PERGAMON

PHYTOCHEMISTRY

Phytochemistry 51 (1999) 125–132

New features of intramolecular copigmentation by acylated anthocyanins

Paulo Figueiredo[a,b,*], Florian George[a], Fumi Tatsuzawa[c], Kenjiro Toki[d], Norio Saito[e], Raymond Brouillard[a]

[a]*Laboratoire de Chimie des Polyphénols, Associé au CNRS, Université Louis Pasteur, Institut de Chimie, 1 rue Blaise Pascal, 67008 Strasbourg, France*

[b]*Universidade Lusófona de Humanidades e Tecnologia, Lisbon, Portugal*

[c]*Faculty of Horticulture, Chiba University, Matsudo, Chiba, Japan*

[d]*Laboratory of Floriculture, Minami-Kyushu University, Takanabe, Miyazaki 884, Japan*

[e]*Chemical Laboratory, Meiji-Gakuin University, Totsuka-ku, Yokohama, Japan*

Received 10 August 1998

Abstract

Three series of structurally related anthocyanins, extracted from the red–purple flowers of *Dendrobium* 'Pramot', x*Laeliocattleya* cv. Mini Purple, *Bletilla striata* and *Phalaenopsis* all belonging to the *Orchidaceae* family and another series extracted from the pink flowers of *Senecio cruentus* (Compositae) allowed the confirmation of the existence of strong intramolecular copigmentation effects. These interactions confer stability to the coloured forms of the molecules, in a wide range of slightly acidic to neutral aqueous media. Moreover, the existence of structural relationships among the four series stressed the different influences exerted by the diverse substituent groups. The existence of a malonylglucoside attached to position 3 of all but three of the molecules put forward a new role for the malonyl residue, in this particular position.

Keywords: *Dendrobium* cv. Pramot; x*Laeliocattleya* cv. Mini Purple; *Bletilla striata*; *Phalaenopsis*; *Senecio cruentus*; Intramolecular copigmentation; Acylated anthocyanins; Malonyl effect.

