

## Photochemistry of anthocyanins and their biological role in plant tissues\*

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**Abstract:** Anthocyanins, the major red, purple, and blue pigments of plants, absorb visible as well as UV radiation and are effective antioxidants and scavengers of active oxygen species. In plant leaves, one of the functional roles proposed for anthocyanins is protection of the photosynthetic apparatus from the effects of excess incident visible or UV-B radiation and photooxidative stress. In essence, a photoprotective role requires that the excited singlet states of both complexed and uncomplexed anthocyanins deactivate back to the ground state so quickly that intersystem crossing, photoreaction, and diffusion-controlled quenching processes cannot compete. Studies of the photochemical properties of synthetic analogs of anthocyanins and of several naturally occurring anthocyanins show that this is indeed the case, uncomplexed anthocyanins decaying back to the ground state via fast (subnanosecond) excited-state proton transfer (ESPT) and anthocyanin-copigment complexes by fast (sub-picosecond) charge-transfer-mediated internal conversion.

**Keywords:** anthocyanins; charge transfer; copigmentation; fluorescence; proton transfer.

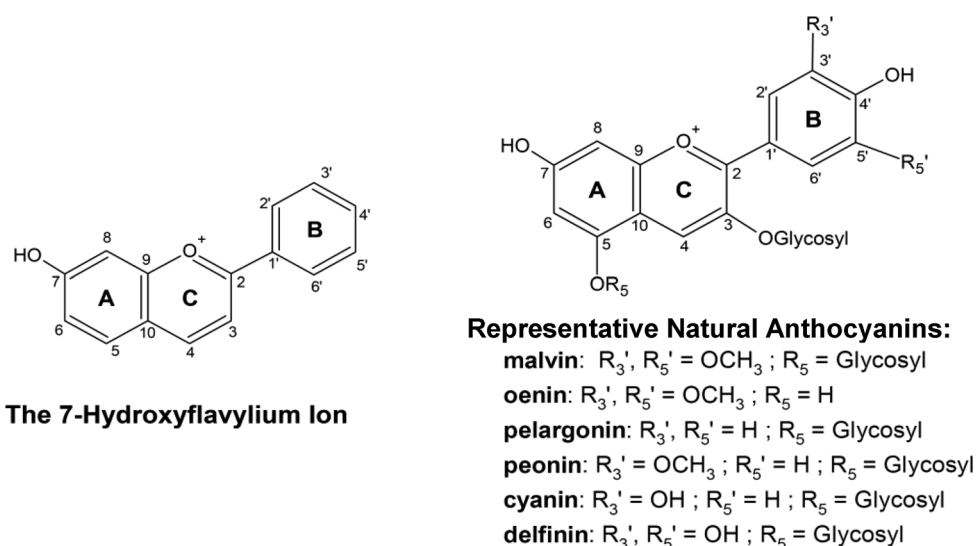
### INTRODUCTION

Anthocyanins (from the Greek words for flower, *anthos*, and blue, *kyanos*) are the principal pigments responsible for the red, blue, and purple colors of terrestrial plants [1,2]. Anthocyanins are omnipresent in our diet, have little or no known toxicity, and are usually quite water-soluble, making them particularly attractive as natural substitutes for synthetic pigments and antioxidants [1,2].

The basic chromophore of anthocyanins, the 7-hydroxyflavylium ion, and the structures of some of the more common naturally occurring anthocyanins are shown in Fig. 1. Naturally occurring anthocyanins typically have hydroxyl substituents at positions 3 (always glycosylated, providing thermal stability) and 5 (occasionally glycosylated), and the 2-phenyl- or B-ring has one or more hydroxy or methoxy substituents [1,2]. Natural anthocyanins in which the 7-hydroxy group is glycosylated or replaced by a methoxy group are quite rare. Although many hundreds of different anthocyanin structures have been reported in the literature [3,4], these differ primarily in the substitution pattern in the B-ring

\*Paper based on a presentation at the XXII<sup>nd</sup> IUPAC Symposium on Photochemistry, 28 July–1 August 2008, Gothenburg, Sweden. Other presentations are published in this issue, pp. 1615–1705.

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**Fig. 1** Structures of the basic anthocyanin chromophoric unit, the 7-hydroxyflavylium cation, and of several typical naturally occurring anthocyanins.

