pigment profile of all other cyclamen carnations examined during the preparation of this study (listed in Materials and Methods). Therefore, two comparable—a simple (monosidic) and a complex (macrocyclic diosidic)—conjugation schemes are affecting two basic anthocyanidins, pelargonidin and cyanidin, each combination being coupled with a specific petal color range. In this regard, the colors originated by the accumulation of these cyclic diosides of both aglycons, cyclamen and purple tonalities, appear to be somewhat unusual for derivatives of pelargonidin and cyanidin, respectively.

On the basis of CIELAB coordinates (for the D_{65}/10° illuminant/observer condition), the attributes of the visually "pure" colors of cyclamen petals are medium lightness ($L^* = 45-50$), a relatively high saturation level (chroma, $C^* = 60-64$), and basic magenta/magenta-red chromatic tonalities (hue angle, $h_{ab} = 358-6°$). By comparison, a typical red is the basic hue ($h_{ab} = 25-30°$) of saturated colors in carnation flowers accumulating simpler pelargonidin derivatives (mainly 3-*O*-malyl-glucoside; Gonnet, 1993), in accordance with the color range currently reported for pelargonidin glycosides (Harborne, 1993). All nine cyclamen carnation cultivars measured had colorimetric coordinates falling in the above-mentioned range: Lanasar ($L^* = 49$, $C^* = 60.2$, and $h_{ab} = 0.1$; RHS code 66A) or Katia ($L^* = 47.5$, $C^* = 60.2$, and $h_{ab} = 3.9$; RHS code 57A), for instance. These colors closely match (by measurement and visually too) those of petals of the *Rosa* cultivars considered for comparison (for instance, Lancôme $L^* = 48$, $C^* = 60.4$, and $h_{ab} = 0.9$, RHS code 66A). However, these cyclamen colors in roses are based on the exclusive presence of cyanin (cyanidin 3,5-di-*O*-glucoside, confirmed by UV—visible spectroscopy, TLC, and HPLC cochromatographies). This is in accordance with the magenta hue displayed by their pigment extract in methanolic 0.1% HCl ($\lambda_{max} = 527$ nm); by contrast, at a comparable concentration, the acidified anthocyanin extract of Red Rox or other cyclamen carnations ($\lambda_{max} = 511$ nm) displays the typical orange-red hue of pelargonidin. Therefore, the final colors of petals of carnations and roses are similar or identical, although their basic anthocyanins are quite different: in other words, within a comparable range of lightness and saturation levels, the color of the complex cyclic pelargonidin glycoside is simulating the one typically attached to the widespread flower pigment cyanin. Besides influential parameters on flower color such as epidermal micromorphology and vacuolar pH, the spectral effects of the copigmentation phenomenon are the probable major parameter explaining these specific modifications of anthocyanin basic colors. Particular and/or stronger copigmentation possibilities probably result from the cyclic structure of the carnation anthocyanin. The subsequent spectral variations—considered throughout the extent perceived by the human visual system, not only at the λ_{max} of the pigment (Gonnet, 1998)—are expected to originate more intense color shifts than those currently observed with simpler glycosidic substitutions. Testing of this hypothesis, especially with regard to the attribute of hue, is in the final stage by means of CIELAB color measurements performed [according to Gonnet (1998, 1999)] on some 150 model copigmented solutions of both the macrocyclic anthocyanins of carnations, the pelargonidin derivative from Red Rox, and the corresponding cyanidin derivative isolated from Nadir and Vanessa purple cultivars. The color variations of these solutions with the pH, the pigment, and copigment concentrations will be reported in a subsequent paper.

Presently, the identification of this pelargonidin glycoside in carnations represents the second report of a cyclic dicarboxylic aliphatic acylated anthocyanin. Because these molecules are undetectable by current electrophoresis procedures and are unstable in acidic medium, they probably occur more frequently than is currently reported in the literature. In particular, the sources of anthocyanin 3,5-diglycosides acylated by dicarboxylic aliphatic acids (malic, malonic, etc.) obtained from extraction and isolation procedures using acidic solvents could be carefully reinvestigated for the presence of these cyclic derivatives with acid-free analytical treatment.

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