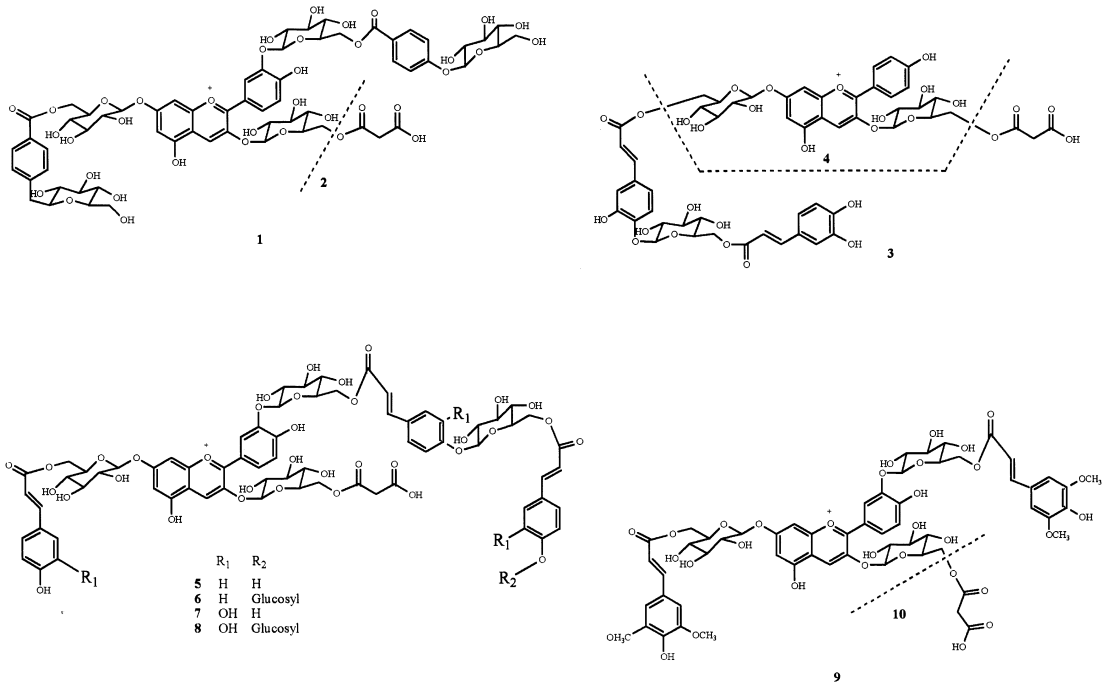
1. Introduction

In the last few years, as extraction, purification and identification techniques improved, several works (Lu, Saito, Yokoi, Shigihara, & Honda, 1992; Saito & Harborne, 1992; Saito, Lu, Yokoi, Shigihara, & Honda, 1993; Saito, Toki, Uesato, Shigihara, & Honda, 1994; Tatsuzawa, Saito, Yokoi, Shigihara, & Honda, 1994, 1996; Toki et al., 1994; Toki, Saito, Imura, Suzuki, & Honda, 1994; Toki, Saito, Kuwano, Shigihara, & Honda, 1995; Saito et al., 1995; Tatsuzawa et al., 1997) have put in evidence the existence of anthocyanins possessing a rather complex substitution pattern. It consists in sequences of glycosyl and aliphatic or aromatic acyl residues linked to the central flavylium chromophore (Scheme 1). The planar aromatic groups, in particular, have the remarkable capacity of folding over the also planar chromophore (Brouillard, 1981; Dangles, Saito, & Brouillard, 1993a,b; Figueiredo, Elhabiri, Saito, & Brouillard, 1996; Figueiredo et al., 1996), interacting with its π-system, protecting it from the nucleophilic attack of water (Kurtin & Song, 1968; Amić & Trinajstić, 1991; Davidović-Amić, Amić, & Trinajstić, 1994). This reaction is the responsible for the disruption of the highly conjugated benzo-pyrylium structure of the flavylium form, AH_2^+. The intramolecular copigmentation will, in fact, prevent the displacement of the equilibria depicted in Scheme 2 towards the formation of the hydrated (and colourless) forms BH_2 (hemiacetal) and C (chalcone). It is this feature that distinguishes these complex molecules from the most common mono- and diglucoside forms represented in Scheme 2, which are characterised for producing colourless solutions in mildly acidic aqueous media.

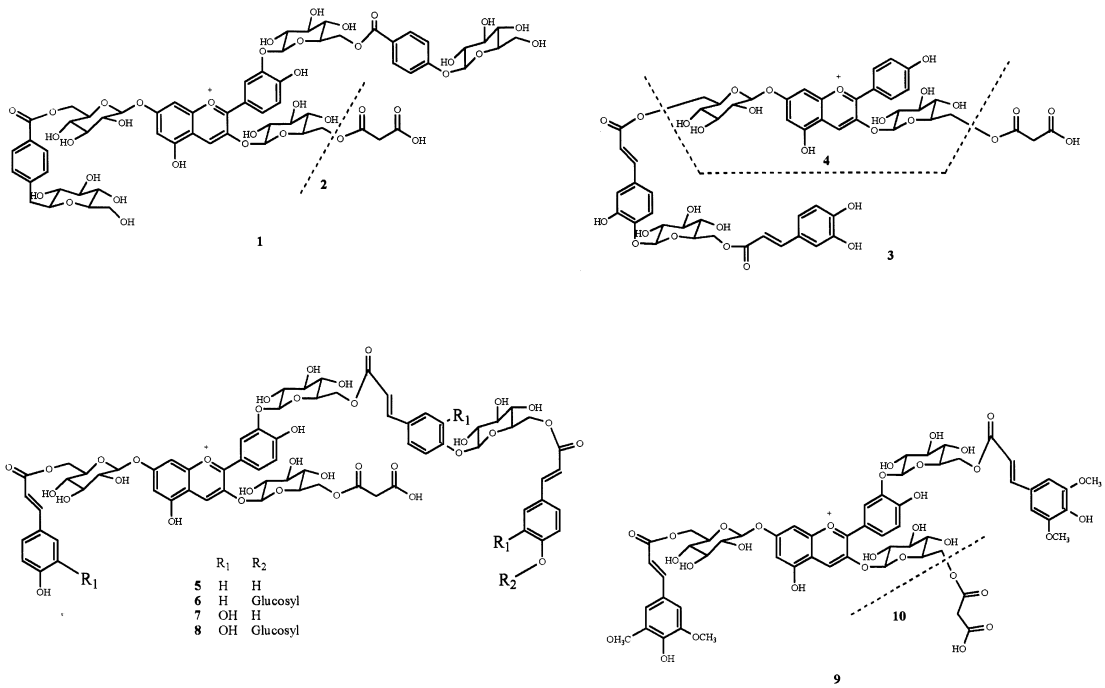
This paper takes a closer look at a series of anthocyanins presenting two substitution patterns that, to the present date, were given only scarce attention (Brouillard, 1981) from the thermodynamic and kinetic points of view. Those features are: (i) a 3,7,3′ glycosyl–acyl substitution and (ii) a malonylglucoside group attached to the position 3 of the chromophore (Scheme 1). The pig-

* Corresponding author. Universidade Lusófona de Humanidades e Tecnologia, Lisbon, Portugal.

0031-9422/99/$ - see front matter
PII: S0031-9422(98)00685-2



Scheme 1.



