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Influence of *trans-cis* isomerisation of coumaric acid substituents on colour variance and stabilisation in anthocyanins

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Abstract

The recently isolated pigments from *Petunia integrifolia* and *Triteleia bridgesii* present a distinct feature that sheds new light on the understanding of intramolecular copigmentation of anthocyanins. These are among the infrequent anthocyanins that naturally present a coumaric acid substituent in both *cis* and *trans* forms. As a consequence, the two isomers demonstrate substantial variations of their thermodynamic and kinetic constants and also colour properties. A possible explanation for these characteristics is presented, making use of molecular modelling and taking into account the three-dimensional structures of the pigments. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Petunia integrifolia*; Solanaceae; *Triteleia bridgesii*; Liliaceae; Acylated anthocyanins; Coumaric acid; *trans-cis* Isomerisation; Intramolecular copigmentation; Molecular modelling

1. Introduction

Progress on extraction and purification techniques has afforded details of several forms of acylated anthocyanins (Toki et al., 1994a,b; Saito et al., 1995; Tatsuzawa et al., 1997). These recently discovered pigments possess, as a common trait, one or more aromatic acyl residues. Depending on the length of the "chains" that link them to the central chromophore, they may fold over the 2-benzopyrylium system and strongly influence its properties as a colourant (Brouillard and Dangles, 1994). A pattern is common to most of these recently discovered pigments: the hydroxycinnamic acid units occur in a *trans* configuration (Saito and Harborne, 1992; Strack and Wray, 1994). To our knowledge, only eight papers report the isolation and characterization of hydroxycinnamic acid residues present in a *cis* configuration, one on the Labiatae (Yoshida et al., 1990), one on the Commelinaceae (Kondo et al., 1991), three on Liliaceae (Hosokawa et al., 1995a,b; Toki et al., 1998) and three on the Solanaceae (Ando et al., 1999a,b; Slimestad et al., 1999) flowers. In all of the above-cited works, the *trans* isomer always predominates in vivo.

The present paper reports, for the first time, on the colour stabilisation and intensity changes brought about by the isomerisation of the coumaroyl residue. The results concern two pairs of acylated anthocyanins (Scheme 1): malvidin 3-*O*-[6-*O*-(4-*O*-(*trans-p*-coumaroyl) -α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-(β-D-glucopyranoside) (**1**) and malvidin 3-*O*-[6-*O*-(4-*O*-(*cis-p*-coumaroyl)-α-L-rhamnopyranosyl)-β-D-glucopyranoside]- 5-*O*-(β-D-glucopyranoside) (**2**) isolated from the red-purple flowers of *Petunia integrifolia* (Ando et al., 1999a,b) and delphinidin 3-*O*-[6-*O*-(*trans-p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside] (**3**) and

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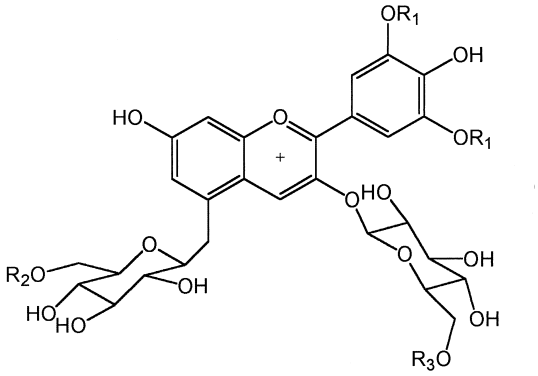

1. $R_1 = CH_3$, $R_2 = H$, R_3 = *trans*-4-*O*-(rhamnosyl)-*p*-coumaric acid
2. $R_1 = CH_3$, $R_2 = H$, R_3 = *cis*-4-*O*-(rhamnosyl)-*p*-coumaric acid
3. $R_1 = H$, R_2 = malonic acid, R_3 = *trans*-*p*-coumaric acid
4. $R_1 = H$, R_2 = malonic acid, R_3 = *cis*-*p*-coumaric acid

Scheme 1.

delphinidin 3-*O*-[6-*O*-(*cis*-*p*-coumaroyl)-β-D-glucopyranoside]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside] (**4**) extracted from the blue-purple flowers of *Triteleia bridgeseii* (Toki et al., 1998).