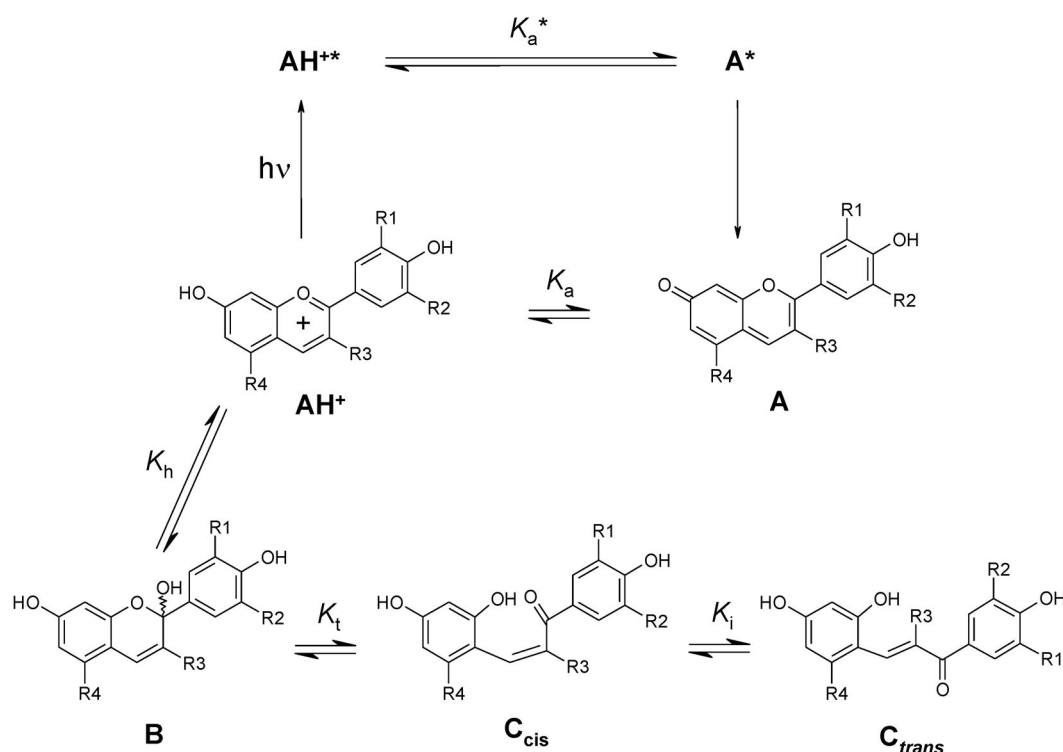
and the nature of the sugars and other molecules that make up the glycosylated portions. The colors of natural and synthetic anthocyanins range from yellow to purple (every color except green has been observed) and depend on the substituents present in the B-ring, the local pH, the state of aggregation of the anthocyanins, complexation by organic molecules, or, particularly in the case of blue colors, complexation by metal cations as well [5–8].

For more than a century, biologists, biochemists, and plant physiologists have hypothesized as to the possible biological roles of anthocyanins in plants [9–12]. In the case of flowers, the colors imparted by anthocyanins presumably serve to attract insects and other pollinators. In fruit, they are often an indicator of ripeness and provide contrast against an otherwise green background, attracting birds and other animals that feed on the fruit and eventually disperse the seeds. In vegetative tissues such as leaves, however, the potential biological significance of the presence of anthocyanins has been the subject of ongoing discussion [9–12], and perhaps no single explanation for all of the possible roles of anthocyanins will prove to be sufficient [10,12]. Since anthocyanins absorb visible as well as UV radiation and are effective antioxidants [13] and scavengers of active oxygen species [10,14], a leading hypothesis is that they protect the photosynthetic apparatus from the effects of excess incident visible or UV-B radiation and photooxidative stress [9–12,14–18]. Indeed, light-sensitive seedlings or plants subjected to excessive doses of light often respond by synthesizing anthocyanins. At the same time, however, anthocyanin synthesis can often be triggered by other external stress factors such as cold, drought, osmotic stress, or wounding of the leaf [10]. A particularly intriguing situation is in senescent autumn leaves [15–18], where newly synthesized anthocyanins accumulate in vacuoles in a layer of cells above the chloroplasts as the dying leaves reabsorb nutrients and their photosynthetic apparatus begins to degrade. Other hypotheses for anthocyanin function, less attractive from the standpoint of plant physiology, include suggestions that anthocyanins may affect leaf temperature or serve as a deterrent to herbivores or other potential predators [9,10,12].