Scheme 2.

ments concerned are: cyanidin 3-*O*-[6-*O*-(malonyl)-*β*-D-glucopyranoside]-7,3′-di-*O*-[6-*O*-(4-*O*-(*β*-D-glucopyranosyl) oxybenzoyl)-*β*-D-glucopyranoside] (**1**) and cyanidin 3-*O*-[*β*-D-glucopyranoside]-7,3′-di-*O*-[6-*O*-(4-*O*-(*β*-D-glucopyranosyl) oxybenzoyl)-*β*-D-glucopyranoside] (**2**) isolated from the red–purple flowers of *Dendrobium* 'Pramot' (Saito et al., 1994), pelargonidin 3-*O*-[6-*O*-malonyl-*β*-D-glucopyranoside]-7-*O*-[6-*O*-(4-*O*-(6-*O*-(*trans*-caffeyl)-*β*-D-glucopyranosyl)-caffeyl)-*β*-D-glucopyranoside] (**3**) and pelargonidin 3,7-diglucoside (**4**) from the pink flowers of *Senecio cruentus* (Toki et al., 1995), cyanidin 3-*O*-[6-*O*-(malonyl)-*β*-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-*p*-coumaryl)-*β*-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-*p*-coumaryl)-*β*-D-gluco-

pyranosyl)-*p*-coumaryl)-β-D-glucopyranoside] (**5**) (Tatsuzawa et al., 1994) and cyanidin 3-*O*-[6-*O*-malonyl-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-caffeyl)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-caffeyl)-β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranoside] (**7**) from x*Laeliocattleya* cv. Mini Purple (Tatsuzawa et al., 1996), cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-*p*-coumaryl)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-4-*O*-(β-D-glucopyranosyl)-*p*-coumaryl)-β-D-glucopyranosyl)-*p*-coumaryl)-β-D-glucopyranoside] (**6**) and cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7-*O*-[6-*O*-(*trans*-caffeyl)-β-D-glucopyranoside]-3′-*O*-[6-*O*-(*trans*-4-*O*-(6-*O*-(*trans*-4-*O*-(β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranosyl)-caffeyl)-β-D-glucopyranoside] (**8**), purified from *Bletilla striata* (Saito et al., 1995) and cyanidin 3-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside]-7,3′-di-*O*-[6-*O*-(sinapyl)-β-D-glucopyranoside] (**9**) and cyanidin 3-*O*-[β-D-glucopyranoside]-7,3′-di-*O*-[6-*O*-(sinapyl)-β-D- glucopyranoside] (**10**), extracted from the red–purple flowers of five species of *Phalaenopsis* (Tatsuzawa et al., 1997).

These anthocyanins may be divided into two categories: pelargonidin based (**3** and **4**) and cyanidin based (**1**, **2**, **5**, **6**, **7**, **8**, **9** and **10**). The results obtained for the second group are compared to the ones obtained with another set of pigments, also cyanidin based, (Figueiredo et al., 1996a) in order to emphasise the consequences, in both thermodynamic and kinetic equilibria, produced by the particular kind of substitution presented by molecules **1**, **2**, **5**, **6**, **7**, **8**, **9** and **10**.

2. Results and discussion

The immense diversity of flower colours is, in great measure, affected by the chemical structure of the pigments residing in the plant vacuoles, the anthocyanins. In fact, these molecules are able to display a wide variety of tints and hues as a function of the nature of some of the substituents attached to the central chromophore. Thus, the diverse colours exhibited by the petals of the flowers, from where the present group of pigments was extracted, is readily explained by the different patterns of substitution (Scheme 2), that characterise each of the anthocyanins here reported. These differences are reflected by the visible maxima wavelength displayed on the UV–visible spectra of the flavylium cation form (AH_2^+) of the anthocyanins as well as by their molar extinction coefficients, reported in Table 1. The λ_{max} obtained for the cyanidin based molecules (**1**, **2** and **5**–**10**) present a pronounced bathochromic shift when compared to the parent compound cyanidin 3,7,3′-triglucoside (506 nm) (Elhabiri, 1997). Moreover, inspection of Table 1 reveals that, for the same pH value, the bathochromic shift increases with the degree of substitution. Also, in the homogeneous sub-series **1**–**2**, **3**–**4** and **9**–**10**, an increase in the ε values is readily noticeable when going from the simpler to the more complex structure. Another striking feature, that results from the analysis of Table 1, is the abnormally low molar extinction coefficient of pigment **4** (8600 mol^{-1} dm^3 cm^{-1}), which has a parallel only with those anthocyanins possessing a disaccharide as substituent in position 3 of the chromophore (Elhabiri, Figueiredo, Fougerousse, & Brouillard, 1995; Figueiredo et al., 1996a,b) or with cyanidin 3,7,3′-triglucoside (ε = 12300) (Elhabiri, 1997). This leads us to tentatively postulate the glycosylation on C-7 as the reason for the verified low ε value. This assumption is further supported by the comparison of the latter pigment with another anthocyanin non-glycosylated on C-7, cyanidin 3,3′-diglucoside (ε = 23700) (Elhabiri, 1997). However, the glycosylation effect on the molar extinction coefficient seems to be cancelled, once the C-7 glucosyl has a more complex substitution pattern (cf. **3**).