The thermodynamic and kinetic results gathered from the two pairs of natural pigments allowed the interpretation of intramolecular copigmentation from a new perspective, even supporting the hypothesis that such *cis*-hydroxycinnamic isomers may be more prolific in nature than has previously been thought, due to their aptitude to positively influence flower colour.

2. Results and discussion

Flower colour is, to a large extent, the consequence of the expression of the colouring properties of anthocyanins, the pigments residing in the vacuoles of plant cells. These pigments are, according to their chemical structure, capable of displaying an immense variety of tints and hues. In particular, acylated anthocyanins were shown to exhibit some of the more astonishing and stable colour capabilities ever found in plant pigments (Brouillard, 1981; Dangles et al., 1993a,b; Figueiredo et al., 1996a,b,1999). In this paper, we look at the changes brought about by the isomerisation around a coumaroyl residue on two sets of acylated anthocyanins (Scheme 1). In the vast majority of acylated anthocyanins extracted from plant material, the hydroxycinnamic acid units commonly appear as *trans* isomers, *cis* isomers being very rare. The two pairs of isomers presented in this work do not undergo isomerisation in aqueous solution, due to the high activation energy needed to convert between *trans* and *cis* forms. However, conversion between the two isomers was shown to take place in vitro, both with artificial (366 nm) and natural (sun) irradiation, for another set of structurally comparable anthocyanins (Yoshida et al., 1990).

The simple change from a *trans* to a *cis* configuration of the terminal coumaric acid on the molecules here studied produced some appreciable modifications both at the level of colour expression and colour stability. Table 1 reports those properties. Although the maximum absorbance wavelength in the visible, for the flavylium cation forms, does not greatly change among the four pigments concerned, their molar extinction coefficients do so (Fig. 1). In fact, *cis* isomers exhibit ε values about 1.5 times greater than *trans* ones, in both pairs of molecules. This increase in colour intensity may be due to differences in co-planarity between the central chromophore and the acyl residue. Computed structures for the two isomers **3** and **4** (Fig. 2), performed both in vacuo, in the AM1 (Dewar et al., 1985) parametrization, and in a simulated water container, with the MM+ (Allinger, 1977) force field, suggest that **4** has the coumaroyl residue almost parallel to the central anthocyanidin moiety, while **3** presents a quasi-perpendicular conformation.

The computed structures for anthocyanins **1**–**4** showed their ability to form folded structures (intramolecular copigmentation) that protect the pigments against the colour damaging hydration reaction. This is particularly true with the *cis* isomers (Fig. 2). The distance of the acyl residue in the *cis* isomer **4** to anthocyanidin moiety is smaller than in the *trans* isomer **3** (Fig. 2).

The hydration reaction is the primary cause of a marked colour loss in non-acylated anthocyanins, due to the formation of colourless equilibrium forms, in aqueous solution. This property is also affected by the change in configuration of the coumaroyl moiety from *trans* to *cis*. Calculated pK'_h values (Table 1) reveal that the *cis* isomers are less prone to undergo hydration than their *trans* counterparts. A closer look at the values obtained for the kinetic constants k_1 (hydration) and k_2 (reverse reaction) helps to understand this behaviour. While k_1 is of the same order of magnitude for both isomers, k_2 is always greater for the *cis* forms. This means that although

Table 1
Spectral characteristics (UV–vis.; pH = 1.0), hydration and deprotonation constant values for pigments **1**–**4**

	Pigment			
	1	**2**	**3**	**4**
λ_{max} (nm)	532	534	528	532
$\varepsilon_{AH2}+$ (mol^{-1} dm^3 cm^{-1})	8500	12550	20900	35000
pK'_h	1.75±0.03	2.03±0.02	2.01±0.03	2.76±0.04
k_1 (min^{-1})	16.1±0.1	12.5±0.1	7.5±0.1	4.0±0.1
k_2 (mol^{-1} dm^3 cm^{-1})	914±7	1325±5	778±3	2300±10
pK_a	3.39±0.02	3.55±0.01	4.43±0.02	4.48±0.03