In the present paper, we examine the evidence from studies performed in our laboratories that bears directly on the question of whether or not the photochemistry of anthocyanins is compatible with the proposed photoprotective role [9–12,14–18] of anthocyanins in vegetative plant tissues.

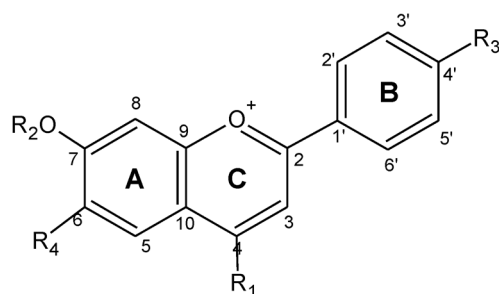
## (PHOTO)CHEMISTRY OF ANTHOCYANINS IN AQUEOUS SOLUTION

Investigations of anthocyanins are complicated by the richness of their ground- and excited-state chemistry. In the ground state, natural anthocyanins can exist in aqueous solution between pH 1–8 in at least five different forms that interconvert via pH-dependent equilibria (Fig. 2) [1,2,19]. At pH < 2, anthocyanins exist predominantly in the reddish-colored flavylum cation form,  $\text{AH}^+$ , which is a weak acid with a first  $\text{p}K_a$  (deprotonation of the 7-hydroxy group) in the range of 4–5 [20]. Deprotonation of  $\text{AH}^+$  produces a bluish quinonoidal base **A**. However, at pH > 3, most flavylum cations also readily undergo attack at C-2 by nucleophiles such as water, resulting in the formation of the colorless or pale yellow hemiacetal (**B**). Tautomerism of the hemiacetal gives the *cis*-chalcone ( $\text{C}_{cis}$ ), followed by slow isomerization to the *trans*-chalcone ( $\text{C}_{trans}$ ). In the first excited singlet state, 7-hydroxyflavylium ions are superphotoacids ( $\text{p}K_a^*$  less than 0.2) [21], transferring a proton to water in ca. 6–20 ps, and the chalcones can undergo both photoisomerization or, in the case of the *cis*-chalcone, exhibit photochromism [22].



**Fig. 2** Ground- and excited-state reactivity of anthocyanins. The ESPT ( $K_a^*$ ) occurs on the picosecond time-scale and ground-state proton transfer ( $K_a$ ) on the micro- to nanosecond time-scale. The ground-state hydration ( $K_h$ ) of the cationic form  $\text{AH}^+$  to form the hemiacetal (**B**) is pH-dependent and moderately fast (fractions of seconds to minutes) at pH above 3–4. Tautomerism ( $K_t$ ) to form the *cis*-chalcone ( $\text{C}_{cis}$ ) is usually slower (minutes to hours) and the subsequent isomerization ( $K_i$ ) to the *trans*-isomer ( $\text{C}_{trans}$ ) even slower.

A key feature of our studies has been the use of synthetic models of the 7-hydroxyflavylium ion chromophore of anthocyanins (Fig. 3) in which part of the reactivity has been blocked or in which the charge or hydrophobicity has been altered [23]. Examples of blocked reactivity include the 4-methyl-7-hydroxyflavylium ion (**HMF**), in which hydration is disfavored, allowing studies of the prototropic reactivity in the absence of the other ground-state multiequilibria, and the 4-methyl-7-methoxy-