The bathochromic shift, referred to above, is evidence of the formation of either inter- or intramolecular complexes by anthocyanins. In the present case, pigments **1**–**3** and **5**–**10** possess chemical structures that allow a folding of the 'side-arms' constituted by the acyl–glycosyl chains attached to positions 3′ and/or 7 of the aglycone. The superposition of one or two of the aromatic cycles of the substituents over the central chromophore, adopting a 'sandwich' type conformation (Fig. 1), precludes the nucleophilic attack of water molecules to positions 2 or 4 of the aglycone (Kurtin & Song, 1968; Amić & Trinajstić, 1991; Davidović-Amić et al., 1994). This fact allows the existence of the coloured flavylium cation or quinonoidal base forms over a larger range of pH (Fig. 2) than that observed with simple mono- or diglucosides, where the colourless hemiacetal and chalcone forms are prevalent in the pH range 2–6.

Eqns. (1) and (2) account, in a general form, for the transformations undergone by anthocyanins in mildly acidic aqueous solutions:

$$AH_2^+ \overset{K_a}{\rightleftharpoons} AH + H^+ \quad (1)$$

$$AH_2^+ + H_2O \overset{K_{h'}}{\rightleftharpoons} (BH_2 + C_E + C_Z) + H^+ \quad (2)$$

In Eqn. (2), the equilibrium is written as a whole, for the sake of simplicity, since the ring opening of form BH_2 to form the *E*-chalcone is a very fast step. The *Z*-form of the chalcone, formed only in minor amounts (Santos et al., 1993) will hereafter be neglected.

The value of UV–visible absorbance read at the visible maximum is a function of only the flavylium cation (AH_2^+) and quinonoidal base (AH) forms existing in solution at a given pH. By combining Eqns. (1) and (2) with the Beer–Lambert law, one can obtain Eqn. (3) (Dangles et al., 1993b), where *A* represents the absorbance of the anthocyanin solution at a given pH,

Table 1
Maximum visible wavelength (λ_{max}) and molar extinction coefficient (ε) for the flavylium cation form (AH_2^+) of the ten anthocyanins[a]

	Pigment									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
λ_{max} (nm)	526	524	502	490	534	534	534	534	532	532
$\varepsilon_{AH_2^+}$ (mol^{-1} dm^3 cm^{-1})	21800	19000	30200	8600	15300	17300	17000	17600	23800	21100

[a] pH = 0.8.

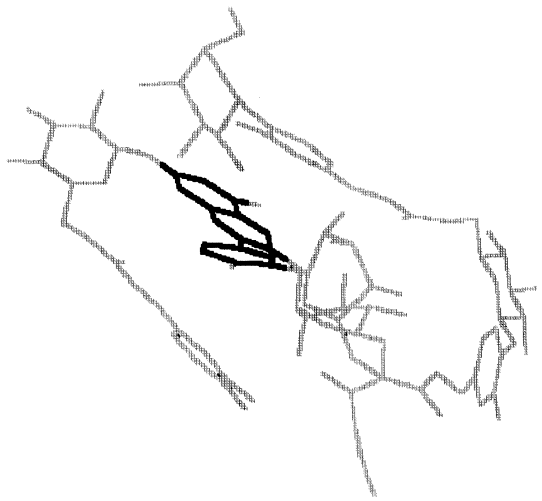


Fig. 1. Structure of **8** optimised by MM+. The central chromophore is represented in black, while substitutes are drawn in grey.

$$\frac{A_0}{A_0 - A} = \frac{K_h' + K_a}{K_h' + K_a(1 - r_A)} + \frac{[H^+]}{K_h' + K_a(1 - r_A)} \quad (3)$$

A_0 stands for the absorption due only to the flavylium cation (obtained from a solution at pH < 1) and r_A corresponds to the ratio $\varepsilon_{AH}/\varepsilon_{AH_2^+}$.

Moreover, if the hydration equilibrium constant K_h (Eqn. (4))

$$AH_2^+ + H_2O \overset{K_h}{\rightleftharpoons} (BH_2 + C_E) + H^+ \quad (4)$$

is expressed as k_1/k_2, where k_1 stands for the hydration rate constant and k_2 is the rate constant for the reverse process, it is possible to write Eqn. (5) (Dangles et al., 1993b):

$$\frac{K_a + K_h + [H^+]}{k} = \frac{1}{k_2} + \frac{K_a}{[H^+]k_2} \quad (5)$$

where k is the first-order apparent rate constant of the hydration reaction delivered by the spectrophotometer.