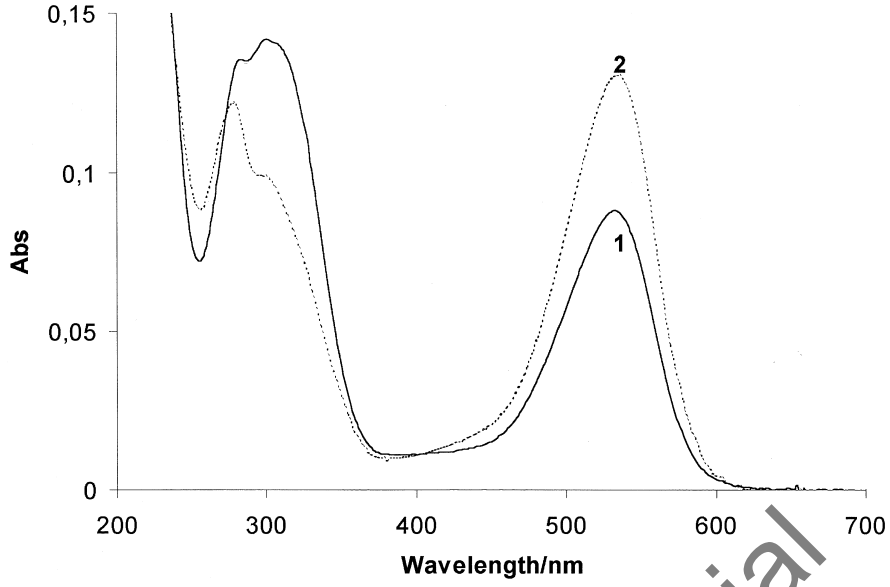

Fig. 1. Visible absorption spectra of pigments **1** (—) and **2** (- - -). pH = 1.0; conc. = 1.04×10^{-5} M.

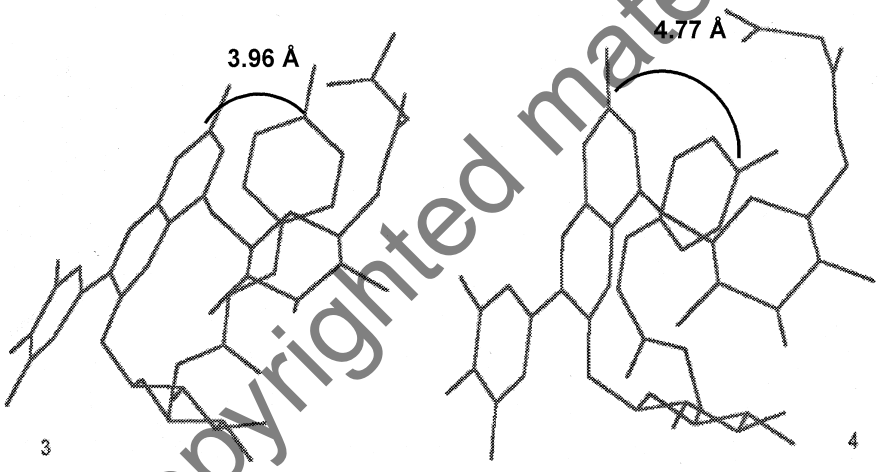

Fig. 2. Conformations of **3** and **4**, optimised by MM+. The arrows point to the coumaroyl moieties.

both forms have the same susceptibility to water addition to the charge deficient positions (C_2 and C_4), the *cis* isomer has a faster back reaction to reform the coloured flavylium cation. This may be due to a more favourable spatial arrangement of the molecules, conferred by *cis* isomerisation around the coumaroyl moiety. The computed (MM+) differences in formation energies between BH_2 (colourless hemiacetal) and AH_2^+ (coloured flavylium) indicate a more pronounced gap for anthocyanin **3** than for pigment **4**, which may account for the faster back reaction of **4**.

The present set of acylated anthocyanins presents pK_a values, for the proton transfer reaction transforming the coloured flavylium form in the also coloured quinonoidal base, within the characteristic range previously estimated for similar molecules (Dangles et al., 1993a,b; Figueiredo et al., 1996a, 1999). The influence of the *trans-cis* isomerisation seems not to be relevant in this particular case (Table 1). There is, however, a small variation in both pairs of anthocyanins, with *trans* isomers exhibiting slightly lower values in pK_a. The AM1 computed electronic charge densities for the atoms on C-7 and C-4' may account for such a difference. In all four molecules, the charge is more positive for C-7 than for C-4', in accordance with previously reported results (Figueiredo et al., 1999), indicating that the hydroxyl in position 7 of the aglycone is always the first to undergo proton transfer. Furthermore, in the present work, *trans* isomers yielded more positive values for the computed C-7 charges than their *cis* counterparts. However, no data clearly indicate that this feature is a consequence of structural variations caused by isomerisation about the coumaroyl moieties of the anthocyanins.