**HMF:**  $R_1 = \text{CH}_3$ ;  $R_2, R_3, R_4 = \text{H}$

**MMF:**  $R_1, R_2 = \text{CH}_3$ ;  $R_3, R_4 = \text{H}$

**DHF:**  $R_3 = \text{OH}$ ;  $R_1, R_2, R_4 = \text{H}$

**CHMF:**  $R_1 = \text{CO}_2\text{H}$ ;  $R_2, R_3, R_4 = \text{H}$

**HHMF:**  $R_1 = \text{CH}_3$ ;  $R_4 = \text{n-Hexyl}$ ,  $R_2, R_3 = \text{H}$

**Fig. 3** Synthetic model compounds for investigating anthocyanin (photo)chemistry.

flavylium ion (**MMF**), in which the acid–base equilibria of Fig. 2 are blocked. Examples of anthocyanins with altered charge or hydrophobicity include 7-hydroxyflavylium-4-carboxylic acid (**CHMF**) [24,25], which is cationic at low pH ( $\text{p}K_1 = 0.73$ ), zwitterionic at intermediate acidic pHs ( $\text{p}K_2 = 4.84$ ), and anionic at neutral pH, and water-insoluble flavylium ions with pendent aliphatic chains [26] such as the 6-(*n*-hexyl)-7-hydroxy-4-methyl-flavylium ion (**HHMF**). Both HMF and these water-insoluble anthocyanins with hydrophobic side chains have proven to be excellent probes of proton-transfer dynamics in the picosecond time domain at the surface of detergent micelles [27–29].

**MMF**, in which proton transfer is blocked, emits quite efficiently and has a relatively long (4.7 ns) fluorescence lifetime [30]. In contrast, the cationic forms, **AH<sup>+</sup>**, of synthetic anthocyanin analogs such as **HMF** and **DHF** are only very weakly fluorescent in aqueous solution and have lifetimes on the picosecond time-scale [31]. This is due to very fast adiabatic excited-state proton transfer (ESPT) from **AH<sup>+</sup>\*** to water in 6–10 ps to produce the excited base form (**A\***), which then decays to its ground state in ca. 100–200 ps [21]. The rapidity of the ESPT from **HA<sup>+</sup>\*** and the fast decay of **A\*** permit one to perturb the position of the ground-state acid–base equilibrium of **HMF** and **DMF** by conventional nanosecond laser flash photolysis [31]. The rate constants for deprotonation ( $k_d$ ) and reprotonation ( $k_p$ ) can then be determined by following the rate of relaxation back to equilibrium as a function of proton concentration [31], taking advantage of the fact that  $k_{\text{obs}} = k_d + k_p[\text{H}^+]$ , where  $k_{\text{obs}}$  is the reciprocal of the lifetime of **A**. Likewise, diglycosylated anthocyanins such as malvin, cyanin, and pelargonin are only weakly fluorescent, while monoglycosylated anthocyanins are practically non-fluorescent [32]. Because the ground-state acid–base equilibrium of these natural anthocyanins could also be perturbed by nanosecond laser flash photolysis and the perturbation is a direct consequence of ESPT, this pointed to the occurrence of fast ESPT in these naturally occurring anthocyanins [31]. Indeed, picosecond time-resolved fluorescence measurements [19,32] confirmed that efficient ultrafast ESPT from **AH<sup>+</sup>\*** to water also occurs in these weakly fluorescent natural anthocyanins.

## PHOTOPHYSICS OF ANTHOCYANINS COPIGMENTED BY ORGANIC MOLECULES

Although free anthocyanins generally begin to lose their color at  $\text{pH} > 2.5\text{--}3$  in aqueous solution due to the onset of hydration, Fig. 2, evolution has developed strategies for stabilizing the color of antho-

cyanins at pH 3.5–5, the typical range of pH values of plant cell vacuoles in which anthocyanins are usually located *in vivo* [7,33]. One of the mechanisms involves the complexation of the anthocyanin (via the hydroxyl substituents in the B-ring [34,35]) by metal ions such as aluminum, magnesium, or ferric ions, often forming self-assembling supramolecular complexes of defined stoichiometry that incorporate colorless “copigment” molecules such as flavones as well. This metal-ion-mediated copigmentation typically results in blue or purple colors, notable examples being blue hydrangeas [33], the blue dayflower [8], the blue cornflower [8], the Himalayan blue poppy [7], and blue salvia [8,36].