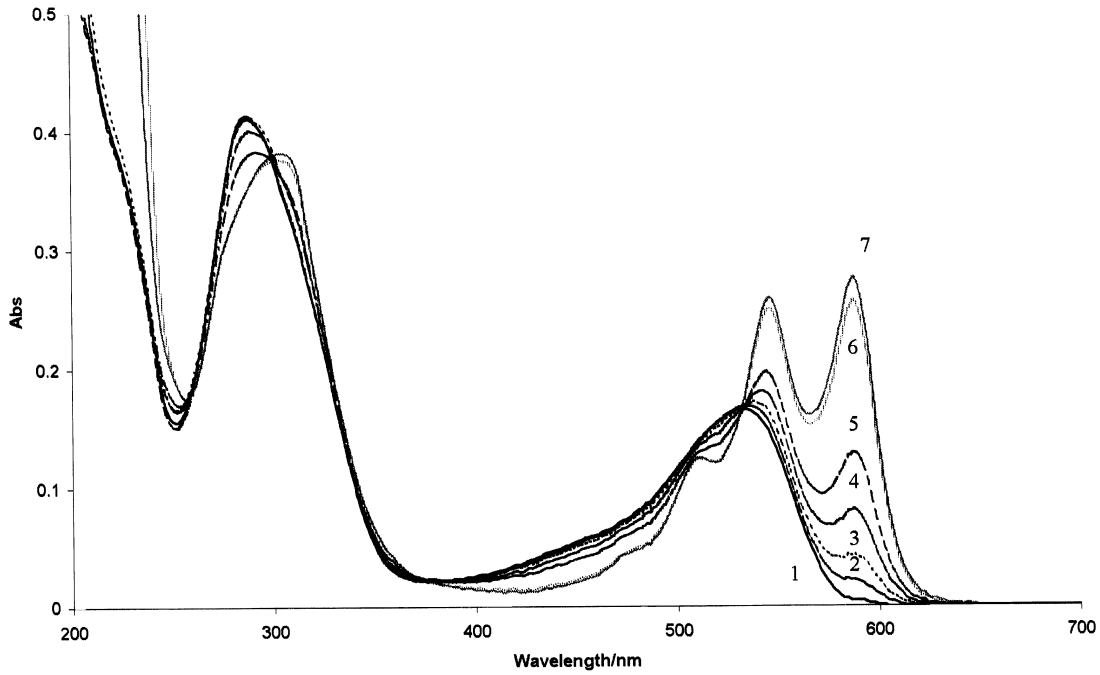


Fig. 2. UV–Visible absorption spectra of pigment **6** as a function of pH. 1: pH = 1.0; 2: pH = 1.8; 3: pH = 2.2; 4: pH = 2.5; 5: pH = 3.0; 6: pH = 4.2; 7: pH = 4.8.

By combining the two expressions it is possible to easily obtain K_a and K_h values for most anthocyanins, through simple spectrophotometric measures.

In the present study, however we were confronted with a group of anthocyanins for which this formulation was not befitting. The reason is that molecules **1**, **2** and **5**–**10** showed no signs of hydration in the usual mildly acidic pH range. Therefore, instead of proceeding according to the above procedure, described in detail elsewhere (Dangles et al., 1993b), we had to use an alternative means to obtain the deprotonation constant without performing kinetic analysis with the referred eight molecules. This may be achieved either through a quasi-static experiment or by means of a thermodynamic equilibrium study. In the first case (Elhabiri, 1997), a pH 2.0–2.5 aqueous solution of the anthocyanin is subject to a pH jump (in a similar manner to what is made in kinetic measurements (Dangles et al., 1993b)), by addition of NaOH or buffer solutions, in a 1:1 proportion, in order to attain a final pH of 3.5–6.0. The value at the maximum of flavylium absorption, measured a few seconds after the jump, plotted against the final pH, allows the calculation of K_a, by curve-fitting with Eqn (6),

$$A = \frac{A_0 + A_1 K_a 10^{pH}}{1 + K_a 10^{pH}}, \quad (6)$$

where K_h is neglected due to the non-existence of hydration. In Eqn. (6), A_1 represents the absorption due to AH, taken at its maximum wavelength.

The alternative method consists of a reasoning similar to the one that led to Eqn. (3), but where K_h' is taken as zero and A_1 stands for the absorption due only to the quinonoidal bases (obtained from a solution at pH ~ 6, where this is the only visible light absorbing form in solution). This leads to Eqn (7),

$$A = \frac{A_1 K_a}{K_a + [H^+]}, \quad (7)$$

which successfully fitted the data obtained during the present study (Table 2).

From analysis of Table 2, it can be stated that, contrary to what is known with all anthocyanins studied until now, pigments **1**, **2** and **5**–**10** present no signs of hydration in mildly acidic aqueous solutions. This fact may be related to the particular structure presented by these molecules, i.e. glycosyl–acyl 'side chains' attached to both positions 3′ and 7 of the chromophore. This led us to propose that this particular placement of the acylated chains favours a better overlap and stronger interaction with the π-system of the central chromophore (Fig. 1), than what is observed with other acylated anthocyanins. In these cases (Dangles et al., 1993a,b; Figueiredo et al., 1996a,b; Elhabiri, 1997) the pigments never present acylated substitutes in the B-ring. The above assessment is supported by molecular calculations performed with the HyperChem program. The data collected this way suggest, as a minimum energy conformation, a 'sandwich' type structure for these eight anthocyanins with the 3′-chain folded 'over' and the 7-chain folded 'under' the chromophore (Fig. 1). This particular structure allows more efficient protection against hydration than the one produced by anthocyanins whose side chains are attached to positions 3 and 5 (Dangles et al., 1993a,b; Figueiredo et al., 1996a,b; Elhabiri, 1997). In the latter cases the folding of the substitutes does not seem to accommodate the same coplanarity of the different π-electron moieties.