The results here reported once more stress the importance of the nature of substituents on colour stability of the anthocyanins. It is demonstrated, for the first time,

that one of the rarest features found in these pigments play an important role in preventing colour loss by nucleophilic water attack. In effect, the change from the common *trans* coumaroyl substituents to the unusual *cis* isomers confers an additional protection against hydration, reflected on an increase in pK'_h values. The more co-planar arrangement allowed by *cis* isomers is postulated as the rationale supporting the enhanced colour stability.

Knowing that *trans-cis* photoisomerisation may take place in vivo (Yoshida et al., 1990), we propose this mechanism as an additional means for preservation of flower colour, but one should bear in mind that the conversion between the two isomers in vivo is certainly rare due to low amounts of the *cis* configuration.

3. Experimental

3.1. Materials

Pigments were extracted according to published procedures and had their purity (always $\geqslant 98\%$) checked by ^{1}H NMR spectroscopy (Toki et al., 1998; Ando et al., 1999a). 3-*O*-[6-*O*-(4-*O*-(*trans*-*p*-coumaryl)-α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-5-*O*-(β-D-glucopyranosyl) malvidin chloride (**1**) and 3-*O*-[6-*O*-(4-*O*-(*cis*-*p*-coumaryl)-α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-5-*O*-(β-D-glucopyranosyl) malvidin chloride (**2**) were isolated from the red-purple flowers of *Petunia integrifolia* and 3-*O*-[6-*O*-(*trans*-*p*-coumaroyl)-β-D-glucopyranosyl]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranosyl] delphinidin chloride (**3**) and 3-*O*-[6-*O*-(*cis*-*p*-coumaryl)-β-D-glucopyranosyl]-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranosyl] delphinidin chloride (**4**) were obtained from the blue-purple flowers of *Triteleia bridgeseii* and used without further purification. All other chemicals used were of analytical grade.

3.2. Absorption spectra

Absorption spectra were recorded with a Hewlett Packard diode-array spectrophotometer fitted with a quartz cell ($d = 1$ cm) equipped with a stirring magnet. A constant temperature of 25 ± 0.1°C, measured with a Comark thermocouple, was maintained in the spectrometer sample cell by use of a Bioblock water-thermostated external bath. Water used in sample preparations was bidistilled.

3.3. Data analysis

UV–visible absorption data were recorded on a Pentium P200 PC using the Hewlett Packard UV–visible Chemstation Programme. Mathematical treatments applied to the data were carried out with the Microsoft Excel 98 programme running on a PowerMacintosh G3/300 machine.

3.4. Thermodynamic measurements

Stock solns ranging from 6.6×10^{-5} M to 1.1×10^{-4} M of the 4 pigments were prepared in 0.1 M HCl and left to equilibrate in the dark for about 3 h. Then, for each pigment, 16 solns were prepared by 1:10 dilutions of the stock solns with different volumes of NaOH 0.1 M soln and H_2O so that the final pH covered a range of 1–3.5. 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, for all the pigments studied, as a function of pH.

3.5. Kinetic measurements

One milliliter of each equilibrated aq. soln of anthocyanin, at different pH values (from 1.0 to 3.0, depending on the pigment) was magnetically stirred in the spectrophotometer cell. The concentrations of pigments **1**–**4** were respectively 8.54×10^{-6} M, 6.61×10^{-6} M, 1.07×10^{-5} M and 7.82×10^{-6} M. To these solns, 1 ml of acetate buffer solns, ranging in pH from 3.0 to 5.0, was quickly added and the visible absorbance at ca. 530 nm (near the visible absorption maxima for all the pigments) was immediately recorded every second over 120 s to guarantee that the hydration equilibrium is attained. The final pH was then measured and ranged from 2.4 to 4.6. The spectrophotometer software automatically computes the first order apparent rate constant of the hydration reaction (k).

3.6. Molecular modelling

Molecular mechanics [MM+ (Allinger, 1977)], inside a simulated periodic box of ca. 350 water molecules and semi-empirical quantum mechanical calculations [AM1 (Dewar et al., 1985)] were performed on a Pentium P350 PC using the Hyperchem programme (version 4, Hypercube, Inc., Ont., Canada).

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