A second copigmentation mechanism, on which we have concentrated our attention [30,37], is the complexation of the flavylium cation in the absence of metal ions by colorless “copigment” molecules such as hydroxylated benzoic and cinnamic acids, hydroxyflavones, and other polyphenols. As in the case of metal ions, the copigmentation of anthocyanins by organic molecules also stabilizes the flavylium cation with respect to the hemiacetal and chalcone forms, but without pronounced modification of the red or purple color of the anthocyanin. An interesting application is the addition of naturally occurring copigment molecules to juices of red berries, which enhances both the color and the stability of the juices [38]. Although the driving force for the copigmentation of anthocyanins by organic molecules was generally assumed to be a hydrophobic effect, perhaps combined with hydrogen-bonding interactions, our studies show that charge transfer from the copigment to the anthocyanin makes a significant contribution to the stability of anthocyanin-copigment complexes [30,37].

Much of our work on copigmentation by organic molecules has been done with **MMF**, which exists in the **AH<sup>+</sup>** form up to at least pH 5. In addition, because the fluorescence lifetime of **MMF** is much longer than that of other anthocyanin analogs, the effect of copigmentation on the photophysics of **MMF** can be conveniently studied by both steady-state and time-resolved fluorescence techniques. In aqueous solution, the fluorescence of **MMF** is efficiently quenched by a number of typical copigment molecules. The quenching is a consequence of the competitive excitation of free **MMF** and of non-fluorescent ground-state **MMF**-copigment complexes, resulting in predominantly static quenching, as shown by the reaction scheme and the comparison of steady-state and lifetime Stern–Volmer plots in Fig. 4. Consequently, fluorescence quenching provides a convenient and straightforward method for determining equilibrium constants,  $K_{\text{cop}}$ , for copigmentation [30,37]. As expected for a process dominated by charge-transfer interactions, values of  $\log K_{\text{cop}}$  for **MMF**-copigment and for several other anthocyanin-copigment pairs correlate linearly with the difference between the ionization potential of the copigment and the electron affinity of the anthocyanin [37]. Quantum chemical calculations [37,39] confirm that, in aqueous solution, the lowest-energy excited singlet states of the **MMF**-copigment complexes with ferrulic (4-hydroxy-3-methoxycinnamic acid) and protocatechuic acids (PCA; 3,4-dihydroxybenzoic acid) have very substantial charge-transfer character.

From the standpoint of stabilization of the color of anthocyanins by complexation with copigments, charge transfer to the anthocyanin should decrease the positive charge at carbon 2 of the flavylium cation, resulting in a reduction in the equilibrium constant for hydration. Thus, charge transfer nicely rationalizes the inhibition of the hydration of the flavylium ion upon complexation with the copigment [37]. From the standpoint of photostability, however, the lowest excited singlet state of **MMF** and other analogous synthetic anthocyanins analogs should be a superb electron acceptor [23], capable of oxidizing molecules with oxidation potentials as high as 2.0–2.5 V. Thus, by analogy to pyrylium salts [40], quenching of the excited state of anthocyanin-copigment complexes via exergonic excited-state electron transfer could produce free radical ions and their reaction products if back electron transfer to give the ground state of the complex were too slow. In this regard, recent femtosecond pump-probe studies of the **MMF**-protocatechuic acid complex have shown that internal conversion back to the ground state is extremely fast, being essentially complete within less than a picosecond [39], ruling out the possibility of significant production of free radical ions from the decay of the excited complex in this case.



thocyanins are powerful oxidants. Ultrafast internal conversion via a low-lying, charge-transfer excited state circumvents the potentially deleterious consequences that could result if reactive radical ions were formed by net excited-state electron transfer in the complex.

In summary, the flavylum cation forms of natural anthocyanins absorb UV and green light strongly and the absorption of anthocyanin-copigment complexes is further enhanced by the presence of strong UV absorption bands derived from the copigment. This combination of absorption in the UV and visible regions, the existence of highly efficient energy-wasting mechanisms and the known antioxidant activity of anthocyanins (dependent on the number of hydroxyl groups in the B-ring [35]) is fully compatible with the proposed protective role of anthocyanins against excess solar radiation [9–12,14–18] in vegetative tissues of plants.

## ACKNOWLEDGMENTS

Collaborative interaction supported by a GRICES-CAPES international cooperation grant. FHQ thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for funding (Projeto Universal) and fellowship support; ALM thanks the Fundação para a Ciência e Tecnologia (FCT) for funding and KS, AAF and RR thank the FCT for the grants SFRH/BPD/38339/2007, SFRH/BPD/34820/2007 and SFRH/BD/27908/2006, respectively.

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