In the two pigments that show hydration (**3** and **4**) it is clear that the one less substituted, **4**, is the most prone to hydration, confirming once more the theory that acyl residues may fold over the chromophore protecting its positions 2 and 4 against the water attack. Moreover, if comparing **4** with pelargonidin 3-glucoside (Sondheimer, 1953; Ioncheva & Tanchev, 1972) and pelargonidin 3,5-diglucoside (Timberlake & Bridle, 1967; Abe, Sakaino, Kakinuma, & Kakisawa, 1977) a rather low pK_h' (1.65) for the new pigment is evident. The values presented by these authors for the related 3-monoglucoside and 3,5-diglucoside of pelargonidin are, respectively, around 2.8 and 2.1. If the value found for pigment **4** once more confirms the tendency of diglucosides to easily undergo hydration when compared to monoglucosides, it is nevertheless an unfamiliar comportment with more common diglucosides of anthocyanins. In order to find a reason to such behaviour, comparative AM1 (Dewar, Zoebisch,

Table 2
Hydration and deprotonation reaction values, obtained from the thermodynamic and kinetic data, for the ten anthocyanins ($T = 25°C$)

	Pigment									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
pK_h'	[a]	[a]	2.83 ± 0.03	1.65 ± 0.02	[a]	[a]	[a]	[a]	[a]	[a]
pK_a	2.85 ± 0.05	2.90 ± 0.08	4.78 ± 0.07	[b]	2.98 ± 0.04	2.93 ± 0.02	3.10 ± 0.04	2.89 ± 0.05	3.28 ± 0.04	3.32 ± 0.04

[a] No hydration was detected.
[b] No AH was formed.

Healy, & Stewart, 1985) calculations on pelargonidin 3,5-diglucoside and **4** were performed. As a result, an increase in the electrophilic character on C-2 of pigment **4** is obtained that may justify, at least partially, this reduction in pK_h'. The charge variance from 0.267 (pelargonidin 3,5-diglucoside) to 0.282 (**4**) may be explained by a difference on the *exo*-anomeric effect (Lemieux & Koto, 1974; Dangles & Elhajji, 1994; David, 1995) produced by replacing an OH group by a β-D-glucopyranosyloxy group in positions C-5 or C-7 of the chromophore. This effect is caused by a delocalization of the electron-density of the *exo*-anomeric oxygen towards the sugar ring, resulting in a reduction of its electron-donating ability towards the pyrylium. The higher electrophilic character of carbon 2 in pigment **4** will thus lead to an increased amount of hydrated forms at equilibrium, hence a lower pK_h' value.

Another interesting feature reported on Table 2 is the rather low values obtained for the deprotonation constants (pK_a) in a majority of the anthocyanins studied (**1**, **2** and **5**–**10**). As a matter-of-fact, values this low, or even lower, were only found by some of us in a small group of four other acylated anthocyanins (Figueiredo et al., 1996a). But unlike those molecules that have free OH groups in both C-4′ and C-7 positions of the chromophore, the present set does possess only the C-4′ and C-5 OH groups as candidates for the deprotonation. Knowing that, in a model anthocyanin (4′,3,5,7-tetrahydroxyflavylium), position 7 is the most prone to deprotonation and also that the former group of pigments (Figueiredo et al., 1996a) has a special structural arrangement that allows an assisted deprotonation through the intervention of an H bond between the 7-OH and a malonyl group on the C-5 acylated chain, a less favourable environment for the stabilization of deprotonated forms at lower pH will be expected with the present set of molecules. Of the two free OH groups on the six molecules, C-4′ will be, according to computed values for the above cited model anthocyanin, the first to lose its proton. Thus, in the ensemble of molecules concerned in this paper it should also be the loss of a proton on the 4′-OH group that is mainly responsible for the formation of quinonoidal forms (AH) in solution. An explanation for the lower values may be found, once again, by the existence of a H bond formed between the C-4′ OH and an OH group of the pyranose ring attached to C-3′. Fig. 3 shows pigment **5** with such a H bond as computed through the use of the MM+ force field (Allinger, 1977). The other seven molecules concerned showed similar results. The formation of these H bonds would increase the charge density in C-4′ and thus facilitate the deprotonation, resulting in low pK_a values. The deprotonation constants here reported compare well with the one found for the *Zebrina pendula* anthocyanin ($pK_a = 3.50$) (Brouillard, 1981), also a 3,7,3′-triacylglycosylated pigment, but in this case with a delphinidin-like structure.

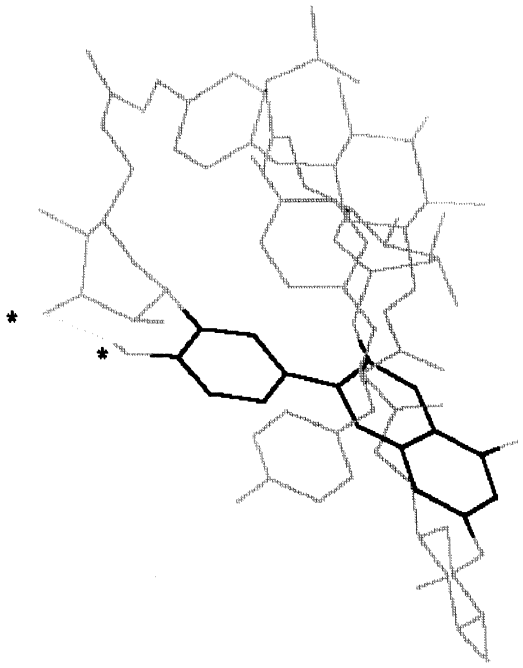


Fig. 3. Structure of **5**, as optimized by MM+, showing the H bond between the C-7 OH group and the vicinal pyranose.

Another interesting feature of this set of anthocyanins is the existence of a malonyl group as terminal function on the C-3 chain in some of the molecules (**1**, **3**, and **5**–**9**). Since pigment **2** is a structural relative of **1** and **10** is demalonyl anthocyanin **9**, it is possible to ascertain a function to this particular substitute. In comparing the behaviour of malonylated anthocyanins **1** and **9** with **2** and **10** at $pH \leq 3$–4 no differences were observed, though at higher pH a remarkable capacity of medium acidification by **2** and **10** was registered, i.e. higher amounts of concentrated NaOH were necessary to attain the same pH as with a solution of **1** or **9** at the same initial concentration. Since the first pK_a of malonic acid is 2.83 (Allinger et al., 1987) we propose that it is the deprotonation of the malonyl group that provides protection against alkalinization of the medium and consequent loss of red–blue colour.

The results reported here further prove the existence of intramolecular associations between the planar pyrylium ring and the acylated chains existing in several natural anthocyanins. These interactions seem to have the purpose of preserving the red to blue colours observed in most of the flowers from where such pigments are extracted.

Moreover, a very important role for the existence of acylated chains on C-3′ and C-7 is presented here for the first time. This special arrangement, specific to the *Orchidaceae* family (the two only known exceptions are *Zebrina pendula* (Brouillard, 1981) and *Senecio cruentus* (Yoshitama, Hayashi, Abe, & Kakisawa, 1975)) seems to completely protect the anthocyanin from hydration

throughout all of the acidic to neutral pH range, hence the expression of flower colour.

We also show a colour stabilization role ascribed to the free malonyl group on the C-3 chain. This colour stabilization results not from an intramolecular complexation but from an increase in medium acidity attributable to this function.

3. Experimental

3.1. Materials

The eight natural anthocyanins were isolated according to published procedures (Lu et al., 1992; Saito et al., 1993). Their purity was verified by ^{1}H NMR spectroscopy and the results are **1**: 95–97%; **2**: 90–95%; **3**: 80–85%; **4**: 95–99%; **5**: 85–90%; **6**: 90–95%; **7**: 85–90%; **8**: 90–95%; **9**: +95% and **10**: +95%. All other reagents used throughout the work were of analytical grade.

3.2. Absorption spectra

Spectra were recorded with a diode-array spectrophotometer fitted with a quartz cell ($d=1$ cm) equipped with a stirring magnet. A constant temp. of 25°C (± 0.1°C) was obtained by use of a water-thermostated bath. The temperature was measured with a thermocouple.

3.3. Thermodynamic measurements

Stock solns of ca. 10^{-4} M of the ten anthocyanins were prepared in 0.1 M HCl and left to equilibrate, in the dark, for at least 2 h. Then, for each pigment, twelve solns were prepared by 1:10 dilutions of the stock solns with different volumes of a NaOH 0.1 M soln and H_2O so that the final pH covered a range of 1.0 to 3.5. For pH values closer to neutrality, acetic acid/sodium acetate buffers were used. After equilibration in the dark, the UV–Vis spectra of these solns were recorded. The values of the global equilibrium constants ($K=K_h'+K_a$) are gained from measuring the relative hyperchromic shift at the visible absorption maxima of the flavylium cation or the quinonoidal base (depending on the adopted strategy, see Section 2), as a function of pH.

3.4. Kinetic measurements

1 ml of an equilibrated aq. soln of **3**, at the pH values used for the thermodynamic measurements, was magnetically stirred in the spectrophotometer cell. To these solns 1 ml of acetate buffer solns, ranging in pH from 3.5 to 6.5, was quickly added and the visible absorbance at 502 nm (the maximum of the flavylium band, see Table 1) was immediately recorded every second over 120 s, to guarantee that the hydration equilibrium was attained. The final pH was then measured and ranged from 3.4 to 6.1. The spectrophotometer software automatically computes the first-order apparent rate constant of the hydration reaction (k).

3.5. Computing

Curve-fitting of the data according to Eqns. (3), (5), (6) and (7) was accomplished with the help of Kaleidagraph software on a Macintosh computer. In vacuo molecular mechanics (MM+) and semi-empirical (AM1) quantum mechanical calculations were performed on a PC using the HyperChem program.

References

Abe, K., Sakaino, Y., Kakinuma, J., & Kakisawa, H. (1997). *Nippon Kagaku Kaishi, 8*, 1197.

Allinger, N. L. (1977). *J. Am. Chem. Soc., 99*, 8127.

Allinger, N. L., Cava, M. P., de Jongh, D. C., Johnson, C. R., Lebel, N. A., & Stevens, C. L. (1987). In *Chimie organique* (p. 196). Paris: McGraw-Hill.

Amić, D., & Trinajstić, N. (1991). *J. Chem. Soc. Perkin Trans., 2*, 891.

Brouillard, R. (1981). *Phytochemistry, 20*, 143.

Dangles, O., Elhajji, H. (1994). *Helv. Chem. Acta, 77*, 1595.

Dangles, O., Saito, N., Brouillard, R. (1993a). *Phytochemistry, 34*, 119.

Dangles, O., Saito, N., Brouillard, R. (1993b). *J. Am. Chem. Soc., 115*, 3125.

David, S. (1995). In *Chimie moléculaire et supramoléculaire des sucres* (pp. 143–46). Paris: CNRS Editions.

Davidović-Amić, D., Amić, D., Trinajstić, N. (1994). *Croat. Chem. Acta, 67*, 163.

Dewar, M. J. S., Zoebisch, E. G., Healy, E. F., & Stewart, J. J. P. (1985). *J. Am. Chem. Soc., 107*, 3902.

Elhabiri, M. (1997). Ph.D. thesis, Université Louis Pasteur de Strasbourg, France.

Elhabiri, M., Figueiredo, P., Fougerousse, A., & Brouillard, R. (1995). *Tetrahedron Lett., 36*, 4611.

Figueiredo, P., Elhabiri, M., Saito, N., Brouillard, R. (1996). *J. Am. Chem. Soc., 118*, 4788.

Figueiredo, P., Elhabiri, M., Toki, K., Saito, N., Dangles, O., Brouillard, R. (1996). *Phytochemistry, 41*, 301.

Ioncheva, N., & Tanchev, S. (1972). *Nauchni Tr. Vissh. Inst. Khranit. Vkusova Prom-st. Plovdiv, 20*, 135.

Kurtin, W. E., & Song, P.-S. (1968). *Tetrahedron, 24*, 2255.

Lemieux, R. U., & Koto, S. (1974). *Tetrahedron, 30*, 1933.

Lu, T. S., Saito, N., Yokoi, M., Shigihara, A., & Honda, T. (1992). *Phytochemistry, 31*, 289.

Saito, N., & Harborne, J.B. (1992). *Phytochemistry, 31*, 3009.

Saito, N., Ku, M., Tatsuzawa, F., Lu, T. S., Yokoi, M., Shigihara, A., & Honda, T. (1995). *Phytochemistry, 40*, 1523.

Saito, N., Lu, T. S., Yokoi, M., Shigihara, A., & Honda, T. (1993). *Phytochemistry, 33*, 245.

Saito, N., Toki, K., Uesato, K., Shigihara, A., & Honda, T. (1994). *Phytochemistry, 37*, 245.

Santos, H., Turner, D. L., Lima, J. C., Figueiredo, P., Pina, F. S., & Maçanita, A.L. (1993). *Phytochemistry, 33*, 1227.

Sondheimer, E. (1953). *J. Am. Chem. Soc., 75*, 1507.

Tatsuzawa, F., Saito, N., Seki, H., Hara, R., Yokoi, M., & Honda, T. (1997). *Phytochemistry, 45*, 173.

Tatsuzawa, F., Saito, N., Yokoi, M., Shigihara, A., & Honda, T. (1994). *Phytochemistry, 37*, 1179.

Tatsuzawa, F., Saito, N., Yokoi, M., Shigihara, A., & Honda, T. (1996). *Phytochemistry, 41*, 635.

Timberlake, C. F., & Bridle, P. (1967). *J. Sci. Food Agric., 18*, 473.

Toki, K., Saito, N., Imura, K., Suzuki, T., & Honda, T. (1994). *Phytochemistry, 36*, 1181.

Toki, K., Saito, N., Kawano, K., Lu, T. S., Shigihara, A., & Honda, T. (1994). *Phytochemistry, 36*, 609.

Toki, K., Saito, N., Kuwano, H., Shigihara, A., & Honda, T. (1995). *Phytochemistry, 38*, 1509.

Yoshitama, K., Hayashi, K., Abe, K., & Kakisawa, H. (1975). *Bot. Mag., 88*, 213.